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Effects of Polyphenol-Rich Foods on Human Health

Edited by
Giuseppe Grosso

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Special Issue Editor

Giuseppe Grosso

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About the Special Issue Editor

Giuseppe Grosso's research focuses on evidence-based nutrition, a recently emerged field as the bottom line of the Health Technology Assessment applied to food and nutrition. The main interests include the impact of dietary and lifestyle habits on common non-communicable diseases. In particular, he produced over 100 papers on the effects of dietary patterns (i.e., Mediterranean diet) and specific antioxidant-rich foods (i.e., coffee, tea), as well as individual antioxidants (i.e., polyphenols, n-3 PUFA) on cardiovascular and metabolic diseases, cancer, and depression. Dr. Grosso conducted his research on cohorts of individuals in both Mediterranean and non-Mediterranean countries collaborating with several research institutions. He is interested in evidence synthesis aimed to generate policy-oriented research in the area of public health nutrition. He is currently working as research fellow at Integrated Cancer Registry of Catania-Messina-Siracusa-Enna, southern Italy. He is a cum-laude graduated MD and PhD.

Preface to "Effects of Polyphenol-Rich Foods on Human Health"

The global burden of non-communicable diseases (NCDs) has been rising over the last century, and among the main NCDs are cardiovascular diseases (CVDs), cancers and diabetes. Besides genetic, environmental, and social factors, exploring dietary factors influencing such conditions is of primary importance to better define effective strategies for reducing the burden of disease. In fact, higher adherence to healthy and equilibrated dietary patterns has been shown to be implicated in prevention of NCDs.

In recent years, polyphenols have received a great deal of attention due to their potential beneficial effects on human health. Contained in foods commonly consumed in all populations worldwide, polyphenols offer a range of beneficial effects and are contained not only in fruits and vegetables, characteristic components of healthy dietary patterns, but also in other plant-derived foods, such as tea, coffee, and cocoa, which only recently have been scientifically exploited as being beneficial for humans. In addition to the numerous biological properties, polyphenols have been indicated as being responsible for a decreased risk of several health outcomes. Numerous epidemiological studies have demonstrated the association between both polyphenols and polyphenol-rich foods intake and human health. For example, recent meta-analyses show that high consumption of polyphenols and polyphenol-rich foods decrease the risk of overall and CVD-related mortality, cancer, CVD, diabetes and neurodegenerative diseases. However, some studies reported null results, which could be at least partially explained by significant differences in dietary intake of polyphenols in different populations, differences in food processing (loss of phenolic content), absorption, bioavailability, and metabolism of polyphenols.

Several molecular mechanisms have been taken into account for the beneficial effects of polyphenols. The antioxidant effects of dietary polyphenols can be attributed to the regulation of redox enzymes through reducing reactive oxygen species (ROS) production and modulation of the II-phase enzymes responsible for the cellular oxidative response. Moreover, several studies have suggested that polyphenols may exert protective effects on cardio-metabolic health by reducing inflammatory response, lowering LDL oxidation and blood pressure, and by improving endothelial function. Finally, polyphenols may exert chemo-preventive effects through a variety of mechanisms, including elimination of carcinogenic agents, modulation of pathways responsible for cancer cell signaling and cell cycle progression, and by promotion of apoptosis.

Even though research is ongoing, further evidence is needed in order to better characterize dietary factors that may exert beneficial effects toward prevention of chronic diseases associated with oxidative stress and inflammation.

Giuseppe Grosso
Special Issue Editor

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Article

Processing 'Ataulfo' Mango into Juice Preserves the Bioavailability and Antioxidant Capacity of Its Phenolic Compounds

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Abstract: The health-promoting effects of phenolic compounds depend on their bioaccessibility from the food matrix and their consequent bioavailability. We carried out a randomized crossover pilot clinical trial to evaluate the matrix effect (raw flesh and juice) of 'Ataulfo' mango on the bioavailability of its phenolic compounds. Twelve healthy male subjects consumed a dose of mango flesh or juice. Blood was collected for six hours after consumption, and urine for 24 h. Plasma and urine phenolics were analyzed by electrochemical detection coupled to high performance liquid chromatography (HPLC-ECD). Five compounds were identified and quantified in plasma. Six phenolic compounds, plus a microbial metabolite (pyrogallol) were quantified in urine, suggesting colonic metabolism. The maximum plasma concentration (C_{max}) occurred 2–4 h after consumption; excretion rates were maximum at 8–24 h. Mango flesh contributed to greater protocatechuic acid absorption (49%), mango juice contributed to higher chlorogenic acid absorption (62%). Our data suggests that the bioavailability and antioxidant capacity of mango phenolics is preserved, and may be increased when the flesh is processed into juice.

Keywords: mango; pharmacokinetics; phenolic acids; human; food matrix; antioxidant

1. Introduction

Mango (*Mangifera indica* L.) is one of the most consumed tropical fruits in the world, due to its sensorial attractiveness and nutritional and phytochemical composition [1,2]. Mexico is the leading mango-exporting country (41%), and among Mexican cultivars, 'Ataulfo' is one of the most consumed, due to its appetizing organoleptic and sensory characteristics [3]. 'Ataulfo' mango has the highest content of phenolic compounds and antioxidant capacity, as compared to other cultivars [2,4]. Because of this, 'Ataulfo' mango can be considered a "natural functional food", whose regular consumption can prevent several chronic diseases, including cardiovascular diseases and some types of cancer [5,6].

To corroborate the health benefits of a functional food, knowledge of its bioactive constituents and their bioaccessibility and bioavailability is required. According to Grundy et al. [7] "Bioaccessibility refers to the proportion of a nutrient or any other substance (i.e., phytochemicals) that is released from the food matrix and is potentially available for absorption in the small intestine", while bioavailability is defined

as the rate and extent to which a compound is absorbed, transported, and becomes available at the site of action [8]. Bioaccessibility precedes bioavailability in nutrient/drug pharmacokinetics and plays a critical role in the final bioefficacy at target organs. The concept of food matrix refers to the fact that nutrients are contained in a continuous medium, where they may interact physicochemically at different length scales with other components and structures, such as proteins, carbohydrates, dietary fiber, lipids, organic acids, and others [9], which governs both the bioaccessibility and bioavailability of nutrients and xenobiotics. Phenolic compounds are generally bound tightly to food matrices [10], consequently, most of the ingested phenolics (67–99%) are not absorbed in the upper gastrointestinal (GI) tract because they are not bioaccessible [11].

The impact of the food matrix on a compound's bioaccessibility can be strongly influenced by food preparation technologies, such as homogenization, pressing, grinding, fermentation, and heating [9,12–14], as well as many intrinsic factors related to the digestive process. Juices are produced by pressing and squeezing the fruit, which releases soluble compounds and those that are not bound to the matrix, resulting in products that differ in nutrient density, sugar profile, pectin, and dietary fiber contents [15]. Phenolic compounds from fruit juices are expected to be more bioaccessible and bioavailable than those from fruit flesh because of their differences in proximate fiber content [5]. Dietary fiber content in 'Ataulfo' mango flesh and juice is approximately 18% and 14%, respectively. However, in vivo evidence on the bioavailability of mango phenolics from different matrices has not been fully documented. This information is important to substantiate the reported health benefits of mango phenolics. The objective of this study was to compare the effects of food matrix (flesh and juice) on the bioavailability and antioxidant capacity of phenolic compounds from 'Ataulfo' mangos in healthy human subjects.

2. Materials and Methods

2.1. Chemicals and Solvents

All solvents, salts, and acids used (methanol, HCl, ascorbic acid, ethylenediaminetetraacetic acid (EDTA,) NaH_2PO_4, 2′,3′,4′-trihydroxyacetophenone, NaCl, and perchloric acid) were purchased from Fisher Scientific Co (Montreal, Canada). Phenolic standards (*p*-coumaric acid, gallic acid, chlorogenic acid, ferulic acid, vanillic acid, protocatechuic acid, gentisic acid, sinapic acid, caffeic acid, and pyrogallol), β-glucuronidase (containing sulfatase from *Helix pomatia*), and creatinine assay kit were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. 'Ataulfo' Mangos and Products

Three hundred (200–300 g) commercially ripe (Stage 4) 'Ataulfo' mangos [16] were obtained from the local market in Leon, Guanajuato, Mexico and transported to the laboratory. They were washed under tap water, sanitized, peeled, and processed to obtain fresh raw slices (flesh), which were administered to the participants during the crossover intervention. Mango juice was prepared from mango flesh as suggested by Santhirasegaram et al. [17], with some modifications. The flesh was blended using a domestic juice extractor (Moulinex, Centri III, A75312V, Groupe SEB, Mexico City, Mexico) and filtered through organza cloth. The juice was prepared fresh, and immediately consumed by the volunteers.

2.3. Phenolic Profile of 'Ataulfo' Mango Flesh and Juice

The phenolic composition of the flesh and juice was determined from freeze-dried samples (Labconco Corporation, Kansas City, MO, USA). One gram of the dried products was homogenized in 20 mL of 80% methanol using an IKA® Works homogenizer (Model T25, Willmington, NC, USA) at room temperature. The homogenate was sonicated for 30 min (Bransonic Ultrasonic Co., Model 2210, Danbury, CT, USA) and centrifuged at 20,000× *g* for 15 min at 4 °C. The pellet was homogenized in 10 mL of methanol:water (80/20, *v*/*v*) and reextracted under the same conditions. The supernatants

were combined, filtered (Whatman No. 1, Springfield Mill, Maidstone Kent, UK), and made up to a final volume of 30 mL with 80% methanol, to generate a phenolic extract. Before its chromatographic analysis, the extract was subjected to an acid hydrolysis, by adding 1 mL of 2.4 M HCl to 1 mL of the phenolic extract, and incubating the mixture for 2 h in the dark at 80 °C. After the incubation period, the mixture was filtered through a 0.22-µm Ultrafree Durapore Centrifugal filter (Merck Millipore, Billerica, MA, USA), and directly injected into a high pressure liquid chromatography (HPLC) system, equipped with an electrochemical detector (HPLC-ECD) for analysis of phenolic compounds. The HPLC analysis was performed as previously described [18].

2.4. Subjects and Study Design

A randomized crossover pilot clinical trial was conducted to evaluate the postprandial plasma bioavailability (0–6 h) and urinary excretion (0–24 h) of phenolic compounds from mango flesh and juice. The protocol was approved by the Bioethics Committee (CE/003/2014) of the Research Center for Food and Development, A.C (CIAD), and was conducted in compliance with the declaration of Helsinki. A graphical representation of the experimental protocol is illustrated in Figure 1. Twelve middle-aged healthy men aged 22 to 34 years were recruited from CIAD. Only male participants were selected to study the bioavailability of mango phenolics, in order to avoid anthropometric variables and menstrual cycle phase-related variability in women that may have affected the absorption, metabolism, and excretion of the compounds of interest. The study was thoroughly explained to the volunteers, and written informed consent was obtained before any experimental activities were performed. All subjects were free of diagnosed heart disease, homeostatic disorders, gastrointestinal disease, or other medical conditions; they were not taking any medication or any vitamin/mineral supplement.

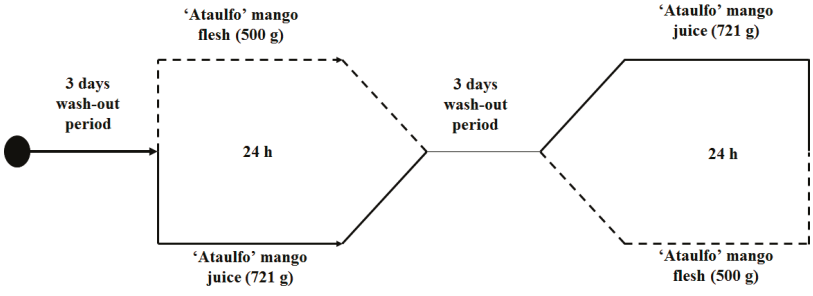

Figure 1. Graphical representation of the randomized crossover clinical trial.

The subjects underwent a 3 day wash-out period to minimize the confounding effect of phenolic compounds from their habitual diets. They were instructed to refrain from consuming foods high in phenolic compounds, such as fruits, berries, vegetables, juices, nuts, tea, coffee, cocoa, olive oil, coffee, wine, and beer. Twenty-four-hour dietary recall was performed, using a slightly modified food frequency questionnaire, to evaluate their compliance to the low phenolic protocol during the wash-out period [19]. At the end of the wash-out period, subjects reported to the study site after a 12 h fast.

After baseline (time 0), blood and urine were collected, subjects were randomly assigned to consume mango flesh slices or juice in a 10-min period, in random order separated by a 3 day wash-out period. The amounts of flesh or juice administered to the subjects (500 g of flesh, 721 g of juice) were chosen because they contained equal amounts of phenolic compounds (45 mg), as determined through chromatographic analyses (see Results). Six additional blood samples were collected at hourly intervals post-consumption, and four additional urine samples were collected at 0–4, 4–8, 8–12, and 12–24 h. Blood was collected in a vacutainer tube containing EDTA as anticoagulant. Plasma was recovered, and 0.5 mL aliquots were immediately acidified using 20 µL of 4 M HCl. Acidified plasma and urine samples were stored at −80 °C until analysis. During blood collection, subjects were only allowed

water, other foods and beverages were not allowed. They were given a hamburger without vegetables and ketchup for lunch. For dinner, they were allowed to eat a meal, but with the same restrictions as those of the wash-out period.

2.5. Determination of Plasma Phenolic Compounds

Phenolics in plasma were quantified according to the method of Chen, Milbury, Lapsley, and Blumberg [18]. Briefly, 20 μL of vitamin C-EDTA (100 mg ascorbic acid plus 1 mg EDTA in 1.0 mL of 0.4 M NaH_2PO_4, pH 3.6), 20 μL of 10 μg/mL internal standard (2′,3′,4′-trihydroxyacetophenone), and 20 μL β-glucuronidase/sulfatase (98,000 kU/L β-glucuronidase and 2400 kU/L sulfatase) were added to 500 μL of plasma, and the mixture was incubated at 37 °C for 60 min. Subsequently, phenolics were extracted with acetonitrile. After centrifugation, the supernatant was removed, dried with nitrogen gas, and reconstituted in the aqueous HPLC mobile phase, filtered using Ultrafree Durapore Centrifugal filter devices (0.22, Merck Millipore, Billerica, MA, USA), and injected into the HPLC- ECD system, using previously optimized chromatographic conditions [18]. The mobile phases were 75 mM citric acid and 25 mM ammonium acetate in 90% water/10% acetonitrile (A), and 75 mM citric acid and 25 mM ammonium acetate in 50% water/50% acetonitrile (B). The concentrations of individual phenolics were calculated based on calibration curves that were constructed using authentic phenolic standards ($R^2 > 0.999$) of *p*-coumaric acid, gallic acid, chlorogenic acid, ferulic acid, vanillic acid, protocatechuic acid, gentisic acid, sinapic acid, and caffeic acid. The intraday coefficient of variation (CV) for standards spiked into plasma was <8%.

2.6. Determination of Urinary Phenolic Compounds

Phenolics in urine were quantified according to the method of McKay et al. [20]. Urine (200 μL) was dissolved in 800 μL of buffer (0.1 M sodium acetate, pH 5) and 30 μL of β-glucuronidase/sulfatase (98,000 kU/L β-glucuronidase and 2400 kU/L sulfatase), and incubated at 37 °C for 60 min. After incubation, 10 μL of 100 μg/mL internal standard (2′,3′,4′-trihydroxyacetophenone) and 200 μL glacial acetic acid were added, and the mixture was briefly vortexed. After the addition of approximately 1 g of NaCl, phenolics were extracted twice with 3 mL of ethyl acetate. The combined ethyl acetate fractions were dried under nitrogen gas and reconstituted in 1 mL of the aqueous HPLC mobile phase, filtered through a 0.22-μm Millex syringe-driven filter (Millipore Corporation, Bedford, MA, USA), and injected (50 μL) onto the HPLC-ECD system. Concentrations of individual phenolics were calculated based on calibration curves constructed using authentic phenolics ($R^2 > 0.999$), with the adjustment of the internal standard. The intraday CV for standards spiked into urine was <7%. Urinary phenolics were normalized to creatinine concentration, which was measured with a commercial kit.

2.7. Antioxidant Capacity

The oxygen radical absorbance capacity (ORAC) assay was performed, according to the method of Prior et al., 2003 [21], using a FLUOstar Optima Microplate reader (BMG Labtechnologies, Inc., Durham, NC, USA). Frozen plasma samples were thawed at room temperature and briefly vortexed. Plasma samples were then mixed with 0.5 M perchloric acid (1:1 *v*/*v*) to obtain a protein-free supernatant. The protein-free supernatants were used in the ORAC assay. Urine samples were analyzed without previous dilution [22].

The ferric reducing antioxidant power (FRAP) assay was performed according to Benzie and Strain [23], using untreated plasma and urine samples. All results are expressed as μmol Trolox equivalents (TE)/mL.

2.8. Statistics

Results are expressed as mean ± standard deviation (SD). The maximum concentration (C_{max}) in plasma and the time to reach C_{max} (T_{max}) were visually identified. The area under the concentration-time curve (AUC) of plasma phenolics was calculated using the linear trapezoidal

integration equation [24]. The differences in C_{max}, T_{max}, and AUC of phenolics between mango flesh and juice treatments were examined using an analysis of covariance, with time 0 values as the covariant, followed by the Tukey-Kramer multi-comparison test. The Number Cruncher Statistical System version 6.0 software (NCSS, NCSS LLC, Kaysville, UT, USA) was used to perform all statistical analyses.

3. Results

3.1. Mango Phenolics

The major phenolics identified in 'Ataulfo' mango flesh and juice were gallic, chlorogenic, *p*-coumaric, vanillic, sinapic, protocatechuic, ferulic, gentisic, and caffeic acids (Figure 2 and Table 1). Among the quantified phenolics, *p*-coumaric, gallic, and chlorogenic acids were the predominant species in both flesh and juice. Chlorogenic acid content in juice was 59% lower than in flesh. The sum of quantified phenolics in flesh and juice was 47.60 mg/500 g fresh weight (FW) and 43.24 mg/721 g FW, and were statistically similar. The values reported in Table 1 are the doses effectively consumed by the volunteers (500 g of flesh and 721 g of juice), which contain equal concentrations of phenolic compounds.

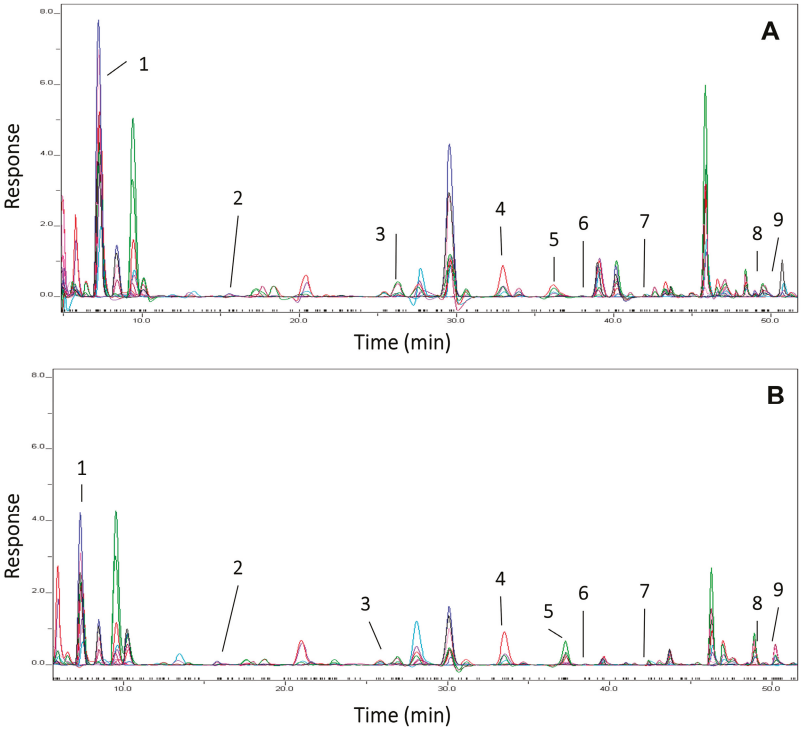

Figure 2. Representative chromatogram obtained by electrochemical detection coupled to high performance liquid chromatography (HPLC-ECD) used to identify and quantify phenolic compounds in mango flesh (**A**) and mango juice (**B**). 1: gallic acid, 2: protocatechuic acid, 3: gentisic acid, 4: chlorogenic acid, 5: vanillic acid, 6: caffeic acid, 7: *p*-coumaric acid, 8: ferulic acid, 9: sinapic acid.

Table 1. Content of phenolic compounds in 'Ataulfo' mango flesh and juice by HPLC-ECD analysis.

No.	Phenolic Compound	Mango Flesh	Mango Juice
		(mg/500 g of Fresh Weight)	(mg/721 g of Fresh Weight)
1	*p*-coumaric acid	19.36 ± 2.46	16.63 ± 1.24
2	gallic acid	16.52 ± 0.95	15.90 ± 0.34
3	chlorogenic acid	17.96 ± 0.50	7.32 ± 0.53
4	ferulic acid	1.94 ± 0.28	1.59 ± 0.02
5	vanillic acid	1.07 ± 0.06	0.87 ± 0.02
6	protocatechuic acid	0.41 ± 0.02	0.53 ± 0.03
7	gentisic acid	0.24 ± 0.01	0.18 ± 0.03
8	sinapic acid	0.06 ± 0.00	0.06 ± 0.00
9	caffeic acid	0.03 ± 0.00	0.03 ± 0.00
Total		47.60 ± 3.72	43.24 ± 0.28

3.2. Plasma and Urine Pharmacokinetic Analyses

Five phenolic acids present in 'Ataulfo' mango flesh and juice were detected in plasma (gallic, chlorogenic, protocatechuic, ferulic, and gentisic acid); these results are presented in Figure 3. The pharmacokinetic parameters of the phenolic compounds are presented in Table 2. The C_{max} of chlorogenic and ferulic acid was significantly higher in subjects that consumed mango juice. The relative absorption was estimated from the AUC and dose-adjusted (AUC/dose); chlorogenic and ferulic acid showed statistically significant differences between matrices (higher values in the juice group). The five phenolic compounds detected in plasma reached C_{max} at 2–4 h after mango consumption. The T_{max} of all detected phenolic acids was statistically similar between matrices, but the T_{max} of the juice group was slightly shorter than that of the flesh. Consistent with mango juice having the highest C_{max}, the AUC of chlorogenic and ferulic acid was higher in the juice group.

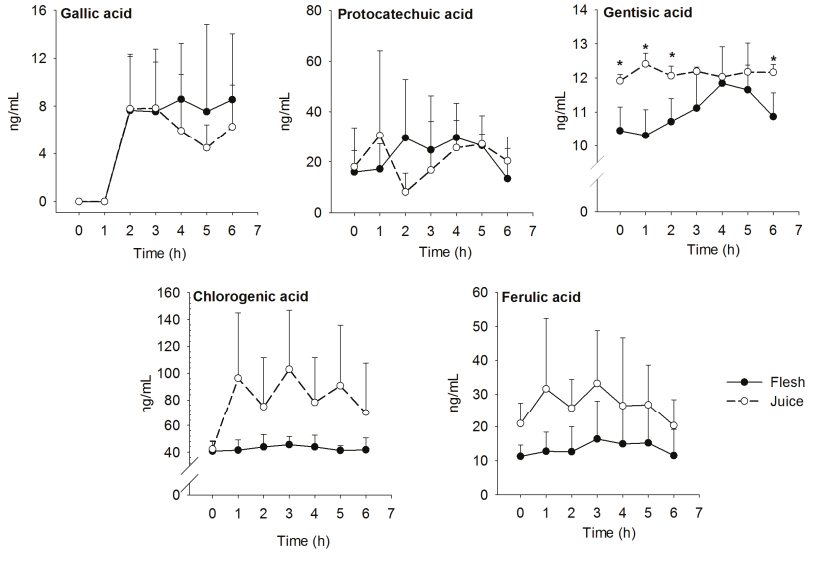

Figure 3. Average plasma concentration of gallic, protocatechuic, gentisic, chlorogenic, and ferulic acid in plasma (ng/mL). Blood samples were collected after consumption of 'Ataulfo' mango flesh (-●-) and juice (-○-). * Statistically significant differences ($p < 0.05$) between mango matrices.

Six major phenolic acids were detected in urine, including chlorogenic, vanillic, ferulic, sinapic, gallic, and *p*-coumaric acid; results are presented in Figure 4. Pyrogallol was not detected in mango samples, but was found in urine. *p*-coumaric and ferulic acids had significantly higher excretions in the juice group at 0–4 and 4–8 h; the remaining metabolites were not significantly different between matrices. All phenolic compounds detected in urine were excreted 8–24 h after mango consumption. Interestingly, the concentration of gallic acid excreted in urine decreased with time, in parallel with an increase in pyrogallol. Pearson's correlation coefficients (r) between gallic acid and pyrogallol were −0.42 for flesh and −0.47 for juice, indicating a linear negative correlation between the urinary excretion of these compounds. Higher AUC/dose excretions were found for vanillic and sinapic acids; the values were higher in the juice group.

Table 2. Pharmacokinetic parameters after consuming 'Ataulfo' mango flesh and juice.

Phenolic Compound	Flesh				Juice			
	C_{max} (ng/mL)	T_{max} (h)	AUC (ng h/mL)	AUC/Dose ((ng h/mL)/mg)	C_{max} (ng/mL)	T_{max} (h)	AUC (ng h/mL)	AUC/Dose ((ng h/mL)/mg)
Chlorogenic acid	49.7 ± 7.3 *	3.5 ± 1.4	208.7 ± 24.5 *	11.5 ± 1.78 *	109.7 ± 0.26 *	2.5 ± 1.8	366.9 ± 130.7 *	50.12 ± 15.5 *
Protocatechuic acid	30.8 ± 13.3	3.5 ± 2.0	141.4 ± 73.9	344.8 ± 138.3	34.5 ± 18.0	3.7 ± 1.7	108.6 ± 5.4	204.90 ± 9.9
Ferulic acid	16.5 ± 3.9 *	2.8 ± 2.1	60.2 ± 22.7 *	31.0 ± 9.63 *	32.7 ± 10.9 *	2.3 ± 1.5	133.4 ± 47.7 *	83.8 ± 30.2 *
Gentisic acid	11.8 ± 2.1	4.0 ± 1.4	53.1 ± 8.3	221.2 ± 34.0	12.2 ± 0.2	2.8 ± 1.9	50.9 ± 11.0	282.7 ± 59.1
Gallic acid	8.7 ± 1.7	4.4 ± 1.1	36.9 ± 24.3	2.2 ± 0.91	7.9 ± 4.7	3.5 ± 1.0	38.5 ± 14.5	2.4 ± 0.94

Values are means ± standard deviation (SD) (n = 12) of plasma phenolic compounds. * Statistically significant differences ($p < 0.05$) between mango matrices. C_{max}: maximum concentration. T_{max}: maximum time. AUC: area under the curve from time 0 to 6 h.

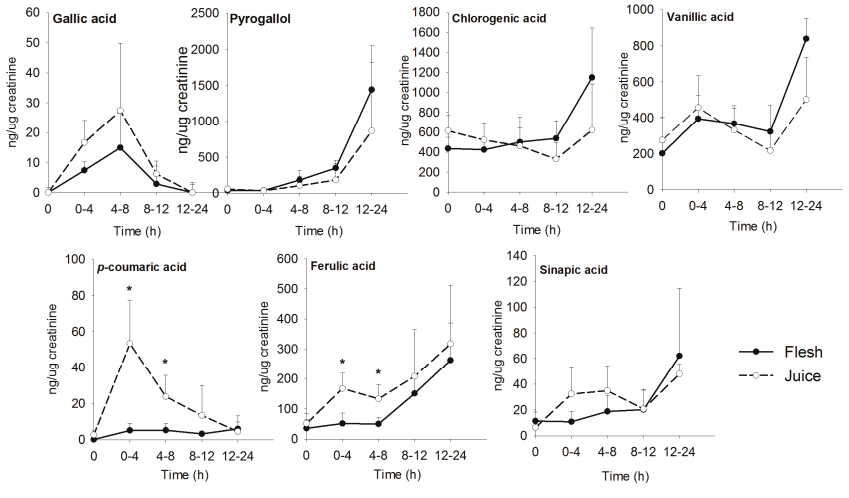

Figure 4. Phenolic compounds detected in urine. Samples were collected after consumption of 'Ataulfo' mango flesh (-●-) and juice (-○-). * Statistically significant differences ($p < 0.05$) between mango matrices.

3.3. Antioxidant Capacity

The results of plasma and urine antioxidant capacity are shown in Figure 5. No significant changes were found in antioxidant capacity values of plasma and urine at any time point. Although no significant differences were found between mango matrices, the antioxidant capacity as measured by two different assays (FRAP and ORAC) showed slightly higher plasma antioxidant capacity values in the juice group. The antioxidant capacity values of urine increased with time, possibly due to the increased excretion of phenolic acids. No significant differences were found with the FRAP assay. The ORAC assay showed significant differences between food matrices at baseline (time 0) and at

the first urine sampling (0–4 h). The highest antioxidant capacity was found at 8–24 h after mango consumption. This is positively associated with the increased concentration of phenolic compounds in urine to these times (FRAP and phenolic compounds from flesh, r = 0.99; ORAC and phenolic compounds from flesh r = 0.73; FRAP and phenolic compounds from juice r = 0.99; ORAC and phenolic compounds from juice r = 0.80).

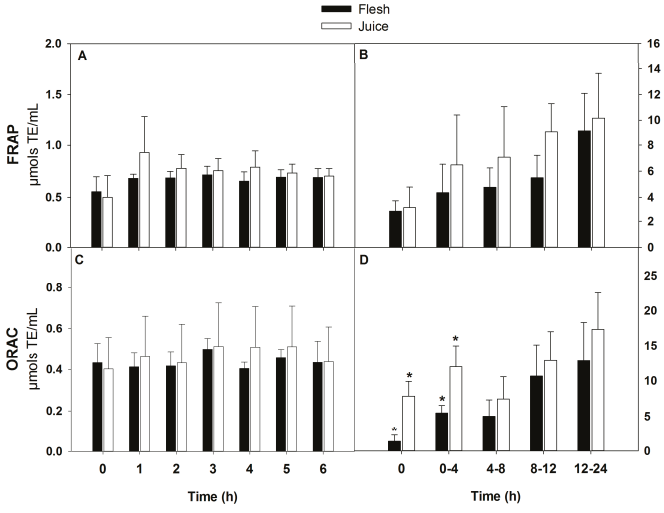

Figure 5. Antioxidant capacity of plasma (**A**) and urine (**B**), as determined with the ferric reducing antioxidant power (FRAP) assay. Antioxidant capacity of plasma (**C**) and urine (**D**), as determined with the oxygen radical absorbance capacity (ORAC) assay. * Statistically significant differences ($p < 0.05$) between mango matrices.

4. Discussion

Phenolics contribute to the inverse correlation documented between consumption of plant foods and chronic diseases, such as metabolic disorders and cancers, through mechanisms that include antioxidant, anti-inflammatory, and anti-proliferative effects [5,25]. A substantial amount of preclinical and clinical data illustrates that phenolics from plant foods are bioavailable and capable of enhancing antioxidant capacity values in humans, while also exerting multi-organ actions that promote health [26,27]. 'Ataulfo' mango fruit has the highest content of total phenolics among other commercial varieties of mango [2]. In this study, we investigated the bioavailability of phenolic acids of 'Ataulfo' in healthy adult men. We also studied whether their bioavailability would differ when they were delivered in different mango forms (flesh vs. juice), because phenolics bound tightly to flesh fiber might be less bioaccessible and bioavailable.

Our results are in agreement with reports by other authors [2,28], where phenolic acids are the major phenolic compounds in 'Ataulfo' mango fruit, and specifically, gallic acid and its derivatives are the most representative. Gallic acids (*m*-digallic acid and *m*-trigallic acids), gallotannins, and mangiferin are among the main phenolic compounds identified in the flesh of different mango cultivars ('Ataulfo', 'Alphonso', 'Kitchener', 'Abu Samaka', 'Keitt') [2,28–30]. Our mango flesh samples contained 33.04 mg/kg of gallic acid, while Ramirez et al. [31] reported 6 mg/kg of gallic acid in 'Pica' mango and 17 mg/kg of gallic acid in 'Tommy Atkins' mango. Our 'Ataulfo' mango juice samples had 31.8 mg/kg of gallic acid.

Five phenolic compounds were detected in plasma after mango flesh and juice consumption, and the concentration of gentisic acid was significantly higher in the juice group. Caffeic acid was detected in the mango samples, but not in plasma or urine. This could be attributed to an oxidative

degradation in the GI tract, low concentration in plasma/urine or quick absorption (<1 h) and tissue distribution, or metabolism into ferulic acid [32]. Phenolic compounds identified in plasma showed a T_{max} of 1–4 h after mango consumption, suggesting that the absorption of these phenolics occurred in the small intestine [33]. Even though some phenolic compounds such as gallic, chlorogenic, caffeic, and *p*-coumaric acids can be absorbed in the stomach via monocarboxylic acid transporters (MCTs) within 5 min after gastric administration [34], the small intestine is known to be the major site for the absorption of phenolic acids. The T_{max} of gallic acid from juice was 3.5 ± 1.0 h, which is less than the T_{max} of the same compound, as compared to its ingestion from tea (T_{max}: 1.39 ± 0.21 h) and red wine (T_{max}: <1.5 h) [35,36]. Both mango matrices contain similar amounts of the individual phenolic compounds, therefore the AUC/dose showed no difference, except for chlorogenic and ferulic acids.

Six phenolic compounds were detected in urine, plus one microbial metabolite, and the excretion of *p*-coumaric and ferulic acid was significantly higher in the juice group. Protocatechuic and gentisic acids were detected in plasma, but not in urine. Vanillic and sinapic acids were detected in urine but not in plasma. Excretion rates were maximum at 8–24 h. Free and polymeric gallic acid is the main phenolic acid in 'Ataulfo' mango. This phenolic acid is absorbed in the small intestine, but a percentage of this compound is not bioaccessible in the small intestine and reaches the colon, where it is apparently decarboxylated into pyrogallol by microbial gallic acid decarboxylase [37–39]. Pyrogallol has been previously reported as a metabolite after consumption of 'Keitt' mango, berry fruits, Concord grape juice, and black tea [37,39,40]. Stalmach, Edwards, Wightman, and Crozier [40] reported urinary pyrogallol at later time points after ingestion of Concord grape juice, but this compound was not observed in the urine of ileostomized patients, which strongly suggests a colonic origin of this metabolite. This trend was expected, as previous studies have identified increased concentrations of gallic acid microbial metabolites in plasma and urine at 6–8 h from baseline [37,41].

As previously mentioned, the matrix of different foods may favor or hinder the bioaccessibility and bioactive responses of phenolic compounds in vivo [9]. In particular, because of the physicochemical properties of dietary fiber, it can delay the release and absorption of macronutrients, some minerals and trace elements, as well as phytochemicals, such as phenolic compounds and carotenoids [42]. Food processing such as macerating, grinding, fermentations, and/or mild heating may improve the bioaccessibility and bioavailability of phenolics, most likely as a result of disruption of the cell walls (dietary fiber) of plant tissues, dissociation of bioactive compound-matrix complexes, or transformation into more active molecular structures [9]. In our study, mango flesh represents the raw food, without any modifications to the matrix, while mango juice is the soluble part that results from the removal of the fiber. Our data showed that processing mango flesh into juice can increase the Cmax of some phenolic acids, while favoring the excretion of others. This could be attributed to complex carbohydrates present in the mango matrix, which are mainly non-starch, of which pectin is the predominant compound, but hemicellulose and arabinogalactans may also be present [43–45]. Other authors have also reported important interactions between phenolic compounds and macromolecules, such as proteins. For example, Quintero-Flórez et al. [46] show that some of the phenolic compounds used in our study (*p*-coumaric acid, caffeic acid, vanillic acid, protocatechuic acid) and specific proteins found in olive oil (mucin) rapidly interact (1 min) to form insoluble precipitates, which suggests that the bioavailability of both phenolics and proteins may change as a consequence of these interactions.

Normal postprandial metabolic and oxidative processes produce reactive oxygen species with oxidative effects, which reduce the plasma antioxidant capacity [47]. This pattern could be related to the plasma antioxidant capacity values found in this study. Some of the absorbed antioxidants (i.e., phenolic compounds or vitamins) could exert their antioxidant power in plasma, and as a result, they can partially attenuate the oxidative stress, maintaining or increasing plasma antioxidant capacity. The higher antioxidant capacity in urine is attributed to the higher concentration of phenolic compounds excreted, but urinary pyrogallol may have also contributed to this effect.

5. Conclusions

We administered 'Ataulfo' mango to healthy subjects in two different forms, flesh or juice. We determined that the bioavailability and antioxidant capacity of the phenolic compounds are preserved, or may increase, when the mango is processed into juice. Consumption of 'Ataulfo' mango has the potential to increase the concentration of phenolic acids in humans, which have been shown to exert anti-inflammatory and anti-carcinogenic effects. The consumption of mango, as flesh or juice, is highly recommended as a source of health-promoting phytochemicals. Nevertheless, further studies are still required to determine the preventive or ameliorating effects of mango bioactives against specific diseases or metabolic aggressions.

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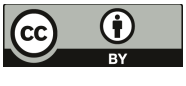

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Phytoestrogen Concentrations in Human Urine as Biomarkers for Dietary Phytoestrogen Intake in Mexican Women

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Abstract: There has been substantial interest in phytoestrogens, because of their potential effect in reducing cancer and heart disease risk. Measuring concentrations of phytoestrogens in urine is an alternative method for conducting epidemiological studies. Our objective was to evaluate the urinary excretion of phytoestrogens as biomarkers for dietary phytoestrogen intake in Mexican women. Participants were 100 healthy women from 25 to 80 years of age. A food frequency questionnaire (FFQ) and a 24 h recall were used to estimate habitual and recent intakes of isoflavones, lignans, flavonols, coumestrol, resveratrol, naringenin, and luteolin. Urinary concentrations were measured by liquid chromatography (HPLC) coupled to mass spectrometry (MS) using the electrospray ionization interface (ESI) and diode array detector (DAD) (HPLC-DAD-ESI-MS). Spearman correlation coefficients were used to evaluate associations between dietary intake and urine concentrations. The habitual consumption (FFQ) of total phytoestrogens was 37.56 mg/day. In urine, the higher compounds were naringenin (60.1 μg/L) and enterolactone (41.7 μg/L). Recent intakes (24 h recall) of isoflavones ($r = 0.460$, $p < 0.001$), lignans ($r = 0.550$, $p < 0.0001$), flavonoids ($r = 0.240$, $p < 0.05$), and total phytoestrogens ($r = 0.410$, $p < 0.001$) were correlated to their urinary levels. Total phytoestrogen intakes estimated by the FFQ showed higher correlations to urinary levels ($r = 0.730$, $p < 0.0001$). Urinary phytoestrogens may be useful as biomarkers of phytoestrogen intake, and as a tool for evaluating the relationship of intake and disease risk in Mexican women.

Keywords: phytoestrogen intake; urinary excretion; biomarker; Mexican women

1. Introduction

Experimental evidence suggests that phytoestrogen intake may modulate the risk of cancer and cardiovascular disease [1–4]. Phytoestrogens are plant-derived, naturally occurring non-steroidal

polycyclic phenols that may have weak estrogenic effects when they are ingested and metabolized [5]. Major classes of phytoestrogens are isoflavones, lignans, and coumestans. Diet is the main source of phytoestrogens in humans. Isoflavones are present in berries, soybeans, and other legumes [6]. Lignans, primarily matairesinol and secoisolariciresinol, are found widely in many fiber rich foods such as fruits, vegetables, cereals, and flaxseeds [7]. The main dietary sources of coumestans are alfalfa sprouts, followed by pinto and pea beans [8]. Resveratrol, from the stilbene group, is found in wine, grape skins, and peanuts [9].

The estimation of dietary consumption of phytoestrogens is limited by the scarcity of data on the content of food; therefore, food composition databases are complex to establish. Phytoestrogen analysis in food is complicated due to the variety of matrices and the different concentrations in foods. Thus, there is a need for a validated biomarker of phytoestrogen intake for epidemiological studies [10]. An alternative approach for determining the intake of phytoestrogens, is measuring them in biological samples, such as urine.

Phytoestrogens in food are mainly in the form of several types of conjugates (β-glycosidic) and in smaller amounts as aglycones. Conjugate forms (inactive) are ingested and hydrolyzed to their aglycone forms (bioactive) by bacterial β-glucosidases in the intestine wall. Then, only the bioactive forms are absorbed by the intestinal tract, and can be further glucuronidated in the intestinal wall and liver [11]. Overall, the main circulating and excreting forms of phytoestrogens are the glucuronidated metabolites. Urinary excretion of phytoestrogens varies with the type of diet, which may be related to differences in both human pharmacokinetics and metabolism by the intestinal bacteria [12].

At population level, little information is available on the excretion of urinary phytoestrogens and their associations with dietary intakes in Western countries. Dietary intake of phytoestrogens has been associated with urinary excretion of related metabolites in observational studies [1,13–15] and controlled trials [16,17]. In Mexico, a study evaluated the phytoestrogens consumption in typical diets [18]; however, a main limitation was the scarcity of foods with phytoestrogen concentrations in food composition databases. We have previously evaluated phytoestrogens in serum as biomarkers of intake in Mexican women, although limited correlations for individual compounds were found [19]. Furthermore, there are no studies on the concentration of urinary phytoestrogens of the general population. Therefore, the ability to monitor objectively exposure to these compounds is important in order to understand the health impact of dietary intake of phytoestrogens in Mexican population. A better understanding of the relationship of phytoestrogen consumption and urine concentration in the population of Mexico is essential for carrying out epidemiological research regarding the association between phytoestrogens and health.

Therefore, the aim of this study was to determine phytoestrogens in urine as biomarkers of phytoestrogen intake, by measuring the association of urinary excretion of these compounds with recent and habitual dietary intakes in Mexican women. Estimates of dietary intakes of isoflavones (genistein, daidzein, equol, glycitein, formononetin, biochanin A), lignans (enterodiol, enterolactone, secoisolariciresinol, matairesinol), flavonols (quercetin, kaempferol), flavanones (naringenin), flavones (luteolin), stilbenes (resveratrol), and coumestans (coumestrol) were compared with their urinary compounds.

2. Materials and Methods

2.1. Study Subjects

Subjects in this cross-sectional study were 100 apparently healthy women, aged 25 to 80 years and with at least 5 years of residence in Northwest México. Women were excluded from the study if they were pregnant or breastfeeding, or have had prior diagnosis of specific chronic illnesses such as diabetes, cancer, or heart disease. Participants were selected randomly from 20 blocks in the city of Hermosillo, Sonora, Mexico, through the Master Sample Frame, which is the basis sample of houses for the surveys that make up the System of National Health Surveys in México.

The protocol for this research was reviewed and approved by the Ethical Committee from Centro de Investigación en Alimentación y Desarrollo (Center for Research on Food and Development). The investigation was carried out following the Declaration of Helsinki; all women provided written informed consent prior to participation. Socioeconomic and demographic indicators were collected at first interview in women's households. Weight and height were measured using standardized procedures to characterize the study population. Weight was measured by a portable electronic scale (and FG-150KBM (up to 150 kg weighing capacity) with 0.05 kg of accuracy). Height was measured using a portable stadiometer (SECA). Body mass index was calculated from weight and height.

2.2. Dietary Assessment

Habitual intake of phytoestrogens was assessed using a food frequency questionnaire (FFQ) validated for women in the region [20] and modified for this study. The questionnaire was applied by trained interviewers, and included 162 food items from 11 categories. Frequency of consumption of each food item was evaluated on a daily, weekly, or monthly basis, and portion sizes (small, medium, large) were specified.

Recent intake of phytoestrogens was assessed using a 24 h dietary recall on the same day that the urine sample was taken. Printed food models, plates, glasses and spoons were used as visual aids to improve recall. Additionally, a second 24 h dietary recall was applied to a subsample of 50 women, with a minimum of three weeks between interviews, to calculate the coefficient of intraindividual variation of daily intake.

Quantification of daily intakes of 16 phytoestrogens considered in this study was performed using a food dictionary that included data from the USDA [6], the National Institute of Nutrition in Mexico, and the food composition database from our Institute. In addition, tables and databases from different studies [7–9,21–24] were used to identify foods that are a source of phytoestrogens. Total phytoestrogen intake was calculated as the sum of lignans, isoflavones, flavonoids, coumestrol and resveratrol intakes.

2.3. Urine Collection

Subjects were provided with supplies and instructed in the total collection of 12 h urine on any day but those of the menstrual period. Participants should not have consumed any antibiotics or other kind of drugs within 7 days prior to urine collection. Total 12 h urine was collected in 0.5 L portable containers with 0.5 g of *L*-ascorbic acid (A4544-100G, Sigma-Aldrich, St. Louis, MO, USA), to prevent microbial contamination and oxidative degradation [25]. Subjects were asked to use a separate container for before-dinner urine, overnight urine, and the first urine excreted in the morning of the following day, and to store containers in their refrigerators at 4–6 °C or cooler until the next morning. Total 12 h urine samples were picked up and transported in a cooler to the laboratory. The total urine volume of each subject was measured, and a sample was filtered using Whatman grade 41 filters. The filtered samples were dispensed into 5 mL aliquots and stored in polypropylene cryogenic vials at −70 °C until analysis of phytoestrogens. One aliquot was sent to a biomedical laboratory for analysis of urinary creatinine.

2.4. Urine Analysis

Urine samples (n = 100) were analyzed for phytoestrogens using liquid–liquid extraction (LLE) based on a protocol published by Bolca et al. [26], followed by a high-resolution chromatography-mass spectrometry (HPLC-MS) analysis using the method described by Wyns et al. [27], previously modified for the analysis of phytoestrogens using HPLC-DAD-ESI-mass spectrometry. Urine samples were analyzed for total concentration of isoflavones (genistein, daidzein, equol, glicitein, formononetin, biochanine A), lignans (enterodiol, enterolactone, secoisolariciresinol, matairesinol), coumestans (coumestrol), stilbenes (resveratrol), flavonols (quercetin, kaempferol), flavanones (naringenin), and flavones (luteolin). Quercetin, luteolin, and naringenin were obtained from Indofine Chemical (Hillsborough, NJ, USA). The remaining phytoestrogens, the internal standard

(4-hydroxybenzophenone), deconjugation internal standards (β-phenolphthalein glucuronide and 4-methylumbelliferone sulfate), and *Helix pomatia*, H1, were obtained from Sigma-Aldrich.

Prior to extraction, urine samples were centrifuged at 3000 rpm for 10 min to remove solids and 2.0 mL of supernatant were diluted with 2.0 mL of sodium acetate buffer (pH 5.0; 0.1 M), spiked with 100 µL of internal standard 4-hydroxybenzophenone (4 µg/mL) and 10 µL of deconjugation internal standards (20 µg/mL). Conjugated analytes were hydrolyzed by the addition of 30 µL β-glucuronidase/sulfatase (*Helix pomatia*, H1, containing 5.14 mg of enzyme in 1 mL of sodium acetate buffer, pH 5.0, 0.1 M) and incubated 4 h at 37 °C. Urine-deconjugated samples were extracted (twice) with 5 mL of diethyl ether and subsequent vortex mixing (30 s). After samples stand for 5 min, the organic phase, containing phytoestrogens, was transferred into a new 15 mL conical tube. Eluates collected were dried under a gentle nitrogen stream, dissolved in a 200 µL injection solvent (40/60, *v*/*v*) (initial mobile phase (65A:35B) and methanol) with a gentle vortexing for 1 min and transferred into an amber vial for HPLC-DAD-ESI analysis. The phytoestrogens in the extracted samples were separated by injecting 50 µL of filtered extract into the Agilent 1100 series HPLC system (Agilent Technologies Inc., Palo Alto, CA, USA).

HPLC analyses were carried out using a Waters XBridge C18 reversed phase (3.5 µm) column (3.0 mm internal diameter × 150 mm) connected to a C18 guard column (3.0 mm internal diameter × 20 mm) and maintained at a temperature of 52 °C. The mobile phase was milliQ H_2O (Solvent A) and MeOH/CH_3CN (80/20, *w*/*w*) (Solvent B), both acidified with 0.025% (*v*/*v*) formic acid. The gradient and flow rate were programmed as follows: 0–5.0 min: 35–40% B (flow: 0.6 mL/min), 5.0–16.0 min: 40–100% B (flow: 0.6 mL/min), 16.0–19.0 min: isocratic 100% B (flow. 0.8 mL/min), 19.0–19.1 min: 100–35% B (flow: 0.6 mL/min) and 2.9 min for re-equilibration of the column before subsequent injection. Analyses were monitored simultaneously by diode array detection at 260, 280, 290, 310, and 360 nm during the entire HPLC run. All sample compounds were ionized by electrospray (ESI) in the negative ion mode and analyzed by single quadrupole mass spectrometry for confirmation of phytoestrogens. The drying temperature was 350 °C. The nebulizer pressure was 60 psi, the capillary voltage 3.5 kV, and the drying gas flow 11 L/min. Quality control samples (spiked urine) were analyzed along with unknown samples.

Linearity of the method (r^2 = 0.995–0.999) was established using eight different concentrations covering from 0.16 to 2400 ng/mL. The limit of detection was from 0.001 to 0.075 ng/mL. In addition, different blanks were run to exclude the presence of interferences and ensure the results. The recovery of phytoestrogens was from 80 to 120%, with the exception of resveratrol (50%) and secoisolarisiresinol (60%).

2.5. Statistical Analysis

Urinary concentrations of phytoestrogens are expressed as micrograms of phytoestrogens per gram of urinary creatinine (µg/g) by adjusting concentrations for urinary creatinine levels [26]. The mean dietary nutrient intake was adjusted for total energy intake using the residual method [28]. Spearman rank correlations were used to assess the relationship of habitual and recent intake of total phytoestrogens and subgroups with urinary phytoestrogen excretion. The raw correlation was first evaluated and then attenuated considering intraindividual variation in daily consumption of food. Statistical analyses were performed using the STATA statistical software package, version 8.0 (StataCorp LP, College Station, TX, USA). Statistical significance was assumed at the level of 0.05.

3. Results

3.1. General Characteristics of Participants

Mean age of the women was 44.7 ± 12.7 years. The majority of women were premenopausal (57%), married (74%), and of low socioeconomic status (62%). Overall, the prevalence of overweight and obesity according to body mass index was 79%. When nutrient intakes were estimated by the 24 h recall, mean energy intake was 1473 kcal/day, and, by the FFQ, energy intake was 2563 kcal/day.

3.2. Intake of Phytoestrogens

There was a wide variation in intake among phytoestrogen groups, values were right-skewed. The mean and geometric mean of phytoestrogens intake estimated by the FFQ are shown in Tables 1 and 2. Intakes of naringenin, quercetin, and coumestrol were the highest. The more consumed isoflavone was genistein, followed by daidzein. On the other hand, estimation of phytoestrogen intakes using the 24 h recall (Tables 3 and 4) showed that naringenin, quercetin, and genistein were the most consumed phytoestrogens. Recent intake of coumestrol (403 µg/day) was lower than its estimated habitual intake (1850 µg/day). In general, estimated recent intakes of phytoestrogens were lower than habitual intakes.

3.3. Phytoestrogens in Urine

Urine volumes ranged from 0.22 to 1.7 L, and the mean concentration of creatinine was 0.63 g/L. The majority of phytoestrogens (75%) were detected in 77% of the urine samples analyzed. Table 5 shows that, from the group of isoflavones, biochanin A and daidzein had respectively the lowest and highest concentrations in urine. From the group of lignans, enterolactone was the more detected phytoestrogen in urine, and the lower concentration was found for matairesinol, which is metabolized to enterolactone. In the flavonoid group, naringenin in urine was 40 times higher than quercetin.

Table 1. Isoflavone, coumestrol, and resveratrol intakes estimated by the FFQ in Mexican women (n = 100).

Phytoestrogens	Mean ± SD	Geometric Mean *	Value	Percentiles		
(µg/Day)		(95% CI)	Min–Max	25	50	75
Daidzein	1151 ± 3211	388.5 (283–533)	30.7–26,249	145.8	232.5	867.9
Genistein	1642 ± 5251	642 (452–912)	38.0–46,147	156.0	296.0	1023.7
Glicitein	14.8 ± 16.9	11.3 (9.5–13.4)	2.6–107.3	7.3	10.4	15.3
Biochanin A	310 ± 279	270.5 (236–310)	23.7–1420.6	105.9	234.8	471.5
Formononetin	53.0 ± 110.2	28.3 (22.6–35.5)	1.6–775.7	14.2	23.9	42.1
Equol	2.4 ± 1.9	1.9 (1.6–2.2)	0.2–13.6	1.1	1.9	3.16
Total isoflavones	3173 ± 8456	1168 (894–1526)	289–73,037	625.8	1025.4	2297.8
Coumestrol	1850 ± 1730	1569 (1333–1847)	96.6–8633.7	541.9	1361.3	2901
Resveratrol	17.4 ± 43.7	5.2 (3.6–7.4)	0.006–290.3	0.9	4.4	14.8

FFQ: Food Frequency Questionnaire; SD: Standard Deviation; 95% CI: 95% Confidence Interval; * Adjusted for energy intake by the residual method [25]. Total isoflavones consist of the sum of daidzein, genistein, glicitein, biochanin A, formononetin, and equol.

Table 2. Lignan, flavonoid, and total phytoestrogen intakes estimated by the FFQ in Mexican women (n = 100).

Phytoestrogens	Mean ± SD	Geometric Mean *	Value	Percentiles		
(µg/Day)		(95% CI)	Min–Max	25	50	75
Secoisolariciresinol	712.8 ± 1448	490.5 (386–623)	69.6–11,771	145.6	222.5	586.9
Matairesinol	20.1 ± 14.0	17.1(15.3–19.1)	3.9–97.3	11.4	17.2	25.1
Enterodiol	67.4 ± 37.6	62.5 (57.2–68.3)	16.4–222.3	42.3	61.8	80.9
Enterolactone	183.2 ± 115.2	156.7 (138–178)	49.9–662.7	102.2	158.9	227.6
Total lignans	1171 ± 1586	872.7 (742–1027)	240.9–13,335	439.9	754.9	1182.3
Naringenin	15,940 ± 16,582	12,429 (10,243–15,082)	737.2–111,747	4887.4	10,997	20,759
Luteolin	1555 ± 1355	1239 (1065–1441)	143.9–8772.0	670.2	1221.0	2019.4
Kaempferol	826.1 ± 685.4	625.5 (538–728)	120.0–4519.6	351.1	649.6	1018.3
Quercetin	13,023 ± 6912	11,353 (10,129–12,724)	1719.3–31,993	7873.9	11,472	17,865
Total flavonoids	31,344 ± 20,862	26,773 (23,600–30,373)	6527–139,335	15,617	28,101	39,288
Total phytoestrogens	37,557 ± 23,909	31,957 (28,322–36,059)	9016–159,239	20,716	32,716	48,554

FFQ: Food Frequency Questionnaire; SD: Standard Deviation; 95% CI: 95% Confidence Interval; * Adjusted for energy intake by the residual method [25]. Total lignans consist of the sum of secosiolariciresinol, matairesinol, enterodiol, and enterolactone. Total flavonoids consist of the sum of naringenin, luteolin, kaempferol, and quercetin. Total phytoestrogens consist of the sum of isoflavones, lignans, coumestrol, flavonoids, and resveratrol.

Table 3. Isoflavone, coumestrol, and resveratrol intakes estimated by the 24 h recall in Mexican women (n = 100).

Phytoestrogens	Mean ± SD	Geometric Mean *	Value	Percentiles		
(μg/Day)		(95% CI)	Min–Max	25	50	75
Daidzein	517 ± 2234	214 (157–289)	0.67–16,220	22.5	86.4	135.7
Genistein	775 ± 3508	262 (184–375)	0.58–26,450	24.17	70.1	173.8
Glicitein	9.46 ± 9.81	6.14 (5.06–7.45)	0.485–57.9	3.01	5.24	14.7
Biochanin A	57.9 ± 81.1	37.8 (29.0–49.2)	0.02–520	4.62	29.9	76.4
Formononetin	19.8 ± 25.6	14.2 (10.8–18.6)	0–159.4	1.38	4.28	31.3
Equol	1.13 ± 1.52	0.67 (0.49–0.92)	0–6.55	0	0.44	1.66
Total isoflavones	1380 ± 5710	567 (423–761)	3.3–40,789	116.5	271.6	441.5
Coumestrol	403 ± 1255	186 (132–264)	0.09–11,643	1.41	3.65	433.4
Resveratrol	2.13 ± 21.1	2.91 (2.15–3.94)	0–211.2	0	0	0

SD: Standard Deviation; 95% CI: 95% Confidence Interval; * Adjusted for energy intake by the residual method [25]. Total isoflavones consist of the sum of daidzein, genistein, glicitein, biochanin A, formononetin, and equol.

Table 4. Lignan, flavonoid, and total phytoestrogen intakes estimated by the 24 h recall in Mexican women (n = 100).

Phytoestrogens	Mean ± SD	Geometric Mean *	Value	Percentiles		
(μg/Day)		(95% CI)	Min–Max	25	50	75
Secoisolariciresinol	307 ± 1767	65.7 (50.0–86.4)	9.12–16,938	31.83	52.2	81.3
Matairesinol	16.0 ± 45.2	9.61 (7.83–11.8)	0.58–438	4.14	7.24	13.9
Enterodiol	18.7 ± 23.0	15.2 (12.5–18.5)	0–133.6	1.99	11.6	26.3
Enterolactone	67.6 ± 83.4	53.4 (42.9–66.4)	0–435.5	8.59	41.7	88.7
Total lignans	409.1 ± 1781	162.4 (130–204)	15.6–17,001	75.0	130	212
Naringenin	5352 ± 18,834	1356 (901–2040)	0–151,179	0	196	1191
Luteolin	471 ± 1081	157 (108–228)	0–6379.3	5.77	46.5	330.8
Kaempferol	476 ± 1274	327 (247–434)	0–8224.2	29.94	116.9	255.9
Quercetin	8170 ± 8954	5202 (4090–6616)	0–56,088	1514	5851	11,277
Total flavonoids	14,471 ± 21,693	8432 (6578–10,807)	0–160,534	2775	8467	18,208
Total Phytoestrogens	16,663 ± 22,858	10,838 (8757–13,414)	54–161,360	3591	9842	20,258

SD: Standard Deviation; 95% CI: 95% Confidence Interval; * Adjusted for energy intake by the residual method [25]. Total lignans consist of the sum of secoisolariciresinol, matairesinol, enterodiol, and enterolactone. Total flavonoids consist of the sum of naringenin, luteolin, kaempferol, and quercetin. Total phytoestrogens consist of the sum of isoflavones, lignans, coumestrol, flavonoids, and resveratrol.

Table 5. Urinary concentrations of phytoestrogens during 12-h urine collection period (n = 100).

Phytoestrogens	Mean ± SD	Geometric Mean *	Percentiles			Geometric Mean *
(μg/L)		(95% CI)	25	50	75	μg/g Creatinin (95% CI)
Daidzein	117 ± 236	31.2 (21.9–44.4)	10.9	38.1	103	56.0 (39.7–79.1)
Genistein	49.2 ± 101	17.4 (12.5–24.1)	4.62	18.4	48.8	30.9 (22.6–42.5)
Glicitein	34.5 ± 88.3	25.5 (16.6–39.4)	0	0	25.8	43.5 (28.7–66.1)
Biochanin A	1.25 ± 1.04	1.02 (0.89–1.17)	0.68	1.08	1.50	1.86 (1.59–2.18)
Formononetin	2.42 ± 1.71	2.31 (2.05–2.61)	1.49	2.16	3.28	4.19 (3.72–4.72)
Equol	42.4 ± 120	29.9 (25.3–35.5)	14.2	23.1	40.3	51.2 (44.0–59.4)
Secoisolariciresinol	3.45 ± 5.73	2.89 (2.36–3.55)	0.69	1.91	5.13	5.49 (4.39–6.86)
Matairesinol	0.64 ± 2.07	1.10 (0.63–1.90)	0	0	0.11	1.82 (1.01–3.26)
Enterodiol	2.86 ± 12.9	10.5 (5.93–18.7)	0	0	0	19.7 (11.9–32.6)
Enterolactone	64.9 ± 66.8	41.7 (33.5–51.9)	24.8	46.7	82.3	75.9 (59.8–96.4)
Coumestrol	1.24 ± 1.09	1.10 (0.91–1.33)	0.55	1.01	1.72	2.07 (1.71–2.50)
Resveratrol	11.9 ± 108	1.32 (0.89–1.96)	0	0.23	1.72	2.51 (1.69–3.73)
Luteolin	6.72 ± 32.5	2.57 (2.12–3.10)	1.40	2.38	4.31	4.61 (3.85–5.53)
Kaempferol	25.8 ± 54.9	12.9 (9.67–17.4)	2.13	10.4	26.1	22.9 (17.2–30.8)
Naringenin	135 ± 213	60.1 (46.3–78.1)	26.2	64.8	127	108 (83.2–140)
Quercetin	2.96 ± 7.14	2.15 (1.77–2.60)	0.71	1.83	3.34	3.92 (3.25–4.73)
Total Phytoestrogens	502 ± 580	336 (284–398)	213	306	5175	603 (511–713)

SD: Standard Deviation; 95% CI: 95% Confidence Interval; * Adjusted for energy intake by the residual method [25]. Total phytoestrogens consist of the sum of total isoflavones, total lignans, coumestrol, total flavonoids, and resveratrol.

3.4. Correlations of Dietary and Urinary Phytoestrogens

The corrected correlation coefficients between phytoestrogen intakes estimated by the FFQ and by the 24 h recall and their concentrations in urine are shown in Table 6. Using the FFQ, only dietary and urinary resveratrol were correlated ($r = 0.337$, $p < 0.01$). Although at the individual level correlations were not found between habitual dietary intakes (FFQ) and urine concentrations, total phytoestrogens showed a high correlation ($r = 0.730$, $p < 0.001$) between FFQ and urine excretion. On the other hand, recent intakes (24 h recall) of genistein ($r = 0.374$, $p < 0.01$), and naringenin ($r = 0.620$, $p < 0.0001$) were correlated with their respective urinary levels. As a group, recent consumptions (24 h recall) of isoflavones ($r = 0.460$, $p < 0.001$), lignans ($r = 0.550$, $p < 0.0001$), flavonoids ($r = 0.240$, $p < 0.05$), and total phytoestrogens ($r = 0.410$, $p < 0.001$) correlated with their urinary excretion.

Enterodiol and matairesinol were detected in only 15% and 26% of the urine samples. Spearman's correlation coefficient between urinary daidzein concentration and its metabolite equol was 0.30 ($p = 0.002$). Additionally, urinary secoisolariciresinol concentration was correlated with its metabolite enterodiol ($r = 0.23$; $p = 0.019$).

Table 6. Corrected correlation coefficients between urinary metabolites and dietary estimates of phytoestrogen intake (Habitual-FFQ and Recent-24 h recall).

Phytoestrogen	Corr CC	*p*-Value	Corr CC	*p*-Value
	Habitual (μg/Day) vs. Urine (μg/g Creatinine)		Recent (μg/Day) vs. Urine (μg/g Creatinine)	
Daidzein	−0.024	0.840	0.099	0.420
Genistein	−0.034	0.784	0.374	0.002
Equol	0.126	0.283	0.079	0.500
Glicitein	0.055	0.657	0.120	0.330
Biochanin A	0.012	0.923	0.160	0.190
Formononetin	−0.024	0.841	0.170	0.160
Total Isoflavones	0.002	0.983	0.460	0.0001
Secoisolariciresinol	0.009	0.934	0.230	0.060
Matairesinol	0.079	0.524	0.028	0.810
Enterolactone	−0.035	0.775	0.067	0.580
Enterodiol	−0.168	0.171	0.130	0.270
Total lignans	−0.111	0.359	0.550	0.000
Naringenin	0.069	0.571	0.620	0.000
Luteolin	−0.104	0.398	−0.200	0.090
Kaempferol	−0.084	0.496	0.040	0.690
Quercetin	0.154	0.212	0.027	0.820
Total Flavonoids	0.055	0.653	0.240	0.043
Coumestrol	−0.035	0.800	0.120	0.320
Resveratrol	0.337	0.005	0.005	0.960
Total Phytoestrogens	0.730	0.000	0.410	0.0007

FFQ: Food Frequency Questionnaire; Corr CC: Corrected correlation coefficient for intra-interindividual variation. Total isoflavones consist of the sum of daidzein, genistein, glicitein, biochanin A, formononetin, and equol. Total lignans consist of the sum of secosiolariciresinol, matairesinol, enterodiol, and enterolactone. Total flavonoids consist of the sum of naringenin, luteolin, kaempferol, and quercetin. Total phytoestrogens consist of the sum of isoflavones, lignans, coumestrol, flavonoids, and resveratrol.

4. Discussion

In this study, we estimated habitual and recent dietary intakes of phytoestrogens, and measured the urinary excretion of these compounds and related metabolites in a group of Mexican adult women. We also evaluated the relationship between dietary and urinary phytoestrogens and observed that total dietary phytoestrogens, estimated by the FFQ, correlated to their urinary excretion. When we estimated dietary intake of phytoestrogens using the 24 h dietary recall, more correlations between dietary and urinary phytoestrogens were found, including genistein, naringenin, isoflavones, lignans, flavonoids, and total phytoestrogens.

Total phytoestrogens in our study included 16 individual compounds. No observational studies have examined simultaneously a wide range of phytoestrogens. The degree of correlation observed

between total urinary phytoestrogens and our estimates of dietary intake were in the magnitude of 0.73 (corrected) for the FFQ, and 0.41 (corrected) for the 24 h recall. Correlations for the FFQ are higher than those obtained in previous study where the correlation between total dietary intake and urinary phytoestrogens was 0.54 [29]. As observed, we obtained a higher correlation of total phytoestrogens using the FFQ, compared to the 24 h recall. This may be because the composition of phytoestrogens in certain food items that were consumed in the previous 24 h were not included in the food database; some phytoestrogens have not been analyzed for particular food components. Since total phytoestrogens consist of the sum of all the individual compounds, the lack of one or more of them contributes to a lower concentration of total phytoestrogens. On the other hand, the FFQ contains a larger list of foods, and many of them may have a complete composition of phytoestrogens in the database.

Some authors attribute the weak or null association of individual compounds to the extensive response categories used in the FFQ, and imprecise interpretation of the interviewer to describe "a few times a week" or "daily or almost daily" [30]. Additionally, composition tables used are not representative of the foods that were consumed. Thus, dietary intake data in our study were based on estimated values and were therefore less accurate than intakes measured by urinary concentrations.

The information available on the dietary intake of phytoestrogens in the general population in Western countries is limited. The estimated habitual (3.2 mg/day) and recent (1.4 mg/day) intakes of isoflavones in women of our study were higher than those reported in previous studies. In postmenopausal White women who participated in the Framingham study, with less than 1 mg/d of isoflavone intakes [31]. Women who participated as controls in a study of ovarian cancer, consumed in average 1.8 mg/day of isoflavones [32], similar to our participants.

Compared to Asian women [33], mean consumption of isoflavones in our study was approximately 8 times smaller. In terms of total lignans and coumestrol, our results from the FFQ were similar to the 1 mg/day and 1.4 mg/day, found in the Bandera et al. study [32]. Naringenin and quercetin were the most consumed flavonoids in our study and their main dietary sources were citrus fruits and onion, respectively.

Overall, our results indicate that the intake of dietary isoflavones exceeded consumption of lignans, which is contrary to the results reported by previous studies in Western diets [34,35]. This discrepancy could be due to the use of more individual phytoestrogens that were added for the assessment by group. Another reason could be the continuous use of soy protein, soy isolated, and soy flour as food additives in the manufacture of soy-based cereal, frozen desserts, energy bars, and particularly meat substitutes [36]. The FFQ included foods such as "Maizoro" cereal, commercial bread, breading, instant soups, and soy beverages, among others, that contributed to the estimates of isoflavone intakes.

Some studies have evaluated the relationship between estimates of dietary intake and urinary excretion of lignans. As in our study, in Australian women, no association between habitual lignan excretion and dietary intake was found for the FFQ [37]. This is likely due to the wide variety of foods containing lignans, making estimations of lignan intake a challenge. The lack of analysis of the different types of lignans in the Western diet contributed to the underestimation of dietary lignans intake in our study, using the FFQ. The lignan or isoflavone concentrations can vary in the same food according to location, variety, crop season, and processing methods [38,39]. Therefore, measuring dietary intakes of phytoestrogens using a food-intake instrument and food composition databases is complicated and may not fully capture the intake of these compounds. Establishing a biomarker of phytoestrogen intake through the present study means that dietary intake can be estimated reasonably accurately from analysis of a 12 h urine collection.

This is the first study on the daily intake of phytoestrogens and their correlation with urinary excretion of these compounds in Mexican women. In a recent study from Northwest Mexico, the authors evaluated the use of serum phytoestrogens as a biomarker of phytoestrogen intake, and found correlations for some individual compounds, such as naringenin, luteolin, genistein, enterolactone, coumestrol, and resveratrol [19]. However, no correlations were found for groups or

for total phytoestrogens. We found correlations between recent intakes and urinary levels for total phytoestrogens, and for groups of isoflavones, lignans, and flavonoids, as well as for genistein and naringenin as individual compounds.

In a study that evaluated serum and urine as biomarkers of intake of phytoestrogens in the general US population, the authors discussed that the concentrations of phytoestrogens were lower in serum than in urine, which points to the fast clearance of these compounds from the body [40]. The time of the collection of the blood sample is an important variable for the determination of phytoestrogens because of their short half-lives. Isoflavones, in the form of glucuronic, predominate in urine, and half-life is approximately 7 to 10 h [41]. Enterolignans appear in circulation approximately 8 to 10 h after the ingestion of lignans derived from plants. In contrast, lignans derived from plants are in blood circulation after 2 h of consumption but their concentrations are lower than those of enterolignans [42]. Thus, urine collections may be more useful for the complete estimation of lignan and isoflavone levels than blood samples. The authors from the study previously mentioned mentioned that the high correlation observed for urinary and serum levels of phytoestrogens validates that noninvasive collection techniques, such as those used for urine, can be used to assess phytoestrogen exposure [40].

Another study in México estimated the dietary intake of phytoestrogens [18]. The authors applied an adapted FFQ to measure the consumption of flavonoids (flavonols, flavones, and flavanols), lignans (secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol), and coumestrol. Only the intake of coumestrol was similar to that obtained in our study. The same estimations were made in a study of Torres-Sánchez et al. [43]. The differences in the intake of phytoestrogens between these two studies and our research could be due to the fact that they used different food composition databases.

In addition to dietary intake, metabolism by intestinal bacteria can also influence an individual's urinary levels of phytoestrogens, especially equol, which is transformed from daidzein by gut microflora [44]. The concentrations of equol that we found in the urine samples from the participants in our study (median, 23.1 ng/mL) were higher than those found in women from Hanoi, Vietnam (19 ng/mL), and exceeded the values of women in Japan (1.4 ng/mL) and USA (2.5 ng/mL) [15]. However, daidzein concentrations were lower in our sample, although we found a significant correlation coefficient between urinary daidzein concentration and its metabolite equol. Diet may contribute to the ability to harbor equol-producing bacteria [45,46]; therefore, not all humans (30–60%) possess the gut flora that produces equol [47].

When we estimated concentration ratios of equol to daidzein in urine samples, as an indicator of conversion efficiency, significantly higher ratios were found for our samples (ratio equol/daidzein = 0.61), compared to samples from women in Hanoi, Vietnam (ratio = 0.53), and Japan (ratio = 0.002) [15]. These results indicate a more efficient biotransformation of daidzein into its metabolite equol by the Mexican women in our study. Equol has a higher estrogenic activity when compared to daidzein, and has been proposed as an important component of isoflavones for disease prevention [48]. Biotransformation of daidzein to equol has been proposed as a key factor in the protective effects of phytoestrogens against breast cancer [49,50]. Thus, our results indicate that the population in Mexico comprised "good" equol producers, and this phenotype could have epidemiological implications for the reduction of some chronic diseases, such as breast cancer risk.

We also observed that the ratio of enterodiol to enterolactone, which can interconvert, was 0.25 (less than 1), indicating that enterolactone was excreted in higher amounts than enterodiol. This finding is in agreement with other human studies [40,51]. The bacterial synthesis of enterolactone occurs via dehydroxylation and demethylation of matairesinol. Enterolactone is also produced by oxidation of enterodiol, which is a product of secoisolariciresinol metabolism [52]. The biological activity of enterolactone and enterodiol is different; enterolactone is a more potent aromatase inhibitor and has approximately 10 times the estrogenic activity than enterodiol [53]. According to Liu et al. [54], enterodiol and enterolactone both have potent inhibitory effects on ovarian cancer, but enterolactone possesses a more effective anti-cancer capability and fewer side effects than enterodiol. Enterolactone also has inhibitory effects on growth and metastasis in human breast

cancer [55]. Thus, differences in metabolism and exposure of these lignans may be of physiological importance in cancer prevention.

On the other side, variations between the estimated intake and the urinary levels of individual or group phytoestrogens, especially when we used the FFQ, could be due to the difficulty in estimating phytoestrogen intakes. The FFQ, by definition, is a semiquantitative questionnaire with a trend toward the overestimation of intake. Methods of dietary assessment are estimations of intake and rely on the veracity or reported intakes. Therefore, the accuracy of dietary data is influenced by memory recall, body size, sex, age, and ethnicity, as well as psychosocial and behavioral factors [56–59].

Studies in developing countries have shown that energy intake is underreported [60,61]. In fact, underreporting intake of some foods is apparent in our study, since energy intake from the 24 h recall was low, 1473 kcal/day. According to Scagliusi et al. [62], individuals with a lower income might have greater difficulties in the reporting tasks. Body mass index is also an important variable. As obesity increases, especially in the female population, underreporting energy intake is more common. Irregular meal habits and low education have been also associated with the under-report of energy intake [63]. In a study in New Zealand, the authors detected 265 unreported foods (often snacks) as revealed by the use of wearable cameras [64].

According to the above, we might think that the under-report of energy intake may be due to the frequency of overweight and obese persons in our study population (37.3% and 38.8% respectively, according to body mass index). Low income and education are factors that could contribute to the underreporting of energy intakes, since 64% of women in our study were from a lower-income level and 58.6% had less than 9 years of schooling. Considering the possibility of the underestimation of intake in our study population, reported consumption of unhealthy foods that are rich in fat, high in sugar, or highly processed may be lower than habitual intake; thus, fruits and vegetables, which represents a source of phytoestrogens, may be overestimated.

A limitation of our study is the use of an FFQ validated for women in the region and modified for the study, although not validated specifically for phytoestrogens. The food list increased in the questionnaire, which could lead to an overestimation of phytoestrogen intake. The collection of 12 h instead of 24 h urine may be another limitation of our study. We used 12 h urine since it is a less burdensome method, and the 24 h urine collection was not well accepted by potential participants. Therefore, women collected their urine samples during the night, and we picked them up in the early morning. As an argument, we may say that, in the study of Grace et al. [65], a spot urine was sufficient for obtaining good results. Because of unpredictable changes in urine flow during the day and since the synthesis and total elimination of creatinine is constant [66], we adjusted the amount of phytoestrogens in urine to the concentration of creatinine. It is desirable that we could have collected urine samples for one or more days in each season of the year to consider seasonal variations in intakes. This could have allowed us to obtain better correlations between phytoestrogens from urine and dietary phytoestrogens estimated from the FFQ, because they represent the usual diet.

An advantage of using urine analysis could mean that we can avoid cases of omission or the overestimation of intake among participants, as well as the problem of not finding a food in the food database. Our quantification method is sensitive and reflects a change due to exposure, since the technique has high specificity and sensitivity. Therefore, the determination of urine levels provides a more accurate and objective measure of intake. The evaluation of 16 individual phytoestrogens, as well as the fact that there is no other study on this subject in Mexican women, is another advantage of our study. The results of our study could motivate other researchers to validate intakes of specific phytoestrogens.

5. Conclusions

In conclusion, this study has shown that the urinary excretion of total phytoestrogens is significantly correlated with habitual dietary intake. In addition, recent intakes of individual, group, and total phytoestrogens were related to their urinary levels. Therefore, phytoestrogens in urine may

be a useful biomarker for the intake of these compounds. The study has furthermore shown the importance of the inclusion of several different phytoestrogens when using total phytoestrogens as a biomarker for intake. The validity of this approach should be extensively investigated. The production of equol by Mexican women is a new avenue of research. This study provides the basis for future studies that look forward to identifying the role of phytoestrogens in reducing the risk of breast cancer and other chronic diseases in the Mexican population.

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Silibinin Restores NAD$^+$ Levels and Induces the SIRT1/AMPK Pathway in Non-Alcoholic Fatty Liver

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Abstract: Nicotinamide adenine dinucleotide (NAD$^+$) homeostasis is emerging as a key player in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) and is tightly linked to the SIRT1/5'-AMP-activated protein kinase (AMPK) pathway. Silibinin, the main component of silymarin, has been proposed as a nutraceutical for the treatment of NAFLD. In this study, we aimed to identify whether silibinin may influence the NAD$^+$/SIRT1 axis. To this end, C57BL/6 mice were fed a high fat diet (HFD) for 16 weeks, and were treated with silibinin or vehicle during the last 8 weeks. HepG2 cells were treated with 0.25 mM palmitate for 24 h with silibinin 25 μM or vehicle. HFD and palmitate administration led to oxidative stress, poly-(ADP-ribose)-polymerase (PARP) activation, NAD$^+$ consumption, and lower SIRT1 activity. In mice fed the HFD, and in HepG2 treated with palmitate, we consistently observed lower levels of phospho-AMPK^{Thr172} and phospho-acetyl-CoA carboxylase^{Ser79} and higher levels of nuclear sterol regulatory element-binding protein 1 activity, indicating de novo lipogenesis. Treatment of mice and HepG2 with silibinin abolished oxidative stress, and inhibited PARP activation thus restoring the NAD$^+$ pool. In agreement with preserved NAD$^+$ levels, SIRT1 activity and AMPK phosphorylation returned to control levels in mice and HepG2. Our results further indicate silibinin as a promising molecule for the treatment of NAFLD.

Keywords: silibinin; NAD$^+$; SIRT1; AMPK; lipogenesis

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) represents the most common liver disease in industrialized countries [1]. Epidemiological studies including prospective cohorts have shown that liver fat accumulation per se precedes the onset of insulin resistance and metabolic syndrome [2]. Beyond metabolic complications, a subset of patients with NAFLD develop non-alcoholic steatohepatitis (NASH), which is emerging as a leading cause of liver transplantation due to progression to cirrhosis and cancer [3]. The most effective therapeutic option to prevent onset and progression of NAFLD is a healthy lifestyle [4], although only few patients are compliant with lifestyle changes.

For this reason, several drugs have been proposed so far for counteracting progression of fatty liver to inflammation and fibrosis, but with unsatisfactory results [5]. Recent translational studies have indicated that natural phenolic compounds may display beneficial effects in NAFLD by modulating hepatic lipid homeostasis [6].

Nicotinamide adenine dinucleotide (NAD^+) is a co-enzyme involved in redox reactions that plays a protective role against metabolic diseases and aging [7,8]. The beneficial effects of NAD^+ can be in part attributed to the activity of the NAD-dependent protein deacetylase sirtuin-1 (SIRT1), an enzyme that regulates metabolic homeostasis by deacetylating lysine residues on histones and transcriptional regulators [9]. SIRT1 activates 5′-AMP-activated protein kinase (AMPK) [10] which controls energy expenditure in health and disease [11]. NAD^+ levels depend on the balance between its biosynthesis and its consumption by several NAD-dependent enzymes, especially poly-(ADP-ribose)-polymerases (PARPs), a family of enzymes that protect cells against oxidative DNA damage [12]. However, although PARPs can be considered as genome guardians, hyperactivation of PARPs leads to NAD^+ depletion and thus cell injury [13]. Recent evidence indicates that NAD^+ repletion reverses fatty liver in mice by restoring SIRT1 activity and modulating the mitochondrial efficiency [14,15].

Silibinin is a flavonolignan that constitutes the main component of silymarin, a lipophilic milk thistle extract, which is consumed worldwide as a nutraceutical for liver diseases [6]. Interest in milk thistle derivatives for the treatment of NAFLD is mirrored by randomized controlled trials on efficacy and safety (www.clinicaltrials.gov). Traditionally, silymarin has been characterized by the potent anti-oxidant activity of its main component silibinin [16]. Recently, beyond antioxidant effects, it was showed that silibinin favourably modulates lipid homeostasis and insulin sensitivity in several models of NAFLD [6]. In this study, we aimed to establish whether silibinin may influence NAD^+ homeostasis and the SIRT1/AMPK pathway in a mouse model of fatty liver and in an in vitro model of hepatocyte lipotoxicity.

2. Materials and Methods

2.1. Cell Culture

The HepG2 cells (American Type Culture Collection, Manassas, VA, USA) were a kind gift from Prof. Maurizio Parola of the University of Torino. Cell viability measurement was performed using a Muse™ Cell Analyzer (Merck Millipore, Milan, Italy) according to the manufacturer's instructions. Treatment of HepG2 with the fatty acid (FA) palmitate was performed as previously described [17]. Briefly, cells at low passages were cultured in DMEM (Sigma-Aldrich, Milan, Italy) supplemented with 10% FBS (EuroClone, Milan, Italy), 100 U/mL penicillin (Life Technologies, Milan, Italy) and 100 μg/mL streptomycin (Life Technologies) at 37 °C in a humidified incubator in a 95% air and 5% CO_2 atmosphere [18]. Upon reaching 80–90% confluence, the HepG2 cells were incubated for 24 h as follows: HepG2 + vehicle (Bovine Serum Albumin (BSA) 5% + DMSO); HepG2 + FA (BSA 5% + DMSO + palmitate 0.25 mM); HepG2 + FA + silibinin (BSA 5% + DMSO + palmitate 0.25 mM + silibinin 25 μmol/L). Silibinin (Indena, Milan, Italy) was dissolved in DMSO at room temperature and administered at a 25 μmol/L concentration. This dose of silibinin was chosen on the basis of previous studies [18,19].

2.2. Animals

All procedures fulfilled the Italian Guidelines for the Use and Care of Laboratory Animals. Eight- to ten-week-old C57BL/6 mice (Charles River Labs, Lecco, Italy) were maintained in a temperature- and light-controlled facility. Animals were fed either a standard diet (SD) or a high fat diet (HFD) for 16 weeks. During the last 8 weeks of the HFD, mice were randomly selected to be daily administered silibinin (Indena, Milan, Italy) by gavage at a dose of 5 mg/kg of body weight or vehicle. Silibinin, as powder, was dissolved in DMSO. Diets were obtained from Harlan Teklad (Madison, WI, USA). The SD provided 3.3 kcal/g with 60% carbohydrates, 23% proteins, and 17% fat. The HFD provided 5.2 kcal/g

with 60% fat, 20% proteins, and 20% carbohydrates. Mice were distributed in three groups: group I included six mice fed the SD (SD); group II comprised eight mice fed the HFD (HFD); group III comprised eight mice fed the HFD and treated with silibinin (HFD + SIL. During the study period, food and beverage consumption was recorded twice weekly; body weight was recorded weekly. After sacrifice by CO_2 asphyxiation, blood and liver samples were obtained, processed, and stored at −80 °C for successive molecular determinations. Ethics approval was obtained from the University of Catania.

2.3. Oil Red O and Hematoxylin/Eosin Staining

Lipid accumulation in HepG2 cells was assessed by Oil Red O staining. In brief, cells were fixed in 4% paraformaldehyde for 30 min, followed by staining with Oil Red O for 30 min. Next, fixed cells were gently washed with isopropanol, and then three times washed with distilled water. Results were determined by fluorescence microscopy imagining and by measurement of absorbance at 500 nm using Synergy HT (BioTek, Milan, Italy). The cells were cultured in 24-well flasks. Six wells were used for each treatment and the experiment was repeated three times.

Liver histopathology was performed on 5 μm liver slices stained with hematoxylin and eosin by standard procedures. Liver slices were examined without knowledge of the type of treatment undergone.

2.4. Biochemical Analyses

Sandwich ELISA for threonine 172-phosphorylated AMPKα (#7959), total AMPKα (#7961), serine 79-phosphorylated acetyl-CoA carboxylase (ACC) (#7986), and total ACC (#7996) were purchased from Cell Signalling Technology (Beverly, MA, USA). Absorbance was read at 450 nm.

PARP activity was assayed using the universal colorimetric PARP assay kit (Trevigen Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. SIRT1 activity was measured using the SIRT1 Fluorometric Drug Discovery Kit (ENZO Life Sciences Inc., Farmingdale, NY, USA) according to the manufacturer's instructions. Changes in PARP1 and SIRT1 activities were calculated against the mean value of PARP1 and SIRT1 activities in control liver and cells, and expressed as percent of control. NAD^+ levels were measured using EnzyChrom™ NAD^+/NADH Assay kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions and as previously described [20]. Sterol regulatory element-binding protein 1 (SREBP-1) activity was measured using a SREBP-1 transcription factor assay kit (Abcam, Cambridge, UK), according to manufacturer's instruction, on nuclear extracts of HepG2 that were obtained by a nuclear extraction kit (Abcam, Cambridge, UK).

Triglyceride content in HepG2 and mouse liver samples was measured using a colorimetric assay according to the manufacturer's instructions (Biovision, Mountain View, CA, USA).

2.5. Real-Time PCR

Gene expression was assessed by quantitative real-time polymerase chain reaction (qRT-PCR). Reverse transcription reaction was carried out on 1 μg of total RNA using oligo (dT) primers (Table S1) and MultiScribe™ Reverse Transcriptase (Applied Biosystems, Milan, Italy), according to the vendors' instructions. Quantitative RT-PCR was performed in a 7900 HT Fast Start real-time PCR system (Applied Biosystems) in a mixture containing SYBR® Green PCR Master Mix (Life Technologies), specific primers, and 50 ng of cDNA in a total volume of 20 μL. The GAPDH housekeeping gene was used as a reference. The ΔCt protocol was used to determine the absolute values of gene expression.

2.6. ROS (Reactive Oxygen Species) Measurement

Reactive Oxygen Species (ROS) production in HepG2 was measured using a Muse™ Cell Analyzer (Merck Millipore) according to the manufacturer's instructions. The Muse® Oxidative Stress Kit (Merck Millipore) allows for the quantitative measurement of Reactive Oxygen Species, namely superoxide radicals in cells undergoing oxidative stress. ROS generation in liver homogenates was

determined by using 2′,7′-dichlorodihydrofluoroscein diacetate as a probe. Dichlorodihydrofluorescein diacetate formation was determined fluorometrically with a Hitachi F-2000 (Hitachi Ltd., Tokyo, Japan) fluorescence spectrophotometer at excitation wavelength of 488 nm and emission wavelength of 525 nm at 37 °C.

2.7. Statistical Analysis

Data are presented as mean ± standard error of mean (SEM). Each determination was performed at least in triplicate. Significance was assessed by ANOVA followed by Bonferroni test; $p < 0.05$ was considered to be statistically significant. Statistical analysis was performed using GraphPad Prism (San Diego, CA, USA).

3. Results

3.1. Silibinin Reduces Lipid Accumulation in HepG2 and Mice Liver

A 24-h culture with 0.25 mM palmitate led to lipid droplet accumulation in HepG2, without affecting cell viability (Figure 1). In cells treated with 25 μM silibinin, lipid accumulation was markedly reduced (Figure 1). Liver sections from mice fed the HFD for 16 weeks showed microvesicular steatosis with a panacinar pattern (Figure 2). By contrast, silibinin treatment during the last 8 weeks of the HFD attenuated steatosis (Figure 2). Consistently with in vitro results, liver triglyceride content was markedly decreased in mice treated with silibinin (Figure 2). Interestingly, these effects on liver steatosis occurred despite no significant effects on body weight (Figure S1).

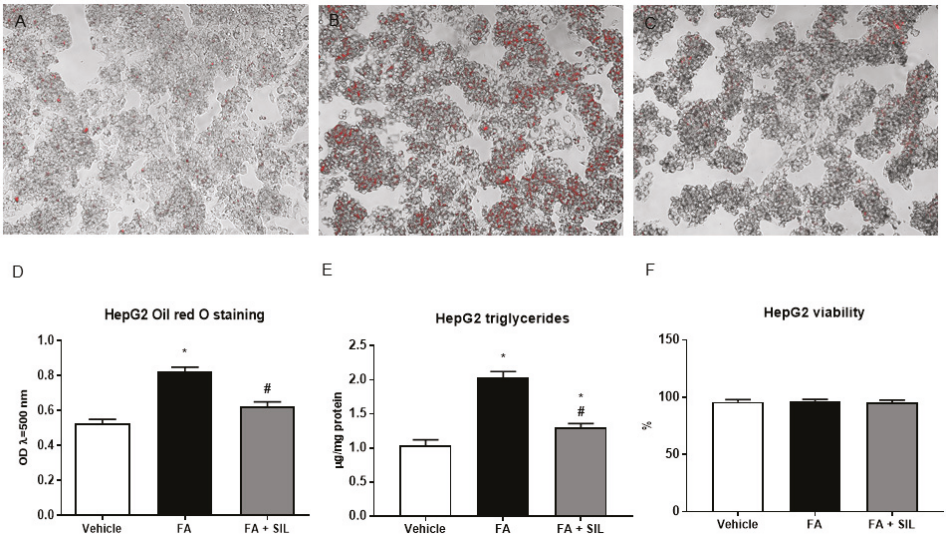

Figure 1. Effects of silibinin on steatosis in HepG2. (**A**–**D**), Following a 24-h culture with 0.25 mM palmitate, HepG2 + vehicle displayed lipid droplet accumulation, as shown by Oil Red O staining. In cells treated with 25 μM silibinin, a reduction of lipid accumulation was observed. The cells were cultured in 24-well flasks. Six wells were used for each treatment and the experiment was repeated three times; (**E**) Consistently with Oil Red O staining, triglyceride content was reduced in HepG2; (**F**) Palmitate or silibinin didn't affect cell viability, assessed using a Muse™ Cell Analyzer. All values are expressed as mean ± SEM of three experiments ($n = 3$) in duplicate. * $p < 0.05$ vs. vehicle; # $p < 0.05$ vs. FA. FA: Fatty acid; OP: Optical density; SIL: Silibinin.

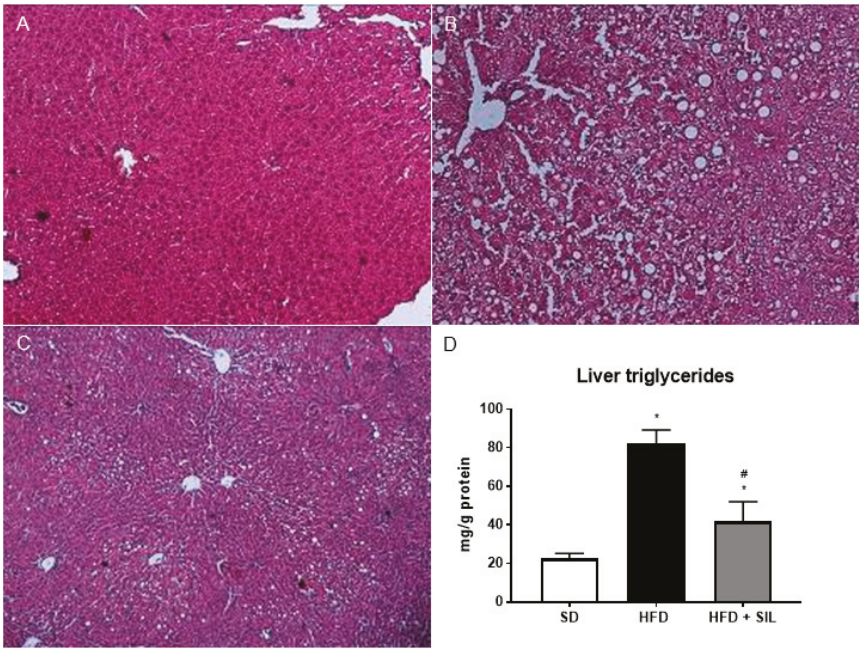

Figure 2. Effects of silibinin on liver steatosis in mice. Liver sections were stained with hematoxylin and eosin. (**A**) Liver sections of mice fed the SD; (**B**) Following a 16-week HFD, the mouse livers displayed microvescicular steatosis with a panacinar pattern; (**C**) An 8-week treatment with silibinin attenuated steatosis; (**D**) Consistently with in vitro results, liver triglyceride content in mice treated with silibinin, as assessed by a colorimetric assay, was markedly reduced. * $p < 0.05$ vs. SD; # $p < 0.05$ vs. HFD. HFD: High fat diet; SD: Standard diet; SIL: Silibinin.

3.2. Silibinin Restores NAD$^+$ Levels and Induces SIRT1/AMPK Signaling

Based on steatosis results, we aimed to verify the hypothesis that silibinin may influence NAD$^+$ levels and the SIRT1/AMPK pathway. As expected, palmitate administration increased the mean number of ROS$^+$ cells and enhanced PARP activity leading to lower NAD$^+$ levels in HepG2 cells (Figure 3). Similarly, the HFD induced oxidative stress and higher PARP activity while lowering the NAD$^+$ pool in mouse livers (Figure 3). As a consequence of PARPs activation and NAD$^+$ consumption, palmitate administration reduced the expression and activity of SIRT1 (Figure 3). The ratio between the levels of AMPKα phosphorylated at the threonine 172 residue and total AMPKα was consistently lower in HepG2 treated with the FA + vehicle compared with the control cells (Figure 3).

Treatment of HepG2 and mice with silibinin abolished oxidative stress and inhibited PARP activation, thus restoring NAD$^+$ levels (Figure 3). In agreement with preserved NAD$^+$ levels, SIRT1 activity and AMPK phosphorylation returned to control levels in HepG2 and in mice (Figure 3).

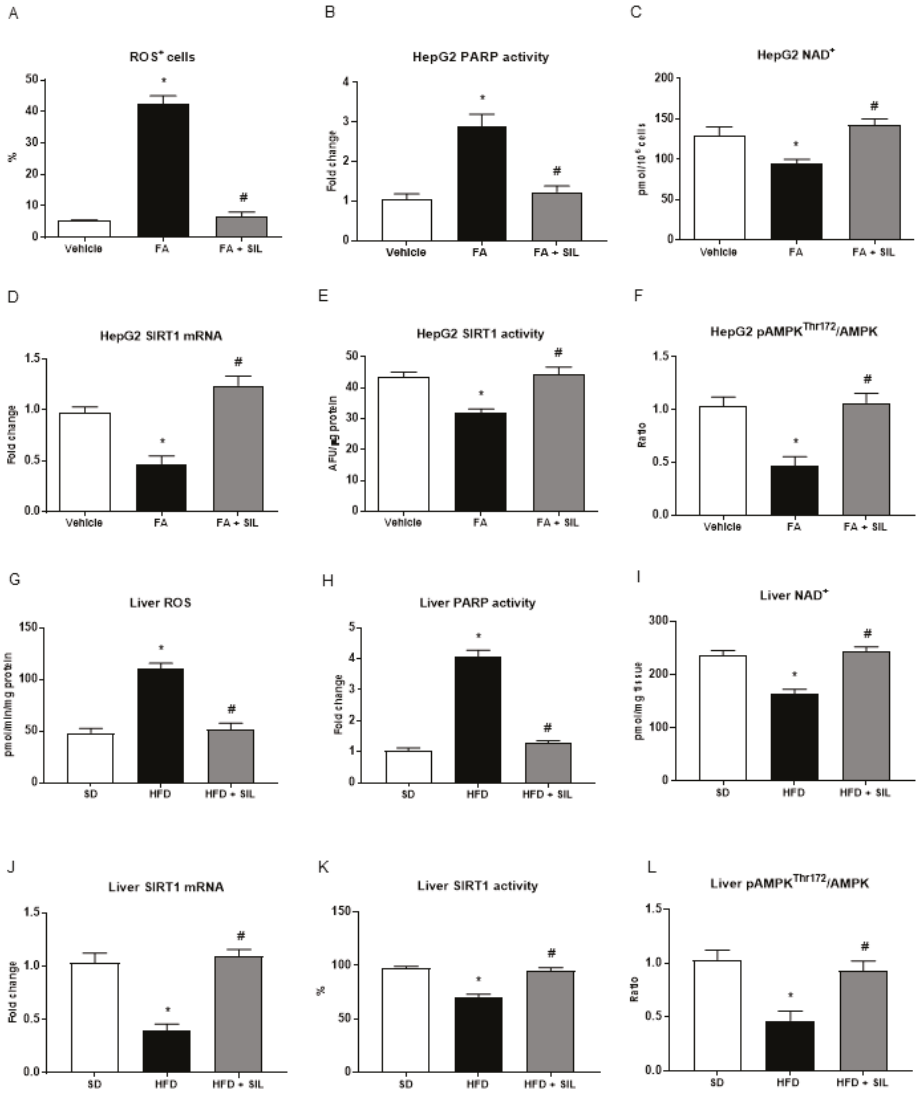

Figure 3. Effects of silibinin on ROS, PARP, NAD^+, SIRT1 and AMPK levels in HepG2 and mice liver (**A**–**C**) Palmitate administration led to higher levels of ROS and PARP activity and lower levels of NAD^+. Treatment of HepG2 with 25 Mm silibinin abolished oxidative stress and inhibited PARP activation, thus restoring NAD^+ levels; (**D**–**E**) SIRT1 activity and mRNA expression were lower in HepG2 treated with 0.25 mM palmitate compared with control cells; (**F**) Levels of phosphorylated and total AMPKα were measured by sandwich ELISA and the ratio is shown; treatment with silibinin restored SIRT1 activity and expression and induced AMPK phosphorylation at Thr172; (**G**–**L**) In vitro results were confirmed in mice fed a 16-week HFD and treated with silibinin during the last 8 weeks. All values are expressed as mean ± SEM of three experiments ($n = 3$) in duplicate. * $p < 0.05$ vs. vehicle; # $p < 0.05$ vs. FA. AFU: Arbitrary fluorescence units; AMPK: AMP-kinase; FA: Fatty acid; HFD: High fat diet; NAD^+: Nicotinamide adenine dinucleotide; PARP: poly-(ADP-ribose)-polymerase; ROS: Reactive oxygen species; SD: Standard diet; SIL: Silibinin.

3.3. Silibinin Inhibits De Novo Lipogenesis and Promotes Mitochondrial β-Oxidation

A 24-h exposure of HepG2 to palmitate led to lower levels of phosphorylation at the serine 79 residue of ACC (Figure 4), the rate-limiting enzyme of de novo lipogenesis [21], which is a direct downstream target of AMPK [22]. Because of AMPKα phosphorylation and activation, cells treated with silibinin displayed an increase in levels of phospho-ACC, indicating lower ACC activity (Figure 4). As expected, a 24 h culture with palmitate was also associated with a higher transcriptional activity of SREBP-1, the main transcription factor promoting lipogenesis [23]. Concomitantly with the phosphorylation of ACC, we observed that silibinin administration reduced the activity of SREBP-1 in nuclear extracts of HepG2 (Figure 4). The expression of fatty acid synthase (FAS), a downstream target of SREBP-1 [23], was similarly down-regulated by silibinin (Figure 4).

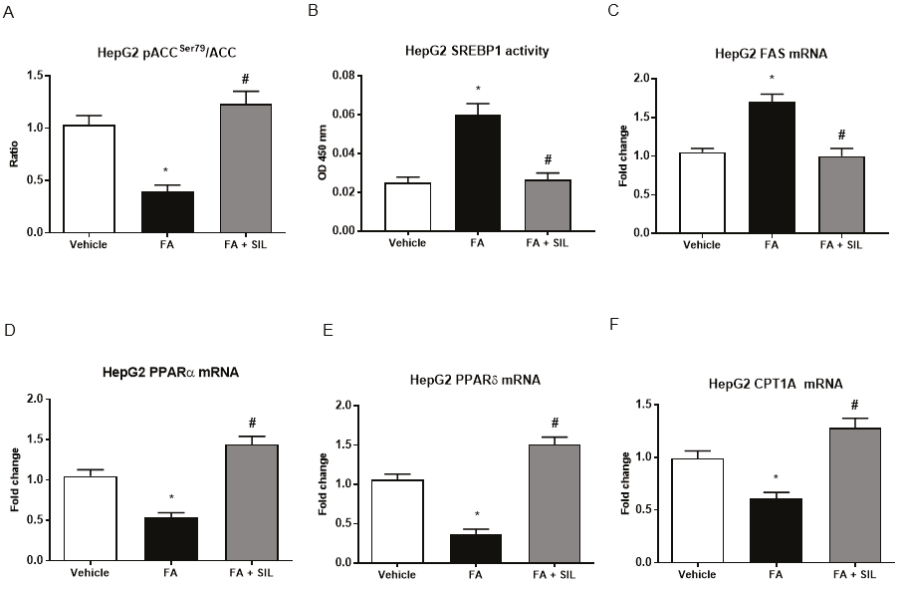

Figure 4. Effects of silibinin on de novo lipogenesis and lipolysis in HepG2. (**A**–**F**) HepG2 exposed to palmitate displayed lower levels of phosphorylation at the serine 79 residue of ACC, the rate-limiting enzyme of de novo lipogenesis. A 24 h culture with palmitate was also consistently associated with higher transcriptional activity of SREBP-1, the main transcription factor promoting lipogenesis, and higher levels of FAS, a downstream target of SREPB-1. Treatment with silibinin stimulated the phosphorylation of ACC, leading to its inactivation and thus to decreased conversion of acetyl-CoA to malonyl-CoA. It also reduced the transcriptional activity of SREBP-1, assessed on nuclear extracts, thus down-regulating the expression of FAS. Concomitantly with the effects on lipogenesis, we observed that treatment with silibinin up-regulated the expression of CPT1A, the rate limiting enzyme of mitochondrial β-oxidation. All values are expressed as mean ± SEM of three experiments ($n = 3$) in duplicate. * $p < 0.05$ vs. vehicle; # $p < 0.05$ vs. FA. ACC: acetyl Co-A carboxylase; CPT1A: carnitine palmitoyltransferase 1A; FA: Fatty acid; FAS: Fatty acid synthase; PPARα: Peroxisome proliferator-activated receptor alpha; PPARδ: Peroxisome proliferator-activated receptor delta; SREBP-1: Sterol regulatory element-binding protein 1; SIL: Silibinin.

Phosphorylation and inactivation of ACC leads to decreased conversion of acetyl-CoA to malonyl-CoA [23], which is an allosteric inhibitor of mitochondrial carnitine palmitoyltransferase 1A (CPT1A), the enzyme responsible for transport of acyl-CoAs into mitochondria for oxidation [24]. In accordance with the inhibition of ACC activity by AMPK and thus with the presumably reduced

levels of malonyl-CoA, we found that silibinin administration increased gene expression of CPT1A (Figure 4). Consistently with lower levels of CPT1A, the gene expression of PPAR-α, the nuclear transcription factor of CPT1A [25], was reduced in HepG2 treated with FA alone and was augmented in cells treated with silibinin (Figure 4). Furthermore, culture of HepG2 with palmitate also led to reduced mRNA levels of PPAR-δ, a main transcription factor promoting FA oxidation in the liver [24], whereas treatment with silibinin also up-regulated PPAR-δ expression (Figure 4). Similarly to what was observed in vitro, we found that, in mice fed the HFD, treatment with silibinin led to the inhibition of lipogenesis and the induction of β-oxidation in the liver (Figure 5).

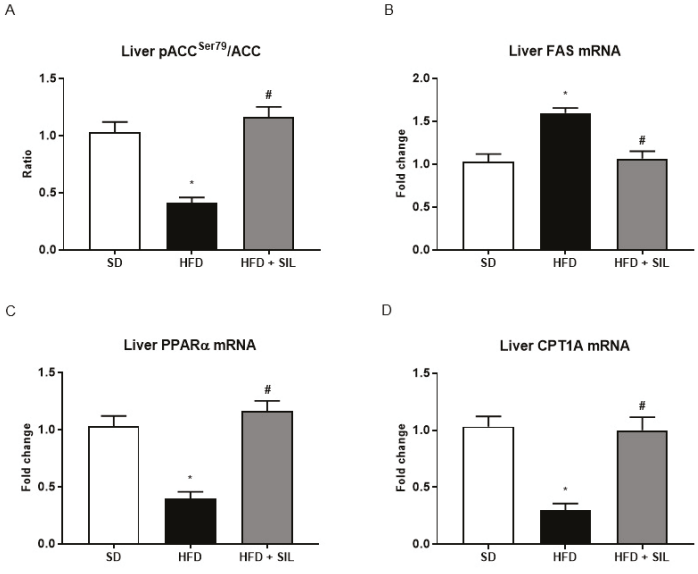

Figure 5. Effects of silibinin on hepatic de novo lipogenesis and lipolysis in mice. (**A**–**D**) Levels of phosphorylated and total ACC, the rate-limiting enzyme of de novo lipogenesis, were measured by sandwich ELISA and the ratio is showed. Phosphorylation at the serine 79 residue inhibits ACC activity, leading to decreased conversion of acetyl-CoA to malonyl-CoA. Following a 16-week HFD, mice displayed lower levels of hepatic phospho-ACC and higher levels of FAS, a main lipogenic enzyme. Treatment with silibinin induced ACC phosphorylation at Ser79 and down-regulated FAS expression. Consistently with these results, gene expression of CPT1A, the rate limiting enzyme of mitochondrial β-oxidation, and gene expression of PPARα, its upstream transcription factor, were downregulated in the livers of obese mice. Concomitantly with the effects on lipid accumulation, we observed that treatment with silibinin up-regulated the expression of PPARα and of CPT1A. All values are expressed as mean ± SEM of three experiments ($n = 3$) in duplicate. * $p < 0.05$ vs. SD; # $p < 0.05$ vs. HFD. ACC: Acetyl-coA carboxylase; CPT1A: Carnitine O-palmitoyltransferase 1A; FAS: Fatty acid synthase; HFD: High fat diet; PPARα: Peroxisome proliferator-activated receptor alpha; SD: Standard diet; SIL: Silibinin.

4. Discussion

In this study, we aimed to investigate whether silibinin, a flavonolignan contained in milk thistle, may exert hepatoprotective effects by modulating NAD^+ homeostasis and the SIRT1/AMPK pathway. In addition to its established protective roles in the context of diabetes and obesity [7,8], NAD^+ homeostasis has been recently shown to play an important role in the onset of fatty liver by modulating mitochondrial efficiency [14]; consistently, it has been showed that AMPK regulation of mitochondrial function may be crucial in the progression of human steatohepatitis [25]. AMPK activation requires

the activity of SIRT1 [26], which controls hepatic lipid metabolism through modulation of PPARs activity [27,28]. In this study, we showed for the first time that silibinin, a potent natural antioxidant, restores NAD^+ levels and induces the SIRT1/AMPK pathway in vitro and in vivo. It is plausible that these effects may be in part dependent on silibinin antioxidant capacity. Overall, we may speculate that lower oxidative stress leads to lower PARPs activation, thus leading to lower NAD^+ consumption. In line with the co-regulation of NAD^+ and SIRT1, a sufficient NAD^+ pool in turn maintains metabolic health by restoring SIRT1 activity and signaling. This may be a common mechanism that explains why several flavonoids are able to inhibit PARPs, elevate intracellular NAD^+ levels, and activate NAD^+-dependent sirtuin-mediated signaling pathways in metabolic and age-related chronic diseases [29]. Nonetheless, since it has been demonstrated that SIRT1 activity is also directly inhibited by oxidative stress [30], we may also speculate that the well-established antioxidant activity of silibinin may also directly influence SIRT1 activity by modulating the redox *status*. Interestingly, we also found that under lipotoxic conditions silibinin induces the expression of PPAR-α and PPAR-δ in HepG2. These last results appear relevant because Elafibranor, a synthetic PPAR-α/δ agonist, was recently shown to be effective in experimental and human NAFLD [31,32]. However, functional studies are needed to establish if silibinin acts as a direct PPAR-α/δ agonist.

It should be underlined that our novel results add to a growing body of experimental evidence showing the beneficial metabolic effects of silibinin in different models of NAFLD [33–38]. Interestingly, some studies have showed that silibinin may influence not only steatosis mechanisms but also mechanisms related to liver inflammation and fibrosis. Our group showed that silibinin inhibits the activity of NF-κB and down-regulates the expression of its pro-inflammatory targets in mice with genetic and diet-induced NAFLD [37,38]. Moreover, silibinin suppresses in vitro activation of rat Kupffer cells [39] and human hepatic stellate cells [19,40]. In this respect, although several SIRT1/AMPK activators have been proposed for NAFLD treatment [41,42], we think that silibinin can be more effective than the others because it can beneficially modulate almost all the molecular mechanisms associated with onset and progression of NAFLD [43].

Besides the experimental data obtained so far, pilot clinical trials have assessed the efficacy of specific nutraceuticals containing silibinin. In a randomized controlled trial of biopsy-proven NAFLD patients, Loguercio et al. showed that a 200 mg daily dose of silibinin not only decreases serum enzymes and insulin resistance, but also led to improvement of histological scores of steatosis, inflammation, and fibrosis [44]. Recently, a small randomized trial showed that supplementation of a 540 mg daily dose of silymarin for three months has better effects on liver steatosis, assessed by non-invasive score, than lifestyle changes [45]. However, translational approaches are hampered by the issue of bioavailability in regards to the plethora of oral nutraceuticals containing silibinin [46]. Thus, the "pharmaceutical" design of a silibinin-based nutraceutical in order to allow adequate bioavailability remains a priority.

5. Conclusions

In conclusion, in this study we provided the first evidence that silibinin restores NAD^+ levels and induces the SIRT1/AMPK pathway in vitro and in vivo. Our results further suggest that silibinin may be a promising molecule for the treatment of NAFLD.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/9/10/1086/s1, Figure S1: Final body weight, Table S1: Primers sequences used for real time PCR analysis.

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Author Contributions: F.S. conceived the study, designed the experiments, analyzed the data, and wrote the manuscript; G.L.V. designed, supervised, and performed the experiments; I.B., J.G., V.L., D.C. performed the experiments; F.M., F.G., N.D., R.A., W.C. analyzed the data and critically reviewed the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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Review

Effects of Olive Oil Phenolic Compounds on Inflammation in the Prevention and Treatment of Coronary Artery Disease

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Abstract: Coronary artery disease (CAD) is responsible for more than 7 million deaths worldwide. In the early stages of the development of atherosclerotic plaques, cardiovascular risk factors stimulate vascular endothelial cells, initiating an inflammatory process, fundamental in the pathogenesis of CAD. The inclusion of potentially cardioprotective foods, such as olive oil, to the diet, may aid in the control of these risk factors, and in the reduction of cytokines and inflammatory markers. The present review aims to address the interaction between phenolic compounds present in olive oil, and inflammation, in the prevention and treatment of CAD. In vitro and in vivo studies suggest that phenolic compounds, such as hydroxytyrosol, tyrosol, and their secoiridoid derivatives, may reduce the expression of adhesion molecules and consequent migration of immune cells, modify the signaling cascade and the transcription network (blocking the signal and expression of the nuclear factor kappa B), inhibit the action of enzymes responsible for the production of eicosanoids, and consequently, decrease circulating levels of inflammatory markers. Daily consumption of olive oil seems to modulate cytokines and inflammatory markers related to CAD in individuals at risk for cardiovascular diseases. However, clinical studies that have evaluated the effects of olive oil and its phenolic compounds on individuals with CAD are still scarce.

Keywords: olive oil; phenols; inflammation; coronary artery disease

1. Introduction

Cardiovascular diseases account for 17.7 million deaths every year, and are the leading cause of death worldwide. Of these deaths, it is estimated that 7.4 million are caused by coronary artery disease (CAD) [1]. More than 90% of the events related to CAD—acute myocardial infarction and death—occur in individuals with at least one of the risk factors for CAD [2,3]. Therefore, primary and secondary prevention strategies aim to reduce traditional risk factors (diabetes mellitus, hypertension, dyslipidemia, obesity) and lifestyle-related disorders, such as inadequate diet, smoking, physical inactivity, and abusive use of alcohol [3,4]. In the early stages of development of atherosclerotic plaques, risk factors stimulate vascular endothelial cells to express chemotactic and adhesion molecules, initiating the inflammatory process, fundamental in the pathogenesis of CAD [5–7].

In the context of a dietary pattern considered to be healthy, the inclusion of potentially cardioprotective foods, including sources of unsaturated fats and phenolic compounds, such as olive oil, may modulate the concentration of pro-inflammatory cytokines and markers of inflammation [8,9], and assist in the control of modifiable risk factors, such as diabetes mellitus [8], hypertension [10],

dyslipidemia [11], and overweight [12–14]. Moreover, the consumption of 50 mL/day of extra-virgin olive oil (EVOO) may reduce the chance of developing CAD by 37%, and the incidence of major cardiovascular events by 30% [15].

The concentration of phenolic compounds in EVOO is influenced, among other factors, by the extraction procedure of the oil. EVOO is obtained by mechanical processes, while refined olive oil (ROO) is subjected to both physical and chemical procedures [16,17]. Although ROO presents a similar composition of fatty acids, due to the low phenolic content, it does not bring the same beneficial effects when compared with EVOO [18].

Phenols such as hydroxytyrosol (HT) and derivatives (oleuropein complex and tyrosol) are mainly responsible for the beneficial effects of olive oil in the prevention and progression of atherosclerosis, by improving endothelial function [19], antioxidant effect [20], and the high density lipoprotein (HDL) function [21], reducing the concentration and the atherogenicity of the low density lipoprotein (LDL) [11,22] and inhibiting platelet aggregation [23]. To ensure the cardiovascular benefits of olive oil, the European Food Safety Authority recommends the daily intake of 5 mg of HT or its derivatives, which can be obtained by the daily consumption of at least 20 g of EVOO [24].

Taking into account the potential benefits of olive oil and its phenolic compounds in preventing the mechanisms and the risk factors that may lead to atherosclerosis, and consequently, to the development of cardiovascular disease, the present review aims to address the effects of phenolic compounds present in olive oil on inflammation, both in the prevention and treatment of CAD.

2. Inflammatory Process in CAD

Atherosclerosis is a chronic disease initiated by the retention and accumulation of cholesterol-rich lipoproteins, particularly LDL, in the artery wall. Processes such as oxidation of lipoproteins, immunity (innate and adaptive) and inflammation are crucially involved in its pathogenesis [5]. The condition can lead to an acute clinical event caused by the rupture of atherosclerotic plaque and thrombus formation [25–27].

Endothelial injury is the event that triggers the formation of atherosclerotic plaques [28]. Risk factors, such as hypertension, dyslipidemia, obesity, diabetes mellitus, smoking, as well as increased oxygen-reactive species, alter the endothelial function and affect the vascular homeostasis by decreasing nitric oxide (NO) synthesis, and altering vessel tone and anti-inflammatory and anticoagulant properties [29–31]. As a consequence, an increase in the adhesion of leukocytes and platelets at the vascular injury site, as well as in the permeability of the intimate layer to atherogenic lipoproteins, mainly oxidized LDL, takes place [32].

Retained in the subendothelial space through the binding of apolipoprotein B100 to proteoglycans of the extracellular matrix, LDL particles undergo oxidation [33–35]. Endothelial cells activated in response to the inflammatory process produce cytokines (interleukin (IL)-1, IL-6 and tumor necrosis factor-alpha (TNF-α)), chemokines (monocyte chemotactic protein (MCP-1)), adhesion molecules (P and E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1)), and by chemotaxis, cause adhesion and migration of leukocytes (monocytes, B and T lymphocytes) [7,36–39].

MCP-1 stimulates the migration and infiltration of monocytes into the subendothelial space, through which the macrophage colony-stimulating factor (M-CSF), differentiates into macrophages [40,41]. Macrophages recognize LDLox and begin to express scavenger and toll-like receptors (TLR). TLR regulate the function of macrophages, promoting their activation. Scavenger receptors bind to oxidized LDL and perform phagocytosis, leading to the formation of foam cells, the main components of the fatty striae—the initial atherosclerotic lesions [42–44].

The immune response plays an important role in the initiation and development of atherosclerosis. The innate response begins with endothelial and monocyte/macrophage activation, and is followed by the adaptive response, which involves CD4+ T lymphocytes [45].

CD4+ T lymphocytes are among the first cells to be recruited. Within the plaque, they interact with macrophages through the presentation of antigens and differentiate into T helper (Th) cells—Th1 and Th2 lineages [45]. The Th1 phenotype develops in the presence of IL-12 and IL-18 [46,47] (pro-atherogenic response), and is characterized by the secretion of mainly interferon-gamma (INF-γ) [48,49]. In the Th2 subtype, the cytokines IL-4 (which inhibits INF-γ), IL-5, IL-10, and IL-13 are produced, and B cells are activated. The Th2 response is atheroprotective through inhibition of IFN-γ by IL-4 [45,50]. In CAD, there is an imbalance between Th1/Th2, with predominance of pro-inflammatory cytokines [51,52].

In response to the stimulation of IL-6, the liver produces acute phase reagents, such as C-reactive protein (CRP) and fibrinogen [42]. Both are used as biomarkers for diagnostic purposes, as they reflect the level of inflammatory activity [53,54]. CRP can stimulate the expression of both VCAM-1 and ICAM-1 by endothelial cells, and mediate the induction of MCP-1 and the uptake of LDL by macrophages [55,56]. Fibrinogen regulates plasma viscosity while inducing platelet aggregation. In the coagulation cascade, fibrinogen is converted into fibrin by the action of thrombin, and promotes platelet aggregation by binding to the glycoprotein IIb/IIa receptor, consequently increasing the reactivity of platelets [54,57].

One of the main regulators of the inflammatory process at different stages of atherosclerosis is the nuclear factor kappa B (NF-κB). NF-κB is responsible for the regulation of genes coding for chemokines, adhesion molecules, cytokines and proinflammatory acute-phase proteins, cyclooxygenase (COX)-2 enzyme, inducible nitric oxide synthase, apoptosis, and cellular proliferation [58,59]. Another important pathway is mediated by mitogen-activated protein kinases, which regulate cellular processes such as cell growth, proliferation, and differentiation [59]. These two pathways, activated by TLR, increase local inflammatory processes, perpetuating the inflammatory response [60].

COX-1 and COX-2 enzymes are responsible for the production of eicosanoids, prostaglandins, and thromboxanes from arachidonic acid (AA), which is derived from the phospholipid membrane, obtained directly from the diet, or synthesized from linoleic acid [61,62]. COX-1 is expressed in most tissues and plays a role in homeostasis (normal arteries); COX-2, in turn, is expressed in inflammatory cells, and is induced by cytokines such as IL-1, IL-6, and TNF-α (atherosclerotic lesion) [63]. Prostaglandins (PG) act in the recruitment of leukocytes and infiltration of immune cells into the inflammatory site. PGE2 is an important mediator of the inflammatory response, inducing the expression and activity of matrix metalloproteinase-9 (MMP-9) in macrophages [64]. MMP-9 plays a role in angiogenesis and in the formation and vulnerability of the atherosclerotic plaque [65]. Thromboxanes (TX) A2 and B2 (TXA2 and TXB2) increase platelet aggregation and potentiate thrombus formation [66]. Another eicosanoid from AA, through lipoxygenases, is the leukotriene (LT) B4 (LTB4), which has a chemotactic effect on neutrophils, directing the cells to the atherosclerotic lesion [67,68].

3. Olive Oil: Classification and Composition

Olive oil is the oil obtained solely from the olive tree fruit (*Olea europaea* L., *Oleacea* family), excluding the use of solvents, re-esterification processes, and mixture with any other types of vegetable oils. Virgin olive oil (VOO) is obtained exclusively by mechanical or other physical means under conditions that do not alter the oil, and is not subjected to any treatment other than washing, decantation, centrifugation, and filtration [16,69].

The virgin olive oils are classified into EVOO, virgin (fine), and lampante, according to the degree of acidity (ratio of free fatty acids to total oleic acid): ≤0.8%, ≤2%, and >2%, respectively. EVOO also differs from fine oil in quality: although both are obtained by physical means, EVOO has superior physicochemical and sensory properties [70].

All virgin olive oils are composed of two fractions: saponifiable and unsaponifiable. The saponifiable fraction (larger components) represents approximately 98% of the oil composition [71], and the oleic monounsaturated fatty acid comprises 55–83% of that fraction. The virgin olive oils also have significant

concentrations of polyunsaturated fatty acids (linoleic fatty acid: 3.5–21%) and saturated fatty acids (palmitic fatty acid: 7.5–20%, stearic fatty acid: 0.5–5%) [16].

The unsaponifiable fraction (minor components) constitutes 1–2% of the total content of the virgin oils, and includes more than 230 compounds: (1) sterols (e.g., β-sitosterol); (2) hydrocarbons (e.g., squalene and carotenoids (β-carotene and lycopene)); (3) volatile compounds; (4) triterpenic and aliphatic alcohols; (5) pigments (e.g., chlorophyll); and (6) phenolic compounds [72,73].

Phenolic Compounds of Olive Oil

Phenolic compounds are secondary plant metabolites synthesized during normal development or in stressful situations [74]. In virgin olive oils, the synthesis of these compounds occurs when the olive fruits are crushed during the industrial process to obtain the olive oil. Thus, the presence of phenolic compounds is directly related to glycosides initially present in the fruit tissue, and the activity of hydrolytic and oxidative enzymes [75]. In terms of chemical structure, they have at least one hydroxyl attached to an aromatic ring [74].

According to their characteristics, phenolic compounds are classified into lipophilic (α, β, and γ-tocopherols and tocotrienols) [76–78] or hydrophilic. Among the lipophilic phenolic compounds present in virgin olive oils, α-tocopherol is the most relevant (>90% of tocopherols) [79], with a mean concentration of 150.7 mg/kg [80], and reaching levels of up to 400 mg/kg [77,79]. At least 36 hydrophilic phenolic compounds have been identified in olive oil and grouped into six categories according to their chemical structure [81] (Table 1).

The phenolic compounds are mainly responsible for the organoleptic characteristics (aroma and flavor) [82,83] and oxidative stability of the olive oil [20,84]. Several factors influence their concentration: plant variety [77,84,85], environmental factors [86,87], olive storage and maturation conditions [88,89], oil extraction conditions [90,91] and commercial storage of the final product [92]. The mean phenolic content in EVOO is 551.4 mg/kg [93,94] (ranging from 50–800 mg/kg) [95]; in fine oil, it is 206.7 mg/kg [94], and ROO has the lowest indices, 198–62.0 mg/kg [17,94]. The phenolic content of EVOO has a wide variation. Montano et al., evaluated eight varieties of plants grown at extreme altitudes, and found that Cornicabra presented the highest concentrations (mean of 632.6 mg/kg) while Arbequina had the lowest values (200.2 mg/kg) [84]. Baiano et al., evaluated the effect of planting location on the content of phenolic compounds and found significant variations, from 195.2 to 32.3 mg/kg for the same type of crop [96].

HT (3,4-DHPEA) and tyrosol (*p*-HPEA), and especially its secoiridoid derivatives—the dialdehydic forms of the decarboxymethyl elenolic acid linked to hydroxytyrosol (oleacein: 3,4-DHPEA-EDA) and tyrosol (oleocanthal: *p*-HPEA-EDA), aglycones of oleuropein (3,4-DHPEA-EA) and ligstroside (*p*-HPEA-EA)—are the most abundant phenolic compounds in olive oil (90% of the total phenolic content) [81,97]. Oleuropein may give origin to oleuropein aglycone, hydroxytyrosol, and elenolic acid by hydrolysis. This process, which occurs during the maturation of the olive fruit and extraction and storage of the oil, is in part responsible for the variety and complexity of EVOO flavors [72]. In addition to dryiridoids, lignans are also present in high concentrations, mainly (+)-1-acetoxypinoresinol and (+)-pinoresinol [98].

The levels of phenolic acids, flavonoids and hydroxy-isocromans are relatively low in virgin olive oil. Phenolic acids were the first compounds identified in the olive oil; at least 14 have already been described and are generally present in amounts of less than 1 mg/kg [99–101]. Luteolin and apigenin are the two flavonoids found in the highest concentration in olive oil; however, this is lower than that of other phenolic compounds [102]. Bianco et al., identified two hydroxy-isocromans [1-phenyl-6,7-dihydroxy-isochroman and 1-(3′-methoxy-4′-hydroxy)-6,7-dihydroxy-isochroman] in commercial virgins olive oils formed from the HT reaction with benzaldehyde and vanillin, respectively [103]. The average concentrations of the phenolic compounds found in the different types of olive oil are presented in Table 1.

Table 1. Classification of the main hydrophilic phenolic compounds found in virgin olive oils and their average concentration in different types of olive oil.

Chemical Structure	Components	ROO mg/kg * (Mean ± SD)	Virgin (Fine) mg/kg * (Mean ± SD)	EVOO mg/kg * (Mean ± SD)
Phenolic acids	benzoic	-	-	-
	gallic	-	-	-
	p-hydroxybenzoic	-	0.37 ± 0.37	-
	protocatechuic	-	1.47 ± 0.56	-
	syringic	-	0.81 ± 1.17	0.25 ± 0.25
	vanillic	-	1.22 ± 2.04	0.64 ± 0.50
	caffeic	-	0.21 ± 0.63	0.19 ± 0.45
	cinnamic	-	-	0.17 ± 0.14
	o-coumaric	-	-	-
	p-coumaric	-	0.24 ± 0.81	0.92 ± 1.03
	ferulic	-	0.19 ± 0.50	0.19 ± 0.19
	sinapic	-	-	-
Phenolic alcohols	hydroxytyrosol (3,4-DHPEA)	6.77 ± 8.26	3.53 ± 10.19	7.7 2 ± 8.81
	tyrosol (*p*-HPEA)	4.11 ± 2.24	5.34 ± 6.98	11.32 ± 8.53
Secoiridoids	oleuropein	-	-	1.65 ± 1.85
	oleuropein aglycone	125.40 ± 41.80	120.57 ± 125.53	36.63 ± 24.34
	ligstroside aglycone	59.93 ± 18.58	82.01 ± 67.78	17.44 ± 18.13
	monoaldehydic form of oleuropein aglycone (3,4-DHPEA-EA)	10.90 ± 0.00	95.00 ± 116.01	72.20 ± 64.00
	monoaldehydic form of ligstroside aglycone (*p*-HPEA-EA)	15.20 ± 0.00	69.05 ± 69.00	38.04 ± 17.23
	dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (oleacein: 3,4-DHPEA-EDA)	57.37 ± 27.04	77.83 ± 256.09	251.60 ± 263.24
	dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol (oleocanthal: *p*-HPEA-EDA)	38.95 ± 9.29	71.47 ± 61.85	142.77 ± 73.17
Flavonoids	flavones			
	luteolin	1.17 ± 0.72	1.29 ± 1.93	3.60 ± 2.32
	apigenin	0.30 ± 0.17	0.97 ± 0.71	11.68 ± 12.78
	flavanonol			
	taxifolin	-	-	-
Lignans	(+)-1-acetoxypinoresinol	7.52 ± 9.10	4.43 ± 21.28	6.63 ± 10.78
	(+)-pinoresinol	24.05 ± 10.02	23.71 ± 17.03	4.19 ± 2.78
Hydroxy-isocromans	1-phenyl-6,7-dihydroxy-isochroman	-	-	-
	1-(3′-methoxy-4′hydroxy)-6,7-dihydroxy-isochroman	-	-	-
Polyphenols, total		198.0 ± 14.85	206.73 ± 150.08	551.42 ± 235.02

Source: Adapted from Cicerale et al., [81] and Rothwell et al., [93,94]. * Fresh weight. ROO: refined olive oil; EVOO: extra virgin olive oil.

After ingestion, the phenolic compounds are metabolized through two phases: hydrolysis (phase 1) that occurs in the stomach and small intestine (where most are promptly absorbed); and conjugation (phase 2), in the small intestine and mainly in the liver—this process basically involves methylation, sulfation, and glucuronidation [104–108]. Oleuropein, specifically, does not follow the same route, as it is degraded by the colon microbiota to HT, which can then be absorbed [106].

In humans, the absorption of phenolic compounds of virgin oils, especially HT and tyrosol, is dose-dependent, and these compounds are excreted in the urine in the conjugated form [109]. Vissers et al., studied healthy subjects (one group submitted to ileostomy and another with intact colon) and estimated that the absorption rate would be at least 55–66% of the dose of ingested olive oil [110]. The absorption of HT also depends on the food matrix: higher percentages of urinary excretion were observed after ingestion of this phenolic compound as a natural component of virgin olive oils (42% of ingested HT), compared to ROO (23%) or to yogurt (5.8%) [111].

4. Phenolic Compounds of Olive Oil and Inflammation: In Vitro and Animal Model Studies

Evidence has shown that regular consumption of foods rich in phenolic compounds may decrease the risk for the development of chronic diseases [112,113], mainly due to their ability to modulate low-grade inflammation [114]. The mechanisms by which these compounds may exert an anti-inflammatory effect, specifically on cardiovascular diseases, involves: (1) antioxidant activity; (2) modification of the signaling cascade and transcription network (blocking the signaling and expression of nuclear factor kappa B); (3) decrease of the adhesion of immune cells (T lymphocytes and monocytes) to the endothelium; and (4) improvement of endothelial dysfunction [114]. Due to the complex chemical composition of the oil, particularly the EVOO, we tried to elucidate which phenols would be more involved in these mechanisms.

In human umbilical vein endothelial cells cultured in vitro and stimulated by lipopolysaccharides (LPS) or cytokines (TNF-α, IL-1β), HT inhibited the endothelial activation and expression of VCAM-1 and ICAM-1 [115,116]. Elenolic acid and tyrosol were also tested, but did not show the same results in reducing VCAM-1 expression [115]. Besides the HT, its phase II metabolites, biosynthesized by intestinal Caco-2 cells (hydroxytyrosol sulfate, hydroxytyrosol 4′-glicuronide, hydroxytyrosol 3′-glicuronide) were also effective in reducing the biomarkers of endothelial dysfunction (MCP-1, E and P- Selectin, ICAM-1, and VCAM-1) [117].

At nutrient-relevant concentrations (<10 μM), close to those found in human fluids following EVOO intake [118], HT inhibited the production of ON and PGE2, but had no effect on the expression of inducible nitric oxide synthase, TNF-α or IL -1β, in granulocytes and monocytes [119]. In peripheral blood mononuclear cells, HT culture reduced MMP-9 concentrations, and inhibited PGE2 production and COX-2 expression, without affecting COX-1 [120]. In endothelial cell culture, HT and oleuropein phenols reduced the inflammatory process in angiogenesis through the inhibition of COX-2 and MMP-9 [121], suggesting that the mechanism of action of HT on the inflammatory process is similar to that of nonsteroidal anti-inflammatory drugs (NSAIDs) [122,123] (inhibition of the COX enzyme results in reduced synthesis of eicosanoids (PG and TX) from AA).

Rosignoli et al., performed an in vitro experiment with the objective of evaluating the effects of different olive oil polyphenols on the modulation of inflammatory mediators in human monocytes. The cells were treated for 24 h with 100 μM HT (3,4-DHPEA), tyrosol (*p*-HPEA) and their secoiridoid derivatives (3,4-DHPEA and *p*-HPEA bound to the dialdehydic form of elenolic acids: 3,4- DHPEA-EDA (oleacein) and *p*-HPEA-EDA (oleochantal), respectively). The evaluated compounds significantly inhibited the production of superoxide anions (O_2^-) by 40% (3,4-DHPEA), 9% (*p*-HPEA), 25% (3,4-DHPEA- EDA), and 36% (*p*-HPEA-EDA). HT significantly reduced COX-2 expression (mRNA and protein level) and release of PGE2, the latter being dose-dependent. HT also increased the production of TNF-α by monocytes. COX-2 mRNA was also inhibited by secoiridoid derivatives [123].

Tyrosol and hydroxyl-isocroman compounds also have an effect on AA. In macrophage culture (RAW 264.7) stimulated by phorbol-12-myristate-13-acetate esters, tyrosol (≥100 μM) inhibited the

release of AA and synthesis of metabolites (PGE2 and LTB4) induced by exogenous oxygen-reactive species. This further reduced the release of NO induced by phorbol-12-myristate-13-acetate stimulus [124]. 1-Phenyl-6,7-dihydroxy-isochroman significantly inhibited the production of TXA2 and PGE2, and of TNF-α in LPS-primed human monocytes; this action was mediated by the suppression of NF-κB activation, leading to a decrease in COX-2 synthesis [125].

In vitro results on AA have also been observed in vivo [126,127]. In healthy subjects, consumption of a meal (150 g of tomatoes) with EVOO (607 mg/kg phenolic content, 300 mg/kg of HT derivatives) reduced the inflammatory markers TXB2 and LTB4 after 2 and 6 h [127].

Oleocanthal is another phenol with anti-inflammatory effects similar to those of NSAIDs. Oleocanthal was able to induce dose-dependent inhibition of COX-1 and COX-2 inflammatory enzymes in vitro, and had higher potential at equimolar concentrations when compared with ibuprofen; 25 mM of oleocanthal inhibited the COX enzyme activity by 41–57% while 25 mM ibuprofen inhibited it by 13–18% [128,129].

In addition to inhibiting the expression of endothelial adhesion molecules (VCAM-1) [115], oleuropein may reduce the inflammatory response by inhibiting TLR and the signaling of mitogen-activated protein kinases in a zebrafish model [130]. Oleuropein administration inhibited proliferation of vascular smooth muscle cells in vitro [131].

Male Sprague Dawly rats were allocated into five groups: (1) the sham group previously treated with vehicle; (2) the acute myocardial infarction group previously treated with vehicle (1 mL of distilled water/day); (3) three acute myocardial infarction groups that received different concentrations of oleuropein (10, 20, and 30 mg/kg) for 7 days before acute myocardial infarction. The groups receiving previous treatment with oleuropein (20 and 30 mg/kg) had lower IL-1β and TNF-α values, when compared to the group with acute myocardial infarction that received only the vehicle [132].

Wister rats were fed, for 9 weeks, a high cholesterol diet or high cholesterol diet supplemented with different types of oils: (1) sunflower oil (SFO); (2) SFO enriched with EVOO phenolic compounds (302 mg/kg) (SFO+); (3) SFO rich in monounsaturated fatty acid (HSFO); (4) SFO rich in monounsaturated fatty acid enriched with EVOO phenolic compounds (341 mg/kg) (HSFO+); (5) EVOO rich in phenolic compounds (168 mg/kg) (EVOO); and (6) EVOO poor in phenolic compounds (28 mg/kg) (EVOO−). In the groups receiving EVOO, EVOO (−), HSFO and HSFO (+), there was attenuation of E-selectin levels in the aorta of the animals. In the EVOO and EVOO (−) groups, the concentration of VCAM-1 (in the aorta) was lower than in those who consumed the high cholesterol diet alone [133].

Hyperhomocysteinemia has been associated with a high risk of cardiovascular disease because it increases vascular endothelial adhesiveness [134]. Phenolic compounds, such as tyrosol and *p*-coumaric acid, may decrease homocysteine-induced cell adhesion and ICAM-1 expression; however, they do not reduce the expression of ICAM-1 induced by TNF-α, demonstrating the potential selective effect of these compounds [135].

Recent studies have suggested the importance of the concomitant presence of non-alcoholic fatty liver disease and systemic inflammation (elevated CRP) in the development of atherosclerosis [136]. The effects of HT were investigated using a high-fat diet in an animal model of insulin resistance and non-alcoholic fatty liver disease. The rats were divided into three groups: (1) control diet (10.5% of lipids); (2) high fat diet (58% lipids); (3) high-fat diet + HT (10 mg/kg/day). After 6 weeks, HT attenuated, significantly, the metabolic impairment induced by the high-fat diet. It had also reduced hepatic inflammation and nitrosative−oxidative stress through decreased protein nitrosylation, lipid peroxidation, and production of oxygen-reactive species [137].

5. Studies on Olive Oil Phenolic Compounds and Inflammation in Individuals at Risk for CAD

Studies on primary prevention have demonstrated the association between consumption of EVOO, naturally rich in phenolic compounds, and reduced risk of major cardiovascular events in patients

at high risk for developing cardiovascular diseases [15,138]. Such an effect may be mediated by the control of modifiable risk factors and potential anti-inflammatory mechanisms of olive oil phenols [9].

Cardiovascular risk factors, such as hypertension, dyslipidemia, diabetes mellitus, and smoking, cause endothelial dysfunction, contributing to the onset of the inflammatory process in atherosclerosis [5]. Obesity and metabolic syndrome are characterized by a chronic and low-grade inflammatory state, increasing the contribution of inflammation to the genesis and evolution of CAD [139,140]. Thus, nutritional strategies and interventions that minimize the inflammatory process in individuals at high cardiovascular risk would help in the primary prevention of CAD.

Studies have shown the anti-inflammatory effects of virgin olive oils supplementation at different stages of development of atherosclerosis. In individuals with endothelial dysfunction, Widmer et al. evaluated the effects of VOO (340 mg/kg total polyphenols) and VOO enriched with epigallocatechin 3-gallate (EGCG), a catechin naturally found in green tea (VOO + EGCG: 300 mg/kg total polyphenols + 280 mg/Kg EGCG) on inflammatory mediators. The main difference in phenolic composition between the two oils was the secoiridoid content (VOO 61% vs. VOO + EGCG 48%), whereas the lignan content was similar (VOO 33% vs. VOO + EGCG 37%). The authors did not find differences between the VOO and VOO + EGCG groups, but concluded that the supplementation of olive oil to the usual diet of the participants for 4 months had a positive effect on the reduction of cell adhesion molecules (sICAM-1), platelets, monocytes, and lymphocytes involved in the inflammatory process [141].

In the PREDIMED (*PREvención con DIeta MEDiterránea*), study, the Mediterranean diet (MeDiet) supplemented with EVOO (1 liter/week), compared to the control group (low-fat diet), was able to significantly reduce proinflammatory cytokines (IL6) (P-selectin, sVCAM and sICAM) in subjects at high cardiovascular risk (type 2 diabetes mellitus alone or $\geq$3 other risk factors: hypertension, HDL $\leq$ 40 mg/dl, LDL $\geq$ 160 mg/dl, overweight or obesity, smoking, family history of premature CAD), at short- (3 months) and long-term (1 year) follow-up [9,142]. The same study group demonstrated the effects of MeDiet supplementation with EVOO on plasma concentrations of inflammatory molecules and atherosclerotic plaque stability. Participants were evaluated at three time points: at baseline, after 3 years, and after 5 years. In contrast to the control group, there were significant reductions in IL-6, IL-8, MCP-1, MIP-1β, IL-1β, IL-5, IL-7, IL-12p70, IL-18, TNF-α, and IFN -γ at 3 and 5 years, compared to the baseline [143].

Two randomized crossover clinical trials evaluated the anti-inflammatory effects of VOO in patients with mild dyslipidemia (no drug treatment). In the VOLOS study (Virgin Olive Oil Study), participants underwent two interventions for 7 weeks each, 40 mL/day of EVOO containing 166 mg/L of HT (free and esterified in oleuropein), and ROO with only 2 mg/L. The predominant fatty acids in the two samples were oleic (70.9% EVOO vs. 72.7% ROO), palmitic (11.5% vs. 10.7%), and linoleic (8.5% vs. 7.6%). The results demonstrated a 20% reduction in serum TXB2 concentration only in the EVOO group containing HT [127]. The second trial compared MeDiet supplemented with three different dietary sources of fats (35–50 g VOO, 40–65 g nuts, and 50–75 g almonds), accounting for 40% of the dietary lipid content (20% of the total energy value). Interventions were performed for 4 weeks, and although VOO contained the highest total polyphenol content (343 mg/kg vs. 13 mg/kg of walnuts and 11 mg/kg of almonds), there was no significant difference in serum levels of CRP and adhesion molecules (sVCAM-1 and sICAM-1) after follow-up [144].

Another short-term intervention (4 weeks), with patients with type 2 diabetes mellitus (without insulin therapy) and overweight, compared the intake of 25 mL/day of EVOO rich in phenolic compounds (577 mg/kg, mainly HT) and ROO (washout period), and did not show differences between the groups (CRP, IL-6, and TNF-α) [145]. On the other hand, women with normal-high blood pressure (systolic blood pressure [SBP] 120–139 mmHg and/or diastolic blood pressure [DBP] 80–89 mmHg) or at stage 1 of essential hypertension (SBP 140–159 mmHg and/or DBP 90–99 mmHg) had reduced CRP concentrations (-1.9 ± 1.3 mg/L; $p < 0.001$) and asymmetric dimethylarginine (-0.09 ± 0.01 µmol/L; $p < 0.01$) by MeDiet supplemented with 60 mL of VOO (564 mg/kg total

polyphenols; 30 mg/day total polyphenols) compared to MeDiet with ROO (polyphenol-free) after 8 weeks [19].

A high-fat meal, in addition to promoting postprandial hypertriglyceridemia, stimulates the intestinal absorption of endotoxins such as LPS. This endotoxin is able to bind TLR4, which in turn triggers various signaling pathways, including NF-κB, leading to transcription of genes related to the inflammatory response [146,147]. With the objective of investigating the mechanisms by which the VOO polyphenols reduce the postprandial inflammatory response, Camargo et al., administered a VOO-rich meal with different concentrations of phenolic compounds (high 398 mg/kg, intermediate 149 mg/kg, and low 70 mg/kg) to subjects with metabolic syndrome. After 4 h, high concentrations of VOO inhibited NF-κB and decreased the expression of IL-1β (vs. intermediate), and IL-6 (vs. low/intermediate). The VOO-rich meal with low phenol concentration promoted increased serum levels of IL-6, as well as the protein NF-κB subunit p65, TLR4, and postprandial LPS. These results suggest that the ingestion of phenolic VOO reduces postprandial inflammation mainly by decreasing the activation of NF-κB, secondary to the reduction of LPS absorption [148].

Obese subjects received a breakfast containing milk and muffins made with different oils: (1) VOO—containing 400 mg/kg of antioxidant phenols (monounsaturated fatty acid 70.5%, polyunsaturated fatty acids 11.1%, saturated fatty acids 18.4%); (2) SFO (monounsaturated fatty acid 34.3%, polyunsaturated fatty acids 58.3%, saturated fatty acids 7.3%); (3) SOD—mixture of oils (30% SFO + 70% canola oil) + 2 mg of dimethylpolysiloxane (artificial antioxidant) (monounsaturated fatty acid 71.8%, polyunsaturated fatty acids 18%, saturated fatty acids 12.2%); (4) SOP—mixture of oils (30% SFO + 70% canola oil) + VOO-phenol compounds (400 mg/kg) extracted from the residue of olive oil production alperujo (monounsaturated fatty acid 76.7%, polyunsaturated fatty acids 17.6%, saturated fatty acids 5.8%). The oils were previously subjected to 20 heating cycles. Interventions with VOO and SOP reduced NF-κB activation, increased NF-κB alpha inhibitor, and reduced plasma LPS concentration (2 h). The results suggest that oils rich in phenolic compounds, both natural (VOO) and artificial (SOP), are capable of modulating postprandial inflammation [149].

In addition to the postprandial anti-inflammatory effect of VOO, the ingestion of a EVOO-rich meal (72% of the caloric intake) (1125 mg/kg total polyphenols and 350 mg/kg tocopherols) resulted in lower elevations of adhesion molecules (ICAM-1 and VCAM-1) in hypertriglyceridemic men, and in healthy subjects after 8 h, and compared to a high ROO breakfast [150].

Table 2 shows the main randomized clinical trials that evaluated the effect of different concentrations of olive oil phenolic compounds on inflammation markers in patients with cardiovascular risk.

Table 2. Clinical trials that evaluated the effect of different concentrations of olive oil phenolic compounds on inflammatory markers in patients with cardiovascular risk and CAD.

Reference	Population	Sample Size	Design	Duration	Intervention Group	Control Group	Outcomes
Visioli [127]	Patients with mild dyslipidemia	22	RCCT	2 × 7 weeks run-in: 3 weeks—ROO washout: 4 weeks—ROO	40 mL EVOO: 166 mg/L total HT	40 mL ROO: 2 mg/L total HT	↓ 20% TXB2 → Urinary excretion (24 h) of F2-isoprostanes (8-iso-PGF2α)
Pacheco [150]	Healthy subjects and patients with hypertriglyceridemia	28	RCCT	post-prandial (8 h) high-fat meal (72%) and 50 g noodles washout: 1 week run-in: 1 week—diet NCEP I + EVOO or ROO	50 g/kg^2 body surface area EVOO: 1125 mg/kg total polyphenols and 350 mg/kg tocopherols	50 g/kg^2 body surface area ROO: without polyphenols or tocopherols	Healthy and hypertriglyceridemia: smaller increment of the area under the sVCAM-1 and sICAM-1 curve
Fitó [18]	Patients with stable CAD	28	RCCT	2 × 3 weeks washout: 2 weeks—ROO	50 mL VOO: 161 mg/kg total phenolic compounds	50 mL ROO: 14.67 mg/kg total phenolic compounds	↓IL-6, ↓CRP, → sVCAM-1, →sICAM-1
Damasceno [144]	Patients with moderate hypercholesterolemia, without drug therapy or hormone replacement	18	RCCT	3 × 4 weeks run-in: 4 weeks MeDiet without washout among interventions	35–50 g MeDiet + VOO: 343 mg/kg total polyphenols	40–65 g MeDiet + walnuts: 13 mg/kg total polyphenols 50–75 g MeDiet + almonds: 11 mg/kg total polyphenols	→ CRP, → sVCAM-1, →sICAM-1
Moreno-Luna [19]	Women at stage 1 of essential hypertension or normal-high BP	24	RCCT	2 × 8 weeks run-in: 16 weeks—MeDiet washout: 4 weeks—MeDiet	60 mL MeDiet + VOO: 564 mg/kg–30 mg/day total polyphenols	60 mL MeDiet + ROO: without polyphenols	↓ CRP ↓ ADMA
Perez-Herrer [149]	Obese subjects	20	RCCT	post-prandial (2 and 4 h)—breakfast containing milk and muffins (made with different types of oils)	0.45 mL of oil/kg of body weight VOO: 400 mg/kg antioxidant phenols	0.45 mL of oil/kg of body weight SFO SOD: sunflower oil/canola oil + 2 mg of dimethylpolysiloxane SOP: sunflower oil/canola oil + 400 mg/kg VOO-phenol compounds extracted from the residue of olive oil production alperujo	VOO and SOP vs. SFO: ↓ NF-kB activation, ↑ protein level IkB-α, ↓ plasma LPS concentration → Levels of subunit mRNAs and NF-kB activators (p65, IKKβ, IKKα), inflammatory molecules (TNF-α, IL-1β, IL-6, MIF, c-Jun N-terminal kinase)
Widmer [141]	Patients with early atherosclerosis (endothelial dysfunction)	82	Paralell RCT	16 weeks usual diet	30 mL VOO + EGCG: 600 mg/kg total polyphenols	30 mL VOO: 340 mg/kg total polyphenols	VOO + EGCG vs. VOO: no differences OO (VOO and VOO + EGCG): ↓ sICAM-1, ↓ monocytes, ↓ lymphocytes, ↓ platelets, ↓ leukocytes ↑ plasma 8-isoprostanes
Camargo [148]	Patients with metabolic syndrome and no drug treatment	49	RCCT	post-prandial (4 h)—breakfast containing white bread and VOO Washout: 6 weeks—low-fat, high-carbohydrate diet	40 mL VOO with concentrations of phenolic compounds: High: 398 mg/kg Intermediate: 149 mg/kg Low: 70 mg/kg		High: → LPS, inhibited NF-κB, → serum levels IL-6, → TLR4 protein High vs. Low/intermediate: ↓ expression IL-6 High vs. Intermediate: ↓ IL-1β expression Low: ↑ NF- κB p65 subunit, ↑ serum IL-6 levels; ↑ TLR4 protein, ↑ LPS Low vs. High: 4 h postprandial, higher levels of LPS ↑ expression of TNF-α independent phenolic content

Table 2. *Cont.*

Reference	Population	Sample Size	Design	Duration	Intervention Group	Control Group	Outcomes
Santangelo [145]	Overweight and type 2 diabetes mellitus patients without insulin therapy	11	RCCT	2 × 4 weeks usual diet	25 mL EVOO: 577 mg/kg total phenolic compounds	25 mL ROO: without phenolic compounds	→ high-sensitive CRP, → IL-6, → TNF-α

↑: increase; → maintenance or no effect; ↓: decrease; RCT: randomized clinical trial; RCCT: randomized crossover clinical trials; OO: olive oil; ROO: refined olive oil; VOO: virgin olive oil; EVOO: extra virgin olive oil; HT: hydroxytyrosol; TXB2: thromboxane B2; NCEP: National Cholesterol Education Program; sICAM-1: soluble intercellular adhesion molecule-1; sVCAM-1: soluble vascular cell adhesion molecule-1; CAD: coronary artery disease; IL-6: interleukin-6; CRP: C-reactive protein; ADMA: asymmetric dimethylarginine; NF-κB: nuclear factor kappa B; SFO: sunflower oil; IκB-α: alpha inhibitor of NF-κB; mRNA: messenger ribonucleic acid; MIF: inhibitory factor of macrophage migration; MeDiet: Mediterranean diet; BP: blood pressure; EGCG: epigallocatechin 3-gallate; LPS: lipopolysaccharide; TLR4: toll-like receptor 4; TNF-α: tumor necrosis factor-alpha.

6. Anti-Inflammatory Effects of Olive Oil Phenolic Compounds in Patients with CAD

The anti-inflammatory effects of VOO phenolic compounds have been extensively investigated in vitro, in animal models and in clinical trials involving subjects at risk for cardiovascular diseases. However, few studies have tested its effects specifically in CAD patients. Fitó et al. evaluated the effect of daily supplementation of 50 mL/day of VOO and ROO in patients with stable CAD for two periods of 3 weeks. The oils used in the study had similar monounsaturated fatty acid content and differed mainly in phenolic content (161 vs. 14.7 mg/kg total; 0.15 vs. 0 mg of β-carotene; 8.73 vs. 5.99 mg of α-tocopherol; 6.53 vs. 0.62 mg of caffeic acid equivalents; respectively). Serum concentrations of sICAM-1, sVCAM-1, CRP, and IL-6 were evaluated. The effects on proinflammatory cytokines (IL-6) and inflammation markers (CRP) were observed only in the VOO group, with a reduction of 0.166mg/dL (95% CI −0.261–0.071; p = 0.002) and 0.063 mg/dL (95% CI −0.119–0.007, p = 0.024), respectively [18] (Table 2).

The main anti-inflammatory effects of olive oil phenolic compounds are briefly presented in Figure 1.

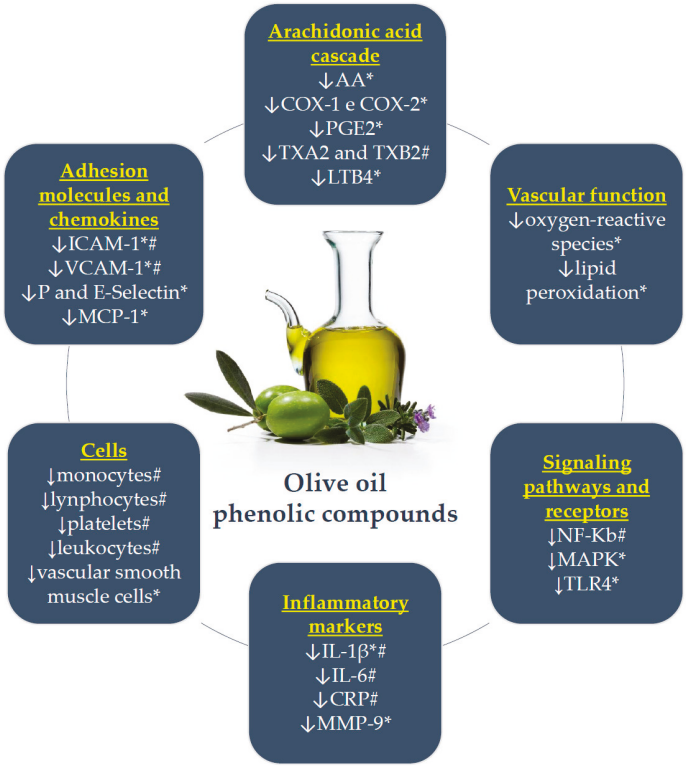

Figure 1. Main anti-inflammatory effects of olive oil phenolic compounds. *: in vitro or animal model; #: individuals at risk for CAD; ↓: decreases or inhibits; AA: arachidonic acid; COX: cyclooxygenase; PGE2: prostaglandins E2; TX: thromboxane; LTB4: leukotriene B4; NF-κB: nuclear factor kappa B; MAPK: mitogen-activated protein kinases; TLR: toll-like receptor; IL: interleukin; CRP: C-reactive protein; MMP-9: matrix metalloproteinase-9; ICAM-1: intercellular adhesion molecule-1; VCAM-1: vascular cell adhesion molecule-1; MCP-1: chemotactic monocyte protein-1.

7. Conclusions

The consumption of VOOs rich in phenolic compounds seems to favorably modulate inflammation, which contributes to the development and progression of CAD. In vitro and animal model studies have suggested mechanisms of action of these compounds in inflammatory activity at the cardiovascular level, including effects on the arachidonic acid cascade and on signaling pathways and receptors, improvement of vascular function, and reduction of adhesion molecules and chemokines. Moreover, these studies allow us to evaluate the isolated effects of VOO phenolic compounds.

Clinical trials conducted in individuals at risk for the development of cardiovascular diseases show positive effects of daily intake of different amounts of olive oil on inflammatory markers. The main findings of the randomized clinical trials included in this review reinforce the results found in in vitro and animal models. In humans, these effects were observed at the cell level (immune cells) and in inflammatory markers. A limitation for the discussion of the results is the great variation in the phenolic content of different types of VOOs. Furthermore, dietary supplementation with olive oil is associated with changes in dietary patterns as a whole, which may improve the inflammatory profile of patients at risk for CAD. It is also important to consider that dietary patterns, like MeDiet, include other sources of phenolic compounds.

As we have seen, the effects of olive oil and/or its phenolic compounds specifically on individuals with established CAD are still scarce. In this sense, more clinical trials, preferably long-term studies, are necessary to evaluate and confirm the beneficial effects of the phenolic compounds present in the olive oil on the inflammatory process, both in the prevention and treatment of CAD.

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Abbreviations

AA	arachidonic acid
CAD	coronary artery disease
COX	cyclooxygenase
CRP	C-reactive protein
EVOO	extra virgin olive oil
HDL	high density lipoprotein
HT	hydroxytyrosol
ICAM-1	intercellular adhesion molecule-1
IL	interleukin
INF-γ	interferon gamma
LDL	low density lipoprotein
LPS	lipopolysaccharides
LT	leukotriene
MCP-1	chemotactic monocyte protein
M-CSF	macrophage colony stimulating factor
MeDiet	mediterranean diet
MIF	macrophage migration inhibitory factor
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NCEP	National Cholesterol Education Program
NF-κB	nuclear factor kappa B
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
PG	prostaglandins

RCT	randomized clinical trial
ROO	refined olive oil
SFO	sunflower oil
sICAM-1	soluble intercellular-1-type adhesion molecule
SOD	mixture of oils (sunflower oil + canola oil)
sVCAM-1	soluble vascular cell adhesion molecule-1
Th	T helper cells
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha
TX	tromboxanes
VCAM-1	vascular cell adhesion molecule-1
VOO	virgin olive oil

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Review

Dietary Anthocyanins against Obesity and Inflammation

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Abstract: Chronic low-grade inflammation plays a pivotal role in the pathogenesis of obesity, due to its associated chronic diseases such as type II diabetes, cardiovascular diseases, pulmonary diseases and cancer. Thus, targeting inflammation is an attractive strategy to counter the burden of obesity-induced health problems. Recently, food-derived bioactive compounds have been spotlighted as a regulator against various chronic diseases due to their low toxicity, as opposed to drugs that induce severe side effects. Here we describe the beneficial effects of dietary anthocyanins on obesity-induced metabolic disorders and inflammation. Red cabbage microgreen, blueberry, blackcurrant, mulberry, cherry, black elderberry, black soybean, chokeberry and jaboticaba peel contain a variety of anthocyanins including cyanidins, delphinidins, malvidins, pelargonidins, peonidins and petunidins, and have been reported to alter both metabolic markers and inflammatory markers in cells, animals, and humans. This review discusses the interplay between inflammation and obesity, and their subsequent regulation via the use of dietary anthocyanins, suggesting an alternative dietary strategy to ameliorate obesity and obesity associated chronic diseases.

Keywords: obesity; inflammation; anthocyanin; flavonoids

1. Introduction

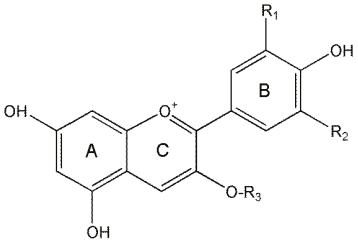
Obesity is accompanied by metabolic disturbances such as dyslipidemia, hyperglycemia and hypertension. The burden of obesity is largely derived from its associated chronic diseases such as cardiovascular diseases, pulmonary diseases, cancer, and type II diabetes mellitus [1,2]. Continuous effort has been made to understand the pathogenesis between obesity and chronic diseases. Interestingly, chronic low-grade inflammation has been linked to the progression of obesity and its related diseases [3,4].

Inflammation is a defense system that responds to harmful stimuli and restores disruptive tissues back to homeostasis [5]. Upon recognizing unfavorable stimuli, including excessive nutrients, immune cells urgently secrete a number of cytokines and chemokines to the damaged area, defined as innate immunity. If the condition is prolonged, antigen-presenting cells (APC) and B- and T lymphocytes perform adaptive immunity which may increase the prevalence of obesity-associated chronic diseases [6]. Thus, preventing this prolonged pro-inflammatory condition can be a valid strategy against obesity-associated metabolic disorders [7,8].

Although there are a number of drugs that are approved for the treatment of obese patients, many of them were withdrawn due to the severe adverse events such as heart diseases and psychiatric disorders [9]. Meanwhile, recent studies demonstrated that consumption of food-derived bioactive

components such as phenolic compounds is positively associated with reducing the risk of obesity and its associated chronic diseases with low toxicity [10,11]. Thus, creating new dietary treatments based on various bioactive components in food has been emerging as a new possible intervention against obesity [12,13].

Anthocyanins are an important subfamily of flavonoids, which are abundant in flowers, fruits, seeds and plant leaves [14]. The basic structural form 2-phenylchromenylium is further classified into six major compounds: cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin, depending on the flavylium B-ring. Sugars (glucose, arabinose, galactose, etc.) can be attached to the main structure of anthocyanins [12,15] (Figure 1). Because anthocyanins are commonly consumed, their biological activities have been extensively studied. Notably, anthocyanins have antimicrobial, antioxidative, anti-inflammatory, and anti-mutagenic properties, which in turn play a role on the prevention and treatment of many chronic diseases such as metabolic disorders, cancer, eye diseases and cardiovascular diseases [12,16,17]. In particular, mixtures of anthocyanins found in food rather than their individual anthocyanin components has been reported to be more beneficial for improving human health [18].



	R1	R2	R3
Cyanidin	OH	H	sugar
Malvidin	OCH_3	OCH_3	sugar
Peonidin	OCH_3	H	sugar
Delphinidin	OH	OH	sugar
Pelargonidin	H	H	sugar
Petunidin	OCH_3	OH	sugar

Figure 1. Structure of the most common anthocyanins.

Collectively, over the last two decades, the link between obesity and inflammation has been highlighted as a new axis to be targeted for intervention. Dietary bioactive components that have beneficial effects against both obesity and inflammation can potentially be a modulator of this axis. Here we discuss the impact of dietary anthocyanins on alleviating obesity and inflammation.

2. Metainflammation and Its Mechanism

Obesity is accompanied by a chronic low-grade inflammatory condition known as metainflammation [19,20]. Pro-inflammatory markers such as interleukin-6 (IL-6), interleukin-1β (IL-1β), C-reactive protein (CRP) and tumor necrosis factor-α (TNF-α) were reported to be persistently expressed in adipocytes of white adipose tissue [19]. It is interesting to note that pro-inflammatory markers were significantly higher in obese patients compared to healthy individuals. Furthermore, obese patients with high IL-6 levels also had increased CRP levels, which is a critical risk factor for cardiovascular diseases and type 2 diabetes [13,21]. In addition, M1 macrophages, T cells, B cells and monocyte chemoattractant protein-1 (MCP-1) were also increased in adipose tissue, whereas there was a reduction in the number of regulatory T cells, M2 macrophages and amount of adiponectin [22]. The possible underlying mechanism is that toll-like receptors (TLRs), in particular TLR4, recognize external stimuli including over-nutrition, and connect them to transcription factors such as nuclear factor Kappa B (NF-κB), activated protein-1 (AP-1) and interferon regulatory factor 3 (IRF3). These inflammation-associated transcription factors enter into the nucleus and bind to the site of target genes that promote inflammation [23]. Due to the coordination between the immune system and metabolism, developing novel immunotherapeutic strategies to simultaneously attenuate obesity-related metabolic disorders seems promising. Indeed, targeting IL-1β, IL-1β receptor, TNF-α, TLRs, T and B lymphocytes were effective in reducing blood glucose levels, insulin resistance and other metabolic markers in

obesity [7,8]. Flavonoids have also been assessed to regulate inflammation and obesity by lowering IL-1α, and IFN-γ compared to control mice [24]. These efforts have diversely been attempted using cell, animal and human models [25].

3. Establishment of an Obesity Model

Cells and animals are essential surrogates for human obesity due to the limitations of promising human studies. In this section, we introduce frequently used cell and animal models of human obesity (Table 1).

Table 1. Frequently used obesity model in vitro and in vivo.

			Advantage	Disadvantage
In vitro	mouse	mesenchymal stem cells embryonic stem cells primary preadipocytes	• easy to study mechanism, • well-established method	• different characteristics of human vs. mouse
	human	preadipocytes adipose-derived stem cells	• easy to study mechanism, • representation of human obesity	• complement of mouse cell lines' disadvantages
In vivo	monogenic	ob/ob mice Zucker fatty rats	• apparent symptoms of metabolic disorders	• monogenic mutation is rare in humans
	polygenic	High energy diets-fed mice Diet-induced obesity	• resemble complexities of human obesity	• complement of monogenic models' disadvantages

Fat accumulation in adipose tissue is critical for obesity. Adipose tissue is composed of adipocytes, macrophages, mesenchymal stem cells, blood cells, fibroblasts, pericytes, and smooth muscle cells [26]. Cells are important in order to identify the mechanism of key adipogenic process. Mouse cell lines to study adipogenesis can be classified into three categories: mesenchymal stem cells, embryonic cells, and primary preadipocytes [27]. Among them, 3T3-L1 is the most frequently used cell line, due to its well-established methodology for mature adipocytes. When adipogenic stimuli such as insulin (1 to 10 μg/mL), dexamethasone, (1 μM) and 3-isobutyl-1-methylxanthine (IBMX) (0.5 mM) are added to medium containing 10% fetal bovine serum, several transcriptional factors such as peroxisome proliferator-activated receptor gamma (PPAR-γ), CCAAT/enhancer-binding proteins (C/EBPs) and sterol regulatory element binding (SREBP) promote adipocyte fate, resulting in fat accumulation in mature adipocyte within 14 days [28]. Mouse cell models can be easily applied to study adipogenesis in obesity, but there are some metabolic differences with human adipocytes [29]. Therefore, human preadipocyte and adipose-derived stem cells were developed, which also require adipogenic stimuli (Insulin, dexamethasone, IBMX, biotin, rosiglitazone) for differentiation to mature adipocytes by transcriptional cascades within 12–14 days [30]. Recent studies of obesity in vitro have utilized not only the above described cell models but also utilized co-culture and three dimensional culture systems [31].

Obese animals can be categorized into two groups, monogenic and polygenic models [32]. In monogenic models, ob/ob mice are C57BL/6 that spontaneously become obese due to a mutation in the leptin gene responsible for controlling appetite [33,34]. Zucker rats are a monogenic obese model where the rats are autosomal recessive for a mutation in leptin receptors, and consequently exhibit metabolic symptoms such as hyperglycemia and insulin resistance [35]. Despite the metabolic disorder phenotype displayed in these monogenic models, these models are limited in their representation of human obesity since they are induced via a mutation of a gene [36]. Diet-induced obesity models have been alternatively suggested to overcome this limitation. These animals are instead fed with high energy diets such as high fat (45% or 60% kcal energy from fat as per difference of lard contents),

high-fructose and high-cholesterol diets. C57BL/6 and A/J mice are commonly used [37]. Wistar, Sprague-Dawley, Long Evans and Osborne mendel rat species are commonly used as high energy diet-induced obese rat model [38]. These polygenic models developed by high energy diets have shown the characteristic symptoms of metabolic disorders such as glucose intolerance and upregulation of cholesterol and triglycerides in the plasma of animals [32]. Diet-induced obese rats are another polygenic model where rats have been bred selectively through several generations to become obese without high energy diet supplementation [39]. These valid models may lead us to predict the mechanism and efficacy of target molecules for human obesity.

4. Biological Functions of Anthocyanins

Anthocyanins are well-known antioxidants that eliminate reactive oxygen species (ROS). It has been proven that anthocyanins, in particular cyanidin-3-glucoside, has great oxygen radical absorbance capacity (ORAC) in vitro [40]. Delphinidin has been revealed as the most active scavenger against superoxide anion [41]. Furthermore various reports have shown that anthocyanin has protective effects against oxidative stress in cell lines [42–44]. Several studies have also demonstrated the antioxidant functions of anthocyanins in vivo. Cyanidin-3-glucoside improved oxidative stress-induced hepatic ischemia-reperfusion in rats [45]. Cyanidin, delphinidin, and malvidin induced antioxidant enzymes have been reported to induce upregulation of antioxidant response element (ARE) pathways [46].

Numerous studies have demonstrated the anti-inflammatory effects of anthocyanins as well. Cyanidin-3-glucoside, delphinidin-3-glucoside and petunidin-3-glucoside inhibited NF-κB activities via mitogen activated protein kinase (MAPK) pathways [47–49], and cyanidins inhibited cyclooxygenase enzyme activities [50]. Additionally, cyanidin-3-glucoside had effects on reducing lung inflammation in rats [51].

These biological activities of anthocyanins are closely related to the incidence of chronic diseases. Indeed, anthocyanins reduced risks factors for cardiovascular diseases [17] and suppressed cell growth in various cancer cell lines, indicating that anthocyanins have anticancer properties [52]. Consumption of anthocyanins reduced body weight and insulin resistance, leading to restored glucose tolerance [53,54].

5. Bioavailability of Anthocyanins

Most bioactive compounds which are well-known for their great health benefits are generally ingested by food, the study of food as a mixture of its bioactive compounds is more relevant. Supplementation of anthocyanin mixtures reduced inflammatory markers in hypercholesterol subjects rather than individual anthocyanins [18]. Combinations of blackberries and raspberries, for instance, have synergistic antioxidant capacity [55].

Besides the total content of bioactive compounds in food, bioavailability including metabolism and excretion is critical as well. Although anthocyanins have been reported to have low bioavailability after evaluating plasma levels after ingestion of anthocyanin-rich food, recent studies have started to focus on anthocyanin metabolites which were not able to be detected previously, using traditional methods. Interestingly, bioavailability of anthocyanin metabolites was reported to be higher than that of parent anthocyanins by 42-fold [56]. Newly identified anthocyanin metabolites contribute to the improvement of human health [57].

Furthermore, various attempts have been made to improve the bioavailability of flavonoids including anthocyanins using a novel delivery system. Nanoparticles, encapsulation of food, liposomes and gel emersions have been studied to enhance intestinal absorption of food [58]. Encapsulated anthocyanins in liposomes and alginate/chitosan microencapsulation of anthocyanins were valuable for increasing bioefficacy, suggesting application of anthocyanins on nutraceutical development [59,60].

6. Studies of Dietary Anthocyanins on Regulation of Inflammation and Obesity in Various Models

Table 2 presents the beneficial effects of dietary anthocyanins on obesity-related metabolic markers and inflammatory markers in cells and animals. Because there are many studies with respect to the effects of anthocyanins on obesity and inflammation, we look at studies of dietary anthocyanins which are clearly defined for their bioactive compounds on metabolism and inflammation in specific models.

It is difficult to demonstrate anti-inflammatory and anti-adipogenic effects in the same cell line due to each cell's different characteristics. One study reported that purple sweet potato extract exerted antilipogenic activities by suppression of adipogenic enzymes and transcription factors, and had an anti-inflammatory effect by down-regulating COX-2, IL-6 and MCP-1 in 3T3-L1 mouse adipocytes [61].

Red cabbage microgreen decreased weight gain, low density lipoprotein (LDL) levels, triacylglycerol, and cholesterol levels in high fat diet-fed mice. Inflammatory cytokines such as CRP and TNF-α were also significantly diminished in mice [62]. Since blueberries contain a distinct amount of anthocyanins, this has attracted several research studies on their health benefits. High fat diets containing blueberry-fed mice have shown reduced body weight and blood glucose levels as well as TNF-α and IL-6 levels than that of high fat diet-fed control mice [63]. In addition, another study demonstrated that whole blueberry improved high fat diet-induced insulin resistance, and decreased TNF-α, IL-6, MCP-1, CD11c+, and inducible nitric oxide synthase (iNOS) [64]. Blueberry juice was also effective in reducing body weight, insulin and leptin levels in serum, cholesterol and triacylglycerol in the liver, and inflammatory markers such as TNF-α, IL-6, iNOS, and NF-κB were decreased in the epididymal adipose tissue of high fat diet-fed mice. This efficacy was also similarly observed in mulberry juice-supplemented obese mice [65]. Other anthocyanin-rich blackcurrant [66], mulberry, cherry [67], black elderberry [68], black soybean [69], freeze-dried jaboticaba peel [70] lowered fat accumulation, triacylglycerol, cholesterol levels in blood serum or liver in high fat diet-fed mice. Rats ingesting fructose rich diets appeared to become obese. However, chokeberry extract was added to the drinking water of the rats, leading to improved metabolic disturbance and inflammation [71]. In addition, tart cherry reduced metabolic and inflammatory factors in Zucker fatty rats [72].

Several clinical studies have also been conducted to evaluate biological functions of dietary anthocyanins on inflammation and obesity, as shown in Table 3. Due to the lack of clinical studies, studies without characterization of anthocyanins were also included in the list. Human subjects whose BMI (body mass index) is over 23 or have a waist circumference over 90 cm (over 85 cm in case of female) were allocated to either placebo or black soybean extract-administered groups. This randomized, double-blinded, clinical study showed reduction in abdominal fat, cholesterol, triacylglycerol and LDL levels, with decreasing TNF-α and MCP-1 levels [73]. Red orange juice was also effective in reducing metabolic markers and inflammatory markers in human subjects.

Table 2. Effects of dietary anthocyanins on obesity and inflammation (cell and animal study).

Food Sources	Identified Bioactive Dose of Anthocyanins	Mediators	Inducer	Metabolic Marker	Inflammatory Marker	Ref.
Purple sweet potato	cyanidnin (3-caffeylferulysophoroside-5-glucoside) peonidin (3-caffeylferulysophoroside-5-glucoside) Dose: 4.28 μg/mL to 12.84 μg/mL [a]	3T3-L1	Stimuli vs. undifferentiated cells	leptin ↓ adipogenic factors ↓	COX-2 ↓ MCP-1 ↓ IL-6 ↓	[61]
Red cabbage microgreen	cyanidin (3-diglucoside-5-glucoside) cyanidin (3-(sinapoyl)-diglucoside-5-glucosides) cyanidin (3-(glucosyl)(sinapoyl)(p-coumaroyl)sophorside-5-glucoside) cyanidin (3-(glucosyl)(sinapoyl)(feruloyl)sophorside-5-glucoside) cyanidin (3-diferuloylsophoroside-5-glucoside) cyanidin (3-(coumaroyl)sophoroside-5-glucoside) cyanidin (3-(feruloyl)sophoroside-5-glucoside) cyanidin (3-diferuloylsophoroside-5-glucoside) cyanidin (3-(sinapoyl)(feruloyl)sophoroside-5-glucoside) cyanidin (3-(sinapoyl)(sinapoyl)sophoroside-5-glucoside) Dose: 139.596 nmol/g [b]	mice (C57BL/6)	high fat-diet vs. normal-diet for 8 weeks	LDL ↓ cholesterol ↓ TG ↓	CRP ↓ TNF-α ↓	[62]
Blueberry	delphinidins cyanidins peonidins malvidins Dose: 1.29 mg/g [b]	mice (C57BL/6)	high fat-diet vs. normal diet for 8 weeks	glucose ↓	TNF-α ↓ IL-6 ↓ MCP-1 ↓ iNOS ↓ IL-10 ↑ CD11c+	[64]

Table 2. *Cont.*

Food Sources	Identified Bioactive Dose of Anthocyanins	Mediators	Inducer	Metabolic Marker	Inflammatory Marker	Ref.
Blueberry	cyanidin (3-galactoside) cyanidin (3-arabinoside) delphinidin (3-arabinoside) delphinidin (3-galactoside) petunidin (3-glucoside) petunidin (3-arabinoside) malvidin (3-galactoside) malvidin (3-glucoside) Dose: 50 to 200 μg/g [b]	mice (C57BL/6)	high fat-diet vs. normal diet for 8 weeks	glucose ↓ TG ↓ cholesterol ↓ insulin ↓ leptin ↓	TNF-α ↓ IL-6 ↓	[63]
Black elderberry	cyanidin (3-glucoside) cyanidin (3-sambubioside) Dose: 3.334, 1.7 μg/g [b]	mice (C57BL/6)	high fat-diet vs. normal diet for 16 weeks	TG ↓ insulin ↓ cholesterol ↓	MCP-1 ↓ TNF-α ↓	[68]
Blackcurrant	delphinidin (3-glucoside) delphinidin (3-o-rutinoside) cyanidin (3-glucoside) cyanidin (3-rutinoside) Dose: 298.1 μg/g [b]	mice (C57BL/6)	high fat/cholesterol-diet vs. normal diet for 12 weeks	adipogenic genes ↓	TNF-α ↓ IL-6 ↓ IL-1β ↓	[66]
Mulberry	cyanidin (3-glucoside) cyanidin (3-rutinoside) pelarginidin (3-glucose) Dose: 200 μg/g [b]	mice (C57BL/6)	high fat-diet vs. normal diet for 16 weeks	glucose ↓ leptin ↓	TNF-α ↓ IL-6 ↓ iNOS ↓ NF-κB ↓	[67]

Table 2. *Cont.*

Food Sources	Identified Bioactive Dose of Anthocyanins	Mediators	Inducer	Metabolic Marker	Inflammatory Marker	Ref.
Cherry	cyanidin (3-2G-glucosylrutinoside) cyanidin (3-rutinoside) pelarginidin (3-rutinoside) Dose: 200 μg/g [b]	mice (C57BL/6)	high fat-diet vs. normal diet for 16 weeks	glucose ↓ leptin ↓	TNF-α ↓ IL-6 ↓ iNOS ↓ NF-κB ↓	[67]
Blueberry juice	cyanidin (3-galactoside) cyanidin (3-arabinoside) delphinidin (3-glucoside) delphinidin (3-galactoside) delphinidin (3-arabinoside) petunidin (3-glucoside) petunidin (3-arabinoside) malvidin (3-galactoside) malvidin (3-glucoside) Dose: 4.09 mg/mL [c]	mice (C57BL/6)	high fat-diet vs. normal diet for 12 weeks	leptin ↓ cholesterol ↓ adiponectin ↑ TG ↓	TNF-α ↓ IL-6 ↓	[65]
Mulberry juice	cyanidin (3-glucoside) cyanidin (3-rutinoside) pelargonidin (3-glucoside) pelargonidin (3-rutinoside) Dose: 21.86 mg/mL [c]	mice (C57BL/6)	high fat-diet vs. normal diet for 12 weeks	leptin ↓ adiponectin ↑	TNF-α ↓ IL-6 ↓	[65]

Table 2. *Cont.*

Food Sources	Identified Bioactive Dose of Anthocyanins	Mediators	Inducer	Metabolic Marker	Inflammatory Marker	Ref.
Black soybean	delphinidin (3-glucoside) cyanidin (3-glucoside) petunidin (3-glucoside) pelargonodin (3-glucoside) peonidin (3-glucoside) Dose: 12.48 mg/g [b]	mice (C57BL/6)	high fat-diet vs. normal diet for 12 weeks	TG ↓ cholesterol ↓	TNF-α ↓ IL-6 ↓ IL-10↑	[69]
Jaboticaba peel	delphinidin (3- *O*-glycoside) cyanidin (3- *O*-glycoside) Dose: 259.9, 519.8, 1039.6 μg/g [b]	swiss inbred mice	high fat-diet vs. normal diet for 6 weeks	insulin ↓	IL-6 ↓ IL-1β ↓	[70]
Chokeberry	total anthocyanin Dose: 10 or 20 mg/kg [d]	Wistar rat	fructose-rich diet vs. normal diet for 6 weeks	glucose ↓ insulin ↓ TG ↓ cholesterol ↓	TNF-α ↓ IL-6 ↓	[71]
Tart cherry	cyanidin (3-sophoroside) cyanidin (3-glucosylrutinoside) cyanidin- (3-glucoside) cyanidin (3-rutinoside) peonidin (3-glucoside) Pelargonidin Dose: 0.6598 mg/g [b]	zucker fatty rats	Spontaneously obese for 90 days	glucose ↓ insulin ↓ cholesterol ↓ TG ↓	TNF-α ↓ IL-6 ↓	[72]

[a]: cyanidin concentration of extract, [b]: the amount of anthocyanidins per weight of diet, [c]: the amount of anthocyanidins per volume of diet, [d]: the amount of anthocyanidin per body weight. CRP: C-reactive protein.

Table 3. Effects of dietary anthocyanins on obesity and inflammation (clinical study).

Food Sources	Bioactives Dose of Anthocyanins	Subject Duration	Metabolic Marker	Inflammatory Marker	Ref.
Black soybean	cyanidin (3-glucosides) delphinidin (3-glucoside) petunidin (3-glucoside) 31.48 mg/day	BMI > 23 WC > 90 for male WC > 85 for female For 8 weeks	TG ↓ cholesterol ↓ LDL ↓	TNF-α ↓ MCP-1 ↓	[73]
Red orange juice	Anthocyanin mixture 250 mg/day	average BMI = 34.4 ± 4.8 for 12 weeks	Δ leptin ↓ Δ adiponectin ↓	CRP ↓ TNF-α ↓	[74]
Red-fleshed sweet orange juice	anthocyanin mixture 750 mL/day	age 23–59 BMI 18.5–24.986.4/74.6	cholesterol ↓	CRP↓	[75]

BMI: body mass index; WC: waist circumstance; CRP: C-reactive protein; Δ: incremental change.

7. Summary

Previously, obesity was regarded as just a matter of excessive energy storage triggered by energy imbalance, however many studies have indicated that chronic low-grade inflammation in adipose tissue can be an important issue in obesity. In particular, this metainflammation could enable pathogenesis of chronic diseases associated with obesity. Anthocyanins have great biological activities and low toxicity in vivo, therefore many scientists are interested in the health benefits of anthocyanins, as well as their application in preventing and treating chronic diseases, including obesity. Here, we describe the positive effects of dietary anthocyanins limited to their well-defined components against metabolic and inflammatory markers in cell, animal and human obesity models. In addition, anthocyanin mixtures found in food such as red cabbage microgreen, blueberry, blackcurrant, mulberry, cherry, black elderberry, black soybean, chokeberry and jaboticaba peel (in whole or extract) interestingly had higher clinical efficacy than single anthocyanins.

8. Conclusions

We reviewed the effects of anthocyanins-rich food on attenuating obesity and inflammation in cells, animals, and humans. Taken together, dietary anthocyanins may be a potential regulator of obesity-derived inflammation and its associated chronic diseases, as presented in Figure 2.

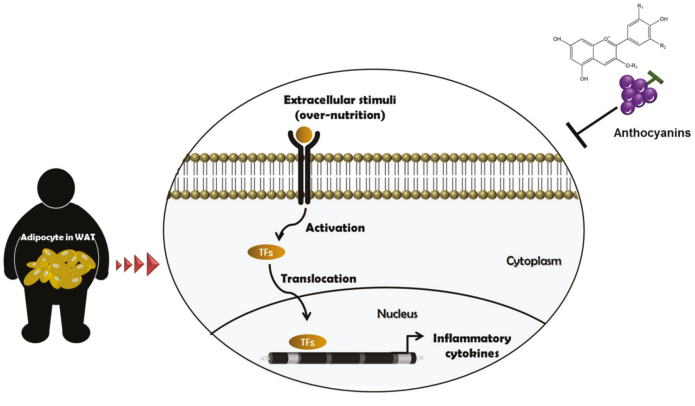


Figure 2. Beneficial effects of anthocyanins on obesity and inflammation. When receptors recognize the status of over-nutrition, they activate various transcription factors such as NF-κB, IRF-3 and AP-1 for translocation into nucleus and bind to the promoter region of target genes. Eventually, inflammatory cytokines are expressed resulting in chronic inflammatory conditions in adipocytes of WAT. Dietary anthocyanins may have preventive effects on these events, leading to health benefits.

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Abbreviation

TG	triacylglycerol
LDL	low density lipoprotein
COX-2	cyclooxygenase-2
TNF-α	tumor necrosis factor-α
IL-6	interleukin-6
IL-1β	interleukin-1β
IL-10	interleukin-10
iNOS	inducible nitric synthase
MCP-1	monocyte chemoattactant protein-1
NF-κB	nuclear factor kappa B
BMI	body mass index
CRP	c-reactive protein
WC	waist circumstance
IRF-3	interferon regulatory factor 3
AP-1	activated protein-1
WAT	white adipose tissue

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Chinese Olive (*Canarium album* L.) Fruit Extract Attenuates Metabolic Dysfunction in Diabetic Rats

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Abstract: Hyperglycemia and dysregulation of lipid metabolism play a crucial role in metabolic dysfunction. The aims of present study were to evaluate the ameliorative effect of the ethyl acetate fraction of Chinese olive fruit extract (CO-EtOAc) on high-fat diet (HFD) and streptozotocin (STZ)-induced diabetic rats. CO-EtOAc, rich in gallic acid and ellagic acid, could markedly decreased the body weight and epididymal adipose mass. In addition, CO-EtOAc increased serum HDL-C levels, hepatic GSH levels, and antioxidant enzyme activities; lowered blood glucose, serum levels of total cholesterol (TC), triglycerides (TG), bile acid, and tumor necrosis factor alpha (TNFα); and reduced TC and TG in liver. We further demonstrated that CO-EtOAc mildly suppressed hepatic levels of phosphorylated IRS-1, TNF-α, and IL-6, but enhanced Akt phosphorylation. The possible mechanisms of cholesterol metabolism were assessed by determining the expression of genes involved in cholesterol transportation, biosynthesis, and degradation. It was found that CO-EtOAc not only inhibited mRNA levels of *SREBP-2*, *HMG-CoAR*, *SR-B1*, and *CYP7A1* but also increased the expression of genes, such as *ABCA1* and *LDLR* that governed cholesterol efflux and cholesterol uptake. Moreover, the protein expressions of ABCA1 and LDLR were also significantly increased in the liver of rats supplemented with CO-EtOAc. We suggest that Chinese olive fruit may ameliorate metabolic dysfunction in diabetic rats under HFD challenge.

Keywords: Chinese olive fruit; high-fat diet; hyperglycemia; metabolic dysfunction; antioxidant activities; proinflammatory cytokines

1. Introduction

Diabetes mellitus (DM) is one of the most common endocrine disorders and the third leading cause of death in developed countries [1]. Type 2 diabetes mellitus (T2DM) accounts for about 90% to 95% of all diagnosed cases of diabetes [2]. Accumulating evidence indicates that obesity contributes to down-regulation of insulin secretion, defecting insulin action or both [3,4], which may disturb carbohydrate and lipid homeostasis [5]. Moreover, T2DM is also associated with the chronic inflammatory state [6]. Until now, among traditional anti-diabetic drugs, metformin is the most used in clinical therapy, but the side effects such as lactic acidosis and permanent nerve damage, limit its application in certain populations [7]. Understanding the molecular mechanism underlying T2DM would definitely facilitate the development of treatment for T2DM [8].

Previous studies showed that obesity-induced diabetes results from defective insulin receptor-mediated signaling, which includes insulin receptor substrate 1 (IRS-1) and protein kinase B (Akt) [9], and is also associated with increased inflammatory response [10]. Dysregulation of inflammatory molecules and lipid profiles usually occurs in the insulin-resistant state of T2DM [11]. Additionally, oxidative stress also leads to insulin resistance and the pathophysiological outcomes of T2DM, such as

β-cell dysfunction and impaired glucose tolerance [12]. Antioxidant enzymes possess the ability to scavenge endogenous free radicals and thus reduce the deleterious consequences that affect glucose and lipid metabolism in vivo [13]. Consistent to this idea, reduced glutathione (GSH) or anti-oxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), exhibit the ability to ameliorate the pathophysiological problems of diabetes [14,15].

Extensive dysregulation of lipid metabolism and cholesterol homeostasis occurs in both obesity and T2DM [16]. Sterol regulatory element-binding protein-2 (SREBP-2) is a transcription factor involved in the regulation of endogenous cholesterol synthesis in the liver [17]. The hepatic enzyme 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoAR) is a downstream target of SREBP-2, responsible for the regulation of cholesterol synthesis. As for the clearance of cholesterol, low-density lipoprotein receptor (LDLR) plays a critical role in uptake of circulating LDL in peripheral tissues, whereas scavenger receptor class B type 1 (SR-B1) uptakes high-density lipoprotein cholesterol (HDL-C) from peripheral tissues to the liver via circulation [18]. Furthermore, adenosine triphosphate-binding cassette (ABC) transporters are abundantly expressed in multiple organs and provide a major function in the regulation of cholesterol homeostasis. ABCA1 is probably the most prominent member of the ABC superfamily and it is crucial for HDL-C formation and works as a cellular efflux transporter of cholesterol and lipids in the liver. Cholesterol can also be removed from hepatocytes by conversion to bile acids through the enzymes, cholesterol 7α-Hydroxylase (CYP7A1) and sterol 27-hydroxylase (CYP27A1). In the liver, ABCG5/G8 transporter is responsible for the efflux of hepatic cholesterol into the bile and subsequently increases the cholesterol metabolite secretion into feces. Thus, overexpression of ABCA1 or ABCG5/G8 in the liver can lower hepatic cholesterol [18].

Epidemiological studies suggest that polyphenols could offer strong protection against metabolic disorders through their anti-oxidative functions [19]. Chinese herbs contain a rich source of bioactive phytochemicals, especially polyphenols and flavonoids, which may provide antioxidant and anti-inflammatory functions as well as hypoglycemic and hypolipidemic potential [20]. It has been reported that Chinese olive (*Canarium album* L.) shows high phenolics content, strong antioxidant capacity and potent free radical-scavenging ability [21]. Chinese olive is a tropical and semi-tropical fruit of the family Burseraceae, is widely cultivated in Taiwan, the southeast China and other regions of Asia. Moreover, it is widely used for the treatment of faucitis, stomatitis, hepatitis and toxicosis [22]. Recent studies have shown that Chinese olive and its related phenolic compounds have a wide spectrum of clinical applications such as in anti-tumor growth, diabetes, and inflammation [21,23]. However, the molecular mechanisms of Chinese olive remain largely unknown. In the present study, we generated a rat model with the combination of high-fat diet (HFD) treatment and low-dose streptozotocin (STZ) injection. This animal model could mimic human type 2 diabetes mellitus that provides the phenotypes of hyperlipidemia, hyperglycemia and increased body weight [24]. Our aim was to evaluate whether treatment with ethyl acetate fraction of Chinese olive fruit extract (CO-EtOAc) had the potential to improve the metabolic abnormalities associated with diabetes under high fat and STZ challenge. Ellagic acid and gallic acid are the main compounds in CO-EtOAc, which may regulate lipid and glucose metabolic pathways. Our findings suggest that Chinese olive is a potential therapeutic herb for the treatment of metabolic disorders.

2. Materials and Methods

2.1. Preparation of Chinese Olive Fruit Extract

Chinese olive fruits were obtained from Baoshan Township, Hsinchu County, Taiwan. The olive fruit extract was prepared by the standard procedure as described [25]. In brief, olive fruit was extracted with 100% methanol and re-suspended with water in a ratio of 1:10 (v/v) and then partitioned with *N*-hexane, ethyl acetate, and *N*-butanol. The CO-EtOAc was stored at −20 °C until use and the same batch of Chinese olive fruit extract was used throughout this study. Composition of CO-EtOAc was determined using high-performance liquid chromatography (HPLC) analysis with a Photodiode Array

Detector (PDA, Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was conducted using a 250 × 4.6 mm (i.d.), 5 μm, C-18 reversed-phase column (MACHEREY-NAGEL, Dueren, Germany). Gradient elution was performed with 0.5% (*v*/*v*) acetic acid (solvent A) and methanol (solvent B) at a constant flow rate of 0.6 mL min^{-1}. The linear gradient profile was as followings: 94% A and 6% B at the start, 38% A and 62% B at 30 min, and 10% A and 90% B at 60 min. UV-Vis absorption spectra were recorded on-line at 200 and 600 nm during HPLC analysis.

2.2. Animals and Diets

Six-week-old male Sprague-Dawley rats (initial weights 130 ± 10 g) were obtained from BioLASCO, Taipei, Taiwan. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the National Taiwan University, Taipei, Taiwan, Republic of China. Rats were randomly divided into four groups: (1) control group; (2) diabetic control (DC); (3) DC + CO-EtOAc (50 mg/kg body weight); and (4) DC + CO-EtOAc (150 mg/kg body weight). The rats besides the control group were fed with high-fat diet (HFD, 60% kcal fat), whereas rats of control group were fed with normal chow diet (12.6% kcal fat) during the whole experimental period. The animals were housed in a room at temperature of 23 ± 2 °C, relative humidity of 55 ± 5% and a 12 h light/dark cycle. In Week 2, HFD-fed rats were treated intragastrically with CO-EtOAc (dissolved in 0.5% carboxymethyl cellulose, 50 or 150 mg/kg/day), control animals were also gavaged with 0.5% carboxymethyl cellulose and this treatment continued daily for eight weeks (n = 10 in each group). To establish a diabetic animal model, the rats were treated with HFD combined with 35 mg/kg STZ (streptozotocin, Sigma-Aldrich, Inc., St. Louis, MO, USA), a compound that is particularly toxic to the insulin-producing beta cells following the modified method described by Srinivasan et al. [26]. In Week 7, experimental rats excluding control group were given a single intraperitoneal injection of STZ. All of the rats in DC group have been verified to be at hyperglycemic stage with fasting blood glucose levels higher than 300 mg/dL (16.7 mmol/L) after STZ administration for 72 h [27]. During the eight-week experimental period, rats were free to access food and water. Animals were sacrificed by CO_2 anesthesia, and serum was collected for biochemical analysis. The liver was dissected and stored at −80 °C for further experiments.

2.3. Biochemical Analysis

Fasting blood samples were collected from the tail vein of the rats after starvation for 12 h. Fasting blood glucose (FBG) levels were determined by the glucose analyzer (Eumed Biotechnology Co., Ltd., Hsinchu, Taiwan). At the end of the experiment, serum samples were collected by cardiac puncture and centrifugation at 1500 g for 10 min. Levels of total cholesterol (TC), triglyceride (TG), HDL-cholesterol (HDL-C), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured by an automatic chemistry analyzer (SPOTCHEM EZ SP-4430, ARKRAY, Inc., Kyoto, Japan). Serum samples were applied into the strips for determination of HDL-C levels. After precipitation of LDL and VLDL fractions by reacting with the polyethylene glycol reagent, cholesterol ester of the HDL fraction was then catalyzed by cholesterol esterase to produce cholesterol and subsequently reacted with cholesterol oxidase and peroxidase to produce a final product of quinoneimine. HDL-C levels were then determined in the chemical analyzer. Insulin levels were determined using commercial kits (Mercodia AB, Uppsala, Sweden). Serum bile acid and TNF-α levels were determined using kits according to the manufacturer's instructions (Cayman, Ann Arbor, MI, USA).

2.4. Quantification of Hepatic Triglyceride and Cholesterol Levels

Lipids were extracted from the liver tissue (1.5 g) according to the method described by Folch et al. [28]. Briefly, total lipids were extracted from the liver samples by homogenizing the tissues using a mixed 8:4:3 chloroform/methanol/0.9% NaCl (*v*/*v*/*v*) to a final dilution of 20-fold original volume of the tissue sample. The organic layer was then separated, evaporated, and reconstituted in

chloroform. The values of TG and TC were measured using a colorimetric assay kit (Sigma-Aldrich, Los Angeles, CA, USA) and the results were expressed as mg per gram of the liver weight.

2.5. Measurement of Hepatic Antioxidant Status and TBARS Levels

Hepatic levels of reduced glutathione (GSH) and activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were determined in the extract of liver homogenates using commercial kits ((Cayman, Ann Arbor, MI, USA). Briefly, SOD activities were measured by detecting the reduction of a tetrazolium salt at 450 nm, which was a superoxide radical generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. This assay measures all three types of SOD (Cu/Zn, Mn, and FeSOD). Glutathione peroxidase (GPx) activity was quantified by a coupled assay with glutathione reductase (GR)-catalyzed oxidation of NADPH. The oxidized glutathione (GSSG) formed after reduction of hydroperoxide by GPx is recycled to its reduced state by GR in the presence of NADPH. Measurements were made at 340 nm and expressed in μmol/min per mg protein. The GSH level was assessed using the protocol for the glutathione assay kit, which contains GSH reductase, 5,5′-dithio-bis-2-nitrobenzoic acid and Ellman's reagent. The 5-thio-2-nitrobenzoic acid (TNB) generated after reaction between the sulfhydryl group of GSH and 5,5′-dithio-bis-2-nitrobenzoic acid can be measured at 405 nm using a microplate reader. Catalase activity was detected in the presence of hydrogen peroxide. The formaldehyde produced was measured with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole and the absorbance was monitored at 540 nm. The level of lipid peroxidation was assayed by determining the production of thiobarbituric acid reactive substances (TBARS). In brief, 0.5 g liver tissues were homogenized in 1% KCl (*w*/*v*). The homogenized solution was mixed with the TBA reagents (20% trichloroacetic acid and 1% butylated hydroxytoluene and incubated at 90 °C for 20 min and then stopped the reaction on ice. After cooling to room temperature, a mixture of solvents *N*-butanol and pyridine (15:1; *v*/*v*) was added. The mixture was mixed thoroughly and separated by centrifugation at 3000 g for 5 min. The organic layer was taken out and TBARS level was measured at 535 nm using a standard curve of thiobarbituric acid (TBA) adduct formation with freshly diluted 1,1,3,3-tetraethoxypropane.

2.6. Histological Analysis

The right medial lobes of rat liver tissues were fixed in 10% neutral formalin, embedded in 100% paraffin, and cut to 4–5 μm-thick sections. Each section was deparaffinized by incubating in xylene, hydrated in a descending series of ethanol (100%, 95%, 80%, and 70%), and washed with distilled water. Afterwards, each section was rapidly dehydrated in an increasing series of ethanol (70%, 95%, and 100%). Finally, the sections were treated with xylene and mounted on glass slides, stained with hematoxylin and eosin (H&E) and examined by a light microscope under 200× magnification. The score counting of histological analyses were following the criteria described by Garcimartin et al. [29]. Interpretation of the scores was following the definition provided by Kleiner et al. [30].

2.7. Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted using TRIZOL reagent (Invitrogen Corp., Carlsbad, CA, USA), following the manufacturer's instructions. The purified RNA (2 μg) was treated with RNase-free DNase I (Invitrogen), and then reverse-transcribed with oligo (dT) primer using the SuperScript First-Strand Synthesis System (Invitrogen) to generate cDNA. The qPCR reaction mixture consisted of 10 μL total volume solution containing 5 μL of 2X KAPA SYBR®FAST qPCR Master Mix ABI Prism™, 0.2 μL of 10 μM of each primer, 10 ng cDNA template and distilled water. The reactions were carried out on a StepOnePlus™ Real-Time PCR Systems (Thermo Scientific, Waltham, MA, USA). The primer sequence of each gene is shown in Table 1. The relative copy number was calculated using the threshold crossing point (Ct) as calculated using the $2^{-\Delta\Delta Ct}$ method [31] and the relative gene expression was normalized to the amount of GAPDH rRNA.

Table 1. Primers used for Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR).

Genes	Sequence of Forward and Reverse Primers (5′ to 3′)	Annealing Temperature (°C)	Accession No.
SREBP-2	FP: AGACTTGGTCATGGGGACAG RP:GGGGAGACATCAGAAGGACA	60 °C	NM_001033694
HMG-CoAR	FP: CCCAGCCTACAAACTGGAAA RP:CCATTGGCACCTGGTACTCT	55 °C	NM_013134
LDLR	FP: CAGCTCTGTGTGAACCTGGA RP:TTCTTCAGGTTGGGGATCAG	55 °C	NM_175762
SR-B1	FP: TGCCCCAGGTTCTTCACTAC RP:CCCTACAGCTTGGCTTCTTG	60 °C	NM_031541
ABCA1	FP: GTACCCAGCGTCCTTTGTGT RP:CCCAAGAGAGTGGAGAGACG	58 °C	NM_178095
ABCG1	FP: CTGCAAGAGAGGGATGAAGG RP:ACAGGAGGGTTGTTGACCAG	58 °C	NM_178095
CYP7A1	FP: CACCATTCCTGCAACCTTTT RP:GTACCGGCAGGTCATTCAGT	60 °C	NM_012942
TNF-α	FP: AAATGGGCTCCCTCTCATCAG RP:TTCTCTGCTTGGTGGTTTGCTACGAC	58 °C	NM_012675
IL-6	FP: TCTCTCCGCAAGAGACTTCCA RP:ATACTGGTCTGTTGTGGGTGG	60 °C	NM_012589.2
GAPDH	FP: AGACAGCCGCATCTTCTTGT RP:CTTGCCGTGGGTAGAGTCAT	60 °C	NM_017008

SREBP-2, sterol regulatory element-binding protein 2; HMG-CoAR, hydroxyl-3-methylglutaryl coenzyme A reductase; LDLR, low density lipoprotein receptor; SR-B1, scavenger receptor class B type I; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP binding cassette transporter G1; CYP7A1, cholesterol 7α-hydroxylase1; TNF-α, tumor necrosis factor alpha; IL-6, Interleukin 6; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

2.8. Western Blotting

Tissue cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 50 mM dithiothreitol, complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and phosphatase inhibitor cocktail I and II (Sigma-Aldrich, Inc., St. Louis, MO, USA)). Cell lysates were centrifuged and the supernatants were collected. The protein concentration was determined using Bradford reagent (Bio-Rad, Hercules, CA, USA) with BSA as the standard. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes after gel electrophoresis. The blots were blocked with 5% (*w*/*v*) skim milk and probed with antibodies against CPT-1 (Abcam, Cambridge, MA, USA), AMPKα, phospho-AMPKα (Thr-172), LDLR, ABCA1 (Gene Tex, Irvine, CA, USA), IRS-1, phospho-IRS-1 (Ser-307), Akt, and phospho-Akt (Ser-473) (Cell Signaling Technology, Beverly, MA, USA) separately, followed by goat anti-rabbit or mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibodies. The protein bands were visualized using enhanced chemiluminescence reagents (ECL, PerkinElmer, Boston, MA, USA). The intensity of each band was quantified by densitometry using Image Quant software (Molecular Dynamics, Sunnyvale, CA, USA). The blots were then stripped for further probing with β-actin or α-tubulin antibodies (Cell Signaling Technology, Beverly, MA, USA) as an internal control.

2.9. Statistical Analysis

All data are expressed as mean ± SEM of at least three independent experiments. Significant differences were analyzed by one-way ANOVA, followed by Duncan's multiple range tests using SAS 9.0 software (Cary, NC, USA). $p < 0.05$ was considered statistically significant between means of two groups. The Mann–Whitney U test, evaluated by the Monte Carlo method for small samples, was used to evaluate NAFLD histological data. Pearson correlations between selected variables were tested.

3. Results

3.1. High Performance Liquid Chromatography (HPLC) Analysis of CO-EtOAc

The major compounds of CO-EtOAc were analyzed by HPLC. As shown in Figure 1C, two major peaks indicated gallic acid and ellagic acid, respectively, as compared to standard references of gallic acid appeared at 17.31 (Figure 1A) and ellagic acid at 53.22 min (Figure 1B). Moreover, the contents (EA and gallic acid) of CO-EtOAc were 7.8 and 11.2 mg/g extract, respectively.

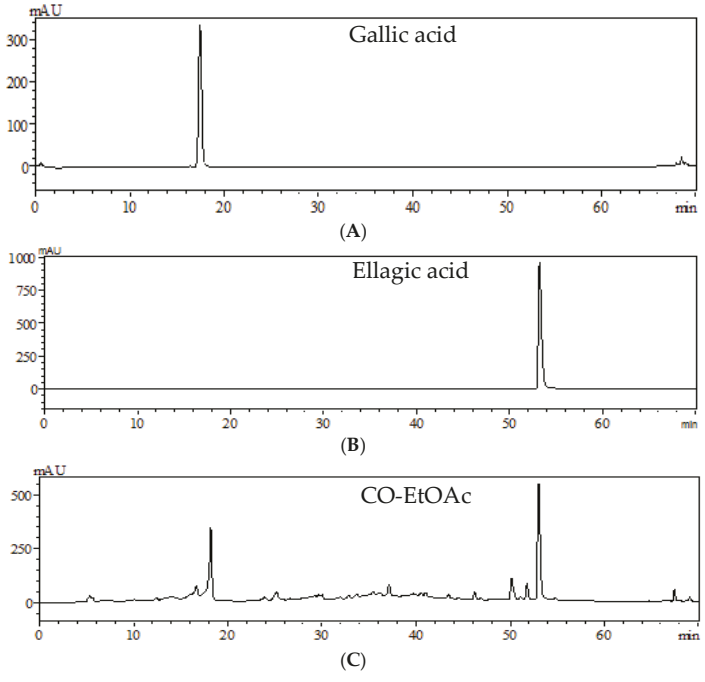


Figure 1. High performance liquid chromatography (HPLC) pattern of polyphenol standards and CO-EtOAc: (**A**) HPLC chromatogram of standard reference: gallic acid (GA, 17.31 min); (**B**) standard reference: ellagic acid (EA, 53.22 min); and (**C**) HPLC chromatogram of CO-EtOAc.

3.2. The Effect of CO-EtOAc on Body Weight, Food Intake and Biochemical Parameters

All of the rats used in this study remained healthy in appearance to the end of the experiment. As shown in Table 2, rats fed with HFD combined with STZ injection (DC group) had a significant increase in body weight, but daily food intake did not significantly differ among groups. Epididymal adipose tissue (EAT) weight and blood glucose levels were significantly increased in DC group. However, administration of 150 mg/kg CO-EtOAc significantly decreased the gain of body weights by 12.9%, the gain of the epididymal adipose tissue weights by 33.4%, and the increase in blood glucose levels by 202% when compared with DC group. The levels of serum TC, TG, bile acid, TNF-α, AST and ALT were significantly increased in the diabetic rats, whereas these biochemical parameters were suppressed to near those in the control rats after the higher dose of CO-EtOAc administration. Moreover, we found that the serum HDL-C levels were significantly increased in rats treated with 50 and 150 mg/kg CO-EtOAc by 27.2% and 53.1%, respectively, when compared with the rats in the DC group.

Table 2. Body weight, food intake and biochemical characteristics in diabetic rats.

Measurements	Control	DC	DC + CO-EtOAc (50 mg/kg)	DC + CO-EtOAc (150 mg/kg)
Body weight				
Wk-0 BW (g)	153.42 ± 6.52 ^{a}	151.87 ± 4.67 ^{a}	159.01 ± 3.55 ^{a}	154.33 ± 5.64 ^{a}
Wk-2 BW (g)	271.13 ± 9.33 ^{b}	308.72 ± 10.33 ^{a}	309.69 ± 11.65 ^{a}	297.45 ± 7.65 ^{a}
Wk-8 BW (g)	414.43 ± 9.74 ^{b}	448.62 ± 15.68 ^{a}	440.11 ± 23.19 ^{a}	411.55 ± 13.95 ^{b}
Body weight gain (g)	143.30 ± 0.41 ^{b}	139.90 ± 5.35 ^{a}	130.42 ±11.54 ^{a}	114.10 ± 6.30 ^{c}
Food intake (g/d)	27.61 ± 2.05 ^{a}	25.64 ± 3.72 ^{a}	24.76 ± 4.63 ^{a}	25.76 ± 3.68 ^{a}
EAT weight (g)	4.51 ± 0.27 ^{c}	7.54 ± 1.71 ^{a}	5.92 ± 1.33 ^{b}	5.02 ± 0.22 ^{b}
BG (mg/dL)	102.75 ± 7.95 ^{d}	309.3 ± 10.56 ^{a}	174.42 ± 10.56 ^{c}	252.55 ±12.10 ^{b}
Serum				
Insulin (ng/mL)	1.25 ± 0.24 ^{b}	0.60 ± 0.18 ^{a}	0.55 ± 0.19 ^{a}	0.53 ± 0.12 ^{a}
TC (mg/dL)	50.43 ± 8.50 ^{b}	71.04 ± 7.01 ^{a}	49.53 ± 12.60 ^{b}	42.88 ± 10.32 ^{b}
HDL-C (mg/dL)	27.38 ± 0.91 ^{b}	22.13 ± 1.16 ^{b}	28.13 ± 0.81 ^{a}	33.88 ± 2.61 ^{a}
BA (mg/dL)	30.53 ± 4.93 ^{c}	50.48 ± 6.55 ^{a}	41.25 ± 5.85 ^{b}	35.75 ± 7.22 ^{b,c}
TG (mg/dL)	75.25 ± 7.51 ^{c}	145.42 ± 18.61 ^{b}	92.45 ± 10.14 ^{a}	72.22 ± 9.22 ^{a}
TNF-α (pg/mL)	4.52 ± 0.25 ^{c}	7.83 ± 1.66 ^{a}	5.27 ± 0.48 ^{b}	4.84 ± 0.80 ^{c}
AST (IU/L)	38.57 ± 4.41 ^{b}	71.30 ± 16.42 ^{a}	40.83 ± 9.46 ^{b}	33.14 ± 7.41 ^{b}
ALT (IU/L)	20.57 ± 7.88 ^{b}	47.30 ± 12.30 ^{a}	25.83 ± 6.46 ^{b}	23.14 ± 4.35 ^{b}

1 BW, body weight; BG, blood glucose; BA, bile acid; DC, diabetic control; CO-EtOAc, ethyl acetate fraction of Chinese olive; EAT, epididymal adipose tissue; TC, total cholesterol; TG, triacylglycerol; TNF-α, Tumor necrosis factor alpha; HDL-C, High-density lipoprotein; AST, aspartate aminotransferase; ALT alanine aminotransferase. 2 Data are expressed as the mean ± SEM (n = 8–10). Values with different letters are significantly different ($p < 0.05$) by using one-way ANOVA coupled with Duncan's multiple range tests. 3 Details of the nutrient contents, feeding, and treatment period are given in the "Materials and Methods" section.

3.3. The Effect of CO-EtOAc on Hepatic Antioxidant Enzyme Activities and TBARS Levels

To evaluate the effect of CO-EtOAc on redox status, we determined the hepatic antioxidant enzyme activities in rats treated with CO-EtOAc (Table 3). HFD combined with STZ resulted in a significant decrease in hepatic GSH levels and activities of SOD, GPx, and CAT, whereas treatment with 150 mg/kg CO-EtOAc reversed the suppression of these antioxidant enzyme activities. This finding reveals that CO-EtOAc significantly restored the HFD and STZ-mediated suppression of antioxidant enzyme activities. On the other hand, we also evaluated the effect of CO-EtOAc on lipid peroxidation, which was estimated by measuring the levels of thiobarbituric acid reactive substances (TBARS). As shown in Table 3, HFD combined with STZ significantly enhanced the TBARS values by 2.8-fold, whereas CO-EtOAc inhibited the induction. These results suggest that CO-EtOAc might reverse the HFD-induced oxidative damage in the liver through the increase in antioxidant enzyme activities and decrease in lipid peroxidation.

Table 3. Antioxidant status and TBARS levels of the liver in diabetic rats.

Measurements	Control	DC	DC + CO-EtOAc (50 mg/kg)	DC + CO-EtOAc (150 mg/kg)
SOD (U mg protein^{-1})	83.33 ± 8.88 ^{a}	59.37 ± 11.96 ^{b}	65.37 ± 12.45 ^{ab}	85.37 ± 10.23 ^{a}
GSH (μmol mg protein^{-1})	32.55 ± 3.72 ^{a}	17.72 ± 2.86 ^{b}	28.64 ± 4.41 ^{ab}	36.72 ± 4.86 ^{a}
GPx (nmol mg protein^{-1})	101.51 ± 10.37 ^{a}	60.5 ± 8.69 ^{b}	76.5 ± 6.43 ^{ab}	92.5 ± 4.69 ^{a}
CAT (U mg protein^{-1})	59.32 ± 6.52 ^{a}	21.5 ± 4.43 ^{cd}	28.5 ± 2.43 ^{c}	50.5 ± 4.38 ^{b}
TBARS (nmol mg protein^{-1})	1.13 ± 0.71 ^{c}	3.12 ± 0.73 ^{a}	1.82 ± 0.35 ^{b}	1.54 ± 0.27 ^{b}

1 DC, diabetic control; CO-EtOAc, ethyl acetate fraction of Chinese olive; SOD, superoxidase dismutase; GSH, reduced glutathione; GPx, glutathione peroxidase; CAT, catalase; TBARS, 2-thiobarbituric acid reactive substances. 2 Data are expressed as the mean ± SEM (n = 8–10). Values with different letters are significantly different ($p < 0.05$) by using one-way ANOVA coupled with Duncan's multiple range tests.

3.4. The Effect of CO-EtOAc on the Regulation of Insulin Signaling

Considering that hyperglycemia apparently occurred in the DC group, we examined whether CO-EtOAc plays a role in the regulation of insulin signaling. Insulin receptor-mediated signaling transduction links the metabolic pathway of insulin actions with the Akt pathway [32]. As shown in Figure 2A, DC rats showed a significant increase in phosphorylated IRS-1 (at Ser-307), which was decreased upon both 50 mg/kg and 150 mg/kg CO-EtOAc treatment. Moreover, 50 and 150 mg/kg CO-EtOAc up-regulated phosphorylated Akt protein expression by 2.4-fold and 1.7-fold compared to the DC group, respectively (Figure 2B).

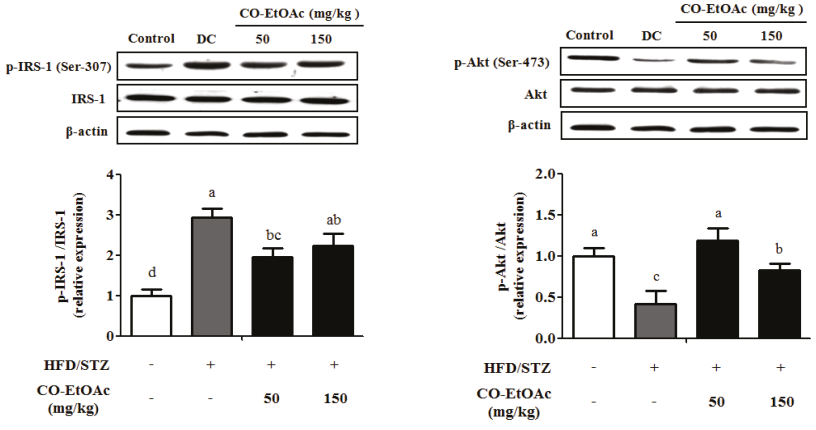


Figure 2. Effects of CO-EtOAc on the regulation of insulin receptor-mediated signaling. Relative protein levels of hepatic: (**A**) IRS-1 (phosphorylated IRS-1 at Ser-307 and total IRS-1); and (**B**) Akt (phosphorylated Akt at Ser-473 and total Akt) were detected by Western blot analysis. Data are represented as mean ± SEM (n = 8–10). Values with different letters are statistically different ($p < 0.05$) by analysis of variance (ANOVA) followed by Duncan's multiple range tests. Relative expression values are expressed as folds of the control group, which is set as 1.

3.5. The Effect of CO-EtOAc on Hepatic Lipid Accumulation

Results from the histological analysis of liver tissue showed that steatosis was occurred in the liver of rats in DC group. Hepatocyte ballooning was clearly found in DC rats, whereas these effects were reversed in the CO-EtOAc-treated rats (Figure 3A). Rats in the DC group developed full spectrum of steatohepatitis, including steatosis, severe inflammation, and hepatocellular ballooning, resulting in a mean non-alcoholic fatty liver disease activity score (NAS) of 5.6, significantly higher than the value in control rats (NAS = 1.9) ($p < 0.05$) (Figure 3A and Table S1). However, treatment with CO-EtOAc significantly improved the phenomenon of steatohepatitis. Furthermore, hepatic triglyceride levels in DC group were increased by 127.4% (vs. control group). Administration of 150 mg/kg CO-EtOAc attenuated the increase of triglyceride levels by 56.8% when compared to the DC group (Figure 3B). These findings indicate that CO-EtOAc might have protective effects against high fat diet-induced lipid accumulation in the liver.

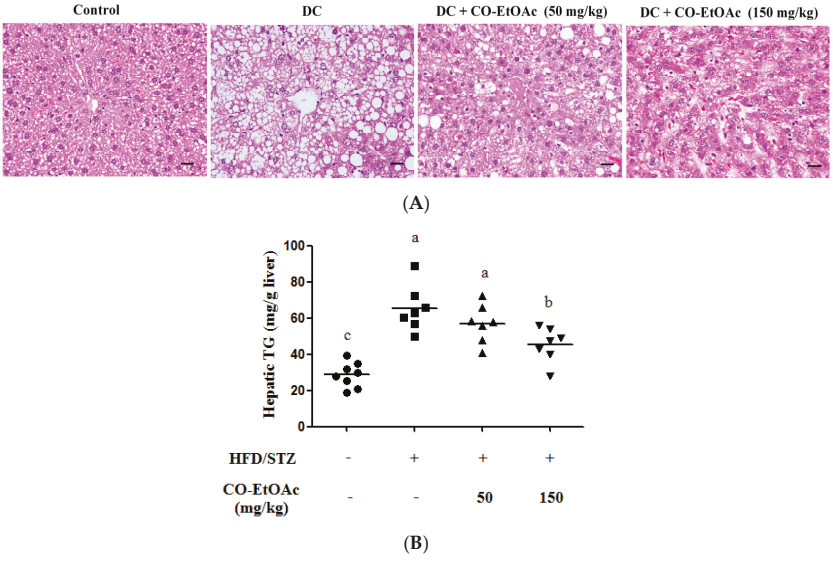
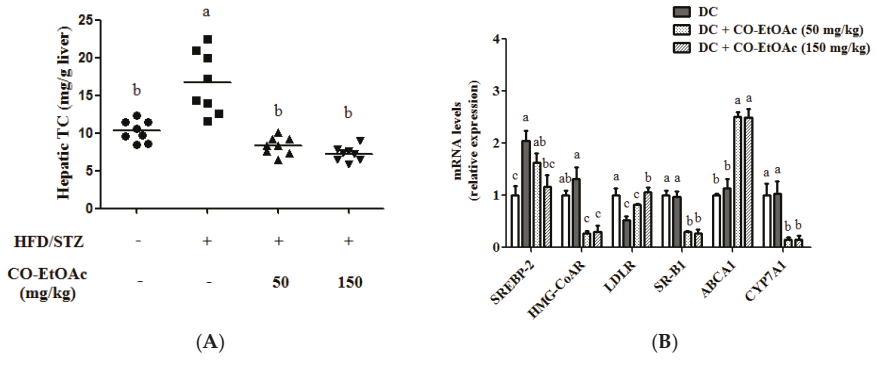


Figure 3. Effects of OE-EtOAc on hepatic lipid accumulation. (**A**) The sections of rat liver tissues were stained by H&E with a representative photograph under 200× magnification; scale bar represents 30 μm. (**B**) The triglyceride content was analyzed using enzymatic assay. Data are represented as mean ± SEM (n = 8–10). Values with different letters indicate statistical difference ($p < 0.05$) by analysis of variance (ANOVA) followed by Duncan's multiple range tests.

3.6. The Effect of CO-EtOAc on Hepatic Cholesterol levels and Gene Expression

Hepatic cholesterol levels were remarkably increased in the DC group by 61.8%. However, treatment with 150 mg/kg CO-EtOAc blocked the increase (Figure 4A). Furthermore, we assessed whether CO-EtOAc was able to affect the expression of genes that govern cholesterol synthesis, transportation and degradation. As shown in Figure 4B, CO-EtOAc apparently suppressed the mRNA levels of *SREBP-2*, *HMG-CoAR*, *SR-B1*, and *CYP7A1*, but increased *LDLR* and *ABCA1* mRNA levels. Consistently, protein levels of LDLR and ABCA1 were significantly increased by the treatment of CO-EtOAc (Figure 4C,D, respectively). These results show that CO-EtOAc exhibits a broad range of actions in the regulation of cholesterol synthesis and transportation in animal livers.



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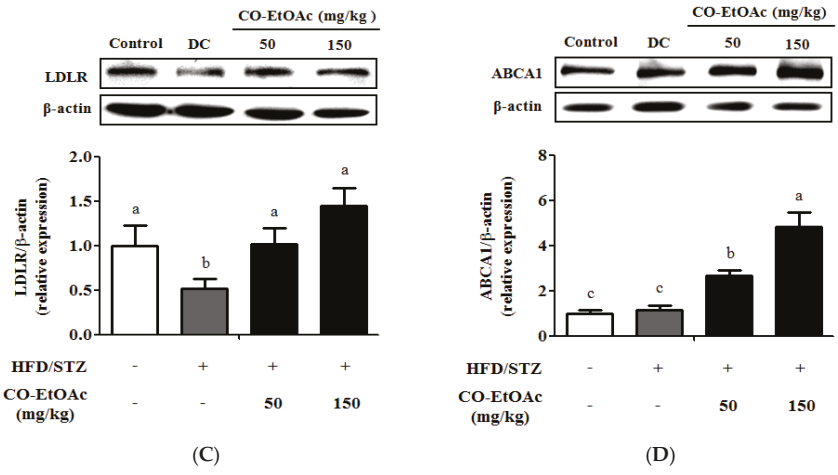
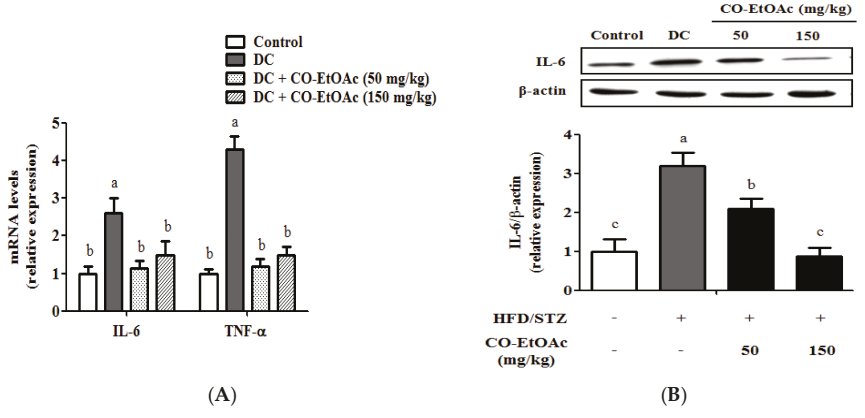


Figure 4. Effects of OE-EtOAc on the levels of hepatic cholesterol and gene expression. (**A**) The content of hepatic cholesterol was analyzed using enzymatic assay. (**B**) Relative mRNA levels of hepatic *SREBP-2*, *HMG-CoAR*, *LDLR*, *SR-B1*, *ABCA1*, and *CYP7A1* were analyzed using RT-qPCR. (**C**) Relative protein levels of hepatic LDLR and ABCA1 were detected by Western blot analysis. Data are represented as mean ± SEM (n = 8–10). Values with different letters show significant difference ($p < 0.05$) by analysis of variance (ANOVA) followed by Duncan's multiple range tests. Quantitative values of mRNA and protein expression are expressed as folds of the control group, which is set as 1.

3.7. The Effect of CO-EtOAc on Expression of Inflammatory Cytokines

Overproduction of inflammatory cytokines is usually a risk factor for metabolic diseases [33]. We thus investigated whether CO-EtOAc administration could modulate the hepatic inflammatory cytokines. As shown in Figure 5A, IL-6 and TNF-α mRNA levels were highly elevated in the DC group. Consistently, the protein levels of IL-6 and TNF-α were significantly increased in the DC group as compared to the control group by 3.1-fold ($p < 0.01$) and 4.2-fold ($p < 0.01$), respectively (Figure 5B). However, administration of CO-EtOAc significantly reduced the IL-6 and TNF-α mRNA and protein levels. The results showed that CO-EtOAc may attenuate hepatic inflammatory stress in the HFD-fed diabetic animals.



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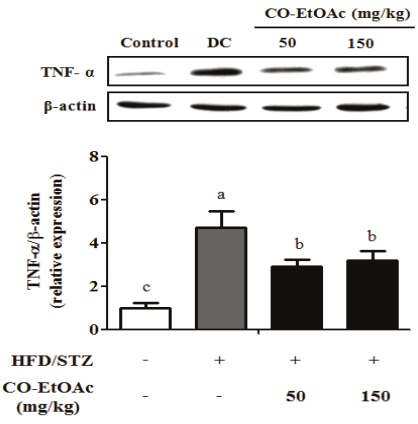

(**C**)

Figure 5. Effects of OE-EtOAc on the expression of hepatic inflammatory cytokines. (**A**) Relative mRNA levels of hepatic IL-6 and TNF-α were determined using RT-qPCR. Relative protein levels of: hepatic IL-6 (**B**); and TNF-α (**C**) were examined by Western blot analysis. Data are represented as mean ± SEM (n = 8–10). Values with different letters are significantly different ($p < 0.05$) by analysis of variance (ANOVA) followed by Duncan's multiple range tests. Quantitative values of mRNA and protein expression are expressed as folds of the control group, which is set as 1.

4. Discussion

A number of phenolic compounds with anti-hyperlipidemia or anti-hyperglycemia potential have been identified in Chinese olive fruit. Our previous study identified gallic acid and ellagic acid as the major compounds in CO-EtOAc [34]. It has been reported that gallic acid not only improves glucose tolerance, triglyceride concentration, total cholesterol, and LDL-cholesterol in diet-induced obesity animals [35,36] but also attenuates high-fat diet fed-streptozotocin-induced insulin resistance via partial agonism of peroxisome proliferator-activated receptor gamma (PPARγ) in experimental type 2 diabetic rats and enhances glucose uptake through translocation and activation of glucose transporter 4 in PI3K/p-Akt signaling pathway [37]. The other phenolic compound, ellagic acid, has been shown to regulate plasma glucose in STZ-induced diabetic rats [38], attenuate high-carbohydrate, high-fat diet-induced metabolic syndrome in rats [39], decrease oxidized LDL uptake and stimulate cholesterol efflux in murine macrophages [40]. Through AMPK activation, ellagic acid stimulates glucose transport in adipocytes and muscles [41], and it downregulates macrophage lipid uptake to block foam cell formation of macrophages and boost cholesterol efflux in lipid-laden foam cells [40].

Excessive intake of energy raises fat accumulation in adipose tissue and liver, which leads to impaired lipid metabolism, inflammatory signaling, and insulin sensitivity [42,43]. It has been reported that HFD induces obesity and insulin resistance, whereas low doses of intraperitoneal STZ induce mild impairment of insulin secretion, which is similar to the feature in the late stage of T2DM [44,45]. Therefore, a T2DM rat model has been established by a standardized HFD treatment and low-dose STZ challenge in rats. Currently, this rat model is widely used to develop therapeutics in diabetes studies [46,47]. Epidemiological studies strongly suggest that intakes of phytochemical-rich foods have beneficial effects on lipid and glucose metabolism as well as the amelioration of inflammation [48]. In addition, medicinal plants are frequently considered to be less toxic and free from side effects than synthetic or clinical drugs [49]. However, the effect of Chinese olive extracts on HFD-mediated metabolic disorder in the liver is still unknown. Herein, we evaluated the role of CO-EtOAc in hepatic lipid accumulation and regulation of genes and proteins involved in lipid and carbohydrate metabolism in HFD-fed rats combined with STZ challenge.

Various evidence has demonstrated the association among obesity, chronic inflammation, and T2MD [50,51]. Two essential inflammatory cytokines, TNF-α and IL-6, affect adipose tissue and the liver, and are involved in the metabolic complications of T2MD [52,53]. Macrophages of adipose tissue are the principal source of pro-inflammatory cytokines and chemokines production. Once macrophages are activated, the inflammatory signaling is subsequently propagated and then insulin sensitivity is interfered in the insulin target tissues to develop IR [54]. Prolonged IR leads to decreased pancreatic function, and increased gluconeogenesis, which increase fasting glucose level, as observed in T2MD patients [55]. Interestingly, HFD-induced obesity causes inflammation in the adipose tissue prior to the liver [56]. In the histological studies (Table S1), we found that both hepatic steatosis and hepatocellular ballooning were not significantly improved upon 50 mg/kg CO-EtOA treatment, while 150 mg/kg CO-EtOAc provided considerable improvement. Decreases in the hepatic levels of TG and serum levels of TG, ALT and AST were coordinate with the improvement of hepatic steatosis caused by HFD. In addition, both severe lobular and portal inflammation were attenuated in rats treated with CO-EtOAc in a dose dependent manner, which is consistent with the improvement in levels of inflammatory cytokines and anti-oxidative capacity.

It has been reported that TNF-α and IL-6 can increase IRS serine/threonine phosphorylation, which negatively modulates insulin signaling transduction and ultimately decreases insulin sensitivity [11,57]. IRS-1 is a cytoplasmic substrate for the insulin receptor and it plays a major role in facilitating glucose uptake and glycogen synthesis [58,59]. The previous report showed that the STZ-induced diabetic rats had increased hepatic p-IRS-1 (Ser^{307}) level, but decreased p-Akt (Ser^{473}) level [60]. Consistently, elevated p-IRS-1 (Ser^{307}) and reduced p-Akt (Ser^{473}) levels were found in the livers of diabetic rats in the present study, and these changes could be significantly ameliorated by CO-EtOAc treatment. However, in contrast to the dose-dependent inhibitory effects of CO-EtOAc on hepatic steatosis, it seems that higher dose of CO-EtOAc did not provide stronger effects on reverting insulin signaling, and the similar phenomenon can also be observed in the blood glucose lowering effects of CO-EtOAc. We propose different compounds within CO-EtOAc might separately regulate insulin signaling and lipid metabolism. It is also possible that different pathways respond to same compounds with different sensitivity.

The major compound GA of CO-EtOAc has been reported to up-regulate the expression of proteins related to insulin signal transduction, including insulin receptor, insulin receptor substrate 1, phosphatidylinositol-3 kinase, Akt/protein kinase B, and glucose transporter 2 [61]. Combined with the results of decreased circulating levels of TNF-α and IL-6, we suggest that CO-EtOAc might be able to improve insulin sensitivity via regulation of inflammatory signaling.

Several studies have shown that hyperlipidemia leads to liver injury and insulin resistance through oxidative stress [62,63]. However, oxidative damage can be attenuated by the enzymatic and non-enzymatic antioxidant defense systems in various tissues. GSH and antioxidant enzymes, such as SOD, CAT, and GPx can scavenge free radicals and reduce oxidative stress in tissues [64]. GSH plays as the first line defense against free radicals in the liver and is also responsible for the maintenance of protein thiols and acts as a substrate for GPx and GST [65]. GSH content has been reported to be depleted in obese rats fed with HFD and the depletion can be restored after the treatment of GA [66]. In this study, we show that CO-EtOAc administration increased GSH levels and activities of SOD, CAT, and GPx in the livers of diabetic rats. Moreover, treatment with CO-EtOAc alleviated the increase in MDA levels in diabetic rats, implying a reduction in hepatic lipid peroxidation. Furthermore, serum levels of AST and ALT were also reduced in the CO-EtOAc-treated rats. We thus propose that CO-EtOAc can protect diabetic rats from liver damage via activation of endogenous antioxidant enzyme activities.

Experiments and clinical cases indicate that T2DM is commonly accompanied by low levels of HDL-C and high levels of LDL-C, which result from cholesterol dysmetabolism [34]. Cholesterol homeostasis is maintained by three different pathways in the liver: de novo cholesterol synthesis, cholesterol uptake, and cholesterol metabolite (bile acids) secretion [67]. In mammals, SREBP-2

is mainly responsible for the regulation of HMG-CoA reductase (HMG-CoAR), a rate-limiting enzyme in de novo cholesterol synthesis, which can be elevated by high-fat diet feeding [68]. Raz et al. reported that inhibitors of hepatic HMG-CoAR are commonly used as drugs for the treatment of hypercholesterolemia and decrease the incidence of dyslipidemia in diabetic subjects [69]. The contribution of de novo cholesterol synthesis versus dietary intake for total body cholesterol has been estimated to be at a ratio of 70:30 [70]. In the present study, the mechanism behind the modulation of CO-EtOAc on reduction of serum and hepatic total cholesterol possibly result from decreasing gene expression of SREBP-2 and HMG-CoAR that regulate de novo cholesterol biosynthesis.

Hepatic nascent HDL-C formation is highly dependent on ABCA1 expression [71], which mediates the efflux of cholesterol and phospholipids to lipid-poor apolipoproteins (apo-A1 and apoE) [72]. The expression of the ABCA1 protein under diabetic states is controversial. A previous study reported that high-fat/high-cholesterol diet results in elevation of hepatic ABCA1 levels and plasma HDL-C levels [73]. However, other studies show decreased ABCA1 mRNA expression in the livers of STZ-induced diabetic mice [74,75]. The differences in regulation of ABCA1 expression under diabetic conditions might be attributed to the animal models used, type of diabetes studied, and its duration. Park et al. reported that ellagic acid increased cholesterol efflux from lipid-loaded macrophages by induction of ABCA1 expression and then transferred cholesterol onto lipid-poor apolipoproteins to form HDL particles [40]. In our study, CO-EtOAc significantly increased ABCA1 and serum HDL-C levels in rats under HFD treatment and STZ challenge, although no significant change was observed in diabetic rats when compared with control rats. Furthermore, biliary secretion of cholesterol, either in the form of free cholesterol or bile acids, is the route for eliminating cholesterol from body in mammals [76]. CYP7A1, the rate-limiting enzyme in bile acid synthesis, could be increased in the liver of animals consuming a high-fat diet combined with STZ administration [77]. Our data showed that hepatic CYP7A1 was significantly increased in diabetic rats, whereas CO-EtOAc decreased CYP7A1 gene expression and circulation of bile acid, which is probably due to the significant reduction of endogenous cholesterol synthesis caused by CO-EtOAc. The results of this study infer that CO-EtOAc could change the metabolism of hepatic cholesterol biosynthesis, transportation, and degradation.

5. Conclusions

This study demonstrated that treatment of CO-EtOAc decreased body weight gain and altered serum lipid and inflammatory profiles in HFD-fed combined with STZ-challenged diabetic rats. We claimed the ameliorative effects of CO-EtOAc on hepatic lipid accumulation and glucose homeostasis, which might be regulated through PI3K/AKT pathway. Furthermore, CO-EtOAc suppressed hepatic gene and protein levels of IL-6 and TNF-α, but enhanced antioxidant enzyme activities. We addressed the molecular mechanisms of glucose and lipid metabolism affected by CO-EtOAc through examining the expression of phosphorylated-IRS-1 and Akt, *SREBP-2*, *HMG-CoAR*, *SR-B1*, and *CYP7A1* (Figure 6). These findings provide insights into the therapeutic potential of Chinese olive fruit extract in the management of metabolic disorders in diabetic animals.

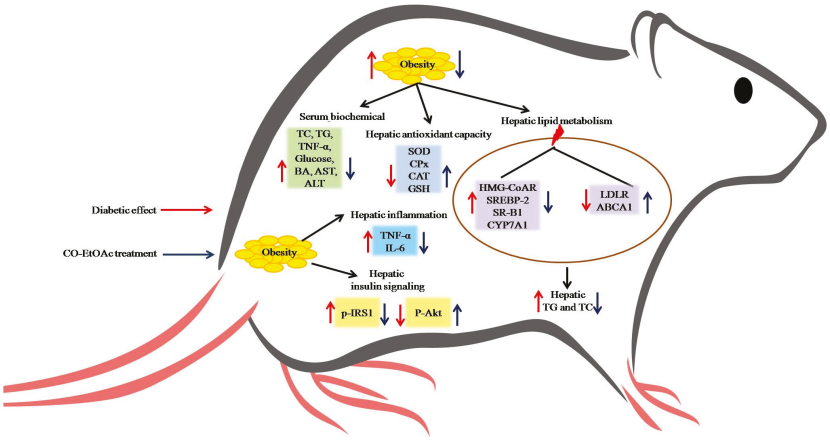

Figure 6. The potential mechanisms of CO-EtOAc protect metabolic dysfunction.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/9/10/1123/s1, Table S1: Histopathological examination results of diabetic rats.

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Author Contributions: S.-C.H and A.-N.C designed the experiments. Y.-T.Y. was responsible for Chinese olive fruit extraction. Y.-T.Y., S.-C.H and A.-N.C performed research, and analyzed and wrote the manuscript. S.-C.H and A.-N.C. had primary responsibility for the final content. All authors have read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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Flavonoid Naringenin Attenuates Oxidative Stress, Apoptosis and Improves Neurotrophic Effects in the Diabetic Rat Retina

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Abstract: Diabetic retinopathy (DR) is one of the leading causes of decreased vision and blindness worldwide. Diabetes-induced oxidative stress is believed to be the key factor that initiates neuronal damage in the diabetic retina leading to DR. Experimental approaches to utilize dietary flavonoids, which possess both antidiabetic and antioxidant activities, might protect the retinal damage in diabetes. The aim of this study was to investigate the potential protective effects of naringenin in the retina of streptozotocin-induced diabetic rats. Diabetic rats were orally treated and untreated with naringenin (50 mg/kg/day) for five weeks and retinas were analyzed for markers of oxidative stress, apoptosis and neurotrophic factors. Systemic effects of naringenin treatments were also analyzed and compared with untreated groups. The results showed that elevated levels of thiobarbituric acid reactive substances (TBARs) and decreased level of glutathione (GSH) in diabetic rats were ameliorated with naringenin treatments. Moreover, decreased levels of neuroprotective factors (Brain derived neurotrophic factor (BDNF)), tropomyosin related kinase B (TrkB) and synaptophysin in diabetic retina were augmented with naringenin treatments. In addition, naringenin treatment ameliorated the levels of apoptosis regulatory proteins; B cell lymphoma 2 (Bcl-2), Bcl-2 associated X protein (Bax) and caspase-3 in the diabetic retina. Thus, the study demonstrates the beneficial effects of naringenin that possesses anti-diabetic, antioxidant and antiapoptotic properties, which may limit neurodegeneration by providing neurotrophic support to prevent retinal damage in diabetic retinopathy.

Keywords: diabetic retinopathy; flavonoid; naringenin; neurotrophic factor; oxidative stress; apoptosis

1. Introduction

Diabetic retinopathy (DR) is one of the most serious complications of diabetes mellitus and the leading cause of decreased vision and blindness in the developed countries. Numerous studies in experimental rodents and in diabetic subjects suggest that diabetes damages both neuronal and vascular components of the retina [1–3]. Furthermore, a large body of evidence suggest that neurons are damaged shortly after the onset of diabetes, which may trigger vascular injury that leads to diabetic retinopathy at the later stages of diabetes [3–5]. The exact pathophysiological mechanism of the neuro-vascular damage in the diabetic retina is still elusive. However, a number of research reports suggest that oxidative stress induced by diabetes is the central factor in initiating the cascade of retinal damage [6,7]. Diabetic induced altered metabolites exert an increase in the level of oxidative stress, which thereby may damage retinal cells [8,9]. These cell damaging effects are mainly caused by

activation of apoptosis and inflammation, and also by lowering neurotrophic support due to increased oxidative stress, which thereby may initiate the development of lesions in diabetic retina [2,5,10].

Molecular mechanisms of retinal damage suggest that high oxidative stress causes profound damage to the diabetic retina by severe lipid peroxidation, protein oxidation, and oxidative DNA damage [8,11]. It has been reported that diabetic retina has lower levels of endogenous antioxidants glutathione, which makes retina vulnerable to damage [6,11]. In addition, high oxidative stress alters signal transduction and expression of pro/anti-apoptotic proteins [6,12]. Under diabetic conditions, oxidative stress induces apoptosis by damaging mitochondrial membrane that translocates proapoptotic protein Bcl-2 associated X protein (BAX) from cytosol to mitochondria and increases the release of cytochrome c which in turn activates caspases, while antiapoptotic Bcl-2 protein level are decreased [6]. Another key factor for neurovascular damage in the diabetic retina is the imbalance of neurotrophic factors [10,13,14]. Oxidative stress is considered the major factor that causes alternations in neurotrophic factors in the diabetic retina [15,16]. Brain derived neurotrophic factor (BDNF) plays an important role in neuronal survival and maintenance [2]. BDNF is initially synthesized in a precursor, proBDNF form, which later undergoes proteolytic cleavage intracellularly to produce the mature form. Several studies have reported decreased levels of BDNF and its downstream neuroprotective signaling through tropomyosin-related kinase B (TrkB) and synaptophysin in the retina of diabetic animals, which cause serious alterations in retinal function [11,15,17,18]. Moreover, increased ratio of proBDNF to BDNF promotes neuronal apoptosis and attenuates synaptic transmission [19]. Therefore, utilization of antioxidant defense strategies may counterbalance oxidative damage and thereby ameliorate neurotrophic imbalance and signaling to protect neurons in the diabetic retina.

A growing number of experimental evidence have emerged, which support the concept that flavonoids with their strong antioxidant activities ameliorate oxidative stress and thereby may prevent damage to the diabetic retina [11,20–22]. Flavonoids being antioxidant can ameliorate apoptosis, and neurotrophic factors, thereby may protect neuronal damage in the diabetic retina [11,21,23]. In this study, we utilized the flavonoid, naringenin to study the neuroprotective effects in the diabetic retina. Naringenin (2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a flavanone, flavonoid, abundantly present in citrus fruits. Naringenin has different pharmacological activities including antioxidant [24], antidiabetic [25], anti-inflammatory [26], and also possesses neuroprotective activities in different experimental models of rodents [27,28]. Recently, our group has reported neuro-protective effect of naringenin in the sciatic nerve of diabetic rats by its antioxidant and anti-inflammatory properties [29]. Despite of a number of beneficial effects of naringenin, to our knowledge, no studies have reported the neuroprotective effects in the diabetic retina. Therefore, in this study we investigated the potential protective effects of naringenin in the retina of streptozotocin-induced diabetic rats, by assessing various oxidative with apoptotic and neurodegenerative markers.

2. Methods

2.1. Animals and Experimental Model

Twelve weeks aged male Wistar albino rats, weighing 250–290 g were received from Experimental Animal Care Center (King Saud University, Riyadh, Saudi Arabia). Diabetes was induced by single intraperitoneal injection of streptozotocin (STZ) (Sigma, St. Louis, MO, USA) at a dose of 65 mg/kg body weight, dissolved in 0.1 mol/L citrate buffered solution (pH 4.5). Three days after STZ injection, blood glucose was measured and blood glucose >250 mg/dL was considered diabetic and included in the study. Naringenin treatment was started orally by gavage (50 mg/kg/day) after two weeks of STZ-injection and continued for five consecutive weeks. A total of 40 rats (n = 10) were randomly divided into four groups as follows; Group 1: Control rats treated with vehicle (C); Group 2: Control rats treated with naringenin (C + N); Group 3: Diabetic rats treated with vehicle (D); Group 4: Diabetic rats treated with naringenin (D + N). At the end of five weeks of treatments,

animals were fasted overnight, then anesthetized and fasting blood samples were collected. Serum was separated and stored at −70 °C till analysis. For retinal studies, only group 1, 3, and 4 were utilized. Retinas were dissected and isolated immediately, rinsed in ice-cold saline, and kept at −70 °C until analysis. All experimental procedures and protocols including anesthesia were in accordance with the Association for Research in Vision and Ophthalmology (ARVO) guidelines to the Care and Use of Experimental Animals as well as the Guidelines of the Experimental Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. The experimental animal protocol number 251-EACC-2015; dated 2 November 2015 to use male Wistar albino rats for the current study has been approved by the Experimental Animal Care Center Review Board, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

2.2. Assay of Glucose and Insulin Levels

Serum glucose levels were measured by using a commercially available kit (RANDOX Laboratories Ltd., Crumlin, Antrim, UK), while insulin serum levels were measured by using ELISA kit (BioSource, Europe S.A., Nivelles, Belgium).

2.3. Estimation of Thiobarbituric Acid Reactive Substances (TBARS) Levels

The lipid peroxidation products TBARS levels were measured in the retina using a commercially available assay kit (ZeptoMetrix Co., Buffalo, NY, USA). Retinal homogenate was prepared by applying short burst of ultrasonication in the 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) lysis buffer, pH 7.4, containing 100 mM NaCl, 1% triton X-100, 0.2% sodium dodecyl sulfate (SDS), and a protease inhibitor cocktail. The homogenates were then centrifuged at 11,000× *g* for 20 min at 4 °C using an ultracentrifuge. Following the centrifugation, supernatants were separated and collected for TBAR quantification. 100 µL of the supernatant was mixed with 2.5 mL of reaction buffer provided in the kit. The mixture was then heated at 95 °C for 60 min. After cooling and centrifugation, the absorbance of the supernatant was measured using a spectrophotometer. The protein concentrations in each sample were estimated using Lowry method [30].

2.4. Glutathione (GSH) Assay

The total GSH levels were measured in the retina of naringenin of treated and untreated diabetic and non-diabetic rats using the method described by Sedlak and Lindsay [31] with slight modification. Retinal homogenate and supernatant were prepared as described above. Retinal supernatant was deproteinized by adding an equal volume of metaphosphoric acid (2.5%, *w*/*v*). After 5 min, the mixture was centrifuged at 12,000× *g* and supernatant collected. In the supernatant, 5 µL of 4 M triethanolamine per 100 µL was added and assay was performed using 50 µL supernatant from the retina. To this mixture, 100 µL of 0.01 M Ellman's reagent, (5,50-dithiobis-(2-nitro-benzoic acid)) (DTNB) was added. The absorbance of the clear supernatants was recorded to measure the concentration of GSH using spectrophotometer at 412 nm within 5 min. A standard curve of GSH was prepared from 0 to 10 µM.

2.5. BDNF Quantification by Enzyme-Linked Immunosorbent Assay (ELISA)

The level of BDNF was measured in the retina using ELISA kits (Quantikine Human Brain-Derived Neurotrophic Factor, R&D Systems, Minneapolis, MN, USA) according to manufacturer's instruction. Retinal homogenate was prepared as described above. Fifty microliter, twofold-diluted retinal supernatant (150 µL/retina) containing approximately 75–100 µg of protein was used for quantitative determination of BDNF in 96-well ELISA plates. Each assay was performed in duplicate. The actual concentration of BDNF in each sample was calculated using a standard curve. The ELISA plate readings were done using Autobio Labtech Instruments Co., Ltd. (Zhengzhou, China). The protein concentrations in each sample were estimated using the Lowry method [30].

2.6. Western Blot Analysis

Western blot was used for the expression of neurotrophic factors, synaptophysin, proapoptotic and anti-apoptotic proteins expression in the retina of control, diabetic and naringenin treated diabetic rats. We analyzed the expression of pro-BDNF, BDNF, TrkB, Bcl-2, Bax and caspase-3 proteins. First, we made retinal tissues homogenate by ultrasonication in the 10 mM HEPES buffer (pH 7.4), containing 100 mM NaCl, 1 mM Na_3VO_4, 10 mM sodium pyrophosphate, 10 mM NaF, 2 mM Ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, 0.2% SDS, and a protease inhibitor cocktail. Samples were centrifuged at 15,000× *g* for 15 min in cooling centrifuge and supernatants collected and the protein concentrations estimated. Protein samples were boiled in Laemmli sample buffer for 5 min, and equal amount of proteins (50 µg/well) were separated on 10–12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. After transferring the proteins, the membranes were blocked for 90 min at room temperature with 5% non-fat milk made in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). The membranes were incubated overnight at 4 °C with anti-proBDNF (1 µg/mL; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-BDNF(1 µg/mL; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-TrkB (1 µg/0.5 mL; Cell Signaling Technology, Danvers, MA, USA), anti-synaptophysin (1 µg/mL; Cell Signaling), anti-caspase-3 (1 µg/mL; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-Bcl-2 (1 µg/mL; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti Bax (1 µg/0.5 mL; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and β-actin (1 µg/mL; Sigma-Aldrich comp, St. Louis, MO, USA), primary antibodies. After overnight incubation with primary antibodies, membranes were washed three times with TBS-T (5 min each) and then incubated with their respective secondary horseradish peroxidase-conjugated antibodies (1:2000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at room temperature for 90 min. Membranes were then washed four times with TBS-T for 5 min each, and the immunoreactivity of bands was visualized on a LI-COR C-DiGit Blot Scanner from Biosciences, Lincoln, NE, USA, using enhanced chemiluminescence (Western blotting luminol reagents (1:1), Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Protein bands were quantified by densitometry analysis using Image-Lab 2.0.1 software (Bio-Rad Laboratories Inc., Hercules, CA, USA). For internal control, membranes were washed and incubated with a mouse monoclonal β-actin antibody (1:2000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and all the steps were followed as described above.

2.7. Statistical Analysis

All statistical analysis was conducted using, Statistical Package for the Social Sciences Version 12 (SPSS 12.0, Chicago, IL, USA). All data are reported as means ± SEM. Analysis of groups were determined using one-way, analysis of variance (ANOVA) with Mann-Whitney test for analysis of pre- and post-treatment measurements between diabetes, control and naringenin treated groups. Statistical significance was accepted when $p \leq 0.05$.

3. Results

3.1. Systemic Effects of Naringenin Treatment on Body Weight, Glucose and Insulin Levels in the Control and Diabetic Rats

Analyses of body weight, glucose and insulin levels in control and diabetic rats were conducted after naringenin treatments. As shown in Table 1, the level of fasting glucose was increased while both insulin levels and body weight were decreased in diabetic rats as compared to control. Oral treatments with naringenin could not correct the body weights. However, naringenin treatment to diabetic rats showed a significant decrease in the levels of fasting blood glucose (250.5 ± 19.5 mg/dL vs. 405.5 ± 21.6 mg/dL; $p < 0.01$) and an increase in the insulin (35.3 ± 3.4 ng/mL vs. 21.3 ± 2.7 ng/mL, $p < 0.05$) compared with untreated diabetic rats. No significant change was observed on body weight, glucose, and insulin levels between the naringenin-treated and non-treated control rats.

Table 1. Effects of naringenin on body weight, blood glucose and serum insulin levels in the rats group as indicated.

	Control	Control + Naringenin	Diabetic	Diabetic + Naringenin
Body weight (gm)	325 ± 16.4	332.2 ± 15.5	225.6 ± 12.3 *	215.7 ± 11.8
Glucose (mg/dL)	92.6 ± 5.6	95.2 ± 6.2	405.5 ± 21.6 *	250.5 ± 19.5 #
Insulin (ng/mL)	45.4 ± 5.4	42.6 ± 3.5	21.3 ± 2.7 *	35.3 ± 3.4 #

Body weights, glucose and insulin were measured five weeks after naringenin treatments. The values are means ± SEM (standard error of mean) of 7–10 rats in each group. (* $p < 0.01$; diabetic vs. control); (# $p < 0.05$; diabetic + naringenin vs. diabetic).

3.2. Effect of Naringenin on Oxidative Stress in the Rat Retina

We measured the levels of GSH and TBARS, as a marker of oxidative stress in naringenin treated and untreated diabetic rat retinas. The level of GSH significantly decreased ($p < 0.01$) in diabetic retinas as compared to controls. However, naringenin treatments increased the retinal GSH level as compared to non-treated diabetic rats ($p < 0.05$) (Figure 1A). Whereas, the level of TBARS increased at-least three fold in diabetic retinas. Though, naringenin treatments significantly reduced the elevated levels of TBARS in the retina of diabetic rats as compared to non-treated diabetic rats ($p < 0.05$) (Figure 1B).

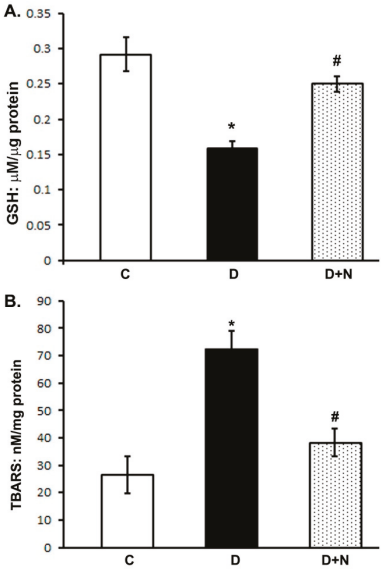

Figure 1. Effects of naringenin on glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) levels in retina of diabetic and non-diabetic animals. (Panel **A**) and (panel **B**) represents measurements of GSH and TBARS respectively. Values are expressed as means ± SEM (standard error of mean); $n = 6$/group. D significantly different from control (C) (* $p < 0.01$); and D + N significantly different from D (# $p < 0.05$). C represents control, D as diabetic, and D + N as diabetic rats treated with naringenin.

3.3. Effects of Naringenin on Retinal BDNF Levels

The level of BDNF in the retina of diabetic rats was significantly lower as compared to corresponding non-diabetic control group (4.5 ± 0.38 vs. 6.42 ± 0.25 pg/µg protein; $p < 0.05$). However, naringenin treatment significantly increased the level of BDNF in the retina of diabetic rats as compared to untreated diabetic rats (6.9 ± 0.4 vs. 4.5 ± 0.38 pg/µg protein), $p < 0.05$) (Figure 2).

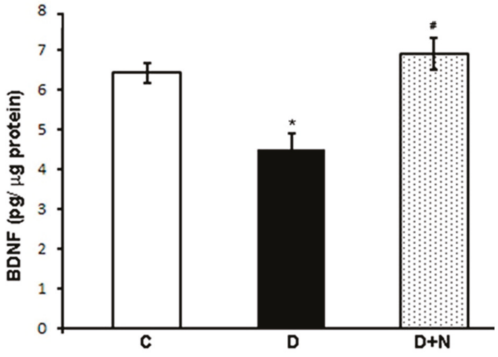

Figure 2. Brain derived neurotrophic factor (BDNF) concentration in the retina of control, naringenin-treated, and non-treated diabetic rats. Values are means ± SEM; $n = 6$/group. $^{*,\#}$ $p < 0.05$ compared to control and diabetic rats, respectively. C represents control, D as diabetic, and D + N as naringenin-treated diabetic rats.

3.4. Effects of Naringenin on ProBDNF and BDNF Protein Expression Levels in the Diabetic Rat Retinas

Western blotting analysis showed a significant reduction in the expression level of retinal BDNF while pro-BDNF level increased in the retina of diabetic rats compared to non-diabetic control group. However, naringenin treatment to diabetic rats caused a significant reduction in the level of proBDNF and improved the BDNF level to almost control level in the retina of diabetic rats ($p < 0.05$) (Figure 3).

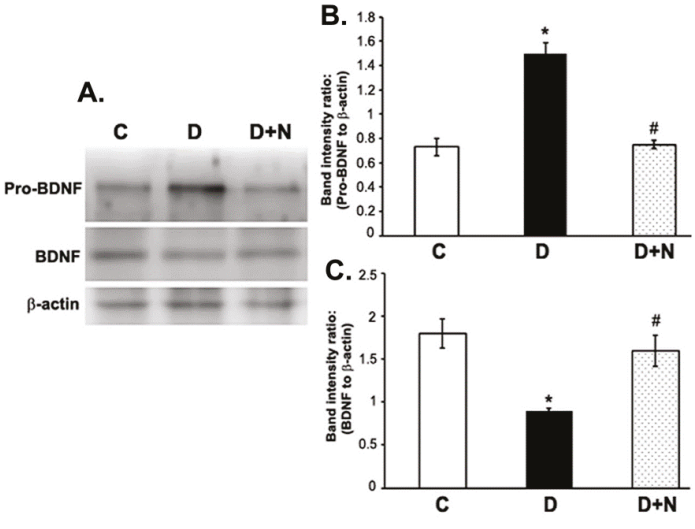

Figure 3. Western blot analysis of the expression of pro-BDNF and BDNF in retinas from control, diabetic, and naringenin-treated diabetic rats. The intensities of the bands were quantified by densitometry. (**A**) Representative immunoblots of pro-BDNF, BDNF and β-actin bands; (**B**,**C**) Data presented as ratios of those protein bands to β-actin. Values are means ± SEM; $n = 6$/group. * $p < 0.05$, significantly different from their controls; $^{\#}$ $p < 0.05$, significantly different from diabetic. BDNF (brain derived neurotrophic factor), pro-BDNF (precursor of brain derived neurotrophic factor). C represents control, D as diabetic, and D + N as diabetic rats treated with naringenin.

3.5. Effects of Naringenin on TrkB and Synaptophysin Protein Expression Levels in the Diabetic Rat Retinas

The expression levels of both TrkB (the specific receptor of BDNF) and synaptophysin were significantly decreased in the retina of diabetic rats compared to non-diabetic control group. However, the decreased levels of TrkB and synaptophysin in diabetic retinas were improved by naringenin treatment (Figure 4).

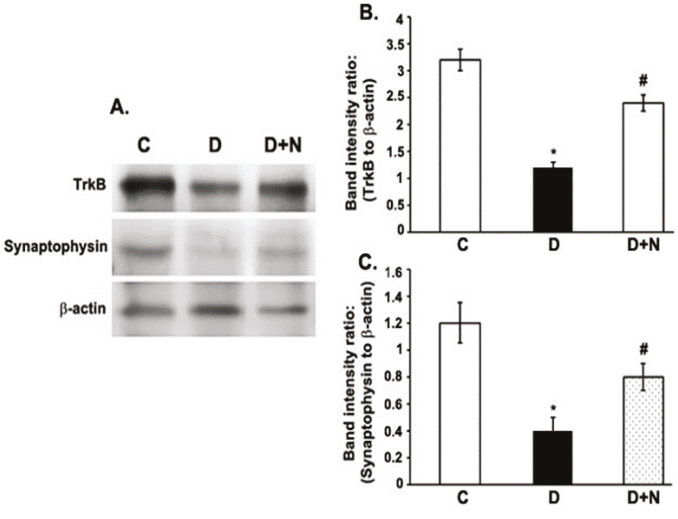

Figure 4. Western blot analysis of the expression of TrkB and synaptophysin in retinas from control, diabetic, and naringenin-treated diabetic rats. The intensities of the bands were quantified by densitometry. (**A**) Representative immunoblots of TrkB and synaptophysin and β-actin bands; Panel (**B**,**C**) Data presented as ratios of those protein bands to β-actin. Values are means ± SEM; $n = 6$/group. * $p < 0.05$, significantly different from their controls; # $p < 0.05$, significantly different from diabetic. TrkB; Tropomyosin receptor kinase. C represents control, D as diabetic, and D + N as diabetic rats treated with naringenin.

3.6. Effects of Naringenin on the Protein Expression Levels of Bcl-2, Bax and Caspase-3 in the Diabetic Rat Retinas

The expression levels of anti-apoptotic Bcl-2 reduced significantly in the diabetic retina compared to controls ($p < 0.01$). However, the decreased level of Bcl-2 in diabetic retinas was significantly augmented when treated with naringenin ($p < 0.05$) (Figure 5A,B). Conversely, the expression levels of pro-apoptotic Bax and caspase-3 proteins increased significantly in the diabetic retinas as compared to controls ($p < 0.05$) (Figure 5C,D and Figure 5C,E). However, naringenin administration to diabetic rats lowered the levels of both caspase-3 and Bax in the diabetic retina to their control levels ($p < 0.05$).

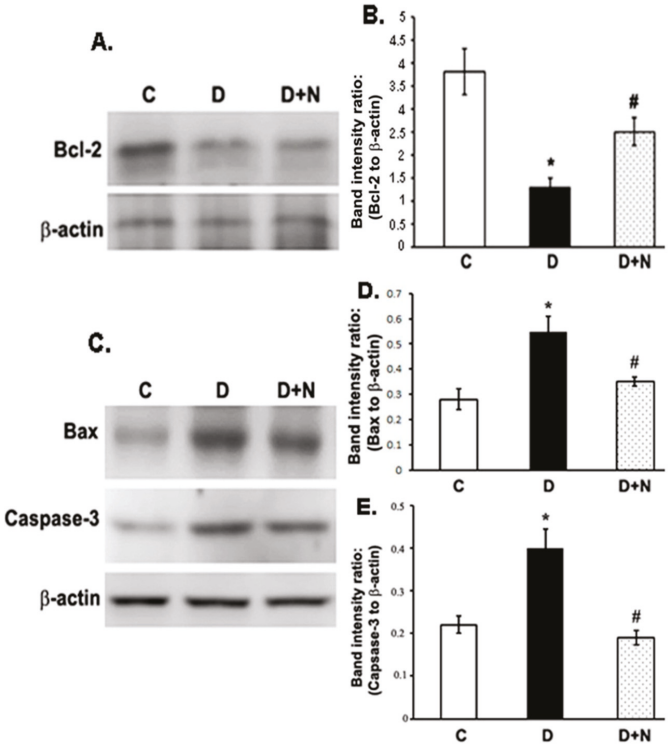

Figure 5. Western blot analysis of the expression of Bcl-2, Bax and caspase-3 in retinas from control, diabetic, and naringenin-treated diabetic rats. (**A**) Representative immunoblots of Bcl-2 and β-actin bands; (**C**) Representative immunoblots of Bax, caspase-3 and β-actin bands; (**B**,**D**,**E**) Data presented as ratios of those protein bands to β-actin. The intensities of the bands were quantified by densitometry. Values are means ± SEM; $n = 6$/group. * $p < 0.01$, significantly different from their controls; $^{\#}$ $p < 0.05$, significantly different from diabetic. Bcl-2; B cell lymphoma 2. Bax; Bcl-2 associated X protein. C represents control, D as diabetic, and D + N as diabetic rats treated with naringenin.

4. Discussion

Naringenin is a flavanone which has been reported to have numerous bioactive effects on human health such as being an antioxidant, an anti-inflammatory, anti-diabetic and anti-neurodegenerative. In this study, we analyzed the potential ameliorative effects of the oral treatment of naringenin in the diabetic retina, especially at the levels of oxidants, caspases and neurotrophic factors; whose dysregulated levels have been found to be damaging in the diabetic retina.

Hyperglycemia and decreased levels of insulin have been considered major factors to cause DR [4,32]. As expected, we found increased level of serum blood glucose and a decreased level of insulin in diabetic rats. Interestingly, treatment of diabetic rats with naringenin exhibited remarkable improvement in insulin and lowering of blood glucose levels. Our results are well supported by a number of previous studies that naringenin protected islets against streptozotocin induced oxidative stress by scavenging free radicals and thereby stimulating the remaining pancreatic β cells to synthesize insulin [33–35]. Hyperglycemia increases the production of reactive oxygen species and depletes cellular antioxidant defense capacities, that contributes towards the cell death and retinal dysfunction in diabetes [8,36]. In this study, we observed a significant increase in retinal TBAR levels, which is usually

a standard marker for lipid peroxidation, whereas the level of endogenous antioxidant glutathione decreased in diabetic retinas compared with control. However, naringenin treatment markedly decreased retinal TBARS to almost control level and returned the levels of GSH towards their control values, suggesting that naringenin may protect the diabetic retina through the inhibition of lipid peroxidation and restoring antioxidant system. Thus, naringenin being an antioxidant might effectively prevent oxidative damage in diabetic retina similar to as observed in various organs affected by diabetes [37,38].

Oxidative stress induced by diabetes is known to activate apoptosis process in the diabetic retina. The increased expression of Bax and caspase-3 early in diabetic retina are correlated with the acceleration of the neuron cell death and reduction of axonal regeneration, which are reliable markers for apoptosis [39,40]. Consistent with the previous studies, we also found increased expression of both Bax and caspase-3 in the diabetic retina. Interestingly, naringenin treatment efficiently decreased their expression, while the drug increased the expression of the survival factor, Bcl-2 protein in the diabetic retina. Previous studies suggest that naringenin activates apoptotic regulatory proteins such as Akt and induce phosphorylation of Erk1/2 which may play an important role in neuroprotection in case of diabetic retina [41,42]. Recently, Kara et al., reported the neuroprotective effect of naringenin by inhibiting the apoptosis of the retinal cells in reperfusion injury and found this drug better than flavonoid, hesperetin for neuroprotection [28]. Therefore, we speculate that antioxidant activity of naringenin in the diabetic retina may be a useful anti-apoptotic intervention for diabetic retina.

It is well documented that BDNF plays an important role in neuronal survival and maintenance [2]. BDNF exerts its action by interacting with TrkB and the downstream signaling cascades after receptor activation that enhances cell proliferation and survival [43,44]. However, proBDNF the precursor of BDNF stimulates neuronal apoptosis and reduces synaptic transmission via activation of p75 receptor [19]. Previously, we and others reported a significant reduction of neurotrophic factors in the diabetic rat retina coincided with the decreased level of retinal TrkB expression [17,45,46]. Consistent with those studies, we found an increased level of proBDNF and a decreased level of BDNF expression, accompanied by a decreased level of TrkB. Remarkably, naringenin treatment to diabetic rats caused a significant decrease in the level of proBDNF while BDNF and TrkB levels were improved. Our results are supported by few previous studies indicating that flavonoids restore the level of neurotrophic factors in the retina of diabetic rats [11,46] and induce the synthesis and secretion of neurotrophic factors in the brain [16,47]. This suggests further that naringenin may improve neurotrophic factors in diabetic retina, which in turn might protect retinal damage.

Synaptophysin is the major synaptic protein necessary for neurotransmission, which is a well-known marker for neurodegeneration in various neurological diseases [48]. Maintaining the level of synaptophysin is necessary for normal synaptic functions such as exocytosis, synaptic vesicle formation, synaptic plasticity, and neurotransmitter delivery [49]. Consistent with previous studies, we found a decreased level of synaptophysin in the diabetic rat retina, which may cause neurodegeneration in the retina [15,50]. Naringenin treatment, significantly increased the level of synaptophysin in the retina of diabetic rats. Our results are supported by Sasaki et al., that antioxidant lutein attenuated oxidative stress and increased the level of synaptophysin in diabetic mice in an attempt to protect retinal neurons [15]. In addition, numerous studies found beneficial effects of flavonoids by protecting the synaptic structure and function in the brain by promoting the expression of synaptophysin [51,52]. Therefore, additionally naringenin might also exert neuroprotective action in the diabetic retina by inducing synaptophysin expression.

5. Conclusions

The results of our current study demonstrates the beneficial effects of naringenin that possesses anti-diabetic, antioxidant and antiapoptotic properties, which may limit neurodegeneration by providing neurotrophic support to prevent retinal damage in diabetic retinopathy as summarized and

depicted in Figure 6. Thus, treatments with naringenin might be effective therapeutics in attenuating retinal neurodegeneration in diabetic retinopathy.

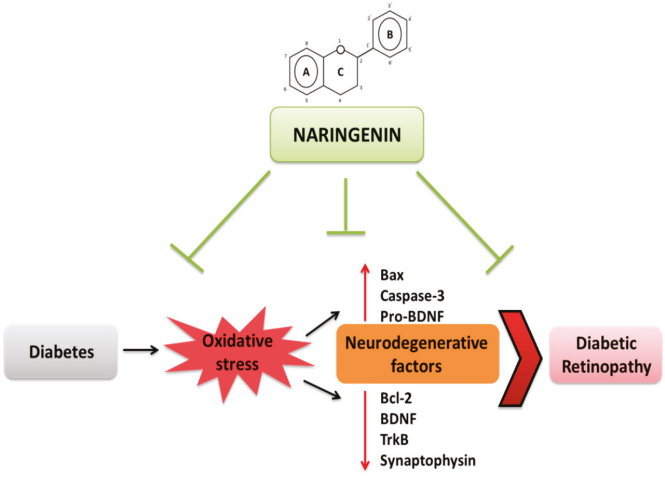

Figure 6. Schematic diagram summarizes diabetes-induced oxidative stress that leads to diabetic retinopathy. The findings of the current study showing that diabetes-induced oxidative stress activates apoptosis and decreases neurotrophic support leading to neuronal damage. Treatments with naringenin that target diabetes, oxidative stress and neurodegenerataive factors might be effective therapeutics in attenuating retinal neurodegeneration in diabetic retinopathy. Bcl-2; B cell lymphoma 2. Bax; Bcl-2 associated X protein. TrkB; Tropomyosin receptor kinase. BDNF; brain derived neurotrophic factor.

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Author Contributions: M.S.O., A.S.A. and S.S.A.-R. conceived and designed the experiments; D.I.A.-D. and M.M.A. performed the experiments; M.S.O. and D.I.A.-D. analyzed the data; M.S.O., S.S.A.-R. and A.S.A. contributed reagents/materials/analysis tools; M.S.O., D.I.A.-D. and A.S.A. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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Review

Coffee Decreases the Risk of Endometrial Cancer: A Dose–Response Meta-Analysis of Prospective Cohort Studies

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Abstract: Aim: The aim of this study was to perform a comprehensive meta-analysis of the association between coffee consumption and risk of endometrial cancer. Methods: Eligible studies were identified by searching the PubMed and EMBASE databases. The dose–response relationship as well as the risk of endometrial cancer for the highest versus the lowest categories of coffee consumption were assessed. Subgroup analyses considering the menopausal and receptor statuses, the smoking status, and the BMI (Body Mass Index) were performed in order to identify potential confounders. Results: We identified a total of 12 studies eligible for meta-analysis. A dose–response meta-analysis showed a decreased risk of endometrial cancer. Moreover, a subgroup analysis indicated that coffee consumption is significantly associated with a decreased risk of postmenopausal cancer. Increasing coffee consumption by four cups per day was associated with a 20% reduction in endometrial cancer risk (relative risk (RR) 0.80; 95% confidence interval (CI) 0.72 to 0.89) and with a 24% reduction in postmenopausal cancer risk (RR 0.76; 95% CI 0.69 to 0.83). Conclusions: Our findings suggest that increased coffee consumption is associated with decreased risk of endometrial cancer, and this association is observed also for postmenopausal cancer.

Keywords: coffee; caffeine; postmenopausal; endometrial cancer; prospective cohort; meta-analysis

1. Introduction

Endometrial cancer is the third most common female cancer, after breast cancer and cervical cancer: in 2012, worldwide, there were over 1.2 million women with a diagnosis of endometrial cancer made in the previous 5 years. Similarly, in Europe there were 370,000 women with the same diagnosis [1]. The burden of non-communicable diseases, including cancers, has been associated with dietary habits, such as the consumption of fibers, sugar, saturated fatty acids, and trans fatty acids [2–4]. Among others, coffee consumption has also been shown to potentially affect human

health [5]. Moderate coffee consumption has been suggested to improve metabolic health and to decrease the risk of mortality [6,7]. Regarding cancer risk, coffee consumption has been associated with decreased risk of liver [8], prostate [9], pancreatic [10], and colon cancer [11], suggesting a potential role of coffee in cancer prevention. Coffee is composed of a variety of compounds, including polyphenols, diterpenes and melanoidins, that have been reported to modulate anti-inflammatory and anti-oxidant body responses, which may explain the potential beneficial effects of coffee in cancer prevention [12,13].

A decreased risk of endometrial cancer in women who regularly drink coffee has been documented in retrospective and prospective studies: Je and Giovannucci performed a meta-analysis including 10 case-control and 6 cohort studies and found a significant inverse association of endometrial cancer with coffee consumption in the highest versus the lowest category of coffee intake [14]. The dose–response analysis showed a decrease of 8% in the risk of endometrial cancer for an increment of one cup of coffee per day [14]. Similar conclusions were reported by Zhou and colleagues in a meta-analysis of prospective studies: they found a significant reduction of endometrial cancer risk associated with increased coffee consumption, with a linear dose–response relationship [15]. Significant results were obtained also in the meta-analysis of Yang and colleagues, who estimated a decreased risk of endometrial cancer of about 10% for every additional daily cup of coffee [16]. Results are promising but evidence is not yet conclusive, especially regarding specific population subgroups (e.g., in relation to body mass index (BMI), age, geographic place of residence, and ethnicity); in light of the high worldwide consumption of coffee, further research is needed [17,18]. Moreover, new cohort studies have been published, and some overlapping cohorts were not considered in the previous meta-analyses [19]. Thus, the aim of this study was to update the current evidence on the association between coffee intake and risk of endometrial cancer, providing insights on potential effect modifiers or confounding factors, including the BMI and the menopausal status.

2. Methods

We followed the Meta-Analysis of Observational Studies in Epidemiology (MOOSE) protocols throughout the design, execution, analysis, and reporting of the current meta-analysis (Supplementary Table S1) [20].

2.1. Search Strategy

We conducted a comprehensive literature search using two medical databases, namely, PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and EMBASE (http://www.embase.com/); the databases were screened from the earliest available online indexing year up to March 2017, with English-language restriction. We included the following search terms: *(coffee OR caffeine OR beverages OR diet Or dietary) AND (endometrial OR endometrium) AND (cancer OR carcinoma OR neoplasm)* (Supplementary Table S2). Two authors evaluated independently the pertinence of the retrieved studies and obtained full texts of the relevant ones. We limited our search to prospective cohort studies that evaluated the association between dietary coffee intake and the risk of endometrial cancer in the overall female population. Studies were included if they provided any of the following risk estimates: relative risks (RRs) or hazard ratios (HRs). We excluded studies that reported insufficient statistics or insufficient coffee consumption categories (less than three). Among the included manuscripts, all references were also examined in order to maximize the number of relevant studies through the addition of studies not previously identified. When duplicate publications from the same study were identified, we chose the report that provided the largest number of cases (or the entire cohort), or the longest follow-up for each endpoint of interest. Full texts of potentially relevant articles were assessed independently for eligibility by two different authors.

2.2. Data Extraction

A standardized extraction form was used to extract data from each study included in the meta-analysis. The following information was collected: (1) first author name; (2) year of publication;

(3) study cohort name; (4) country; (5) number of participants; (6) sex of participants; (7) age range of the study population at baseline; (8) categories of consumption; (9) follow-up period; (10) endpoints and cases; (11) distributions of cases and person-years, RRs, HRs, and 95% CIs (confidence interval) for all categories of exposure; (12) covariates used in adjustments. Two authors independently performed such process, and discrepancies were discussed and resolved by consensus. Each study was critically appraised through the use of a quality scale, namely, the Newcastle-Ottawa Quality Assessment Scale [21], which consists of three variables of quality as follows: selection (4 points), comparability (2 points), and outcome (3 points), for a total score of 9 points (9 representing the highest quality).

2.3. Statistical Analysis

In this meta-analysis, HRs were deemed equivalent to relative risks (RRs) [22]. For each study, extracted statistical estimates were RRs and HRs with 95% CI for all categories of exposure. Random-effects models were used to calculate pooled RRs with 95% CI for the highest versus the lowest categories of exposure (such analysis aimed to assess the presence of a relationship between coffee intake and risk of endometrial cancer). In performing such analysis, for each study, we used the risk estimate derived from the most fully adjusted models (analysis of the pooled RR). Heterogeneity was assessed using the Q test and I^2 statistic. The level of significance was set equal to 0.10 for the Q test. The I^2 statistic explains the amount of total variation that could be attributed to heterogeneity. I^2 values $\leq$25%, 25–50%, 50–75%, and >75% indicated no, small, moderate, and significant heterogeneity, respectively. To evaluate the stability of the results and potential sources of heterogeneity, a sensitivity analysis by exclusion of one study at a time was performed. Moreover, a subgroup analysis was performed in order to check for potential sources of heterogeneity according to the geographical area. To test for potential confounders and effect modifiers, other subgroup analyses were performed (by menopausal status, receptor status, BMI, smoking status, and coffee type). The publication bias was evaluated by a visual investigation of the funnel plots for potential asymmetry.

To better explore the relationship between exposure and outcome, a dose–response meta-analysis was performed (a dose–response analysis was preferred over other designs, e.g., meta-regression, to better compare our results with previous studies and to better draft potential recommendations whether evidence would support the present findings). Extracted data were stratified by the level of coffee intake, and distributions of cases and person-years (when available), and RRs or HRs with 95% CIs for $\geq$3 exposure categories were included. The median or mean intake of coffee in each category was assigned to the corresponding RR or HR with the 95% CI for each study. If coffee consumption was reported in a range of intake, we used the midpoint of the range. Similarly, when the highest category was open-ended, we assumed that the width of the category would be the same as the adjacent category. In the case of an open-ended lowest category, we clearly set the lower boundary to zero. Two-stage random-effects dose–response meta-analysis was performed to examine linear and non-linear relationship between coffee intake and risk of endometrial cancer. In the first stage, the method of Greenland and Longnecker (generalized least-squares, GLS) was used to calculate study-specific coefficients on the basis of results across categories of coffee intake, taking into account the correlation within each set of retrieved RRs and HRs [23,24]. Through the use of restricted cubic splines with three knots at fixed percentiles (25%, 50%, and 75%) of the distributions, non-linear dose–response analyses were modelled [25], and the coefficients (that had been estimated within each study by performing random-effects meta-analysis) were combined. To estimate the relative risks, we used the method of DerSimonian and Laird in linear dose–response meta-analyses, and the multivariate extension of the method of moments in non-linear dose–response meta-analyses. We calculated a *p*-value for non-linearity by testing the coefficient of the second spline as equal to zero. We performed all analyses with R software version 3.0.3 (Development Core Team, Vienna, Austria).

3. Results

3.1. Study Characteristics

The search identified 598 studies, of which 511 were excluded after reviewing the title, and 70 on the basis of the abstract (Figure 1). Of the 17 publications selected, five were excluded for the following reasons: (1) the article did not provide risk measurements with confidence intervals; (2) the article did not have a prospective design; (3) the article provided data only on genetic polymorphism. For the analysis on the association between coffee consumption and endometrial cancer risk, 12 studies were eligible [16,26–36]. Two cohorts, NOWAC and VIP [27,33], were excluded from the main analysis because part of theirs cases are included in the multicenter study EPIC [32]. However, an alternative analysis was performed by including these cohorts and excluding the EPIC study. One article was used only for subgroup analysis [31]. Studies eligible for the main analysis comprised 1,404,541 participants and 10,548 endometrial cancer cases. The main characteristics of the studies included in the meta-analysis are described in Table 1. Seven studies provided relative risk measurements for the postmenopausal [26–31,35] status, and two for the premenopausal status [27,31]. Five studies were conducted in the USA [28–31,35], five in Europe [16,26,27,33,36], one in Asia [34], and one on a cohort from Europe and North America [32]. The follow-up in prospective cohort studies ranged from about 6 to 26 years, and the age range at study baseline was between 25 and 74 years (with almost all studies covering the age range between 40 and 60 years).

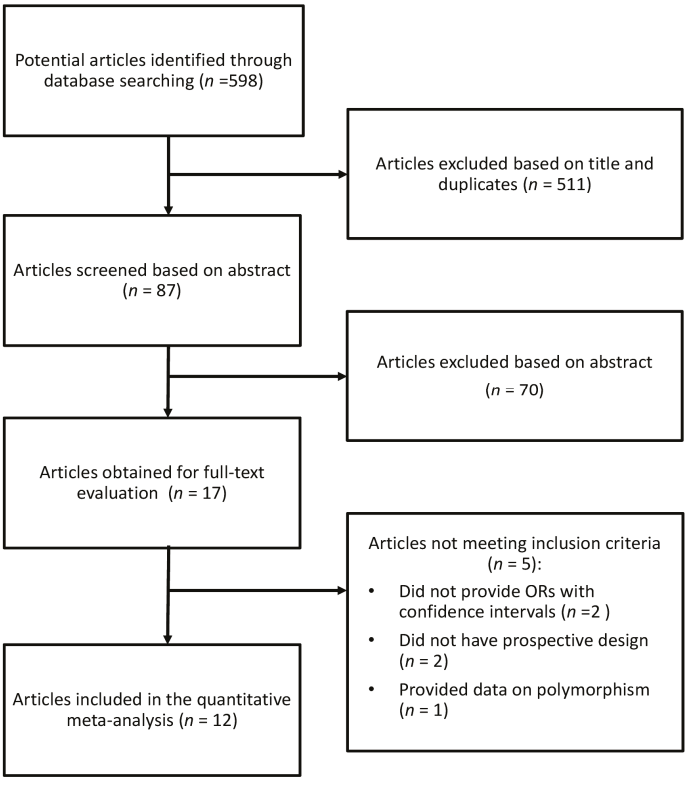

Figure 1. Selection process of relevant studies reporting on the association between coffee consumption and endometrial cancer risk.

Table 1. Characteristics of the studies included in the meta-analysis.

Author, Year	Cohort Name, Country	Years of Study, Follow-Up	Cases; Total Population	Age Range	Adjustments
Shimazu, 2008	JPHC, Japan	1990–2005, 15 years (maximum)	117; 53,724	40–59 years	Age, study area, BMI (body mass index), menopausal status, age at menopause for postmenopausal women, parity, use of exogenous female hormones, smoking status, green vegetable consumption, beef consumption, pork consumption, and green tea consumption.
Friberg, 2009	SMC, Sweden	1992–2007, 17.6 years (mean)	677; 60,634	40–76 years	Age, BMI, smoking.
Nilsson, 2010	VIP, Sweden	1985–2007, 6 years (median)	108; 32,178	30–60 years	Age, sex, BMI, smoking, education, recreational physical activity.
Giri, 2011	WHI, USA	1993–2005, 7.5 years (average)	427; 45,696	50–79 years	Age, ethnicity, unopposed estrogen use, progestin + estrogen use, smoking, BMI.
Je, 2011	NHS, USA	1980–2006, 26 years (maximum)	672; 67,470	34–59 years	Age, BMI, age at menopause, age at menarche, parity and age at last birth, duration of oral contraceptive use, postmenopausal hormone use, pack-years of smoking, alcohol intake, and total energy intake, tea analysis.
Gunter, 2011	NIH-AARP, USA	1995–2006, 9.3 years (mean)	1486; 111,429	50–71 years	Age, smoking, BMI, age at menarche, age at first child's birth, parity, age at menopause, HT (hormonal therapy) use, oral contraceptive use, diabetes and physical activity.
Ucella, 2013	IWHS, USA	1986–2005, 20 years (maximum)	542; 23,356	55–69 years	Age, diabetes, duration of HT use, hypertension, age at menarche, age at menopause, BMI, waist-to-hip ratio, smoking status, pack years of smoking, total energy and alcohol use.
Gavrilyuk, 2011	NOWAC, Norway	1991–2007, 10.9 (average)	462; 97,926	30–70 years	Parity, smoking status, BMI, duration of OC (oral contracception) and HRT use.
Weiderpass, 2014	WLH, Sweden	1991–2009, 18 years (maximum)	144; 42,270	30-49 years	Age, education, duration of hormonal contraceptive use, parity, duration of breastfeeding, smoking status and number of cigarettes/day, menopausal status, BMI, and diabetes mellitus.
Merritt, 2015	EPIC, Multicentre; NHS/NHSII USA	EPIC 1992-NA, 11 years (mean); NHS 1976–2010, 25 years (mean); NHSII 1989–2011, 25 years (mean)	EPIC 1303; 301,107; NHS/NHSII 1531; 155,406	EPIC 25–70 years; NHS 30–55 years; NHSII 25–42 years	BMI, total energy intake, smoking status, age at menarche, oral contraceptive use, a combined variable for menopausal status and postmenopausal hormone (PMH) use, parity, and was stratified by the age of recruitment, and the study centre.
Owen Yang, 2015	MWS, UK	1996–2001, 9.3 years (average)	4067; 560,356	~60 years (mean)	Age, region, socioeconomic status, height, age at menarche, parity, duration of oral contraceptive use, age and status of menopause at study baseline, duration of hormone therapy for menopause, BMI, smoking, alcohol consumption, strenuous exercise, tea consumption, and other nonalcohol fluid intake.
Hashibe, 2015	PLCO, USA	1992–2011, 13 years (maximum)	254; 50,563	55–74 years	Age, sex, race, education, smoking status, smoking frequency, smoking duration, time since stopping smoking for past smokers, and drinking frequency.

Abbreviations: EPIC (*European Prospective Investigation into Cancer and Nutrition*); IWHS (Iowa Women's Health Study); JPHC (Japan Public Health Center-based Prospective Study); NHS (The Nurses' Health Study); MWS (Million Women Study); NIH-AARP (NIH-AARP Diet and Health Study); NOWAC (The Norwegian Women and Cancer study); PLCO (*Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial*); SMC (*The Swedish Mammography Cohort*); WHI (Women's Health Initiative); WLH (Women's Lifestyle and Health); VIP (Västerbotten intervention project cohort).

3.2. Summary Relative Risk for Highest vs. Lowest Category of Coffee Consumption

The summary Relative Risk (RR) of endometrial cancer for the highest versus the lowest category of coffee consumption was RR = 0.79, 95% CI: 0.73, 0.87, with small heterogeneity I^2 = 28%, p = 0.19, (Figure 2); no publication bias was found after a visual inspection of the funnel plot (Supplementary Figure S1). When we performed the alternative analysis that included the NOWAC and VIP cohorts instead of the EPIC study (used in the main analysis), the RR was even lower and equal to 0.73 (95% CI: 0.64, 0.84), with moderate heterogeneity (I^2 = 44%, p = 0.0375).

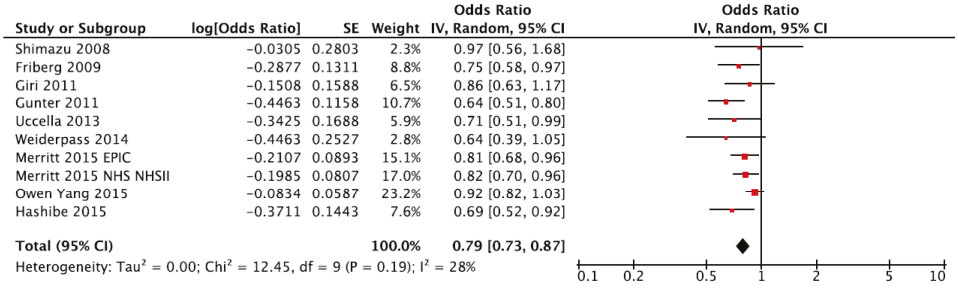

Figure 2. Forest plot of summary relative risks (RRs) of endometrial cancer for the highest versus the lowest (reference) category of coffee consumption. Exposure categories are reported as identified in the original studies; in the dose–response analysis they were harmonized (range: 0–9 cups).

The associations for caffeinated and decaffeinated coffee were consistent with the aforementioned relationship (RR = 0.65, 95% CI: 0.50, 0.85 for caffeinated coffee, and RR = 0.76, 95% CI: 0.62, 0.93 for decaffeinated coffee, Table 2).

Table 2. Subgroups and additional analyses of studies reporting the risk of endometrial cancer for the highest versus the lowest (reference) category of coffee consumption (analyses based on 12 studies consisting of 10 databases).

Subgroup/Additional Analysis	No. of Datasets	RR (95% CI)	I^2	$P_{heterogeneity}$
Total	10	0.79 (0.73, 0.87)	28%	0.19
Geographical area				
North America	5	0.75 (0.67, 0.84)	6%	0.37
Europe	4	0.84 (0.74, 0.94)	29%	0.24
Asia	1	0.97 (0.56, 1.68)	NA	NA
Menopausal status				
Postmenopausal	7	0.70 (0.63, 0.78)	0%	0.60
Premenopausal	2	0.76 (0.49, 1.19)	16%	0.27
Coffee type				
Caffeinated	4	0.65 (0.50, 0.85)	64%	0.04
Decaffeinated	4	0.76 (0.62, 0.93)	0%	0.72
BMI				
<25 kg/m^2	7	0.99 (0.86, 1.14)	0%	0.58
>25 kg/m^2	7	0.79 (0.61, 1.01)	66%	0.004
>30 kg/m^2	5	0.75 (0.63, 0.88)	22%	0.27
Smoking status				
Never smoker	8	0.78 (0.68, 0.88)	7%	0.38
Ever smoker (former/current)	8	0.74 (0.57, 0.98)	68%	0.003
Adjusted for smoking				
No	0	NA	NA	NA
Yes	10	0.79 (0.73, 0.87)	28%	0.19
Adjusted for BMI				
No	1	0.69 (0.52, 0.91)	NA	NA
Yes	9	0.80 (0.74, 0.88)	27%	0.20
Adjusted for education				

Table 2. *Cont.*

Subgroup/Additional Analysis	No. of Datasets	RR (95% CI)	I^2	$P_{heterogeneity}$
No	8	0.81 (0.74, 0.89)	30%	0.19
Yes	2	0.68 (0.53, 0.87)	0%	0.80
Adjusted for alcohol intake				
No	8	0.77 (0.71, 0.84)	0%	0.56
Yes	2	0.85 (0.67, 1.07)	52%	0.15

When taking into account the menopausal status, a significant decrease in the risk of endometrial cancer (RR = 0.70, 95% CI: 0.63, 0.78; I^2 = 0%, p = 0.60) was found for postmenopausal women, but not for premenopausal women (RR = 0.76 95% CI: 0.49, 1.19; I^2 = 16%, p = 0.27). No differences were observed between ever-smokers and non-smokers, i.e., in both subgroups the risk was significantly reduced for the highest category of coffee consumption compared to lowest category (RR = 0.78, 95% CI: 0.68, 0.88 for non-smokers, and RR = 0.74, 95% CI: 0.57, 0.98 for ever-smokers, respectively, Table 2). Finally, the analysis of the highest versus the lowest category of coffee consumption according to body mass index (BMI) categories showed a significant decrease in the risk of endometrial cancer among the obese (BMI > 30 kg/m^2) women (RR = 0.75, 95% CI: 0.63, 0.88).

3.3. Dose–Response Meta-Analysis

Nine studies [16,26,28–30,32,34–36] were eligible for dose–response meta-analysis of prospective cohort studies on coffee consumption and endometrial cancer risk. Six studies [26–31] provided risk estimates for postmenopausal women only. In both the non-linear and the linear dose–response meta-analyses, a significant association between coffee consumption and endometrial cancer risk was found (Figure 3, Table 3). Compared to no coffee consumption, the pooled relative risks for endometrial cancer were: 0.95, 95% CI: 0.92, 0.97, for one cup/day; 0.90, 95% CI: 0.85, 0.94, for two cups/day; 0.85, 95% CI: 0.78, 0.92, for three cups/day; 0.80, 95% CI: 0.72, 0.89, for four cups/day; 0.76, 95% CI: 0.67, 0.86, for five cups/day; 0.72, 95% CI: 0.61, 0.84, for six cups/day; 0.68, 95% CI: 0.57, 0.81, for seven cups/day. Two cohorts, NOWAC and VIP, were excluded from the main analysis, as part of theirs cases are included in the multicentre study EPIC. However, an alternative analysis was performed by including these cohorts and excluding the EPIC study, confirming the results of the main analysis (Supplementary Table S3). Finally, the association between coffee intake and endometrial cancer was stronger when taking into consideration postmenopausal women.

Table 3. Dose–response meta-analysis of prospective cohort studies on coffee consumption and endometrial cancer risk.

	No. of Datasets (No. of Studies)	Coffee Intake (Cups/Day)								I^2(%)	$P_{heterogeneity}$	$P_{non\text{-}linearity}$
		0	1	2	3	4	5	6	7			
Total analysis												
Non-linear	11 (9)	Ref.	0.91 (0.85, 0.97)	0.85 (0.76, 0.94)	0.81 (0.73, 0.91)	0.79 (0.70, 0.89)	0.76 (0.67, 0.87)	0.74 (0.64, 0.86)	0.72 (0.61, 0.85)	30.98	0.09	0.09
Linear	11 (9)	Ref.	0.95 (0.92, 0.97)	0.90 (0.85, 0.94)	0.85 (0.78, 0.92)	0.80 (0.72, 0.89)	0.76 (0.67, 0.86)	0.72 (0.61, 0.84)	0.68 (0.57, 0.81)	59.21	0.01	NA
Postmenopausal												
Non-linear	7 (6)	Ref.	0.92 (0.83, 1.01)	0.85 (0.73, 0.99)	0.80 (0.69, 0.92)	0.75 (0.65, 0.87)	0.71 (0.61, 0.83)	0.67 (0.56, 0.79)	0.63 (0.52, 0.76)	0	0.64	0.67
Linear	7 (6)	Ref.	0.93 (0.91, 0.95)	0.87 (0.83, 0.91)	0.81 (0.76, 0.87)	0.76 (0.69, 0.83)	0.71 (0.63, 0.79)	0.66 (0.58, 0.76)	0.62 (0.53, 0.72)	0	0.46	NA

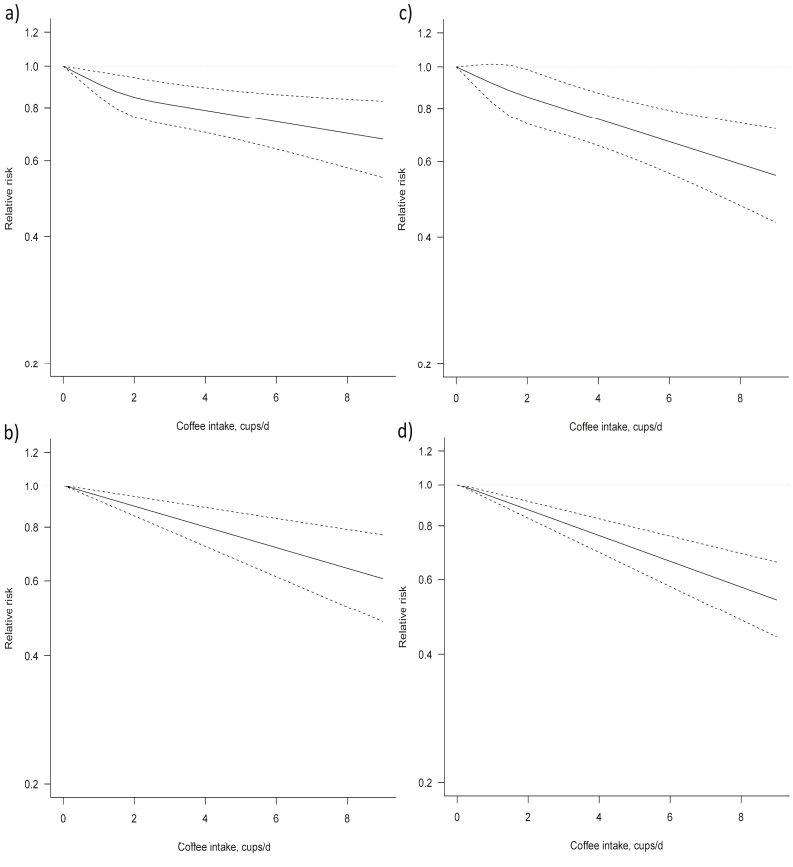

Figure 3. Dose–response association between coffee consumption and endometrial cancer risk (**a**) non-linear, total analysis; (**b**) linear, total analysis (**c**) non-linear, postmenopausal; (**d**) linear, postmenopausal.

4. Discussion

The present meta-analysis, including 12 prospective cohort studies, showed that coffee consumption is associated with a lower risk of endometrial cancer; such association was stronger for postmenopausal endometrial cancer and in obese women (BMI > 30).

Various meta-analyses on the association of coffee consumption and endometrial cancer risk have been conducted so far: for instance, in 2011 Je and Giovannucci [14] documented an inverse dose–response association, with a geographical gradient (stronger association in Japan, followed by the USA and then Europe). Subgroup analyses were later published in 2015 by Yang and colleagues [16] and by Zhou and colleagues [15]: both groups limited their analyses to prospective studies and found a linear inverse association. The first research group found some evidence of heterogeneity among BMI subgroups (overweight women, with a BMI higher than 25, showed a more pronounced inverse association than women with a lower BMI). The second group confirmed these results and pointed out a similar pattern for women without a history of hormone therapy, while documenting no differences with respect to study location, smoking status, and menopausal status. Compared to the previous literature, our results, especially on subgroup analyses for postmenopausal status and BMI (overweight vs obese), are in line with the reported observations, yet provide original findings.

Coffee constituents have been associated with several biological mechanisms related to carcinogenesis, both in vitro and in vivo. These mechanisms include: DNA methylation, oxidative damage, activation of proto-oncogenes and inactivation of onco-suppressor genes, loss of apoptosis and growth control, and induction of angiogenesis [37,38]. Active coffee constituents that have been identified include not only caffeine (mainly known for its ability to increase blood pressure and for its psychostimulatory and diuretic properties) [39], but also polyphenols (e.g., chlorogenic acids, which produce catechins, caffeic, ferulic and coumaric acids), lipids in the form of diterpenes (e.g., cafestol and kahweol), melanoidins, and trigonelline [40,41]. There is evidence that dietary polyphenols might be associated with decreased mortality and cancer risk, and may be the mediators of the potential effects of coffee on cancer prevention [42,43].

With particular referral to female reproductive cancers, several mechanisms have been proposed: for instance, caffeine and coffee intake have been positively associated to sex hormone-binding globulin (SHBG) in postmenopausal women [44]. SHBG is the major carrier of estrogens and testosterone, thus lowering the circulating levels of free hormones; the positive relationship between coffee or caffeine intake and SHBG has been proved in many studies [45,46]. Another possible mechanism resulting in lower levels of circulating estrogens after coffee intake is through the inhibition of the enzyme converting androgens into estrogens, i.e., CYP19 or aromatase [44]. A low level of estrogens is considered a protective factor against endometrial cancers acting through the down-regulation of endometrial proliferation [45], and the inverse association between coffee or caffeine consumption and estrogens has been widely documented [47,48].

Additional effects of coffee and caffeine intake on hormonal functions have been seen in improved insulin sensitivity as a result of the stimulation of insulin-mediated uptake of glucose [49]. Coffee could therefore have a protective role against type 2 diabetes development: most epidemiological studies have documented an inverse association between the intake of caffeinated coffee, decaffeinated coffee, and caffeine and type 2 diabetes in a dose–response manner compared with no or infrequent coffee consumption [50]. In turn, type 2 diabetes has been associated with an increased risk of endometrial cancer, and more specifically with an increased risk of type 1 endometrioid endometrial adenocarcinoma [51]. In recent epidemiological studies, such risk has been associated not only with insulin resistance and diabetes, but also with the metabolic syndrome that is characterized by the coexistence of various factors, such as abdominal obesity, low levels of high density lipoprotein, elevated levels of triglycerides and low levels of density lipoprotein, hypertension, and insulin resistance [52,53].

As already pointed out, we observed a stronger association between coffee consumption and endometrial cancer in women with high BMI (above 30) compared with women with a BMI of 25 or lower. Overweight and obesity have been associated with the development of cancers [54], and various mechanisms have been proposed, including: (i) chronic inflammation and oxidative stress; (ii) cross-talk between tumor cells and surrounding adipocytes; (iii) migration of adipose stromal cells; (iv) obesity-induced hypoxia; (v) genetic susceptibility; and (vi) immunological dysfunctions [55]. In several studies, coffee intake has been inversely associated with metabolic syndrome [56–66]. Metabolic risk factors, such as obesity, impaired glucose tolerance, dyslipidemia, and hypertension have been linked to elevated systemic inflammation and oxidative stress. Thus, impaired metabolism may induce inflammation and oxidative stress, which in turn may lead to carcinogenic transformation. Within these pathways, four main components have been identified: insulin, insulin-like growth factor-I, sex steroids, and adipokines, and coffee consumption has been associated, directly or indirectly, with most of them [55]. For instance, an in vitro study showed that exposure to coffee reduced the accumulation of lipids inhibiting adipocytic differentiation [67]. In animal models, coffee consumption has been related to changes in transcription factors and lipogenesis-related proteins, and, in epidemiological studies, decreased body weight and decreased visceral fat, in relation to coffee consumption, have been observed [68].

The present meta-analysis has some limitations. First, despite we provided insights on underrated factors potentially affecting the association between coffee consumption and endometrial cancer risk, other variables (such as the type of coffee seeds, the roasting method, and the type of preparation) remained largely unexplored. Second, we cannot rule out the possibility of changes in dietary habits (i.e., increased or decreased consumption of coffee) over time, leading to the risk of reverse causation in the event that an individual changed coffee intake due to a newly diagnosed medical condition.

5. Conclusions

In conclusions, our findings suggest that increased coffee consumption is associated with decreased risk of endometrial cancer, especially in postmenopausal, obese women.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/9/11/1223/s1.

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Article

WNT Inhibitory Activity of *Malus Pumila miller* cv Annurca and *Malus domestica* cv Limoncella Apple Extracts on Human Colon-Rectal Cells Carrying Familial Adenomatous Polyposis Mutations

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Abstract: Inhibitors of the Wingless-related Integration site (WNT)/β-catenin pathway have recently been under consideration as potential chemopreventive agents against Familial Adenomatous Polyposis (FAP). This autosomal-dominant syndrome is caused by germline mutations in the gene coding for the protein APC and leads to hyperactivation of the WNT/β-catenin signaling pathway, uncontrolled intestinal cell proliferation and formation of adenocarcinomas. The aim of the present work was to: (i) test, on in vitro cultures of cells carrying FAP mutations and on ex vivo biopsies of FAP patients, the WNT inhibitory activity of extracts from two common southern Italian apples, *Malus pumila Miller* cv. 'Annurca' and *Malus domestica* cv 'Limoncella'; (ii) identify the mechanisms underpinning their activities and; (iii) evaluate their potency upon gastrointestinal digestion. We here show that both Annurca and Limoncella apple extracts act as WNT inhibitors, mostly thanks to their polyphenolic contents. They inhibit the pathway in colon cells carrying FAP mutations with active dilutions falling in ranges close to consumer-relevant concentrations. Food-grade manufacturing of apple extracts increases their WNT inhibitory activity as result of the conversion of quercetin glycosides into the aglycone quercetin, a potent WNT inhibitor absent in the fresh fruit extract. However, in vitro simulated gastrointestinal digestion severely affected WNT inhibitory activity of apple extracts, as result of a loss of polyphenols. In conclusion, our results show that apple extracts inhibit the WNT pathway in colon cells carrying FAP mutations and represent a potential nutraceutical alternative for the treatment of this pathology. Enteric coating is advisable to preserve the activity of the extracts in the colon-rectal section of the digestive tract.

Keywords: nutraceuticals; apple polyphenols; WNT pathway inhibitors; familial adenomatous polyposis; colon cancer

1. Introduction

Familial adenomatous polyposis (FAP) is an inherited gastrointestinal syndrome, characterized by the formation of a large number of adenomas throughout the small intestine and the large bowel. FAP has a birth incidence of about 1/8300, manifests equally in both sexes, and accounts for less than 1% of colorectal cancer (CRC) cases [1]. The birth frequency of FAP patients in European populations is estimated at roughly 1 in 11,300–37,600 live births and its progression to colorectal cancer is close

to 100% by the age of 35–40 years [2]. While colectomy remains the optimal prophylactic treatment, the identification of chemopreventive agents represents one of the major challenges for the future [2].

FAP is caused by a germline mutation in the *Adenomatous Polyposis Coli (APC)* gene on chromosome 5q21–q22. This locus contains a tumor suppressor gene encoding for the protein APC, that functions intracellularly as a scaffold in a large protein complex, known as "β-catenin destruction complex" [3]. This includes the serine/threonine kinase, glycogen synthase kinase-3 β (GSK-3β), Axin, and casein kinase I [4]. The complex represents an important intracellular checkpoint. In virtue of its ability to target β-catenin for proteasomal degradation, it reduces its intracellular levels. This activity avoids β-catenin translocation into the nucleus, binding to the transcription factors, TCF and LEF, and induction of oncogenes, like *c-myc* and *cyclin D1* [5,6].

In wild type cells, APC counterbalances the activity of the Wingless-related Integration site (WNT) pathway, a signaling cascade regulating development in embryos and tissue homeostasis in adult organs. In the gastrointestinal (GI) tract, WNT supports the self-renewal capacity of epithelial stem cells and allows GI organs to be the most intensively self-replenishing in mammals [7]. The class F G-Protein-Coupled Receptor (GPCR) family members, Frizzleds (FZDs), act as a WNT receptors. Upon activation, these recruit and disassemble the β-catenin destruction complex, inhibiting its function and causing β-catenin intracellular accumulation and nuclear translocation [5]. This is the reason for why FAP mutations, by abolishing APC function, lead to constitutively active WNT signaling and, in turn, to uncontrolled proliferation of colon cells, formation of polyps and adenocarcinomas [8].

Apple extracts have been shown to mediate several biological cellular effects that might be of interest with respect to chemoprevention of colorectal diseases [9–12]. Such activity mostly relies on the high number of polyphenols they contain [13–15]. Polyphenol-rich apple extracts have been shown to suppress human colon cancer cell growth in several in vitro culture models [16–18]. Moreover, in $APC^{Min/+}$ mice (a murine model of FAP), the consumption of beverages containing apple polyphenol extracts has been shown to affect the number and growth of colon polyps and reduce colorectal bleeding and high-grade dysplasia [19]. So far, the biological activity of apple polyphenols has been mainly ascribed to their antioxidant potential [20]. However, the exact mechanisms underpinning WNT's inhibitory activity of apple extract is not yet clear. Recently, several polyphenols have been proposed of being endowed with modulatory activities toward specific protein targets; this includes, among others, many of the components WNT signaling pathway [11,14,21].

The aim of the present work was to test the WNT inhibitory activity of two apple cultivars, native to Southern Italy, namely "Annurca" and "Limoncella", identify the polyphenols mainly responsible for their inhibitory activity and determine their mechanism of action.

Malus pumila Miller cv. Annurca is a widespread apple and accounts for 5% of Italian apple production. It is listed as a Protected Geographical Indication (PGI) product from the European Council (Commission Regulation (EC) No. 417/2006)). This apple has been already shown to possess nutraceutical potential in virtue of its ability to reduce cellular glucose levels and lipid uptake [22–25]. *Malus domestica* cv 'Limoncella' is a juicy and aromatic variety of apple, known since ancient Roman times [26]. It is resistant to long time storage and can survive cold winters. In contrast to Annurca, Limoncella's nutraceutical potential has not yet been documented [27].

In the current study, we tested, on in vitro cultures of cells carrying FAP mutations and on ex vivo biopsies of FAP patients, the WNT inhibitory activity of Annurca and Limoncella apple extracts, aimed to identify the mechanism underpinning their activity and evaluate their potency upon in vitro simulated GI digestion.

2. Materials and Methods

2.1. Reagents

Chemicals and reagents used were either analytical-reagent or HPLC grade. The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA) before use.

The standards used for the identification and quantification of phenolic acids and flavonoids were chlorogenic acid, (+)-catechin, (−)-epicatechin, phloretin, phloridzin, procyanidin B2, quercetin, rutin (quercetin-3-*O*-rutinoside) and isoquercetin (quercetin-3-*O*-glucoside), (Sigma Chemical Co., St. Louis, MO, USA). Chemicals and reagents used to simulate the GI digestion were potassium chloride (KCl), potassium thiocyanate (KSCN), monosodium phosphate (NaH_2PO_4), sodium sulphate (Na_2SO_4), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), urea, α-amylase, hydrochloric acid (HCl), pepsin, pancreatin and bile salts (Sigma Chemical Co., St. Louis, MO, USA). Acetonitrile and methyl alcohol were of HPLC grade (Carlo Erba, Milano, Italy). The Nuclear Factor of Activated T-cells (NFAT) inhibitor, cell permeable (sequence: RRRRRRRRRRRGG GMAGPHPVIVITGPHEE; #592517-80-1, Tocris, Bristol, UK) was dissolved in water; Bisindolymaleimide II (3-(1H-Indol-3-yl)-4- [1-[2-(1-methyl-2-pyrrolidinyl)ethyl]-1H-indol-3-yl]-1H-pyrrole-2,5-dione, #137592-45-1, Tocris, Bristol, UK) was dissolved in DMSO.

2.2. Fruit Collection and Sample Preparation

Annurca (*Malus Pumila Miller* cv Annurca) apple fruits and Limoncella (*Malus Domestica* cv Limoncella) were collected from Valle di Maddaloni (Caserta, Italy), in October 2016, when fruits had just been harvested. Annurca fruits were reddened for about 30 days [23], and then analyzed. Lyophilised peels and flesh (10 g) of Limoncella and Annurca apple samples were treated with 60 mL of 80% methanol (0.5% formic acid), homogenized for 5 min by ultra-turrax (T25-digital, IKA, Staufen im Breisgau, Berlin, Germany) and shaken on an orbital shaker (Sko-DXL, Argolab, Carpy, Italy) at 300 rpm for 15 min. Then, the samples were placed in an ultrasonic bath for another 10 min, before being centrifuged at 6000 rpm for 10 min. The supernatants were collected and stored in darkness, at 4 °C. The pellets obtained were re-extracted, as described above and with another 40 mL of the same mixture of solvents. Finally, the extracts obtained were filtered under vacuum, the methanol fraction was eliminated by evaporation, and the water fraction was lyophilized. To obtain polyphenol-enriched fractions of Annurca apple extract (AAE) and Limoncella apple extract (LAE) (in the text referred to as PEF(AAE) and PEF(LAE), respectively) the dry extracts were dissolved in distilled water and slowly filtered through an Amberlite XAD-2 column, packed as follows: Resin (10 g; pore size 9 nm with particle sizes of 0.3–1.2 mm; Supelco, Bellefonte, PA, USA) was soaked in methanol, stirred for 10 min and then packed into a glass column (10 × 2 cm). The column was washed with 100 mL of acidified water (pH 2) and 50 mL of deionized water for removal of sugars and other polar compounds. The adsorbed phenolic compounds were extracted from the resin by elution with 100 mL of methanol, which was evaporated by flushing with nitrogen. The extracts were stored at −80 °C until HPLC analysis. Before performing the biological tests, each extract was dissolved in DMSO at a final concentration of 300 mg/mL. Food grade Limoncella apple extracts (IndLAE) were produced at MB-Med (Turin, Italy) starting from fresh Limoncella Apples. Upon lyophilization of peels and flesh of Limoncella apples, samples were treated with ethanol/water (70:30 v/v) for 24 h at 40 °C to extract phenolic compounds and generate food grade Limoncella Apple Extracts (in the text referred to as IndLAE).

2.3. In Vitro Simulated GI Digestion of Apple Extracts

The assay was performed according to the procedure described by Raiola et al. [28] and by Tenore et al. [23], with few modifications. For GI digestion, the apple samples (20 g) were mixed with 6 mL of artificial saliva composed of KCl (89.6 g/L), KSCN (20 g/L), NaH_2PO_4 (88.8 g/L), Na_2SO_4 (57.0 g/L), NaCl (175.3 g/L), $NaHCO_3$ (84.7 g/L), urea (25.0 g/L) and 290 mg of α-amylase. The pH of the solution was adjusted to 6.8 with HCl 0.1 N. The mixture was introduced in a plastic bag containing 40 mL of water and homogenized in a Stomacher 80 Microbiomaster (Seward, Worthing, UK) for 3 min. Immediately, 0.5 g of pepsin (14,800 U) dissolved in HCl 0.1 N was added, the pH was adjusted to 2.0 with HCl 6 N, and the solution was incubated at 37 °C in a Polymax 1040 orbital shaker (250 rpm) (Heidolph, Schwabach, Germany) for 2 h. Then the pH was increased to 6.5 with NaHCO3 0.5 N and

5 mL of a mixture of pancreatin (8.0 mg/mL) and bile salts (50.0 mg/ mL) (1:1; *v*/*v*), dissolved in 20 mL of water, was added and incubated at 37 °C in an orbital shaker (250 rpm) for 2 h. Supernatants were extracted with an acetonitrile/water (84:16; *v*/*v*) mixture, and then either analyzed by HPLC or dried and dissolved in DMSO for biological tests.

2.4. HPLC-DAD Analysis

HPLC separation and quantification of phenolic compounds in apple extracts and samples obtained from in vitro digestion, were performed according to earlier studies [23] with some modifications. Identification was possible by comparing spectra and retention times with those of commercial standards and with those reported in previous works [29]. The column selected was a Hypersil BDS C18 column (250 mm, 4.6 mm, 5 μm) (Thermo, Bellefonte, PA, USA). Analyses were run on a Finnigan HPLC system (Thermo Electron Corporation, San Jose, CA, USA) provided with a photodiode array detector (DAD). The identity of phenolic acids and flavonoids was confirmed with LC-ESI/MS/MS experiments, as already reported [24].

2.5. Cell Cultures

HEK293, CaCo-2, and U87MG cells were grown in DMEM (#41965-039, GIBCO, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (#10270, GIBCO), Glutamax (#35050-061, GIBCO) and Pen/Strep (#15070-063, GIBCO). HEK293 transfection was performed using Polyethylenimine (Sigma Chemical Co., St. Louis, MO, USA). CaCo-2 transfection was performed using Lipofectamine (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Both cell cultures and human biopsies were analyzed for viability using Trypan blue, Propidium Iodide (PI) and Acridine-Orange (AO) staining. A pre-mixed AO/PI solution was directly added to cell samples for viability analysis, using a fluorescent cell counter.

2.6. Excision and Culturing of Human Biopsies from FAP Patients

Biopsies from Familial Adenomatosis Poliposis patients were kindly provided by Prof. G.B. Rossi. The study was approved by the Ethics Committee of the University Federico II of Naples. For all the patients enrolled in this study, polypectomy was part of their clinical treatment plan and it was scheduled and performed independently from this research. Immediately after excision, biopsies were rinsed in physiological saline. Samples were then digested with Trypsin for 10 min at room temperature (RT) and centrifuged at 400 rpm for 10 min at RT. Isolated cells were then counted and cultured at the confluency of 100,000 cell per mL in DMEM, supplemented with 10% FBS, Glutamax and Pen/Strep. When indicated, apple extracts were added at a concentration of 400 mg/L. After 24 and 48 h of incubation, cell viability was measured with using Trypan blue, Propidium Iodide (PI) and Acridine-Orange (AO) staining.

2.7. DNA

All DNA constructs were synthesized at GeneScript (Piscataway, NJ, USA). The cDNA coding for N terminally HA tagged FZD4wt (HA-FZD4-wt), was cloned in the expression vector, pCDNA3.1 (Invitrogen). For the reporter construct, WNT reporter Green Fluorescent Protein GFP construct (TCF-wt GFP), 8 repeats of the optimized TCF/LEF binding sequence [5′-AGATCAAAGGGG-3′] (interspaced by the triplet 5′-GTA-3′) were positioned upstream to a minimal TATA box promoter [5′-tagagggtatataatggaagctcgaattccag-3′] and a KOZAC region [5′-cttggcattccggtactgttggtaaaaagcttggcattccggtactgttggtaaagccacc-3′]. The sequence was cloned in the vector pCDNA 3.1 (+) GFP between the restriction sites for NruI and HindIII. This replacement substitutes the cytomegalovirus (cmv) promoter of the original vector with the TCF/LEF responsive sequences. The correctness of the sequences were verified by DNA sequencing. The reporter construct (TCF-mut GFP) was used as a negative control. This was obtained by the mutagenesis of TCF-wt GFP and presented the 8 repeats of the TCF/LEF binding sequence mutated to [5′-AGGCCAAAGGGG-3′].

The reporter construct, cmv GFP, used in this manuscript as the transfection control, was obtained by replacing the TCF/LEF consensus region in TCF-wt GFP with a cmv promoter.

2.8. WNT Pathway Activity Measurement in HEK293 Cells Using the TCF-GFP Constructs

HEK293 cells were seeded (5×10^3 per well) in 96-well black Optyplates (Perkin Elmer, Waltham, MA, USA). After 24 h, cells were co-transfected with both WNT reporter GFP construct and HA-FZD4-wt. Transfection mixtures were prepared as follows: for each well, 0.25 μg of PEI (pH 7.0) was supplemented with 0.08 μg of DNA (both diluted in 4 μL of DMEM). The mixture was incubated at room temperature (RT) for 30 min, to be then diluted in culture medium and added to the cells. Twenty-four hours after transfection, the medium was replaced and cells were incubated with WNT5A conditioned medium. Human glyoblastoma U87MG cells were used as a source of WNT5A conditioned medium [30]. U87MG cells endogenously express FZD4, which has been shown to be necessary for the activity of WNT/β-catenin pathway in these cells [31]. Confluent 6 cm plates of U87MG cells were incubated for 3 days in DMEM, 10% FBS and Glutamax, in the absence of antibiotics. Conditioned medium was subsequently collected and stored at −20 °C. Cells were then rinsed with fresh medium and cultivated for a further period of 3 days. The conditioned medium obtained after the second incubation was pooled with the first one. The pooled conditioned medium was used to stimulate TCF/LEF activity in HEK293 cells. It was used undiluted. When required, the conditioned medium was supplemented with apple extracts at the indicated concentrations. When reported, cells were supplemented with LiCl, Epidermal Growth Factor (EGF), NFAT inhibitor (10 μM) or Bisindolylmaleimide II (7.5 μM). After 48 h, cells were fixed in 3.7% formaldehyde, in PBS (pH 7.4), for 30 min. Formaldehyde was quenched by incubating the cells for 30 min in 0.1 M glycine in PBS 1x. The activity of the apple extract was evaluated by measuring GFP expression.

2.9. WNT Pathway Activity Measurement in CaCo-2 Cells Using the TCF-GFP Constructs

CaCo-2 cells, growing on glass coverslips, were transfected by Lipofectamine , according to the to the manufacturer's procedures. Twenty-four hours after transfection, the medium was replaced and cells were incubated with the indicated extracts for 24–48 h. The cells were then fixed in a solution of 3.7% formaldehyde, in PBS with a pH 7.4, for 30 min. Formaldehyde was quenched by incubating the cells for 30 min in 0.1 M glycine in PBS.

2.10. Immunofluorescence

CaCo-2 cells were grown on glass coverslips. Cells were fixed in 3.7% formaldehyde/PBS (pH 7.4) for 30 min. Formaldehyde was quenched by incubating the cells for 30 min in 0.1 M glycine in PBS 1x. Then, cells were permeabilized in 0.1% Triton/PBS, pH 7.4, for 10 min at 25 °C and then incubated with a rabbit polyclonal anti-β-catenin antibody (H-102, sc-7199, Santa Cruz, Dallas, TX, USA) diluted 1:200 in PBS and a goat anti-rabbit IgG (H&L), DyLight 594 conjugate, (ImmunoReagents, Raleigh, NC, USA) (diluted 1:500 in PBS), for 1 h and 45 min, respectively.

2.11. Statistical Analysis

Unless otherwise stated, all of the experimental data are shown as mean ± standard deviation (SD) of at least three replications [32]. Statistical analyses of data were performed using the Student's *t*-test or two-way ANOVA, followed by the Tukey–Kramer multiple comparison test, to evaluate significant differences between a pair of means. For dose-response data, half maximal effective concentrations (EC_{50}) were calculated using nonlinear regression analysis with Prism software 6.0 (GraphPad, GraphPad Software, San Diego, CA, USA, www.graphpad.com). EC_{50} are indicated as mean ± standard error mean (SEM). All data were analyzed using the two-tailed Student's *t*-test and $p < 0.05$ indicated a statistically significant result.

3. Results

3.1. WNT Inhibitory Activity of AAE and LAE

The total (flesh and peel) polyphenolic content in AAE and LAE prepared for this study was determined by HPLC-DAD analysis and is reported in Table 1. AAE and LAE presented a qualitatively similar polyphenolic repertoire.

Table 1. Polyphenolic content of AAE and LAE.

Compound	AAE *	LAE *	PEF (AAE) **	PEF (LAE) **
Chlorogenic Acid	3.9 ± 0.2	4.7 ± 0.1	10.2 ± 0.6	9.7 ± 0.9
(+) Catechin	0.8 ± 0.1	1.3 ± 0.1	3.0 ± 0.2	5.5 ± 0.4
(–) Epicatechin	0.9 ± 0.2	1.3 ± 0.1	2.8 ± 0.1	4.0 ± 0.2
Isoquercetin	1.4 ± 0.1	2.9 ± 0.2	3.4 ± 0.3	2.7 ± 0.3
Rutin	11.2 ± 0.2	0.4 ± 0.3	34.7 ± 0.1	1.7 ± 0.2
Phloridzin	2.1 ± 0.3	1.2 ± 0.5	7.5 ± 0.2	3.0 ± 0.1
Procyanidin B2	1.3 ± 0.1	2.8 ± 0.1	4.1 ± 0.1	10.8 ± 0.1
Phloretin	n.d.	n.d.	n.d.	n.d.
Quercetin	n.d.	n.d.	n.d.	n.d.

Polyphenolic content present in: Annurca Apple Extract (AAE), Limoncella Apple Extract (LAE), a polyphenolic enriched fraction of AAE (PEF(AAE)), a polyphenolic enriched fraction of LAE ((PEF(LAE)). * mg/100 g of Fresh Weight (FW); ** mg/100 mg Dry Weight (DW); n.d., not detected.

They contained a similar amount of chlorogenic acid, the most abundant hydroxycinnamic acid in apples, as well as of the flavan-3-ols, (+) catechin and (–) epicatechin. The quercetin-glycoside, rutin and the phloretin–glycoside, phloridzin, were more abundant in AAE than in LAE. The latter, on the contrary, contained higher amounts of procyanidin B2 and isoquercetin [24,26]. The aglycones, quercetin and phloretin were absent in both the extracts.

The activity of the apple extracts on WNT/β-catenin signaling was assayed in a reconstituted recombinant system. We used, as a biological platform, human embryonic HEK293 cells, transiently expressing both the WNT receptor Frizzled 4 (FZD4) [33] and a WNT pathway reporter DNA construct. Three different WNT reporter constructs were used. The first, hereinafter referred to as TCF-wt GFP, presents the coding sequence of GFP under the control of an optimized WNT pathway responsive promoter. In the second, hereinafter referred to as TCF-mut GFP, the WNT responsive promoter was mutagenized to become unresponsive to WNT (see methods for details). Finally, a third reporter construct (cmv GFP) presents the coding sequence of GFP under the control of a constitutive cmv promoter. The TCF-mut GFP and cmv GFP constructs were here considered as negative controls, and used to monitor WNT unrelated change in GFP expression.

The FZD agonist, WNT5A, induced GFP expression in HEK293 cells, transiently transfected with TCF-wt GFP (Figure S1). On the contrary, WNT5A did not affect GFP expression in cells transfected either with TCF-mut GFP or with cmv GFP (Figure S1), confirming the specificity of the platform. At the endogenous level, HEK293 cells express several other FZD receptors. However, differently from FZD4, these did not respond to WNT5A stimulation. As shown in Figure S1, in the absence of FZD4, WNT5A stimulation does not influence GFP expression in HEK293.

WNT5A has been shown to activate both the WNT/β-catenin pathway as well as one of the "non-canonical" branches of WNT signaling, the WNT/Ca^{2+} pathway [5]. In our biological system, WNT5A increased GFP expression, mainly as a consequence of WNT/β-catenin pathway activation.

Inhibitors of Nuclear Factor of Activated T-cells (NFAT) and Protein Kinase C (PKC) (both key elements of the WNT/Ca^{2+} pathway) did not affect GFP expression induced by WNT5A (Figure S1). The combination of WNT5A and a recombinantly expressed FZD4 allowed us a clear interpretation of the effect of apple extracts on the WNT/β-catenin signaling pathway.

AAE and LAE both worked efficiently as WNT inhibitors and reduced WNT activity elicited by WNT5A. The EC_{50} of WNT pathway inhibition were 140 ± 16 mg/L and 330 ± 23 mg/L for AAE

and LAE, respectively (Figure 1A,B). AAE and LAE did not affect GFP expression in cells transfected either with TCF-mut GFP or with cmv GFP (Figure S2), confirming that the two extracts affect the WNT/β-catenin pathway.

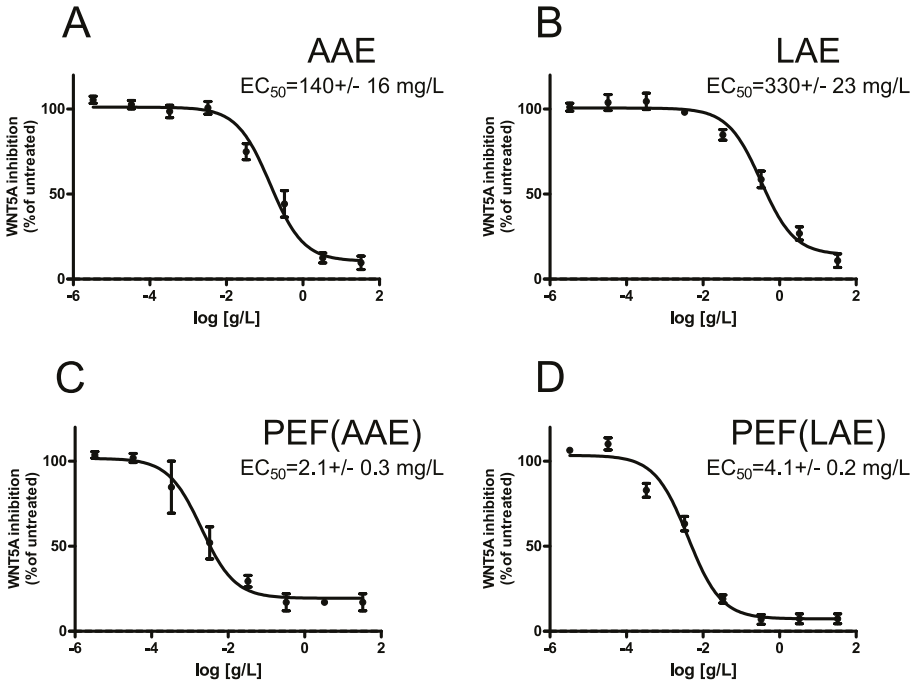

Figure 1. AAE and LAE act as WNT pathway inhibitors. (**A**,**B**) Dose-response curves represent AAE (**A**) and LAE (**B**) modulation of WNT/β-catenin pathway in HEK293 cells co-expressing FZD4 and the WNT reporter construct (TCF-wt GFP). Values indicate changes in GFP expression (expressed as mean percentage change over untreated samples). (**C**,**D**) Dose-response curves represent PEF(AAE) (**C**) and PEF(LAE) (**D**) modulation of WNT/β-catenin pathway. Values are reported as mean ± SD (n = 3 replicates). EC_{50} values for each sample are shown on the corresponding graph and are reported as mean ± SEM (n = 5 independent experiments).

The WNT inhibitory activity of both the extracts mainly depends on their polyphenolic content. Compared to total fresh fruit extracts, polyphenolic enriched fractions of AAE and LAE, (hereinafter indicated as PEF(AAE) and PEF(LAE)) (Table 1) were associated with increased WNT inhibitory activity (2.1 ± 0.3 mg/L and 4.1 ± 0.2 mg/L for PEF(AAE) and PEF(LAE), respectively) (Figure 1C,D). The WNT inhibitory activity of both full extracts and polyphenolic fractions cannot be ascribed to their toxicity, which occurs at dilutions higher than 5.0 g/L (Figure S2).

3.2. WNT Inhibitory Activity of Pure Polyphenols

In an attempt to identify which of the polyphenols contained in AAE and LAE were mostly contributing to the WNT inhibitory activity of the apple extracts, we tested them singularly, as pure molecules (Figure 2 and Table 2). HPLC grade, pure epicatechin and catechin both showed very weak WNT5A inhibitory activities. Chlorogenic acid had a WNT inhibitory activity of 3.4 ± 0.2 μM. Rutin and isoquercetin inhibited WNT5A with EC_{50} of 1.8 ± 0.1 μM and 3.1 ± 0.4 μM, respectively. Phloridzin and procyanidin B2 showed EC_{50} of 12.9 ± 0.5 μM and 1.4 ± 0.3 μM, respectively. Considering their low abundance in both the apple extracts, none of the abovementioned molecules would reach effective

concentrations in AAE and LAE solutions diluted at their EC_{50} for WNT inhibition (Table 2). Thus, while polyphenols surely contribute to the WNT inhibitory activityof the apple extracts, none of the polyphenols by themselves can account for the full activity of AAE and LAE, which seem to require, on the contrary, the presence of the whole polyphenolic fraction.

Table 2. WNT inhibitory activity of pure polyphenols.

Compound	[a] EC_{50} (M)	[b] M
Chlorogenic Acid	$3.4 \pm 0.2 \times 10^{-6}$	$\approx 5.3 \times 10^{-8}$
(+) Catechin	$2.5 \pm 0.3 \times 10^{-4}$	$\approx 1.8 \times 10^{-8}$
(–) Epicatechin	$>>10^{-4}$	$\approx 2.6 \times 10^{-8}$
Isoquercetin	$1.8 \pm 0.1 \times 10^{-6}$	$\approx 7.8 \times 10^{-9}$
Rutin	$3.1 \pm 0.4 \times 10^{-6}$	$\approx 2.6 \times 10^{-9}$
Phloridzin	$1.2 \pm 0.2 \times 10^{-5}$	$\approx 1.1 \times 10^{-8}$
Procyanidin B2	$1.4 \pm 0.3 \times 10^{-6}$	$\approx 1.9 \times 10^{-8}$

[a] EC_{50} of WNT pathway inhibition of the indicated compounds. Values are reported as mean ± SEM (n = 3); [b] Molarity of the indicated compounds in a solution of LAE, diluted at its EC_{50} of WNT pathway inhibition.

3.3. Mechanism Underpinning AAE and LAE WNT Inhibitory Activity

We thus moved to identifying the WNT pathway branches inhibited by AAE and LAE. This is very important, especially when searching for therapeutic agents to use for FAP patients. Since APC is a midstream component of the WNT pathway, its mutations make the manipulation of most of the upstream signaling components therapeutically ineffective. To be active in FAP patients, WNT inhibitors should act either downstream to APC or on "non-canonical" WNT pathway branches. One of the most active non-canonical branches positively contributing to WNT signaling is the one involving the EGF Receptor (EGFR) [34,35]. Once activated, the EGFR pathway bypasses APC and leads, via AKT, to β-catenin activation.

We thus challenged AAE and LAE to compete with LiCl and EGF, two inducers of the WNT pathway, both acting downstream to APC. LiCl binds directly to GSK-3β and inhibits the β-catenin destruction complex. In contrast, EGF activates the EGFR pathway, that, via AKT, promotes β-catenin detachment from the Plasma Membrane and its nuclear translocation. In our biological system, both LiCl and EGF induced, in a dose-response manner, GFP expression in HEK293 transfected with TCF-wt GFP (Figure 3A–C). On the contrary, they both did not have an effect on cells transfected either with TCF-mut GFP or with cmv GFP (Figure 3A–C).

AAE and LAE failed to inhibit activation of the WNT pathway induced by 15 and 30 mM LiCl. However, they both reduced WNT pathway activity induced by 5 and 10 mM LiCl (the results for LAE are depicted in Figure 3B). Moreover, at all the tested concentrations, the extract abolished the WNT stimulatory activity of EGF [34] (Figure 3D). These results prove that the apple extracts inhibit WNT pathway activation induced by LiCl and EGF and are thus suitable WNT inhibitors for FAP cells carrying APC mutations.

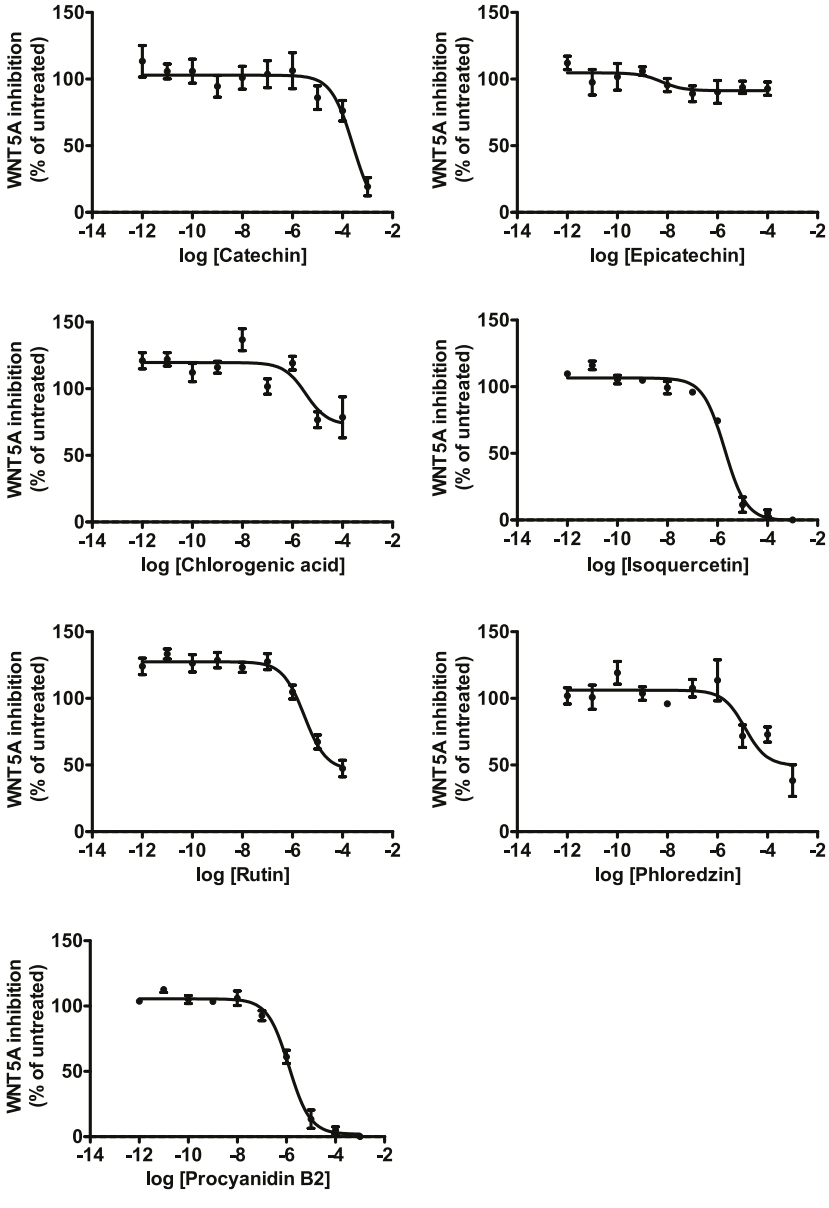

Figure 2. WNT inhibitory activity of apple polyphenols. Dose-response curves represent the indicated compounds modulation of WNT pathway in HEK293 cells. Values are reported as mean ± SD (n = 3). EC_{50} values for each compound are reported in Table 2.

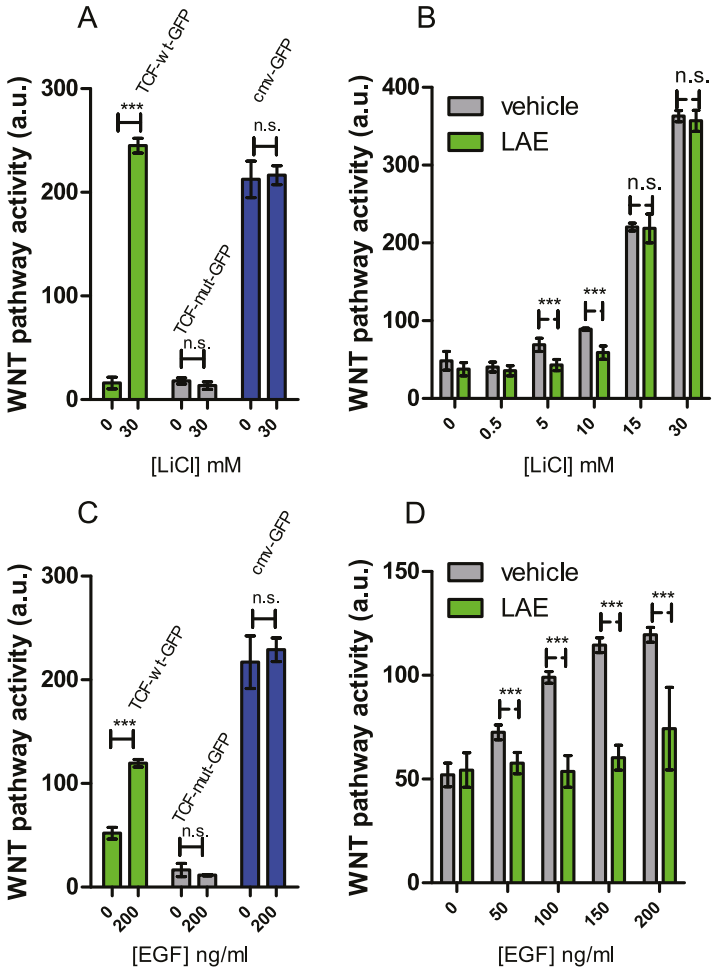

Figure 3. LAE inhibits the WNT pathway, acting downstream to APC. (**A**) The histogram shows the WNT pathway activity induced by LiCl (30 mM) in HEK293 cells transfected with TCF-wt GFP (green bars), TCF-mut GFP (grey bars) and cmv GFP (blue bars); (**B**) WNT pathway activity of cells treated with the indicated concentration of LiCl in the presence (green bars) or in the absence (grey bars) of LAE (400 mg/L); (**C**) WNT pathway activity induced by EGF (200 ng/mL) in HEK293 cells transfected with TCF-wt GFP (green bars), TCF-mut GFP (grey bars) and cmv GFP (blue bars); (**D**) WNT pathway activity of cells treated with the indicated concentration of EGF in the presence (green bars) or in the absence (grey bars) of LAE (400 mg/L). Values are reported as mean ± SEM ($n = 5$). *** $p < 0.05$, n.s. indicates non-statistical.

3.4. WNT Inhibitory Activity of LAE and AAE on CaCo-2 Cells

The suitability of LAE and AAE for APC treatment was further proved in vitro, by testing the WNT inhibitory activity of AAE and LAE on CaCo-2 cells. This colon cancer cell line presents a mutation in the APC gene and is commonly used as in vitro cell culture prototype for FAP cells [36,37]. Despite presenting a mutation in APC, CaCo-2 cells are heterogeneous in regard to the WNT pathway activity. This is due to the strong adhesion established among neighbor cells. Cadherins sequester

β-catenin at the plasma membrane of the cells, avoiding its translocation to the nucleus (Figure S3). CaCo-2 cells were transiently transfected with TCF-wt GFP (Figure 4A). Thanks to the GFP reporter construct, the small percentage of WNT active cells can be easily followed (Figure 4A). Upon treatment with AAE or LAE (400 mg/L, 48 h), GFP expression decreased in CaCo-2 cells (Figure 4A,B), indicating that the extracts efficiently inhibited the WNT pathway in these cells. Similar to what we measured in HEK293 cells, the extract affected the viability of the cells at dilutions higher than 5.0 g/L, (Figure 4B).

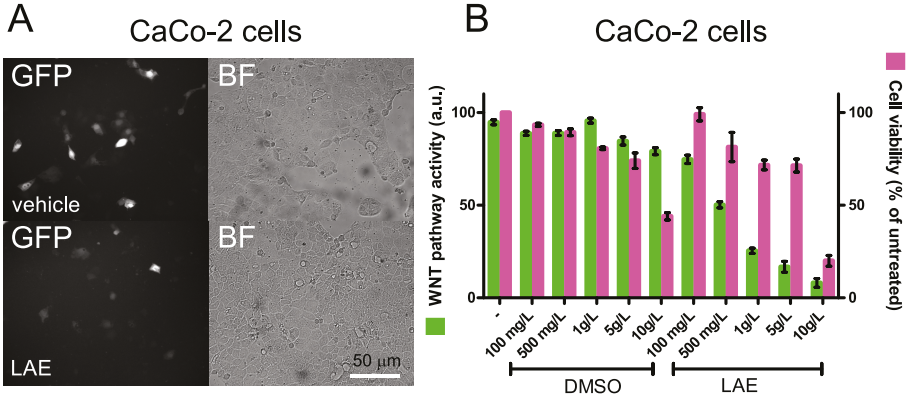

Figure 4. AAE and LAE act as WNT inhibitors in CaCo-2 cells. (**A**) Activity of the WNT reporter construct TCF-wt GFP in CaCo-2 cells cultivated for 48 h in the presence or in the absence of LAE (400 mg/L) (representative of five experiments) (BF = Bright Field; scale bar is shown); (**B**) WNT pathway activity (green bars) and cell viability (magenta bars) of CaCo-2 cells transfected with TCF-wt GFP and cultivated in the absence (−) or in the presence of the indicated concentration of LAE (or of the corresponding dilution of DMSO). Values on the left axes indicate changes in GFP expression (a.u.). Values on the right axes indicate changes in cell viability expressed as percentage of untreated cells (−).

3.5. WNT Inhibitory Activity of LAE and AAE on Human Biopsies

Finally, AAE and LAE were tested in an ex vivo system of FAP cells. Human colon biopsies were cultured in vitro soon after their resection from FAP patients (Figure 5). In unsupplemented media, these ex vivo samples survived and duplicated for up to two days (Figure 5A), and then underwent growth arrest. Treatment with AAE or LAE resulted in a decreased proliferation rate and survival of the ex vivo cultures (Figure 5A–C). The extracts arrested proliferation and induced cell death at dilutions of 400 mg/L and 5 g/L, respectively.

3.6. WNT Inhibitory Activity of Food Grade LAE

Food-grade manufacturing of fruit extracts can often affect patterns and structures of polyphenols. This is because extraction procedures for alimentary purposes only allow water and ethanol as solvents. Thus, the industry makes use of high temperatures and harsher conditions to increase the yield of extraction. We here tested the WNT inhibitory activity of apple extracts obtained from industrial-scale food-grade preparation of LAE (hereinafter referred as IndLAE). IndLAE presents a WNT inhibitory activity increase compared to LAE (EC_{50} of WNT pathway inhibition were of 47.4 ± 0.9 mg/L) (Figure 6A). A HPLC-DAD analysis of the flavonoid content of the IndLAE did not reveal major changes in the number of polyphenols compared to AAE and LAE (Table 3). However, we realized the presence of a discrete amount of quercetin, a molecule that was absent in the apple extracts, which probably arose from the conversion of quercetin glycosides into their aglycone form. Tested in our WNT inhibitory assay and as already demonstrated [38,39], quercetin had a strong inhibitory activity on the WNT pathway (EC_{50} at 110 ± 5 nM, Figure 6B) compared to the other

polyphenols. This suggests that the molecule may contribute to the increased WNT inhibitory activity of the industrial extract.

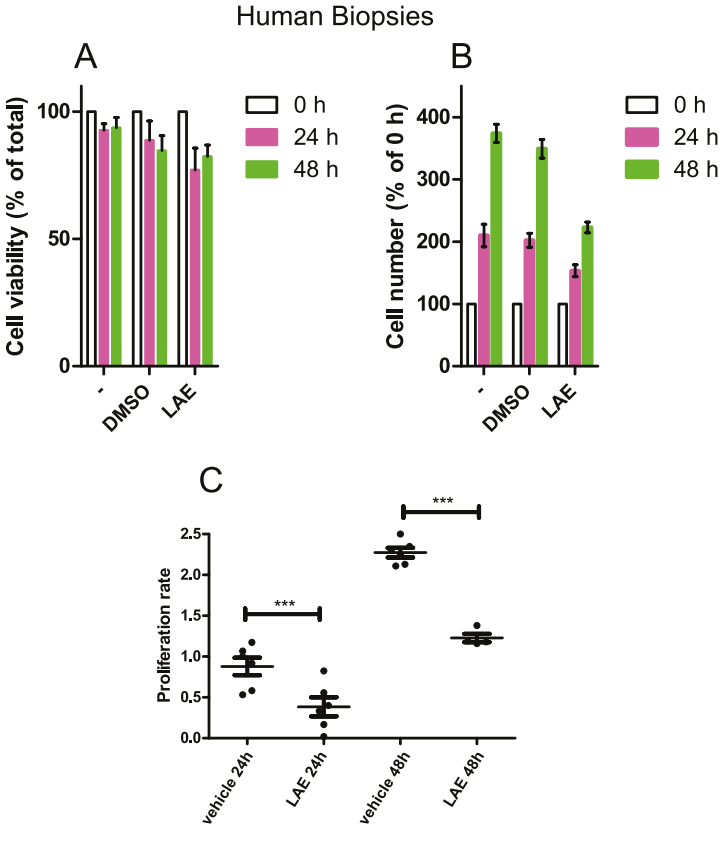

Figure 5. AAE and LAE affect ex vivo cultures of cells carrying FAP mutations. Cell viability, cell number and proliferation rate of human colonic biopsies cultured for 24 h and 48 h in a culture medium supplemented with LAE (400 mg/L) or with vehicle (DMSO). Values are expressed as mean ± SEM ($n = 9$). *** $p < 0.05$.

Table 3. Polyphenolic content of industrial-scale food-grade LAE.

Compound	Ind(LAE) *
Chlorogenic Acid	8.6 ± 0.1
(+) Catechin	1.4 ± 0.2
(–) Epicatechin	0.7 ± 0.1
Isoquercetin	0.5 ± 0.1
Rutin	2.6 ± 0.3
Phloridzin	7.0 ± 0.4
Procyanidin B2	2.5 ± 0.1
Quercetin	1.2 ± 0.1
Phloretin	n.d.

Polyphenolic content of Industrial Limoncella Extract (IndLAE). * mg/100 g DW; Values are reported as mean ± SD.

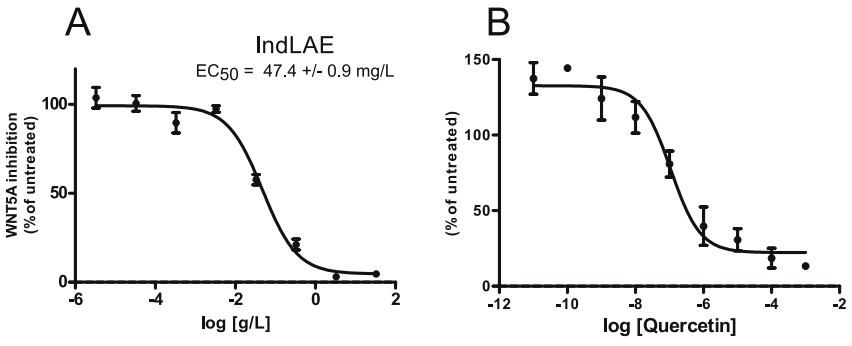

Figure 6. WNT inhibitory activity of IndLAE and quercetin. (**A**) The dose-response curve represents Ind(LAE) modulation of WNT/β-catenin pathway. Values are reported as mean ± SD (n = 3). The EC_{50} value for Ind(LAE) is shown on the graph and is reported as mean ± SEM (n = 5); (**B**) The dose-response curve represents quercetin modulation of the WNT pathway in HEK293 cells. Values are reported as mean ± SD (n = 3). EC_{50} value for quercetin is reported in the text.

3.7. WNT Inhibitory Activity of LAE after In Vitro Simulated GI-Digestion

To achieve WNT inhibitory activity in the colon, apple polyphenolic must be bio-accessible, i.e., they should be extracted from the food matrix, resist to GI-digestion and reach the colon-rectal section of the intestine. However, GI digestion may affect substantially native apple polyphenolic patterns and concentrations as well as induce drastic structural changes of the food constituents [40]. During GI digestion, polyphenols may be further degraded [41] or hydrolyzed, deglycosylated or cleaved by esterases [42]. To measure the bio-accessibility of the WNT inhibitory pool of apple polyphenol, we subjected apple extracts to in vitro simulated digestion [23]. Upon digestion, AAE and LAE (hereinafter referred to as digAAE and digLAE) drastically lost their WNT inhibitory activity (Figure 7A,B). Results of the HPLC-DAD analysis of the polyphenolic contents of digAAE and digLAE are reported in Table 4. As expected, in vitro digestion decreased the overall polyphenolic content of around 40–50%. For both cultivars, digestion converted procyanidins into monomeric catechins, while total amounts of chlorogenic acid, rutin, epicatechin and isoquercetin were drastically reduced. Interestingly, GI digestion reduced the content of phloridzin in AAE but, not in LAE, where the molecule was almost completely resistant to GI. These results indicate that, most likely, the polyphenols ingested by consumption of fresh fruit or fresh fruit extract, will not reach the colon-rectal segment of the intestinal tract and strongly points towards the encapsulation of the extract in gastro-resistant tablets as an essential requirement to preserve apple extract WNT inhibitory activity.

Table 4. Polyphenolic content of in vitro digested AAE and LAE.

Compound	digAAE *	digLAE *
Chlorogenic Acid	0.9 ± 0.1	0.7 ± 0.1
(+) Catechin	2.5 ± 0.1	2.8 ± 0.1
(–) Epicatechin	0.1 ± 0.1	0.3 ± 0.1
Isoquercetin	0.1 ± 0.1	0.1 ± 0.1
Rutin	0.3 ± 0.2	n.d.
Phloridzin	n.d.	0.3 ± 0.1
Procyanidin B2	n.d.	0.2 ± 0.1
Phloretin	n.d.	n.d.
Quercetin	n.d.	n.d.

Polyphenolic content of: in vitro GI-digested Annurca Apple Extract (digAAE), in vitro GI-digested Limoncella Apple Extract (digLAE); * mg/100 g of DW. Values are reported as mean ± SD.

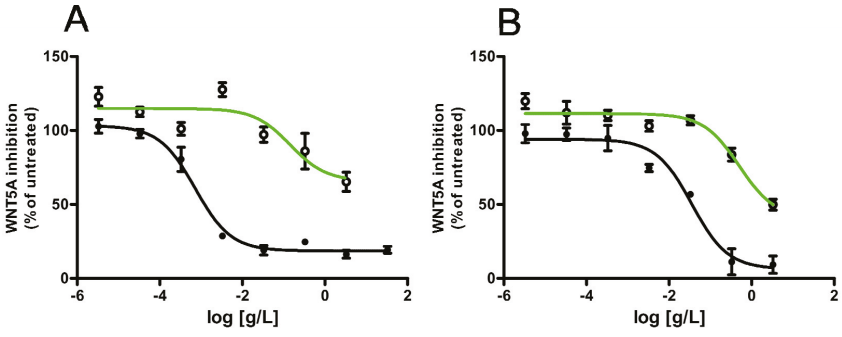

Figure 7. In vitro simulated GI digestion reduces WNT inhibitory activity of AAE and LAE. Dose-response curves for AAE (**A**) and LAE. (**B**) Modulation of the WNT/β-catenin pathway before (black lines) or after in vitro simulated GI digestion (green lines). Values are reported as mean ± SD (n = 3).

4. Discussion

Polyphenol consumption has been related to several health beneficial effects, such as reduced overall mortality [43], incidence of cancer [10] and cardiovascular diseases [12,13,20,44]. The diet is the principal human source of polyphenolic compounds. Polyphenol-rich foods include fruits, vegetables, whole grains, nuts and olive oil.

We here showed that apple extracts from both Annurca (AAE) and Limoncella (LAE) are endowed with inhibitory activity toward WNT/β-catenin signaling, an intracellular pathway involved in many forms of cancer. Moreover, we showed that both the extracts are potentially suitable for the treatment of FAP, a WNT related disease. In this syndrome, a mutation in the protein APC, leads to hyperactivation of the WNT/β-catenin signaling pathway and, in turn, to uncontrolled intestinal cell proliferation and a high risk for the formation of adenocarcinomas. We showed that both AAE and LAE are able to inhibit WNT signaling in cells carrying APC mutations. In both in vitro cultures of cells as well as in ex vivo biopsies of FAP patients, AAE and LAE blocked the proliferation and duplication rates of colon-rectal cancer cells.

AAE and LAE WNT inhibitory activity can be partially ascribed to their polyphenolic content. However, none of the major constituents of their polyphenolic fractions, tested as pure molecules, exerted strong WNT inhibitory activities, suggesting the importance of the whole pool of polyphenols for the activity of the extracts. WNT pathway inhibitory activity has been described for many individual polyphenols. Epigallocatechin gallate (EGCG) [45], isoquercetin [46], anthocyanins [47], theaflavin [48], ellagic acid derivatives [49], caffeoylquinic acids [50] and polymeric polyphenols [51] have all been shown to act as WNT inhibitors in many in vitro cultures of cancer cells. Some of them, like EGCG and ellagic acid, are undetectable in AAE and LAE [24]. We cannot exclude that these could indeed account, alone, for the entire WNT inhibitory activity of the extracts in which they are contained. However, regarding the polyphenols we detected in AAE and LAE, our results are in line with previous reports describing, for these compounds, EC_{50} for WNT pathway inhibition in the micromolar range [35,52,53]. According to this and other studies [54], upon consumption of apples at consumer-relevant concentrations [55–57], none of the polyphenols contained in AAE and LAE (even assuming a full bioavailability) would reach active concentrations for WNT pathway inhibition in the colon-rectal segment of the intestine. This is in line with epidemiologic studies that, more and more, show that supplementation with mixtures of bioactive compounds more than with single components, may be effective in exerting biological activity [10,44,58–60].

Our preliminary results suggest that the WNT inhibitory activity of AAE and LAE can be ascribed either to the antioxidant activity of the entire polyphenolic mixture or to the effects that the latter may

exert on its own components, protecting them from detrimental chemical modifications occurring in physiological environments.

We here showed that food-grade large-scale manufacturing alters the flavonoid composition of LAE, drastically increasing the WNT inhibitory activity of the extract. This could be ascribed to the presence of quercetin, a potent β-catenin inhibitor, absent in the original extract, which probably originated from the conversion of quercetin-glycosides into their aglycone form. This result should encourage researchers to test the activity of the extract after industrial manufacturing. The latter drastically alters the composition of the extract, even if this event, as we have here shown, does not always have to be detrimental to the properties of the extract.

We finally showed that both LAE and AAE lose activity upon in vitro simulated GI digestion as a result of a severe loss of polyphenols. This strongly points towards encapsulation in gastro-resistant tablets (for example enteric coatings) as an essential requirement to preserve apple extract WNT inhibitory activity in the colon-rectal section of the intestinal tract.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/9/11/1262/s1. Figure S1: Description of the biological platform used to measure the activity of canonical WNT/β-catenin signaling in HEK293 cells, Figure S2: AAE and LAE inhibit WNT/β-catenin signaling in HEK293 cells, Figure S3: WNT/β-catenin pathway activity in CaCo-2 cells.

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Author Contributions: G.R., S.B., N.B. conducted all the biological experiments. M.M. and G.C.T. performed the fruit extraction and the characterization of the polyphenols. G.B.R. performed the resection of biopsies. M.S. and E.N. designed the experiments and wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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Study of Structure and Permeability Relationship of Flavonoids in Caco-2 Cells

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Abstract: Flavonoids exhibit a broad range of biological activities. However, poor absorption of some flavonoids is a major limitation for use of flavonoids as nutraceuticals. To investigate the structure requirements for flavonoids intestinal absorption, transepithelial transport and cellular accumulation (CA) of 30 flavonoids were determined using the Caco-2 cell monolayer. The bilateral permeation of five types of flavonoids followed the order: flavanones $\geq$ isoflavones > flavones $\geq$ chalcones > flavonols. The concentration of flavonoids accumulated in cells did not correlate with cell penetration since the correlation coefficient between the apparent permeability coefficient (P_{app}) and their corresponding CA was poor ($R^2 < 0.3$). Most flavonoids exhibited a ratio of 0.8–1.5 for $P_{app\ A\ to\ B}/P_{app\ B\ to\ A}$, suggesting passive diffusion pathways. However, luteolin, morin and taxifolin may involve the efflux mechanisms. The quantitative structure-permeability relationship (QSPR) study demonstrated that the intestinal absorption of flavonoids can be related to atomic charges on carbon 3′ ($Q_{C3'}$), molecule surface area (*SlogP_V3*), balance between the center of mass and position of hydrophobic region (*vsurf_ID1*) and solvation energy of flavonoids (*E_sol*). These results provide useful information for initially screening of flavonoids with high intestinal absorption.

Keywords: Caco-2; flavonoids; intestinal absorption; permeability; QSPR

1. Introduction

Flavonoids which are produced by plants as secondary plant metabolites are one of the most widely distributed polyphenols. Structurally, flavonoids are derived from a parent nucleus, a diphenylpropane (C6-C3-C6) skeleton. Flavonoids are mainly divided into eight subclasses depending on the structure difference on C ring, including flavones, flavonols, flavanones, flavanonols, isoflavones, flavan-3-ols, flavan-3, 4-diols (flavandiol) and anthocyanins [1]. Flavonoids exhibit health benefits due to their various biological activities including antioxidant, anti-cancer [2], antimicrobial [3], anti-inflammation [4] and others. However, poor oral bioavailability of some flavonoids, caused by low epithelial transport and extensive metabolism, is a major limitation for use of flavonoids as nutraceuticals. Low bioavailability has been associated with flavonoid interactions at various stages of the digestion, absorption and distribution process, which are strongly affected by their molecular structures. Hence, an increase in absorption of flavonoids is one way to improve their oral bioavailability. Previous studies have shown that the molecular structures play an important role on the absorption of flavonoids, including the degree or position of substitution of hydroxyl and alkyl group [5], methoxyl group [6], prenyl group [7], and glycosidic group [8].

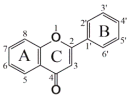
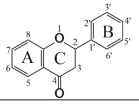
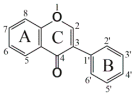
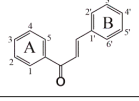
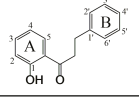
Numerous structure permeability relationship (SPR) studies of flavonoids have been investigated by comparing the difference of permeability/absorption data and structure of analogues.

However, the application of SPR study has been limited due to lack of prediction for untested compounds. The quantitative structure activity relationship (QSAR) for flavonoids is gaining interest by quantitatively correlating the molecular structures or properties with variation in biological activity. But, few QSPR studies have been performed to investigate the intestinal absorption of flavonoids [9] and the absorption mechanisms for flavonoids are not well established.

Caco-2 monolayer model is widely used [10,11] to estimate and predict the intestinal permeability of various flavonoids [5,12], because it is derived from human colonic adenocarcinoma and shares many morphological (e.g., microvilli) and functional properties with mature enterocytes. Caco-2 cells also exhibit a well-differentiated brush border on their apical surfaces and express many typical transporters and enzymes found in the small intestine [13,14]. An excellent correlation was found between the oral absorption of flavonoids in human and the apparent permeability (P_{app}) in Caco-2 monolayer model when the P_{app} is more than 10^{-6} cm/s [15].

In this study, 30 flavonoids were investigated for their intestinal permeability and cellular accumulation (CA) by using the Caco-2 monolayer model. First, the tested flavonoids with diverse structures including flavones, flavonols, flavanones, flavanonols, isoflavones and chalcones were studied to obtain their SPR. To observe the effect of structure of flavonoids on their intestinal absorption, a 2D-QSPR model was developed using the P_{app} values from apical to basolateral side ($P_{app\ A\ to\ B}$) as the dependent variable and molecular descriptors as independent variables. The chemical structures of the 30 flavonoids tested are presented in Table 1.

Table 1. The chemical structures of 30 flavonoids.

No.	Flavonoids	Core Structure	R_3	R_5	R_6	R_7	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$
1	Flavone		H	H	H	H	H	H	H	H	H
2	Tangertin		H	OMe	OMe	OMe	OMe	H	H	OMe	H
3	Wogonin		H	OH	H	OH	OMe	H	H	H	H
4	Baicalein		H	OH	OH	OH	H	H	H	H	H
5	Luteolin		H	OH	H	OH	H	H	OH	OH	H
6	Apigenin		H	OH	H	OH	H	H	H	OH	H
7	Chrysin		H	OH	H	OH	H	H	H	H	H
8	Schaftoside		H	OH	Cglc	OH	Carb	H	H	OH	H
9	Galangin		OH	OH	H	OH	H	H	H	H	H
10	Quercetin		OH	OH	H	OH	H	H	OH	OH	H
11	Morin		OH	OH	H	OH	H	OH	H	OH	H
12	Kaempferol		OH	OH	H	OH	H	H	H	OH	H
13	Kaempferide		OH	OH	H	OH	H	H	H	OMe	H
14	Myricetin		OH	OH	H	OH	H	H	OH	OH	OH
15	Isorhamnetin		OH	OH	H	OH	H	H	OMe	OH	H
16	Quercitrin		Orha	OH	H	OH	H	H	OH	OH	H
17	Rutin		ORG	OH	H	OH	H	H	OH	OH	H
18	Hesperetin		H	OH	H	OH	H	H	OH	OMe	H
19	Naringenin		H	OH	H	OH	H	H	H	OH	H
20	Naringin		H	OH	H	ONG	H	H	H	OH	H
21	Liquiritigenin		H	H	H	OH	H	H	H	OH	H
22	Taxifolin		OH	OH	H	OH	H	H	OH	OH	H
23	Formononetin		-	H	OH	H	H	H	OMe	H	H
24	Puerarin		-	H	H	OH	Cglc	H	H	OH	H
25	Glycitein		-	H	OMe	OH	H	H	H	OH	H
26	Daidzein		-	H	H	OH	H	H	H	OH	H
27	Genistein		-	OH	H	OH	H	H	H	OH	H
28	Biochanin A		-	OH	H	OH	H	H	H	OMe	H
29	Isoliquiritigenin		OH	OH	-	-	-	H	H	OH	H
30	Neohesperidin dihydrochalcone		NG	OH	-	-	-	H	OH	OMe	H

Cglc: -C-glucopyranosyl; Carb: -*O*-(α-L-Arabinopyranosyl); Orha: -*O*-α-L-rhamnopyranosyl; RG: -(6-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyloxy); NG: -(2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl).

2. Materials and Methods

2.1. Materials and Methods

Human colon adenocarcinoma cell line Caco-2 (ATCC #HTB-37) was purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Gen-View Scientific Inc. (Calimesa, CA, USA). Compound **1** and **2** were purchased from BBT INC. (Tianjin, China) (purity > 98%). Compound **8** was purchased from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China) (purity > 98%). Compound **16** (purity > 95%) and Lucifer yellow carbohydrazide (CH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The remaining flavonoid compounds were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China) (purity > 98%). The flavonoids were dissolved in stock with a concentration of 100 mM anddiluted to a final concentration of 0.1% (v/v) dimethyl sulfoxide. Fetal bovine serum (FBS) was obtained from Gibco Laboratories (Life Technologies Inc., Grand Island, NY, USA). Minimum essential medium (MEM) and non-essential amino acids (NEAA) were purchased from Hyclone (Logan, UT, USA). Millicell hanging cell culture inserts of 12 wells (PET, 12 mm, pore size 0.4 μm) were purchased from Millipore (Boston, MA, USA) and 12-well plates were purchased from Corning Costar (Cambridge, MA, USA). All other chemicals were of analytical grade.

2.2. Cell Viability Assay

The cytotoxicity of flavonoids and colchicine was evaluated by MTT assay. The cells were grown in 96-well plates at a density of 1×10^4 cells/well. After incubation with 40 μM flavonoid for 24 h, the cells were further incubated with an MTT solution (0.5 mg/mL) for 4 h at 37 °C. Finally, after removal of the supernatant, 150 μL dimethyl sulphoxide was added to each well to dissolve crystallized MTT. Absorbance was read at 570 nm with a multiskan spectrum microplate reader (Thermo Labsystems, Waltham, MA, USA). The percentage cell viability relative to that of the control cells was used as the cytotoxicity measure.

2.3. The Permeability of Flavonoids in Caco-2 Cell Monolayers

Caco-2 cells were cultured in MEM supplemented with 10% FBS, 1% NEAA, penicillin (100 U/mL) and streptomycin (100 μg/mL) in an atmosphere of 5% CO_2 and 90% relative humidity at 37 °C. All cells used in this study were between passages 35 and 45 [16].

Caco-2 cells were seeded at a density of about 8×10^4 cells/cm^2 on a 12 wells Millicell hanging insert and left to grow for 19–21 days to reach confluence. Culture medium was replaced every other day for 14 days and daily thereafter. The integrity and transport ability of the Caco-2 cells monolayer were examined by measuring the trans-epithelial electrical resistance (TEER) with a Millicell voltammeter (Millicell ERS-2, Merck Millipore, Billerica, MA, USA) and running standard assays using Lucifer yellow CH as paracellular flux marker. Only cell monolayers with a TEER above 600 Ω·cm^2 and P_{app} of Lucifer yellow CH flux less than 0.5×10^{-6} cm/s were used for the transport assays. Differentiation of Caco-2 cells was checked after 4 and 12 days by determining the activity of alkaline phosphatase with an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and after 21 days transmission electron microscopy was performed on Caco-2 monolayer to evaluate differentiation [17].

The Caco-2 cell monolayer was washed with D-Hank's buffer (pH 7.4). Then, the flavonoid (40 μM) was added to either the apical (AP, 0.4 mL) or basolateral (BL, 1.95 mL) side, while the receiving chamber contained the corresponding volume of D-Hank's buffer and incubated for 1 h at 37 °C. Solutions from both AP and BL sides of Caco-2 cell monolayer were collected and immediately frozen, lyophilized and preserved at −80 °C for high performance liquid chromatography (HPLC) analysis. P_{app} was calculated from Equation (1), where $\Delta Q/\Delta t$ is the rate of the flavonoid on the

accepting chamber (μM/s), A is the surface area of the hanging insert (cm^2) and C_0 is the initial concentration of tested flavonoid in donating chamber (μM/mL).

$$P_{app} = \frac{\Delta Q/\Delta t}{AC_0} \quad (1)$$

2.4. The CA of Flavonoids in Caco-2 Cell Monolayer

After the collection of samples from both AP and BL sides, the hanging insert was rinsed twice with ice-cold D-Hank's buffer to stop further transport, and lysed with 0.1% Triton X-100 in D-Hank's buffer then placed in an ultrasonic bath for 15 min. Cell lysates were collected and centrifuged at 7000× *g* for 10 min. Supernatants were analyzed by BCA kit (Dingguochangsheng Biotechnology, Beijing, China) for protein content. After protein content assay, supernatants were immediately frozen, lyophilized and preserved at −80 °C. The flavonoid concentration in lysis solution was determined by HPLC and normalized with cellular protein content.

2.5. HPLC Assays of Flavonoids

To determine both the permeability and CA of flavonoids in Caco-2 cells, the lyophilized samples were dissolved in running 120 to 200 μL methanol, and centrifuged at 7000× *g* for 5 min. Aliquot of 20 μL of the supernatant solution was used for assay using Waters e2695 HPLC system (Milford, MA, USA) fitted with an Amethyst C18-H column (250 mm × 4.6 mm i.d., 5 μm; Sepax, Newark, DE, USA). The mobile phase consisted of A (0.01 M phosphoric acid (H_3PO_4))/B (acetonitrile) with 1.0 mL/min flow rate. The assay was performed using the following gradient: 0–5 min: 95% A; 5–10 min: 95–50% A; 10–20 min: 50% A; 20–22 min: 50–95% A; 22–30 min: 95% A. Elution peaks were monitored with a diode-array detector at the wavelength of maximum absorption of each flavonoid (200–400 nm). Peak area measurement was used to obtain standard calibration curves to determine concentration of each flavonoid.

2.6. 2D-QSPR Study

The structures of 30 flavonoids were drawn by Chembiodraw ultra 12.0 and energy minimization was performed by Sybyl X-2.0 (Tripos Inc., St. Louis, MO, USA). The geometric structures of these compounds were optimized using the density functional methods (DFT) calculations at the level of Becke's-parameter hybrid functional (B3LYP) and polarized basis sets 6-31G (*d*, *p*). The electronic and topological descriptors and molecular properties were calculated by Gaussian 09 program package, Sybyl X-2.0 and molecular operating environment (MOE) 2009.

The $pP_{app\ A\ to\ B}$ ($-\log(P_{app\ A\ to\ B})$) was used as the dependent variable and descriptors were used as independent variables in the 2D-QSPR study. Twenty-two compounds were chosen by random as the training set to build the QSPR model and the remaining six compounds (**9**, **12**, **14**, **20**, **27** and **30**) were used as the test set. Correlation analysis was performed by stepwise linear regression and bivariate methods to select the molecular descriptors affecting the permeability of flavonoids. Collinear descriptors, with the absolute value of inter-correlation coefficient (*R*) higher than 0.7, were omitted [18]. The 2D-QSPR model was achieved using the partial least squares (PLS) algorithms with default parameters in Sybyl X-2.0. The resulting model was validated by using cross-validation (leave-one-out) procedures. Cross-validation coefficient (Q^2) should be more than 0.5 for a reliable model [3]. The root-mean-square error (RMSE) for the training set was measured as shown in Equation (2) to evaluate the predictability of the developed model [19].

$$\mathrm{RMSE} = \sqrt{\frac{\sum_{i=1}^{n}\left(Y_{iexp.} - Y_{ipred.}\right)^2}{n}} \quad (2)$$

2.7. Statistical Analysis

Statistical differences were determined by student's *t* test on SPSS 16.0 and *p* values less than 0.05 and 0.01 were considered significant and very significant, respectively.

3. Results

3.1. Cell Viability

Cell viability was evaluated in Caco-2 cells treated with 40 μM flavonoid. As shown in Table 2, there is no significant difference ($p < 0.05$) between the Caco-2 cell viability of the control and each flavonoid. These results indicated that the flavonoids at the tested concentration showed no cytotoxicity to Caco-2 cells.

Table 2. The cell viability of 30 flavonoids in Caco-2 cells.

No.	Cell Viability (%)	No.	Cell Viability (%)	No.	Cell Viability (%)
1	96.32 ± 9.79^{a}	**12**	100.22 ± 4.53^{a}	**23**	94.60 ± 3.60^{a}
2	97.52 ± 3.53^{a}	**13**	101.77 ± 6.15^{a}	**24**	105.49 ± 6.13^{a}
3	97.68 ± 8.39^{a}	**14**	100.79 ± 3.15^{a}	**25**	98.35 ± 4.60^{a}
4	96.72 ± 6.31^{a}	**15**	93.64 ± 1.22^{a}	**26**	105.02 ± 4.56^{a}
5	100.66 ± 4.19^{a}	**16**	98.38 ± 3.86^{a}	**27**	103.27 ± 3.37^{a}
6	101.09 ± 4.56^{a}	**17**	105.78 ± 5.48^{a}	**28**	105.78 ± 6.44^{a}
7	103.02 ± 4.94^{a}	**18**	100.12 ± 2.70^{a}	**29**	99.18 ± 7.94^{a}
8	103.40 ± 4.77^{a}	**19**	95.60 ± 3.47^{a}	**30**	102.04 ± 6.35^{a}
9	106.20 ± 7.79^{a}	**20**	99.06 ± 4.92^{a}	**C**	100.00 ± 4.38^{a}
10	102.16 ± 5.19^{a}	**21**	100.74 ± 4.27^{a}		
11	101.86 ± 3.62^{a}	**22**	99.48 ± 2.00^{a}		

C: Control. Results are presented as means ± Standard Deviation (SD), $n = 5$. Statistical comparisons were made using the one way ANOVA and a Duncan test. [a]: Same letters mean there is no significance between the tested flavonoid and control in Caco-2 cells.

3.2. Transport of Flavonoids

Based on Caco-2 cell viability, the same nontoxic concentration (40 μM) was used for all flavonoids in the transport studies to minimize error resulting from the concentration-dependent effect shown by some flavonoids.

The flavonoids permeated across the membrane to the acceptor compartments in both the apical to basolateral (A to B) and the basolateral to apical (B to A) assays. The results of bidirectional transport are summarized in Table 3. The flavonoid with the highest P_{app} is **26** (a isoflavonoid) for both $P_{app\ A\ to\ B}$ and $P_{app\ B\ to\ A}$ with a value of $(33.90 \pm 3.55) \times 10^{-6}$ cm/s and $(42.19 \pm 3.11) \times 10^{-6}$ cm/s, respectively, followed by **19**, **21** and **27** with P_{app} more than 30×10^{-6} cm/s. Other isoflavonoid aglycones (**23**, **25**, and **28**) also showed good permeability with P_{app} more than 10×10^{-6} cm/s. Flavanone aglycones (**18** and **22**) also showed good intestinal absorption with high P_{app} more than 20×10^{-6} cm/s. These results indicate that isoflavonoids and flavanones show higher permeability than other flavonoids. Flavone aglycones display modest good intestinal absorption with P_{app} greater than 6×10^{-6} cm/s while the low permeability ($<2 \times 10^{-6}$ cm/s) of compound **4** results from the easily oxidized pyrogallic group. The flavonols with P_{app} values less than 10×10^{-6} cm/s were poorly absorbed. The permeability of flavonols was lower than flavones by comparing **5** versus **10**, **6** versus **12** and **7** versus **9**, respectively. The hydroxyl group at position 3 may be unfavorable for flavonoids to pass through the cell membrane, leading to low permeability for flavonols from both apical and basolateral sides. Flavonoid glycosides (**8**, **16**, **17**, **20**, **24** and **30**) showed low P_{app} values ($<6 \times 10^{-6}$ cm/s), suggesting that the presence of glycosidic group is also unfavorable for the absorption of flavonoids.

Table 3. Apparent permeability coefficients (P_{app}) of flavonoids in Caco-2 monolayer.

No.	$P_{app\ A\ to\ B} \times 10^{-6}$ cm/s	$P_{app\ B\ to\ A} \times 10^{-6}$ cm/s	$Ratio_p$	No.	$P_{app\ A\ to\ B} \times 10^{-6}$ cm/s	$P_{app\ B\ to\ A} \times 10^{-6}$ cm/s	$Ratio_p$
1	22.35 ± 1.37	21.73 ± 1.21	0.97	**16**	4.24 ± 1.25	5.96 ± 1.20	1.41
2	21.86 ± 0.585	23.94 ± 0.45	1.10	**17**	4.04 ± 0.07	4.28 ± 0.44	1.06
3	16.43 ± 1.20	18.07 ± 1.62	1.10	**18**	23.50 ± 0.85	27.92 ± 2.76	1.19
4	1.95 ± 0.53	0.71 ± 0.21	0.37	**19**	32.13 ± 2.98	35.34 ± 1.16	1.10
5	10.10 ± 7.33	21.45 ± 2.12	2.12	**20**	3.73 ± 0.73	5.09 ± 1.55	1.36
6	17.12 ± 1.72	16.92 ± 1.02	0.99	**21**	30.97 ± 0.80	36.96 ± 1.97	1.19
7	6.78 ± 0.12	8.41 ± 0.62	1.24	**22**	6.32 ± 1.16	26.08 ± 2.08	4.13
8	3.28 ± 0.32	2.81 ± 0.24	0.86	**23**	17.42 ± 1.78	18.31 ± 1.83	1.05
9	1.94 ± 0.41	1.75 ± 0.44	0.90	**24**	2.25 ± 0.96	2.70 ± 0.41	1.20
10	2.55 ± 1.45	4.68 ± 0.41	1.84	**25**	12.45 ± 0.91	6.23 ± 0.46	0.50
11	8.23 ± 0.87	21.58 ± 0.32	2.62	**26**	33.90 ± 3.55	42.19 ± 3.11	1.24
12	6.68 ± 0.93	5.92 ± 0.45	0.89	**27**	31.07 ± 2.13	30.48 ± 2.13	0.98
13	0.35 ± 0.06	0.23 ± 0.03	0.66	**28**	11.11 ± 0.51	10.70 ± 2.98	0.96
14	0.29 ± 0.01	0.44 ± 0.01	1.50	**29**	13.76 ± 0.73	20.04 ± 1.29	1.46
15	1.09 ± 0.02	0.90 ± 0.42	0.83	**30**	4.25 ± 0.53	5.08 ± 1.08	1.20

$P_{app\ A\ to\ B}$: Transport of a flavonoid from apical to basolateral side; $P_{app\ B\ to\ A}$: Transport of a flavonoid from basolateral to apical side; $Ratio_p$: the ratio of $P_{app\ B\ to\ A}$ to $P_{app\ A\ to\ B}$. Data are means ± SD (n = 3–6). P_{app} value of Lucifer yellow carbohydrazide (CH) was about (3.58 ± 0.12) × 10^{-7} cm/s. The incubation time was 60 min.

Most of the flavonoids exhibited a $ratio_p$ ranging 0.8–1.5 (Table 3), suggesting that transport of these flavonoids is by passive diffusion. However, compounds **5**, **11** and **22** with $ratio_p$ >2.0, exhibit efflux in Caco-2 monolayer. The absorption for compounds **4** and **25** ($ratio_p$ < 0.5) is good, and also indicates higher absorption from apical to basolateral side.

3.3. CA of Flavonoids in Caco-2 Cell Monolayers

The CA in cells from apical to basolateral side ($CA_{A\ to\ B}$) and basolateral to apical side ($CA_{B\ to\ A}$) of flavonoids after transport are summarized in Table 4. The CA from both sides ($CA_{A\ to\ B}$ and $CA_{B\ to\ A}$) of flavonoids **14**, **16**, **17**, **22**, and **24** were not detected. The other two flavonoid glycosides **8** and **30** also showed very low CA (<0.26 μmol/g), suggesting that both the accumulation of flavonoid glycosides and their transport (low P_{app}) were poor. This may result from the low hydrophobicity of these flavonoids, which makes penetration through the phospholipid bilayer cell membrane difficult. Compound **14**, with an unstable pyrogallic group, is easily autoxidized, resulting in low concentration both in cells (not detected) and transport chamber (P_{app} < 0.5 × 10^{-6} cm/s). Compounds **19**, **21**, **26** and **27** showed low CA values (<5 μmol/g) compared with their highest P_{app} (>30 × 10^{-6} cm/s). Compounds **12** and **28** showed highest CA (>6 μmol/g) with modest P_{app}. These results suggest that the concentration of flavonoid aglycones accumulated in cells is not correlated with cell penetration. This is confirmed by the poor correlation coefficient (R^2 < 0.3) between the P_{app} and their corresponding CA.

Table 4. The cellular accumulation (CA) of flavonoids in Caco-2 monolayer.

No.	$CA_{A\ to\ B}$ (μmol/g)	$CA_{B\ to\ A}$ (μmol/g)	$Ratio_c$	No.	$CA_{A\ to\ B}$ (μmol/g)	$CA_{B\ to\ A}$ (μmol/g)	$Ratio_c$
1	1.932 ± 0.051	3.183 ± 0.504 [a]	1.65	**16**	ND	ND	-
2	2.706 ± 0.174	2.423 ± 0.543	0.90	**17**	ND	ND	-
3	1.038 ± 0.212	1.586 ± 0.045	1.53	**18**	1.293 ± 0.086	1.644 ± 0.300	1.27
4	1.182 ± 0.341	3.272 ± 0.445 [a]	2.77	**19**	1.935 ± 0.305	4.436 ± 0.299 [b]	2.29
5	3.876 ± 0.741	8.639 ± 1.275 [b]	2.23	**20**	0.058 ± 0.013	0.068 ± 0.011	1.17
6	4.415 ± 0.467	7.610 ± 0.694 [b]	1.72	**21**	0.635 ± 0.146	1.234 ± 0.249 [a]	1.94
7	3.902 ± 0.251	8.828 ± 0.213 [b]	2.26	**22**	ND	ND	-
8	0.024 ± 0.002	0.044 ± 0.007	1.83	**23**	3.819 ± 1.320	4.842 ± 0.424	1.27
9	3.780 ± 0.711	9.286 ± 0.859 [b]	2.46	**24**	ND	ND	-
10	NT	NT	-	**25**	1.821 ± 0.774 [a]	0.158 ± 0.021	0.09
11	0.129 ± 0.008 [b]	0.060 ± 0.011	0.464	**26**	0.500 ± 0.104	1.236 ± 0.117 [b]	2.47
12	8.368 ± 1.039	11.887 ± 1.247 [a]	1.42	**27**	1.415 ± 0.276	2.777 ± 0.220 [b]	1.96
13	2.746 ± 0.478 [b]	0.496 ± 0.249	0.18	**28**	6.766 ± 1.583	14.087 ± 1.287 [b]	2.08

Table 4. *Cont.*

No.	CA $_{A\ to\ B}$ (μmol/g)	CA $_{B\ to\ A}$ (μmol/g)	Ratio$_c$	No.	CA $_{A\ to\ B}$ (μmol/g)	CA $_{B\ to\ A}$ (μmol/g)	Ratio$_c$
14	ND	ND	-	**29**	4.393 ± 0.790	8.804 ± 1.576 [a]	2.00
15	0.332 ± 0.066 [a]	0.076 ± 0.048	0.23	**30**	0.096 ± 0.010	0.251 ± 0.012	2.61

CA $_{A\ to\ B}$: the content of the flavonoids that accumulated in the cell monolayer after transport from apical to basolateral side. CA $_{B\ to\ A}$: the content of the flavonoids that accumulated in the cell monolayer after transport from basolateral to apical side. Ratio$_c$: the ratio of CA $_{B\ to\ A}$ to CA $_{A\ to\ B}$. Data are means ± SD, $n = 3$. [a]: means $p < 0.05$, [b]: means $p < 0.01$ in t test. ND: not detected. NT: not tested.

The ratio$_c$ was within the range of 0.09–2.77, as shown in Table 4. The ratio$_c$ values for most of the compounds (except compounds **2**, **13**, **15** and **25**) were more than 1.00, implying greater accumulation of flavonoids in the basolateral to apical direction. The CA $_{A\ to\ B}$ exhibited significant ($p < 0.05$ or $p < 0.01$) differences from the CA $_{B\ to\ A}$ for most of the flavones (except compound **2** and **3**), isoflavones (except compound **23**) and all of the flavonols. These results indicated that flavonoids accumulated in Caco-2 cells to a different extent during bilateral transport.

3.4. Stability of Flavonoids

In our initial study (data not shown), we found that methanol is a good solvent for the dissolution of flavonoids, even better than the mobile phase B (acetonitrile) used in HPLC assay. Hence, we evaluated the stability of flavonoids in different solvents with methanol as a standard solvent. The relative stability was expressed as the relative appearance of flavonoids in different solvents. Each flavonoid (20 μM) was dissolved in two different solvents, D-Hank's and methanol. The relative concentration of flavonoid in D-Hank's was calculated by Equation (3) with results summarized in Table 5. The relative concentration was more than 50% for most of the tested flavonoids, suggesting that most flavonoids are stable in D-Hank's buffer. However, the values of **4**, **13** and **14** were lower than 40%, suggesting that these flavonoids are unstable in D-Hank's buffer. That could explain the low experimental P_{app} of these three flavonoids.

$$\text{Relative concentration in D-Hank's (\%)} = \text{Peak area in D-Hank's/Peak area in methanol} \times 100\% \quad (3)$$

Table 5. Flavonoid stability.

Relative Concentration of Flavonoids					
No.	D-Hank's/methanol (%)	**No.**	D-Hank's/methanol (%)	**No.**	D-Hank's/methanol (%)
1	60.10	**10**	52.10	**19**	113.93
2	12.09	**11**	79.06	**20**	64.99
3	51.64	**12**	98.60	**21**	71.02
4	34.12	**13**	7.25	**24**	30.31
5	87.51	**14**	ND	**25**	85.60
6	41.76	**15**	90.67	**26**	74.83
7	47.63	**16**	116.67	**27**	88.16
8	72.75	**17**	66.82	**28**	81.33
9	105.96	**18**	115.83	**29**	131.85

Recovery of 7 Flavonoid Glycosides				
No.	Recovery (B to A, %)	RSD (%)	Recovery (A to B, %)	RSD (%)
8	61.83 ± 0.72	1.16	72.31 ± 18.76	25.96
11	90.12 ± 1.39	1.56	81.45 ± 6.70	8.22
16	76.77 ± 9.36	12.19	90.69 ± 8.85	9.76
17	96.95 ± 1.67	1.72	98.18 ± 4.74	4.84
20	82.88 ± 10.99	13.26	93.17 ± 7.62	8.18
24	85.25 ± 0.60	0.70	87.42 ± 3.31	3.79
30	79.50 ± 1.24	1.58	78.66 ± 1.30	1.65

B to A: Recovery of transport of a flavonoid from basolateral to apical side. A to B: Recovery of transport of a flavonoid from apical to basolateral side. RSD: relative standard deviation. ND: not detected.

The recovery of the flavonoid glycosides during the transport assays was determined by mass balance and was measured as total amount of the flavonoids on both sides of the insert and in the Caco-2 cells. The recovery rate and relative standard deviation (RSD) values of seven flavonoid glycosides are shown in Table 5. Recoveries were all greater than 60% for these flavonoid glycosides, indicating that flavonoid glycosides are stable during the transport assay, and the low permeability for these flavonoids is not due to their stability.

3.5. Construction and Validation of the QSPR Model

To evaluate the relationship between intestinal absorption and structure of flavonoids, a QSPR model was constructed with $pP_{app\ A\ to\ B}$ as the dependent variable and physio-chemical properties descriptors as independent variables. Stepwise regression analysis was used for variable selection to obtain the best equation. At the beginning of modeling, compounds **4** and **13** with high residuals (experimental $pP_{app\ A\ to\ B}$ minus predicted $pP_{app\ A\ to\ B} > 0.600$) were omitted as outliers. Several factors may contribute to the outlier status of these two compounds, including low permeability and structure uniqueness. For compound **4**, a specific pyrogallic group makes it unstable, as illustrated by its low relative concentration (34.12%) in D-Hank's, and it has been shown to be metabolically susceptible [20]. For compound **13**, the low relative concentration (7.25%) and highest predictive residual (1.011) led to the outlier of this compound. The best QSPR equation was obtained from 22 compounds in training set as shown in Equation (4).

$$pP_{app\ A\ to\ B} = 4.715 + 1.358Q_{C3}, + 0.059E\_sol + 0.020S\log P\_V3 + 0.056vsurf\_ID1 \quad (4)$$

$$R^2 = 0.881,\ R^2_{adj} = 0.875,\ Q^2 = 0.810,\ F = 44.509,\ p < 0.01,\ \text{RMSE} = 0.141,$$
$$R^2_{pred} = 0.842,\ R^2_{adj\text{-}pred} = 0.802,\ \text{SEE} = 0.156$$

The correlation matrix between $pP_{app\ A\ to\ B}$ and the respective molecular properties are shown in Table 6. The correlation matrix showed that the properties are independent, demonstrating that the model in this study is robust. Moreover, the QSPR model was validated by cross-validation (leave-one-out) and a high cross-validated coefficient ($Q^2 = 0.810$) was achieved. The $pP_{app\ A\ to\ B}$ both in training and test set is predicted by this QSPR model with a particularly high degree of accuracy (Figure 1). The predicted deviations of the tested compounds (except **14** and **15**) both in training and test set were lower than 0.5 as shown in Table 7. The QSPR is considered of high predictive ability with a high value (0.841) of square of predictive correlation coefficient (R^2_{pred}) for the test set. In addition, the low RMSE value (0.242) also confirmed the accuracy of the developed model. These results illustrate that the QSPR model is robust and predictive, and could be used to predict the intestinal absorption of flavonoids.

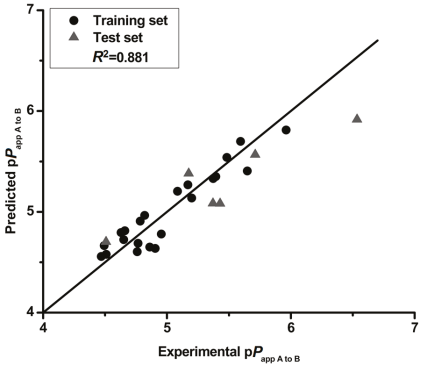


Figure 1. The experimental $pP_{app\ A\ to\ B}$ versus predicted $pP_{app\ A\ to\ B}$.

Table 6. The Pearson Correlation between $pP_{app\ A\ to\ B}$ and related descriptors.

	$pP_{app\ A\ to\ B}$	$Q_{C3'}$	*E_sol*	*SlogP_V3*	*vsurf_ID*1
$pP_{app\ A\ to\ B}$	1.000	0.576 **	0.045	0.738 **	0.407
$Q_{C3'}$		1.000	−0.273	0.223	0.214
E_sol			1.000	−0.103	−0.363
SlogP_V3				1.000	−0.148
*vsurf_ID*1					1.000

**: Correlation is significant at the 0.01 level.

Table 7. Calculated results using the QSPR model.

	No.	$pP_{app\ A\ to\ B}$ (Exper)	$pP_{app\ A\ to\ B}$ (Pred)	*E_sol*	*SlogP_V3*	$Q_{C3'}$	*vsurf_ID*1	Residuals
Training set	**1**	4.651	4.756	−0.789	0	0.010	0.736	−0.105
	2	4.660	4.947	−1.802	0	−0.019	4.097	−0.287
	3	4.784	4.803	1.526	0	0.011	1.561	−0.019
	5	4.820	4.663	−3.225	0	0.305	0.474	0.157
	6	4.766	4.751	−0.893	0	−0.007	0.636	0.015
	7	5.169	5.197	0.986	0	0.011	8.594	−0.028
	8	5.484	5.709	−2.750	41.853	−0.004	2.774	−0.225
	10	5.594	5.278	0.378	25.386	0.301	0.822	0.316
	11	5.085	5.243	0.104	25.386	−0.033	0.359	−0.158
	15	5.962	5.322	1.610	25.386	0.322	1.034	0.640
	16	5.373	5.041	−2.100	0	0.272	6.626	0.332
	17	5.394	5.363	−10.491	20.927	0.276	8.232	0.031
	18	4.629	4.750	1.351	0	−0.028	0.685	−0.121
	19	4.493	4.754	−1.043	0	−0.018	0.669	−0.261
	21	4.509	4.731	−2.117	0	−0.019	0.228	−0.222
	22	5.199	4.755	−0.789	0	0.302	1.064	0.444
	23	4.759	4.735	−1.910	0	−0.012	0.334	0.024
	24	5.648	5.370	1.320	20.927	−0.034	4.292	0.278
	25	4.905	4.749	−1.314	0	−0.023	0.553	0.156
	26	4.470	4.730	−2.317	0	−0.023	0.190	−0.260
	28	4.954	4.753	0.600	0	−0.006	0.690	0.201
	29	4.861	4.765	−1.601	0	−0.013	0.861	0.096
Test set	**9**	5.712	5.347	3.668	25.386	0.005	2.195	0.365
	12	5.175	5.254	2.066	25.386	0.005	0.548	−0.079
	14	6.535	5.376	3.979	25.386	0.295	1.045	1.159
	20	5.429	5.514	−6.238	20.927	−0.034	6.492	−0.085
	27	4.508	4.745	−0.205	0	−0.022	0.527	−0.237
	30	5.372	5.062	−10.370	20.926	0.291	3.024	0.310

QSPR: quantitative structure-permeability relationship.

3.6. QSPR Study

In this study, four descriptors, namely $Q_{C3'}$, *E_sol*, *SlogP_V3* and *vsurf_ID*1, were selected to build the QSPR model. This model showed that higher values for the descriptors results in a decrease in intestinal absorption of flavonoids. The significant Pearson coefficients between $Q_{C3'}$ or *SlogP_V3* and $pP_{app\ A\ to\ B}$ suggests that these two descriptors play major roles in the transport of flavonoids. The electronic properties of flavonoids are important for their absorption. The molecule charge of a flavonoid is electro-neutral, while, the electronic delocalization for each atom in a molecule varies with the number and position of substituents. The value of $Q_{C3'}$ for each flavonoid is shown in Table 7, representing the atomic charges of carbon 3′ of the molecule. By comparing the $Q_{C3'}$ and structure difference for tested flavonoids (Tables 1 and 7), the π–π-conjugation on B ring makes this ring in a stable electron distribution and carbon 3′ with small positive atomic charges (0.011) when without substitution at this position (**1**, **3**, **4**, **7** and **9**). The atomic charges increased to higher positive atomic charges (approximately 0.300) with the presence of hydroxyl or methoxyl group (**5**, **10**, **14**, **15**, **16**, **17**, **22** and **30**). The oxygen of hydroxyl/methoxyl group withdraws the electron(s) to the oxygen atom side leading to a loss of carbon 3′ electron(s) resulting in positive atomic charges for carbon 3′. This can be confirmed by comparing compound **27** and **29**, with same *SlogP_V3* and similar *vsurf_ID*1,

although **29** has a lower *E_sol* which favors absorption, **27** with a lower $Q_{C3'}$ showed better absorption. The hydroxylation or methoxylation of adjacent carbon 4′ (**2**, **6**, **8**, **11**, **19**, **20**, **21**, **24**, **25**, **26**, **27**, **28** and **29**) results in carbon 4′ having positive charges, and carbon 3′ having negative charges. Similarly, it can be presumed that substitution on carbon 2′ will also increase absorption. This is verified when evaluating flavonoids **11** and **12**. Flavonoid **11** having negative charges on carbon 3′, with substitution both on carbon 2′ and carbon 4′ showed better absorption than **12** with substitution only on carbon 4′. These results suggest that the substitution by an electronegative group (such as a hydroxyl group) on carbon 3′ is unfavorable for flavonoid absorption while the substitution on adjacent carbon (2′ and 4′) favors absorption.

SlogP_VSA descriptors aim to capture hydrophilic and hydrophobic effects in the receptor and the atomic contribution to logP was calculated by the Wildman and Crippen SlogP model [19]. *SlogP_VSA*3 (*SlogP_V*3) represent the Van der Vaals surface area of the atoms contributing to the logP (o/w) of the molecule in the range (0, 0.1). High *SlogP_V*3 increases the molecular surface area, resulting in low absorption of flavonoids. For flavonols, the substitution of a hydroxyl group on position 3 increases *SlogP_V*3 to 25.386. Flavonoid glycosides (**8**, **17**, **20**, **24** and **30**), with substitution of a glycosidic group also showed an increase in *SlogP_V*3 (>20.93). Flavonoid **8**, having two glycosidic groups at two different positions (6 and 8), showed the highest *SlogP_V*3 value (41.853). These results suggest that the substitution of 3-OH or glycosidic group(s) is unfavorable for the absorption of flavonoids.

The vsurf descriptors relating to structure connectivity and conformation are similar to the VolSurf descriptors [21]. *vsurf_ID*1 is a hydrophobic integy moment vector pointing from the center of mass to the center of a hydrophobic region. The descriptor represents the unbalance between the center of mass of a molecule and the position of the hydrophobic regions around it at a −0.2 kcal/mol energy level. A high integy moment indicates that the hydrophobic regions are clearly concentrated in only one part of the molecular surface. A small integy moment indicates that the barycenter is close to the center of the molecule. Compounds **25** and **26** displayed same *SlogP_V*3 and $Q_{C3'}$ and similar *E_sol* however **26** with lower *vsurf_ID*1 showed better absorption because **25** had an external methoxyl group on position 6. The flavonoid glycosides (**8**, **16**, **17**, **20**, **24** and **30**) showed high integy moment (>2.7) resulting from bulky glycosidic group(s), which could aggravate the unbalance between the barycenter and hydrophobic region position. Flavonoids (**5**, **11**, **21**, **23** and **26**) showed small integy moment (<0.5). The numbers of substitution on A and B ring are the same for these flavonoids so that the barycenter is close to the center of the molecule. For compound **7**, two hydroxyl groups (position 5 and 7) are far away from the center of mass leading to a high integy moment (8.594) which is similar to compound **2** also having a high integy moment (4.097). In summary, when the distribution of substituents is equal between A and B ring, the balance between the center of hydrophobic region and the center of mass will increase, resulting in high absorption of flavonoids. However, the substitution of a balky group (such as a glycosidic group) decreases the balance between the center of hydrophobic region and the center of mass, thereby decreasing the absorption of flavonoids.

E_sol is a 3D molecular descriptor, representing the solvation energy of flavonoids in the solvation process. The higher the energy is, the lower the resulting absorption. By comparing compounds **20** and **24**, with same *SlogP_V*3 and $Q_{C3'}$ and similar *vsurf_ID*1, compound **20**, with lower *E_sol* showed better absorption. Similarly, with lower *E_sol*, compound **27** had higher absorption than **25**. For flavonoids **18** and **19,** having similar *SlogP_V*3 and *vsurf_ID*1, **19** with much higher *E_sol*, showed better absorption than **18** despite having a slightly lower $Q_{C3'}$. These results indicate that *E_sol* is an important parameter related to the absorption of flavonoids.

4. Discussion

The bilateral permeation across the Caco-2 monolayer of selected five types of flavonoids (Table 1) followed the order: flavanones ≥ isoflavones > flavones ≥ chalcones > flavonols, which is consistent with previous results reported for the bioavailability of flavonoids (isoflavones > flavanols > flavanones > flavonols > anthocyanins) [22]. The permeability for most of

tested flavonoids in this study is similar with the literature results [5,16,23,24] since their bidirectional permeability was in the same order of magnitude. The bilateral permeation of flavonoids followed the order: **12** > **10** > **15**, consistent with a previous study [16]. The permeability of Lucifer yellow CH is also consistent with that reported in a previous study [16]. Our results indicate that the Caco-2 monolayer in this study is well suited to represent flavonoid transport and the data are valid.

Flavonoid glycoside **20** naringin, ($P_{app} < 6 \times 10^{-6}$ cm/s) showed much lower permeability than **19** naringenin, ($P_{app} > 32.13 \times 10^{-6}$ cm/s). The other flavonoid glycosides tested also showed low permeability ($P_{app} < 6 \times 10^{-6}$ cm/s). These results are in agreement with studies that concluded that glycosides are poorly absorbed compounds [23,25]. Poor lipid solubility and the presence of multiple hydroxyl groups are reported reasons contributing to the poor absorption of flavonoid glycosides [25]. However, the P_{app} value reported is lower ($<1 \times 10^{-6}$ cm/s) in previous studies [23,26] than in our study. The discrepancy could be associated in part to the poor membrane permeability of these glycosides, as well as to different experimental conditions used. The CA of glycosides in this study are low (<0.3 μmol/g) or not detected which is agreement with a literature result [23]. Although the absorption of flavonoid glycosides in vitro is poor or not detected, it was confirmed that the absorption of quercetin glycosides in a human study with ileostomy subjects was higher than quercetin, implying that absorption of glycosides in the small intestinal is possible [27].

Flavonols showed lower permeability than corresponding flavones when comparing **5** versus **10, 6** versus **12** and **7** versus **9**, suggesting 3-OH is unfavorable for flavonoid permeability. In addition, flavonoids **4**, **9**, **10**, **13**, **14** and **15**, sharing three or more hydroxyl groups showed low permeability. The P_{app} values increased adversely with the number of hydroxyl groups in a sub class of flavonoids as reported previously [22]. Possible explanation include excess free hydroxyl groups are easily autoxidized, especially the presence of a pyrogallic group which can easily bind hydrogen bonds with its aqueous environment [28] and rapid conjugation by glucuronidation and sulfation [29].

An influential predictive model for intestinal absorption is the "Lipinski rule-of 5", which resulted from the analysis of the World Drug Index [30]. Compounds that are likely absorbable through the intestines contain no more than 5 H-bond donors, 10 H-bond acceptors, have a molecular weight of <500 Da and log*P* (lipophilicity index) <5. According to this rule, flavonoids, with higher numbers of hydroxyl, or glycosidic moieties are less likely to be absorbed through the intestines which are in agreement with our results. In contrast, methoxylated flavonoids lose their H-bond acceptor/donor properties and have higher log*P* values, which make them highly absorbable. Compound **2** containing methoxyl groups showed high permeability, consistent with the literature that concluded methylation greatly improved intestinal absorption [31]. For flavonoids with only one methoxyl group, it is not certain that absorption will increase. Our study found that methoxylation increases the absorption when comparing compound **3** and **7**, but decreases absorption by comparing **10** versus **15**, **25** versus **26** and **27** versus **28**, respectively.

Most flavonoids exhibited a ratio of $P_{app\ A\ to\ B}/P_{app\ B\ to\ A}$ from 0.66 to 1.50, indicating that they permeate across the Caco-2 monolayer via passive diffusion. For compounds **5**, **11** and **22**, however, the hypothesis of active transport should not be excluded since the ratio$_p$ is higher than 2 [15]. For **22**, the ratio$_p$ (4.13), is much higher than 2, suggesting an efflux mechanism which was also concluded in a previous study [32]. The transport mechanism of compound **10** with a high ratio$_p$ (1.84), warrants further study since this compound has been reported to be pumped back by breast cancer resistance protein (BCRP) in the form of glucuronidated metabolites [33]. Chrysin (compound **7**) has been shown to exhibit efflux in Caco-2 monolayer [34]. In this study, efflux of chrysin was not observed probably due to the design of HPLC assays for which only parental compound were determined with standard of chrysin. The relative concentration of chrysin in D-Hank's is less than 50%, indicating that it is not stable in the buffer solution. Besides the rule of ratio$_p$ [15], it is more concise and popular to use inhibitor of transporters to distinguish flavonoid absorption, especially, for poorly absorbed flavonoids. Further study is needed to focus on the efflux transport for flavonoids since it has been

reported that passive diffusion and carrier-mediated efflux are known to be the two major pathways for a molecule to permeate across the intestinal epithelium [35].

Due to the wide range and variability in structure within each group, it is difficult to generalize the absorption of flavonoids based only on structure. Physio-chemical properties need to be taken into account to ascertain structure relationship to permeability. In this study, QSPR model was built to construct a mathematical relationship between the molecular structural descriptors and permeability ($P_{\text{app A to B}}$). QSPR devoted to flavonoid absorption is limited in the literature. 2D and 3D QSPR models were constructed to explain and predict intestinal absorption of flavonoids using $P_{\text{app A to B}}$ in Caco-2 cells [9]. However, the data consisted of the log of the P_{app} (transformed to $P_{\text{app}} \times 10^{-7}$), which omitted the part of 10^{-7} was not precise in the previous study [9]. The data were transformed to pP_{app} ($-$log) in this study is more acceptable than the previous study [9]. Moreover, the atom-type electrotopological state (E-state) and weighted holistic invariant molecular (WHIM) descriptors used to develop QSPR model were reported to be cumbersome to interpret [9]. Therefore, no structure and permeability relationship was discussed with those models and those models only lies on their utility in screening of emerging flavonoids [9]. In addition, the 3D model (comparative molecular similarity index analysis, CoMSIA) reported in the literature was not suitable since CoMSIA is usually used for a target receptor (such as enzyme) [36] but not for permeability properties. Because the permeability of flavonoids can be influenced by several factors, such as pumped out by efflux transporters [24], interaction with membrane [5], stability, solubility and hydrophobicity of the compounds [37].

In QSPR analysis, hydrophobic, electronic and steric are the three major descriptors that must be included in analysis [38]. In our study, the descriptors used to build QSPR model were derived from more than one molecular modeling package, Gaussian 09, SYBYL X-2.0 and MOE 2009 including physical, electrostatic, topological, hydrophobic, and energetic descriptors. Physical 3D structure of flavonoids is indeed a determining factor for intestinal absorption since our QSPR model captured three 3D descriptors among four descriptors. $Q_{C3'}$ used in our study representing the atomic charge on carbon 3′ is easily to interpret when comparing with those descriptors in previous study [9]. The substitution (methoxylation or hydroxylation) on carbon 3′ or adjacent positions affects $Q_{C3'}$, and also affects flavonoid absorption. Hydroxylation or methoxylation at carbon 3′ will decrease absorption of a flavonoid while substitution on adjacent carbon 2′ or 4′ can increase the absorption. This type of descriptor has been successfully used in generating a quantitative structure-activity relationship (QSAR) model to predict antibacterial capacity against *E. coli* for flavonoids [3]. *SlogP_V3* was proven to have a negative effect on absorption, which is consistent with a study that reported that an increase in polar surface area (*PSA*) was unfavorable for absorption [39]. Such descriptor has been successfully used in QSAR modeling of HIV-1 reverse transcriptase inhibition by benzoxazinones [36] and inhibition of potassium channel blockers [40]. It was found in this study that the presence of 3-OH or glycosidic group decreases flavonoid absorption according to *SlogP_V3*. *vsurf_ID*1 representing the balance of the molecule, indicates favorable absorption when the substitution is equal on A and B ring. Descriptors of this type were used to develop a model for discriminating P-gp substrates based on Caco-2 efflux ratio values [41]. *E_sol* describes the process of solubilisation of flavonoids and is utilized in the prediction of solubility [42]. The increasing of the energy will decrease the solubility thereby decrease flavonoids absorption. These descriptors generate a direct link between the molecular parameters of the model to the actual structural properties that govern the permeability and they are easy to interpret.

5. Conclusions

Oral bioavailability remains one of the most critical issues for flavonoids in nutrition and health. In this study, 30 flavonoids were tested for transepithelial permeability in Caco-2 cell monolayer. QSPR model was built to predict intestinal absorption of flavonoids by using structural characteristics and the model showed good predictive power. Four descriptors, $Q_{C3'}$, *SlogP_V3*, *vsurf_ID*1 and *E_sol*, were found to relate to flavonoid absorption. Substitution of a negative group (such as -OH

or -OCH_3) on carbon 3′ decreases the flavonoid absorption while substitution on adjacent carbon 2′ or/and carbon 4′ increases the absorption. The presence of 3-OH or glycosidic group decrease the absorption of flavonoids. The equal substitution on A ring and B ring is also favorable for absorption. The results of this study are important in ascertaining the structural features important to flavonoid intestinal absorption.

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Abbreviations

CA	cellular accumulation
CoMSIA	comparative molecular similarity index analysis
FBS	fetal bovine serum
HPLC	high performance liquid chromatography
MEM	minimum essential medium
NEAA	non-essential amino acids
P_{app}	the apparent permeability coefficient
PLS	partial least squares
SPR	structure-permeability relationship
QSPR	quantitative structure-permeability relationship
QSAR	quantitative structure-activity relationship
RMSE	the root-mean-square error
TEER	trans-epithelial electrical resistance

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Protective Effects of Hesperidin (Citrus Flavonone) on High Glucose Induced Oxidative Stress and Apoptosis in a Cellular Model for Diabetic Retinopathy

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Abstract: The aim of this study was to investigate the protective effects and mechanisms of hesperidin, a plant based active flavanone found in citrus fruits, under the oxidative stress and apoptosis induced by high levels of glucose in retinal ganglial cells (RGCs). RGC-5 cells were pretreated with hesperidin (12.5, 25, or 50 μmol/L) for 6 h followed by exposure to high (33.3 mmol/L) D-glucose for 48 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was adopted to evaluate cell viability. Mitochondrial function was estimated by measuring the mitochondrial membrane potential (ΔΨm). A fluorescent probe was employed to evaluate the intercellular production of reactive oxygen species (ROS). Colorimetric assay kits were used to evaluate lipid peroxidation, antioxidant enzyme activities, and protein carbonyls formation. The expression of apoptosis-related proteins and mitogen-activated protein kinase (MAPK) were measured with Western blotting. Hesperidin inhibited high glucose-mediated cell loss and restored mitochondrial function including a reversion of ΔΨm loss and cytochrome c release. Treated with hesperidin, high glucose-induced increase in ROS, malondialdehyde, and protein carbonyl levels were blocked in RGC-5 cells. Hesperidin was found to elevate the activities of superoxide dismutase, catalase, glutathione peroxidase, and to recover glutathione levels. Hesperidin inhibited high glucose-induced cell apoptosis by attenuating the downregulation of caspase-9, caspase-3, and Bax/Bcl-2. Furthermore, the phosphorylation of c-Jun N-terminal kinases (JNK) and p38 MAPK triggered by high glucose were attenuated in RGC-5 cells after their incubation with hesperdin. We concluded that hesperidin may protect RGC-5 cells from high glucose-induced injury since it owns the properties of antioxidant action and blocks mitochondria-mediated apoptosis.

Keywords: hesperidin; high glucose; retinal ganglial cells; apoptosis

1. Introduction

Diabetes mellitus (DM) and its complications are becoming an increasingly serious global public health issue, with a great increase in the number of patients worldwide. Diabetic retinopathy (DR) is one of the major and most common microvascular complications of DM, and is the most important cause of blindness occurring in adults [1]. Studies have shown that the primary pathology in DR is related to the dysfunction and death of retinal ganglion cells (RGCs), the only efferent neurons that

transmit visual information from the retina to the visual center [2,3]. A high glucose level may alter the structure and function of RGCs, leading to visual field defects, and ultimately blindness [3]. Continuous hyperglycemia during diabetes incurs a redox imbalance caused by the overproduction of reactive oxygen species (ROS) [4]. Sustaining high levels of ROS damages mitochondrion, which ultimately leads to the apoptosis of RGCs [5]. Several biochemical changes have been proposed as essential events that commit a cell to undergo mitochondria-associated apoptosis, which can be mediated by activating pro-apoptotic Bcl-2 family proteins, increasing oxidative stress, initiating a caspase cascade, loss of mitochondrial membrane potential ($\Delta\Psi m$), and cytochrome c redistribution [6,7]. Hence, the regulation of hyperglycemia-induced oxidative stress and associated cell death in RGCs would be essential prevention and treatment strategies for DR.

Epidemiological studies have shown that a large number of phytochemicals in fruits and vegetables might protect the human body against ROS-related pathologies [8]. Therefore, phytochemicals extracted from plant sources have drawn increasing attention since they are potential agents in the prevention and treatment of oxidative stress-related diseases [9]. Hesperidin (hesperetin 7-rutinoside) is an inexpensive flavone glycoside rich in citrus fruits and isolated from the ordinary orange *Citrus aurantium* L. and other plants of the family *Rutaceae* [10]. Hesperidin improves the health of capillaries by reducing the capillary permeability [11]. Evidence presented in numerous in vitro and in vivo studies has shown that hesperidin possesses analgesic, anti-hypertensive, and diuretic activity; is hypolipidemic; and exhibits obvious anti-inflammatory activity [12]. Hesperidin inducing apoptosis has been reported in various cancer cells including colon, liver, and mammary cancer cells [13–15]. On the contrary, this flavanone displays anti-apoptotic properties in neuroblastoma and human keratinocyte cell lines [16,17]. Another potential therapeutic application of hesperidin is in ocular diseases. It has been demonstrated that hesperidin possesses the ability to attenuate hyperglycemia-mediated oxidative stress and pro-inflammatory cytokine production in high fat fed/streptozotocin-induced type 2 diabetic rats [18]. Similar results have been documented where hesperidin possesses the ability to attenuate retina abnormalities via anti-angiogenic, anti-inflammatory, and antioxidative effects, as well as the inhibitory effect on the polyol pathway and advanced glycosylation end product accumulation in diabetic rats [19]. Based on different studies, hesperidin is considered as a potential therapeutic compound for DM and related complications. Thus, it is valuable to clarify whether the protective effects of hesperidin on retinal abnormalities under high glucose is involved in the amelioration of oxidative stress and associated cell death.

However, the role of hesperidin on the amelioration of mitochondrial dysfunction and death of RGCs caused by high glucose has not clarified thus far. To understand the influence of hyperglycemia on the pathogenesis of diabetes, a common model is to expose high glucose concentrations in vitro [20]. Thus, we elucidated the retina-protective effect of hesperidin on RGC-5 model of DR in vitro under high-glucose conditions by analyzing its effect on high glucose mediated oxidative stress generation, mitochondrial dysfunction, and apoptosis.

2. Materials and Methods

2.1. Cell Culture

The retinal ganglion cell 5 (RGC-5) cells, purchased from American Type Culture Collection (Manassas, VA, USA), have been previously characterized as expressing ganglion cell markers and exhibiting ganglion cell-like behavior in culture [21]. RGC-5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM: L-glutamine, 110 mg/L sodium bicarbonate and 1 g/L D-glucose) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), and penicillin-streptomycin (100 U/mL and 100 µg/mL, Sigma-Aldrich, St. Louis, MO, USA) in a humidified incubator with 5% CO_2 at 37 °C. The doubling time of these cells was approximately 20 h under these conditions. The medium was changed every other day, and the cells were passaged at a ratio of approximately 1:8 every 2–3 days. RGC-5 cells of passages 10–20 were used in these studies.

2.2. High-Glucose Stimulation

Cells were seeded at a density of 2×10^6 cells/well in 6-well plates. Upon confluence, cultures were passaged by dissociation in 0.05% (*w*/*v*) trypsin (Sigma-Aldrich) in phosphate-buffered saline (PBS) pH 7.4. For high-glucose-induced functional studies, cells were maintained in fresh medium containing 1% fetal bovine serum for 2 h prior to use in the experiments. Later, cells were pretreated with hesperidin (purity $\geq$ 97%; Cat. No. 50162; Sigma-Aldrich) at a variety of concentrations (12.5, 25, or 50 μmol/L), or vehicle for 6 h followed by further exposure to normal (5.5 mmol/L), or high (33.3 mmol/L) D-glucose for 48 h without medium change. The dosage regimen was selected based on the previous reports demonstrating that hesperidin protected human keratinocytes from ultraviolet B radiation-induced apoptosis and was potentially effective in decreasing vascular formation in endothelial cells [17,22]; never RGCs be exposed to these concentrations. Hesperidin powder was dissolved in dimethyl sulfoxide (DMSO) (purity $\geq$ 99%; Cat. No. W387520; Sigma-Aldrich) to create a stock at a concentration of 10 mg/mL, subsequently diluted in culture medium to the corresponding concentration for the following experiments. DMSO was used as the vehicle control in the experiments. The final DMSO concentration did not exceed 0.1% (*v*/*v*), a concentration that did not affect cell viability. Afterwards, cell viability, antioxidant enzyme activities, reactive oxygen species (ROS) generation, and several apoptotic indexes were assessed. Triple wells were evaluated in each experiment, and each experiment was performed at least five times independently.

2.3. Cell Survival Assay

The viability of RGC-5 cells exposed to normal or high glucose plus either hesperidin was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay, as previously described with modifications in Reference [23]. Typically, 10,000 cells suspended in 100 μL of media were incubated with 10 μL of MTT reagent for approximately 3 h, followed by the addition of a detergent solution to lyse the cells and solubilize the colored crystals. The absorbance of the samples was measured at 570 nm using a microplate reader (MTP-800; CORONA, Tokyo, Japan). The cell viability in the control medium without any treatment was represented as 100% and the cell viability percentage of each well was calculated. Each experiment was conducted in three wells and was duplicated at least five times.

2.4. Detection of Intracellular Reactive Oxygen Species (ROS)

The generation of intracellular ROS was assessed using 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA), a non-fluorescent probe, which upon oxidation by ROS and peroxides is converted to the highly fluorescent derivative dichlorofluorescin (DCF) [24]. We used a DCFDA cellular ROS detection assay kit (Abcam, Cambridge, MA, USA) as per the manufacturer's instructions. In brief, an aliquot of the isolated cells (8×10^6 cells/mL) was made up to a final volume of 2 mL in normal PBS (pH 7.4). Then, a 1 mL aliquot of cells was taken to which 100 μL DCFDA (10 μmol/L) was added and incubated at 37 °C for 30 min and washed twice with PBS. Cells were solubilized in Triton-X100 1% (*v*/*v*) in distilled water. The cells were then examined under a fluorescence microscope (C1-T-SM; Nikon, Tokyo, Japan). The percentage of fluorescence-positive cells was measured at an excitation wavelength of 488 nm and an emission wavelength of 535 nm by a fluorescence spectrophotometer (F-2500; Hitachi, Tokyo, Japan).

2.5. Estimation of Lipid Peroxidation and Protein Carbonyl Content

Lipid peroxidation was studied by measuring the amount of malondialdehyde (MDA) in the cell homogenates using the lipid peroxidation assay kit (Abcam) as per the manufacturer's instructions. Briefly 1 mmol/L ethylene diamine tetraacetic acid (Sigma, St. Louis, MO, USA) was added to a 0.5 mL cell lysate (6×10^6 cells/mL) and was mixed with 1 mL cold 15% (*w*/*v*) thiobarbituric acid to precipitate proteins. The supernatant was treated with 1 mL 0.5% (*w*/*v*) TBA in a boiling water bath for

15 min. After cooling, the absorbance was read at 535 nm, and the concentration of the thiobarbituric acid reactive substance was calculated by using malondialdehyde as a standard [25]. Results were expressed as mmol thiobarbituric acid reactive substances/mg of protein. Protein carbonyl contents in the cell homogenates were determined with a protein carbonyl content assay kit (Abcam) according to the manufacturer's procedures. The assessment of carbonyl formation was done based on the formation of protein hydrazone by reaction with 2,4-dinitrophenylhydrazine [26]. The absorbance was measured at 370 nm. The carbonyl content was calculated using the absorption coefficient of 22,000/M/cm. Results were expressed as mmol/mg of protein. The protein content of the sample was assessed using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) as per the manufacturer's instructions.

2.6. Assay of Antioxidant Enzymes

Cells were seeded at a density of 2×10^6 cells/well in 6-well plates. The activities of antioxidant enzymes were assayed in cells treated with test samples as following.

Superoxide dismutase (SOD) activity was assayed using a SOD activity colorimetric assay kit (BioVision Inc., San Francisco, CA, USA) which utilizes a highly water soluble tetrazolium salt, 2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, that produces a water soluble dye on reduction with O_2^-. The absorbance was measured at 450 nm. The rate of inhibition of the activity of xanthine oxidase by SOD was measured and expressed as the enzyme required for a 50% inhibition of xanthine oxidase activity per minute per milligram protein.

A glutathione peroxidase (GPx) activity assay was performed by using a GPx colorimetric assay kit (BioVision Inc.) following the manufacturer's instructions. The kit is based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPx, which is then coupled to the recycling of GSSG back to GSH utilizing the glutathione reductase and nicotinamide adenine dinucleotide phosphate. Cell extracts were prepared with a glutathione peroxidase assay buffer with 50 mmol/L Tris HCl, adjusted with HCl to pH 7.0, and contained 0.5 mmol/L EDTA, and used in the assay using the protocol described in the assay kit. The rate of nicotinamide adenine dinucleotide phosphate oxidation by H_2O_2 was monitored at 340 nm and GPx activity was expressed in unit/mg protein.

The catalase (CAT) activity in cells treated with test samples was assayed using the catalase assay kit by Cayman Chemical (Ann Arbor, MI, USA). The assay was based on the reaction of methanol and the enzyme in the presence of an optimal concentration of H_2O_2. The formaldehyde produced was measured colorimetrically at 540 nm using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald; Sigma-Aldrich, St. Louis, MO, USA) as the chromogen. CAT activity was expressed in unit/mg protein.

The levels of glutathione (GSH) in the cells were determined by using a glutathione colorimetric assay kit (BioVision Inc.). Briefly, cells were washed and taken to determine GSH levels by following the procedures provided by the manufacturer. The sample was first deproteinized with the 5% 5-sulfosalicylic acid solution. The GSH content of the sample was then assayed using a kinetic assay where the catalytic amounts of glutathione caused a continuous reduction of 5,5′-dithiobis-(2-nitrobenzoic) acid to 2-nitro-5-thiobenzoic acid (TNB). The oxidized GSH formed was recycled by the GSH reductase and NADPH. TNB was assayed colorimetrically at 412 nm. The GSH level was expressed in nmol/mg protein.

2.7. Measurement of Mitochondrial Membrane Potential ($\Delta\Psi m$)

A mitochondrial membrane potential assay kit containing JC-1 (Cat. No. ab113850; Abcam) was used to measure the $\Delta\Psi$m in RGC-5 cells. Briefly, 1×10^4 cells were laid in a 96-well plate and incubated with 20 μmol/L JC-1 in growth medium at 37 °C for 30 min. Monomeric JC-1 green fluorescence emission (530 nm) and aggregate JC-1 red fluorescence emission (590 nm) were determined using a fluorescence spectrophotometer (F-2500; Hitachi). The $\Delta\Psi$m in each group was calculated as the fluorescence ratio of red to green. This value was adjusted by subtracting the value obtained from

the carbonyl cyanide 3-chlorophenylhydrazone incubation and expressed as a percentage of the vehicle control.

2.8. Measurement of Cytochrome C Release

A mitochondrial fractionation kit (Active Motif Inc., Carlsbad, CA, USA) was utilized to isolate the mitochondrial and cytosolic fractions from cells under the manufacturer's instructions. Briefly, the cells (8×10^6 cells/mL) were scraped and spun twice at $600\times$ *g* for 5 min. Ice-cold $1\times$ cytosolic buffer was added into the cell pellet, which was then resuspended and incubated on ice for 15 min. Cells were homogenized and the lysate was spun twice at $800\times$ *g* for 20 min. The resultant supernatant was centrifuged at $10{,}000\times$ *g* for 20 min to pellet the mitochondria. The mitochondrial fraction of protein was obtained from the mitochondrial pellet lysed by adding complete mitochondria buffer, and incubating on ice for 15 min. Meanwhile, the supernatant was centrifuged at $16{,}000\times$ *g* for 25 min to obtain the cytosolic fraction. Protein concentration was determined by the Bradford assay. After isolating the mitochondria and cytosolic fraction, the cytochrome c enzyme-linked immunosorbent assay kit (Abcam) was used to measure the level of cytochrome c as per the manufacturer's instructions.

2.9. Western Blot Analysis

Proteins were prepared from the cytosolic and mitochondrial samples or whole-cell lysis of cells (8×10^6 cells/mL). The mitochondrial fractionation kit (Cat. No. 40015; Active Motif Inc., Carlsbad, CA, USA) was used to isolate cytosolic and mitochondrial fractions from cells according to the manufacturer's instructions. In brief, cells were harvested and centrifuged at $800\times$ *g* at 4 °C for 10 min, and the cell suspension was then placed into a glass homogenizer and homogenized for 30 strokes by using a tight pestle on ice. Homogenates were centrifuged at $800\times$ *g* at 4 °C for 10 min to collect the supernatant, which was further centrifuged at $10{,}000\times$ *g* at 4 °C for 20 min to obtain the cytosol (supernatant) and mitochondria (deposition) fraction. Protein concentrations were determined using the Bradford protein assay (Bio-Rad Laboratories). Equal amounts of protein (30 μg/lane) were resolved by electrophoresis for Western blot analysis. Protein was separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline Tween (20 mmol/L Tris, pH 7.6, 137 mmol/L NaCl, and 0.1% Tween 20) for 3 h at room temperature, accompanied by an overnight incubation at 4 °C with primary antibodies against cytochrome c (Cat. No. 13156; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), cleaved caspase-3 (Cat. No. 9661; Cell Signaling Technology, Beverly, CA, USA), cleaved caspase-9 (Cat. No. 9501; Cell Signaling Technology), Bcl-2 (Cat. No. sc-492; Santa Cruz Biotechnology Inc.), Bax (Cat. No. sc-526; Santa Cruz Biotechnology Inc.), c-Jun N-terminal kinases (JNK) (Cat. No. sc-137020; Santa Cruz Biotechnology Inc.), p-JNK (Thr 183/Tyr 185) (Cat. No. sc-6254; Santa Cruz Biotechnology Inc.), p38 (Cat. No. 9212; Cell Signaling Technology), or p-p38 (Thr180/Tyr182) (Cat. No. 9211; Cell Signaling Technology). The β-Actin antibody (Cat. No. sc-130656; Santa Cruz Biotechnology Inc.) was used as an internal control to exclude the putative contamination of mitochondrial fraction by cytosolic proteins. The cytochrome c oxidase subunit IV isoform 1 (CoxIV) antibody (Cat. No. 4850; Cell Signaling Technology) was used as a mitochondrial loading control. All antibodies were used at a dilution of 1:1000. After being washed three times with Tris-buffered saline Tween 20 (TBST), incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies was performed for one hour at room temperature. After three additional TBST washes, the immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK) as per the manufacturer's instructions. Band densities were calculated with ATTO Densitograph Software (ATTO Corporation, Tokyo, Japan) and quantified as the ratio to β-actin or CoxIV.

The mean value for samples from the vehicle treated cells cultured under normal glucose on each immunoblot, expressed in densitometry units, was adjusted to a value of 1.0. All experimental sample

values were then expressed relative to this adjusted mean value. All determinations were performed in triplicate, and each experiment was repeated at least five times.

2.10. Statistical Analysis

Data were expressed as the mean ± standard deviation (SD). Statistical analysis and graphics were made with a SigmaPlot 12.3 program (version 2016, Systat Software Inc., San Jose, CA, USA). Statistical analysis was conducted with one-way analysis of variance (ANOVA). Dunnett range post-hoc comparisons were employed to determine the source of significant differences where appropriate. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Effect of Hesperidin on High Glucose-Induced Cell Death

The incubation of normal glucose-cultured RGC-5 cells with hesperidin at 12.5, 25, or 50 μmol/L for 48 h had little or no effect on cytotoxicity (>90% viability remaining) (Figure 1A). The protective effects of hesperidin against high glucose-induced cell death in RGC-5 cells are shown in Figure 1B, where cell viability was about 30.2% in high-glucose cultured cells, whereas hesperidin recovered the cell death caused by high glucose in a concentration-dependent manner with almost 94.2% of the cells surviving at a 50 μmol/L.

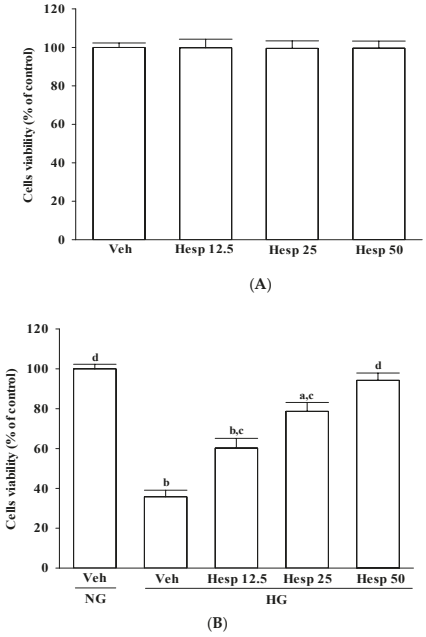

Figure 1. Cytotoxicity of hesperidin to RGC-5 cells. The RGC-5 cells were cultured with normal (NG) or high glucose (HG) plus hesperidin at concentrations of 12.5 (Hesp 12.5), 25 (Hesp 25), or 50 (Hesp 50) μmol/L for 48 h. (**A**) Effects of treatments on cell viability in RGC-5 cells cultured with normal glucose concentration; (**B**) Effects of treatments on cell viability in RGC-5 cells cultured with high glucose concentration. The cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results are presented as the mean ± standard deviation (SD) of five independent experiments (n = 5), each of which was performed in triplicate. [a] $p < 0.05$ and [b] $p < 0.01$ when compared with the normal-glucose vehicle (Veh)-treated group. [c] $p < 0.05$ and [d] $p < 0.01$ when compared with the high-glucose vehicle-treated group.

3.2. Effects of Hesperidin on ROS Production, Lipid Peroxidation, Protein Carbonylation, and Antioxidant Enzymatic Activities in RGC-5 Cells under High Glucose Environment

The intracellular levels of ROS, MDA, and protein carbonyl in RGC-5 cells under a high glucose environment were higher by about 2.1-, 3.8-, and 3.3-fold, respectively in high glucose cultured cells when compared to the normal-glucose vehicle-treated group (Figure 2A–C). The higher levels of ROS, MDA, and protein carbonyl in RGC-5 cells under high glucose were downregulated by hesperidin (50 μmol/L) treatment with a decrease of 42.9%, 55.3%, and 59.7%, respectively, relative to those observed in their vehicle-treated counterparts (Figure 2A–C).

In high glucose conditions, the activities of SOD, GPx, and CAT were decreased sharply in RGC-5 cells (Figure 2D–F). In high glucose-cultured RGC-5 cells with a concentration-dependent manner, hesperidin recovered the reduced activities of SOD, GPx, and CAT (Figure 2D–F). Compared with the normal-glucose group, the concentration of GSH was 40.5% lower in the high glucose cultured RGC-5 cells (Figure 2G). In their vehicle-treated counterpart group, hesperidin concentration (50 μM) upregulated GSH level to 2.2-fold of the levels (Figure 2G).

3.3. Effects of Hesperidin on the Mitochondrial Membrane Potential (ΔΨm) and Cytochrome C Release in RGC-5 Cells under High Glucose Conditions

The ΔΨm in RGC-5 cells exposed to high glucose was reduced to 51.3 ± 3.6% of that in the normal-glucose group (Figure 3A). Treatment of high glucose-cultured RGC-5 cells with hesperidin increased ΔΨm in a concentration-dependent manner (Figure 3A).

It was shown that decreased mitochondrial concentrations of cytochrome c were followed by increased cytosolic concentrations in RGC-5 cells cultured under high glucose, while hesperidin inhibited the release of cytochrome c from mitochondria to cytoplasm in a concentration-dependent manner (Figure 3B).

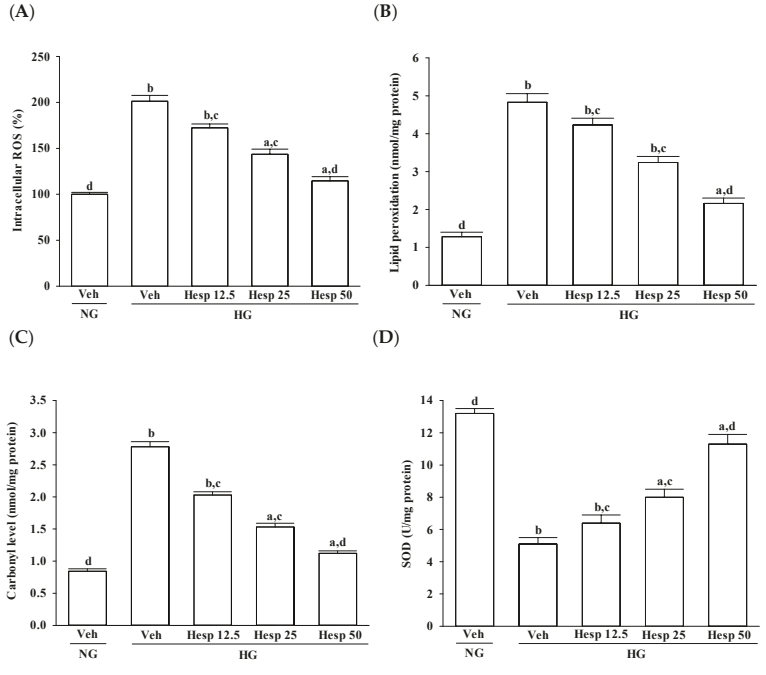

Figure 2. *Cont.*

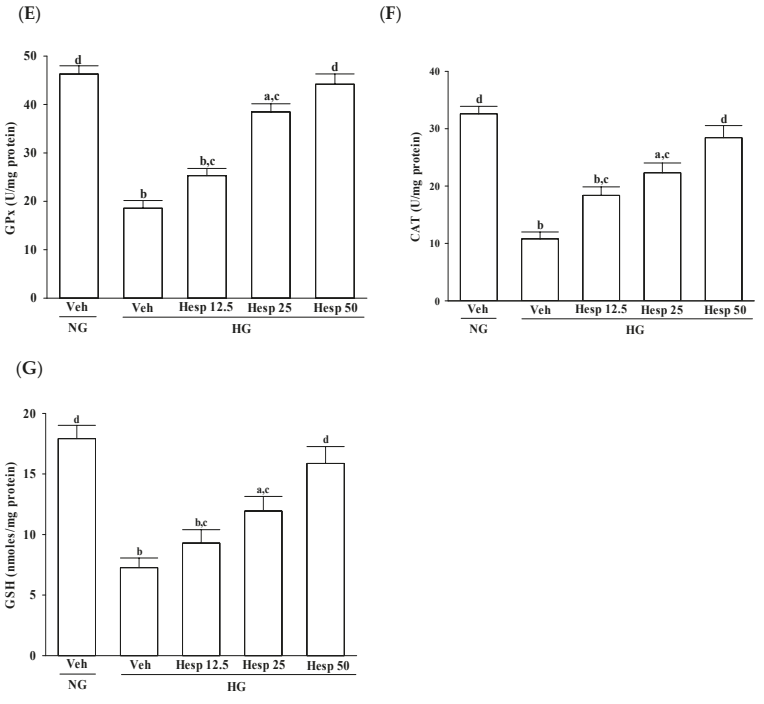

Figure 2. Effects of hesperidin on reactive oxygen species (ROS)production, lipid peroxidation, protein carbonylation, and endogenous antioxidant defense in RGC-5 cells following high-glucose challenge: (**A**) ROS levels; (**B**) lipid peroxidation levels; (**C**) total protein carbonyl content; and (**D**) levels of: superoxide dismutase (SOD); (**E**) glutathione peroxidase (GPx); (**F**) catalase (CAT); and (**G**) glutathione (GSH). The RGC-5 cells were cultured with normal (NG) or high glucose (HG) plus hesperidin at concentrations of 12.5 (Hesp 12.5), 25 (Hesp 25), or 50 μmol/L for 48 h. The experiments were performed in triplicate and data are presented as mean ± SD of five independent experiments ($n = 5$). [a] $p < 0.05$ and [b] $p < 0.01$ when compared with the normal-glucose vehicle (Veh)-treated group. [c] $p < 0.05$ and [d] $p < 0.01$ when compared with the high-glucose vehicle-treated group.

3.4. Effects of Hesperidin on Protein Expression Related to Apoptosis in RGC-5 Cells under High Glucose Conditions

High glucose caused a 4.6-fold and 3.7-fold increase in cleaved caspase-9 and cleaved caspase-3 protein expression in RGC-5 cells (Figure 4A,B). The protein expression of cleaved caspase-9 and cleaved caspase-3 in high glucose cultured RGC-5 cells was sharply decreased (58.3% and 50.9% reduction, respectively) by treatment with hesperidin (50 μmol/L) when compared to those of the vehicle-treated counterparts (Figure 4A,B).

High glucose greatly increased the Bax/Bcl-2 ratio in RGC-5 cells (by 8.4-fold relative to that seen in the normal-glucose vehicle-treated group; Figure 4C,D). This high glucose-induced up-regulation in the Bax/Bcl-2 ratio was reversed in the RGC-5 cells after treatment with 50 μmol/L hesperidin, with a 76.2% decrease when compared to that of their vehicle-treated counterpart group (Figure 4C,D).

3.5. Effects of Hesperidin on JNK and p38 Phosphorylation in RGC-5 Cells under High Glucose Conditions

The immunoblot results showed that the ratio of p-p38/p38 (Figure 5A,B) and p-pJNK/pJNK (Figure 5A,C) were 4.1- and 3.6-fold greater in the high glucose cultured RGC-5 cells than those in the normal-glucose vehicle-treated group, respectively. Compared to those in the normal-glucose

vehicle-treated group, treatment of high glucose cultured RGC-5 cells with hesperidin (50 μmol/L) apparently downregulated the ratios of p-p38/p38 and p-pJNK/pJNK to 1.9- and 1.6-fold (Figure 5B,C). Whether in the presence of hesperidin or not, the expression of total p38 and JNK protein did not change in RGC-5 cells treated with high glucose (Figure 5A).

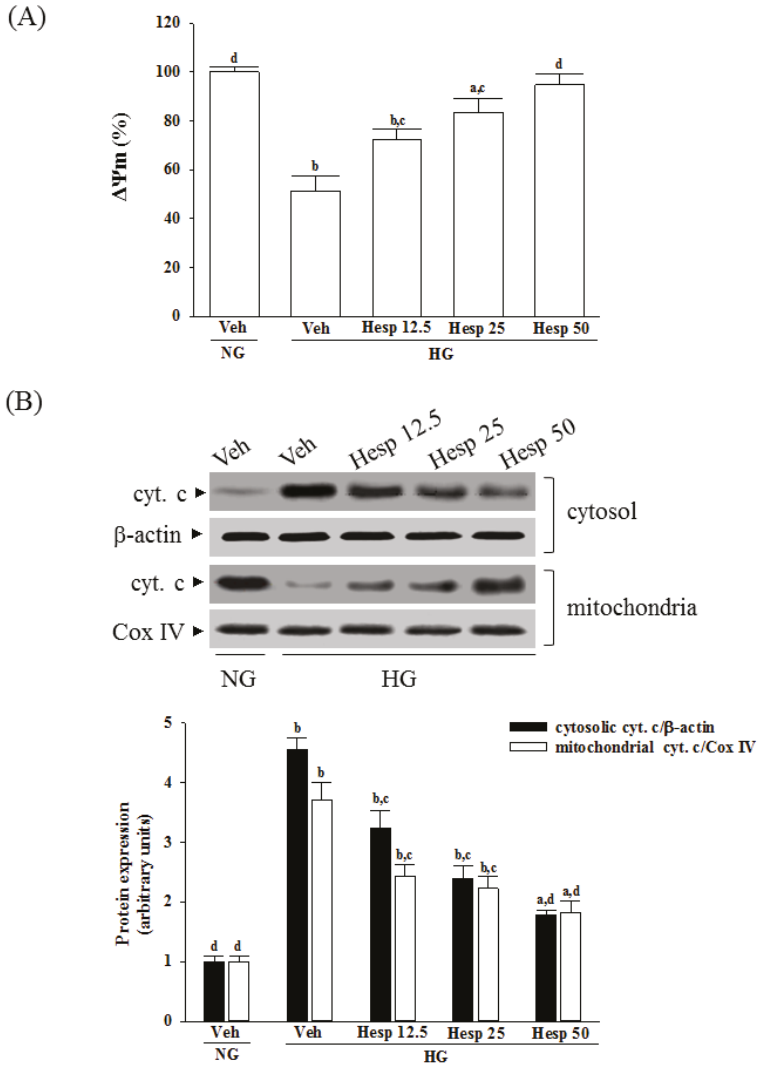

Figure 3. Effects of hesperidin on mitochondrial function in high glucose-treated RGC-5 cells. The RGC-5 cells were cultured with normal (NG) or high glucose (HG) plus hesperidin at concentrations of 12.5 (Hesp 12.5), 25 (Hesp 25), or 50 μmol/L (Hesp 50) for 48 h. (**A**) Effects of treatments on high glucose-induced reduction of mitochondrial membrane potential (ΔΨm); (**B**) Effects of treatments on high glucose-induced release of cytochrome c (cyt. c) from mitochondria. The experiments were performed in triplicate and data are presented as the mean ± SD of five independent experiments ($n = 5$). [a] $p < 0.05$ and [b] $p < 0.01$ when compared with the normal-glucose vehicle (Veh)-treated group. [c] $p < 0.05$ and [d] $p < 0.01$ when compared with the high-glucose vehicle-treated group.

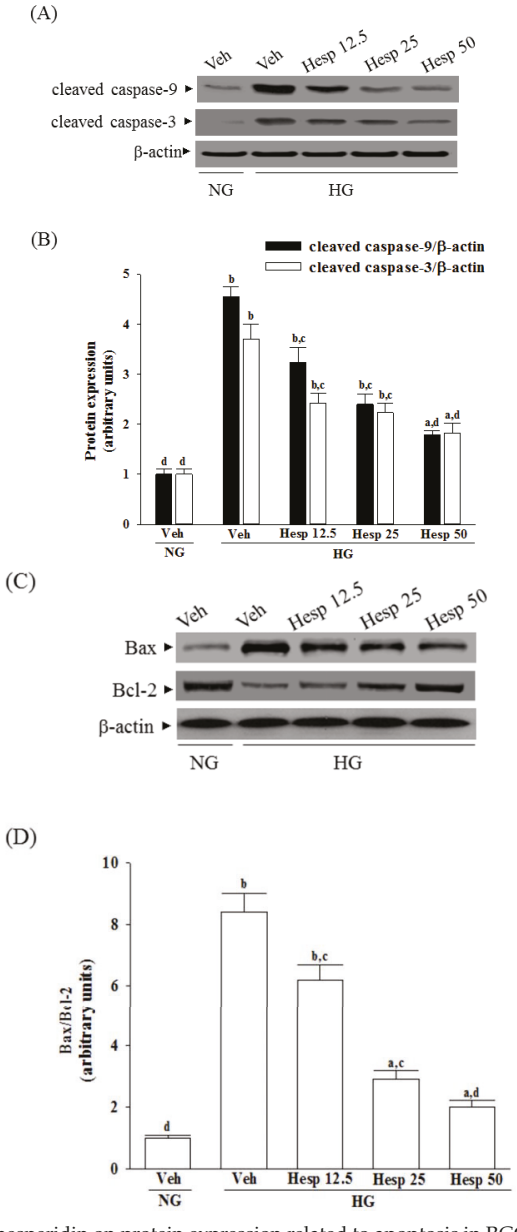

Figure 4. Effects of hesperidin on protein expression related to apoptosis in RGC-5 cells under high glucose conditions. The RGCs were cultured with normal (NG) or high glucose (HG) plus hesperidin at concentrations of 12.5 (Hesp 12.5), 25 (Hesp 25), or 50 μmol/L (Hesp 50) for 48 h. (**A**) Protein bands of cleaved caspase-9 and cleaved caspase-3 in RGC-5 cells detected by Western blotting; (**B**) Quantitative densitometric analysis of caspase-9 and cleaved caspase-3; (**C**) Protein bands of Bax and Bcl-2 in RGC-5 cells detected by Western blotting; (**D**) Changes of the ratios of Bax/Bcl-2 are displayed in the bottom panel. The results are presented as the mean ± SD of five independent experiments (n = 5), each of which was performed in triplicate. [a] $p < 0.05$ and [b] $p < 0.01$ when compared with the normal-glucose vehicle (Veh)-treated group. [c] $p < 0.05$ and [d] $p < 0.01$ when compared with the high-glucose vehicle-treated group.

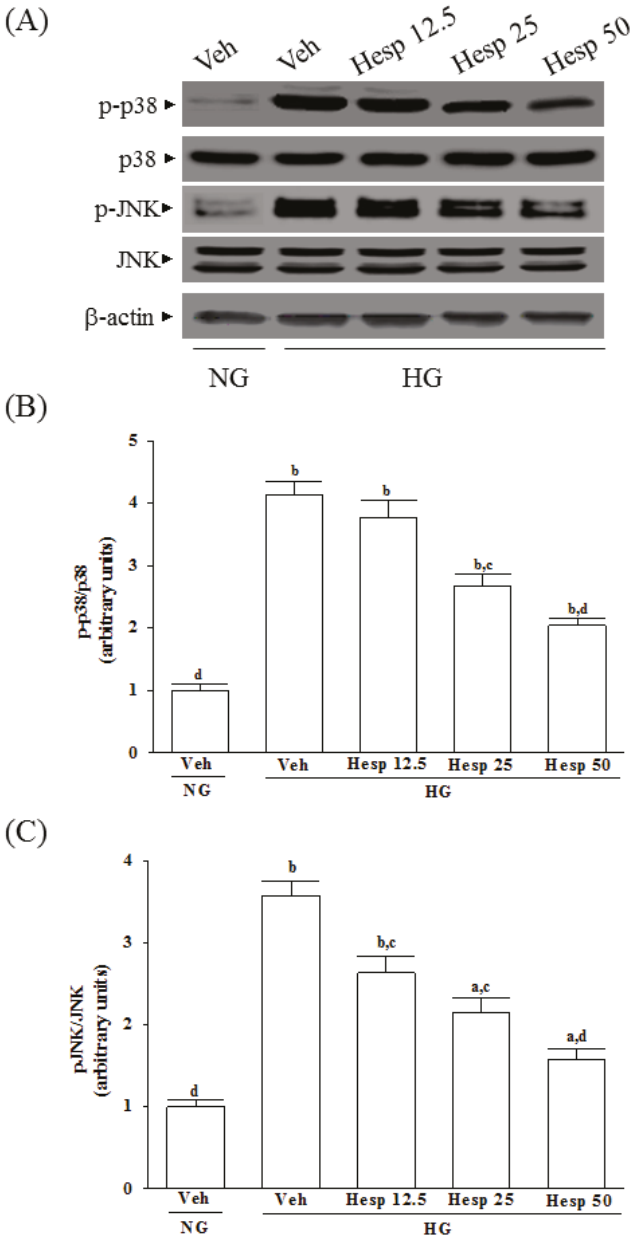

Figure 5. Effect of hesperidin on p38 and c-Jun N-terminal kinases (JNK) activation in high glucose cultured RGC-5 cells. (**A**) The photographs were representatives the Western blots for p-p38, p38, p-JNK, JNK and β-actin. Five western blots in each group were tested; (**B**) The ratio of p-p38/p38 was calculated; (**C**) The ratio of p-JNK/JNK was calculated. The results are presented as the mean ± SD of five independent experiments ($n = 5$), each of which was performed in triplicate. [a] $p < 0.05$ and [b] $p < 0.01$ when compared with the normal-glucose vehicle (Veh)-treated group. [c] $p < 0.05$ and [d] $p < 0.01$ when compared with the high-glucose vehicle-treated group.

4. Discussion

The generation of ROS under high glucose stress conditions has been established in many cell types [27,28]. When the ROS level exceeds the capacity of the antioxidant defense system, ROS initiates chain reactions by oxidizing cellular macromolecules like lipids and proteins, which in turn interrupts cellular activities, ultimately causing apoptosis [7]. In this study, high glucose-mediated oxidant injury was utilized as an in vitro model to examine the protective effects of hesperidin in RGCs. In accordance with previous studies described in Reference [29], higher MDA and carbonyl contents in RGC-5 cells cultured under high glucose were observed. The increase in MDA level is associated with the oxidative damage of membrane lipids since ROS in cells are elevated [30]. Increased carbonyl levels are also involved with ROS-mediated damage of proteins [31]. From this observation, an increase in high glucose-induced oxidative damage of the cell membrane was suggested. Treatment with hesperidin resulted in a reduction of elevated ROS and MDA levels and at the same time the level of protein carbonyl became higher. These data showed that hesperidin has an antioxidant property that protects RGC-5 cells against high glucose-induced oxidative stress.

In the progression of diabetes, accumulated oxidative stress and depleted antioxidant defenses play a major part. SOD, CAT, and GPx are enzymes that ruin peroxides and have an impact on the antioxidant defenses of an organism [32]. SOD acts to dismutate superoxide radicals to hydrogen peroxides, which is then acted upon by GPx [33,34]. CAT catalyzes the reduction of hydrogen peroxides and protects the tissues from highly reactive hydroxyl radicals [35]. Furthermore, GSH acts as a cosubstrate for GPx activity and as a cofactor for many enzymes [36]. A decreased GSH content may predispose the cells to weaken their defense against the condition of oxidative stress during several degenerative disease conditions including diabetes [37]. Phytochemicals with antioxidant activity are known to abrogate oxidative stress [8]. Our data are in accordance with the hypothesis that during high glucose-induced stress to RGC-5 cells, hesperidin has a large influence on the control of ROS levels through upregulating both the enzymic and nonenzymic antioxidant defense systems. Consequently, it is expected that the mechanisms of hesperidin to protect against hyperglycemia-induced changes in RGC-5 cells and its capacity to counter oxidative stress will be studied in depth.

Although hesperidin could induce apoptosis through either an extrinsic or an intrinsic in several cancer cells [13–15], flavonoid can act as pro-apoptotic or anti-apoptotic agents depending on their concentration, the cell system, the type or stage of the degenerative process [16,17]. High glucose-induced oxidative stress mediated cell damage plays a significant role in the reduction of mitochondrial membrane potential, disruption of the mitochondrial membrane, and the release of cytochrome c into the cytosol, which in turn triggers caspase cascades and initiates the intrinsic apoptotic pathway [38]. Our results showed that high-glucose stimulation caused serious impairment of the $\Delta\Psi m$ and indeed fostered the release of cytochrome c, and the involvement of mitochondria in high glucose-mediated RGCs apoptosis was confirmed. It also showed that treatment of high glucose exposed RGC-5 cells with hesperidin could restore mitochondrial function by blocking the dissipation of $\Delta\Psi m$ and releasing cytochrome c into the cytosol. A strong inverse association between cell viability and apoptosis rates was observed. In fact, there was no significant cytotoxicity effect produced by hesperidin in the cell under these experimental conditions. At each concentration, hesperidin increased the survival rate of RGC in high glucose; in particular, the action of hesperidin was most obvious at 50 μmol/L. Data gained from the MTT assay in this study also suggested the direct neuroprotective action of hesperidin against high glucose-mediated mitochondrial dysfunction in RGC-5 cells as a model of DR.

In mitochondria-dependent apoptosis, the released cytochrome c activates caspase-9 and sequentially activates the downstream effector caspase-3 [39]. Moreover, mitochondrial membrane potential is regulated by a specific category of proteins known as the Bcl-2 family proteins [40]. The anti-apoptotic Bcl-2 family proteins such as Bcl-2 are basically localized into mitochondrial outer membranes and can block the membrane permeabilization, whereas proapoptotic members of this family like Bax are usually translocated to the mitochondrial outer membrane from the cytosol,

and facilitates the permeabilization of the mitochondrial membrane and release of cytochrome c [40]. As a result, because hesperidin blocks high glucose-induced elevation of Bax and decreases Bcl-2 in high glucose exposed RGC-5 cells, it inhibits enzymatic activity of caspase-9/3 and lowers the ratio of Bax/Bcl-2. These clarified that hesperidin protected RGC-5 cells from high glucose-induced cell apoptosis through mitochondrial-mediated pathway.

It is known that the mitogen-activated protein kinases (MAPKs), a family of protein kinases, are related to cell growth and ROS-mediated death [41]. It also shows that antioxidants can prevent either ROS accumulation or MAPK activation under oxidative stress conditions [42]. Three major classes of MAPKs are extracellular-signal regulated kinases 1 and 2 (ERK1/2), p38 MAPK/stress-activated protein kinases, and c-Jun N-terminal kinases (JNKs) [43]. The p38 and JNK MAPK pathways are generally recognized as pro-apoptotic pathways in response to cellular stresses such as hypoxic, or oxidative stress, whereas ERKs respond to mitogens and growth factors that regulate cell proliferation and differentiation [43]. Therefore, regulating the activity of the MAPK signaling pathway, particularly the activation of p38 and JNK, is vital for protecting cells from ROS injury and cellular death [44]. In the present study, the phosphorylation degree of p38 and JNK in high glucose exposed RGC-5 cells were greatly decreased under hesperidin treatment. Our findings support a protective role of hesperidin in RGC exposed to a high glucose challenge through the inhibition of p38 and JNK activation.

Human experiments should be designed based upon prior animal experimentation, it is difficult to directly convert the concentration of hesperidin into human intake dose in our in vitro study. It has been demonstrated that hesperidin at the daily oral dosage of 100 and 200 mg/kg could attenuate retina and plasma abnormalities in diabetic rats [19]; this is approximately 16 to 32 mg/kg oral intake for an adult human. Thus, animal studies will be arranged in the future to find out the effective dose of hesperidin for protection the retinal ganglion cell from high glucose-induced damage in vivo; the results can be used as a reference for the dosage of human body.

5. Conclusions

The current results provide evidence that the protective effect of hesperidin on RGC-5 cells against high glucose-induced oxidative are associated with properties relating to the inhibition of oxygen free radicals and the oxidation of lipids and protein. It plays a role in the enforcement of endogenous antioxidant defense mechanisms and the protection of mitochondrial function by the modulating Bcl-2 family members, as well as by inhibiting caspases activation via a ROS-dependent p38 and JNK signaling pathway. The findings of this study shed light on the pharmacological application of hesperidin to prevent and offer therapy to the high-glucose-induced damage of retinal ganglion cells and provides the theoretical basis for further development of hesperidin for preventing blindness in patients with diabetes and retinal eye diseases.

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Conflicts of Interest: The authors declare no conflicts of interest in relation to this work.

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Bioavailability of Bergamot (*Citrus bergamia*) Flavanones and Biological Activity of Their Circulating Metabolites in Human Pro-Angiogenic Cells

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Abstract: Myeloid angiogenic cells (MACs) play a key role in endothelial repairing processes and functionality but their activity may be impaired by the lipotoxic effects of some molecules like stearic acid (SA). Among the dietary components potentially able to modulate endothelial function in vivo, (poly)phenolic compounds represent serious candidates. Here, we apply a comprehensive multidisciplinary approach to shed light on the prospects of Bergamot (*Citrus bergamia*), a citrus fruit rich in flavanones and other phenolic compounds, in the framework of lipotoxicity-induced MACs impairment. The flavanone profile of bergamot juice was characterized and 16 compounds were identified, with a new 3-hydroxy-3-methylglutaryl (HMG) flavanone, isosakuranetin-7-*O*-neohesperidoside-6″-*O*-HMG, described for the first time. Then, a pilot bioavailability study was conducted in healthy volunteers to assess the circulating flavanone metabolites in plasma and urine after consumption of bergamot juice. Up to 12 flavanone phase II conjugates (sulfates and glucuronides of hesperetin, naringenin and eriodyctiol) were detected and quantified. Finally, the effect of some of the metabolites identified in vivo, namely hesperetin-7-*O*-glucuronide, hesperetin-3′-*O*-glucuronide, naringenin-7-*O*-glucuronide and naringenin-4′-*O*-glucuronide, was tested, at physiological concentrations, on gene expression of inflammatory markers and apoptosis in MACs exposed to SA. Under these conditions, naringenin-4′-*O*-glucuronide and hesperetin-7-*O*-glucuronide were able to modulate inflammation, while no flavanone glucuronide was effective in curbing stearate-induced lipoapoptosis. These results demonstrate that some flavanone metabolites, derived from the in vivo transformation of bergamot juice phenolics in humans, may mitigate stearate-induced inflammation in MACs.

Keywords: bergamot; citrus fruits; phenolic compounds; hesperetin; naringenin; conjugated phase II metabolites; myeloid angiogenic cells; inflammation; lipotoxicity; endothelial dysfunction

1. Introduction

Endothelial health relies on a dynamic equilibrium between injury induced by a relevant number of cues and *noxae* and its self-repair capacity [1]. Myeloid angiogenic cells (MACs), bone

marrow-derived cells also referred to as early endothelial progenitor cells (EPCs), are key mediators of endothelial renewal, acting mainly by secreting pro-angiogenetic growth factors [2]. The number and function of MACs are unfavorably influenced by the presence of metabolic stressors, such as insulin-resistance (IR) and cardiovascular (CV) risk factors and diseases, leading to endothelial dysfunction [3,4].

Lipotoxicity, an hallmark of insulin-resistance (IR) states, is a well-known mechanism underlying the association among IR, endothelial dysfunction and increased CV risk [5,6]. Lipotoxicity is characterized by chronically elevated free fatty acid (FFA) levels, mainly saturated free fatty acids (SFAs), palmitic (16:0) and stearic acid (SA, 18:0), which may damage vessel wall by enhancing inflammation/oxidative stress [7,8] and endothelial cell apoptosis [9,10]. We recently demonstrated [11] that SA at physiological concentrations increased inflammation and apoptosis in MACs, suggesting that lipotoxicity may directly and negatively also affect endogenous endothelial repairing processes. This experimental model might be particularly suitable to assess the effects of molecules/drugs in reducing IR-associated lipotoxicity.

Dietary (poly)phenols are known to ameliorate IR [12,13] and counteract vascular cell injury, pointing to their defensive role in preventing endothelial dysfunction and ultimately CV disease development and progression [14–17].

Citrus plants are rich sources of flavanones [18], a class of flavonoids that may display anti-inflammatory/-oxidant properties and have been reported to play a protective role in CV diseases [19,20]. Specifically, *Citrus bergamia* Risso et Poiteau, an endemic plant of the Calabria region (Italy) cultivated along the southern coast, commonly named "bergamot" and belonging to the Rutaceae family, is particularly rich in glycosides of hesperetin, naringenin and eriodictyol [18]. Bergamot juice (BJ) has been considered for long time merely a byproduct of essential oil extraction, which is widely used in the cosmetic and food industries. Nevertheless, over the last decade, new evidence emerged on the possible favorable anti-inflammatory [21–23], antiproliferative [24], neuroprotective [22] and hypolipidemic [25] properties of BJ derivatives. However, despite this body of evidence, there is a lack of information on the specific phenolic metabolites that might be responsible of the biological activity of bergamot.

To the best of our knowledge, no studies to date have assessed (a) the bioavailability of BJ flavanones and (b) whether their beneficial effects on the endothelial/CV system may be mediated, at least in part, by an improvement in vascular regenerative capacity. Notably, the majority of in vitro studies addressing the effects of bergamot juice may be fraught with methodological issues by testing the juice as such [22–24], instead of the circulating derivatives resulting of phase II metabolic transformations. Therefore, the objectives of the present study were: (a) to assess the bioavailability of *Citrus bergamia*-derived phenolic metabolites in healthy humans following BJ intake; and (b) to evaluate the potential role of the identified metabolites in curbing lipoapoptosis and inflammation in MACs obtained from healthy donors, following SA exposure in vitro.

2. Materials and Methods

2.1. Chemicals

Hesperetin-7-*O*-rutinoside (hesperidin), naringenin-7-*O*-rutinoside (narirutin) and isosakuranetin-7-*O*-rutinoside (didymin) were purchased from Extrasynthese (Genay CEDEX, France). Naringenin-7-*O*-glucuronide and naringenin-4′-*O*-glucuronide were purchased from Bertin Pharma (Montigny le Bretonneux, France), while hesperetin-7-*O*-glucuronide and hesperetin-3′-*O*-glucuronide from Santa Cruz (Santa Cruz, CA, USA). Quercetin-3′-*O*-sulfate was kindly provided by Alan Crozier (University of California, Davis, CA, USA). HPLC grade solvents and stearic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Ethics Statement

The in vitro study was approved by the local Ethics Committee, with protocol n. 42170. The in vivo bioavailability study was ethically approved by the Institutional Review Board (IRB) of the University of Parma with protocol n. 0003/2016 and was conducted in accordance with the Declaration of Helsinki. Relevant principles of Good Clinical Practice were followed throughout the study. All subjects gave written informed consent for the bioavailability study. Conversely, no informed consent was required for the cell culture study, as blood donor material was fully anonymized.

2.3. Bioavailability Study

Three healthy adults (2 females and 1 male, mean age 38 $\pm$ 3 years) were recruited for the bioavailability study with BJ. Exclusion criteria were age <18 or >50 years, body mass index <18 or >25 kg/m^2, pregnancy or lactation, celiac or other metabolic diseases, past or current CV diseases, any chronic medication and/or hormone replacement therapy, cigarette smoking, alcohol intake > 80 g/day and use of dietary supplements. Subjects were asked to avoid the consumption of flavonoid-rich foods (e.g., berries, citrus fruits, dark chocolate, green tea and red wine) for the 2 days before entering the study.

The day of the study, participants were invited to drink 400 mL of BJ (Bergasterol®) after an overnight fasting. Venous plasma samples were obtained at baseline (before juice consumption) and at 1 h and 4 h after juice intake to assess the circulating phenolic derivatives coming from BJ flavanones. Urine samples were also collected at baseline and at different collection times (0–2 h and 2–6 h). During this period of time, subjects avoided the consumption of flavonoid-rich foods. Blood samples were centrifuged and plasma was collected, aliquoted and stored at −80 °C until further processing. Urine samples were also aliquoted and stored at −80 °C until further processing.

2.4. Extraction and UHPLC-MS Analysis of Phenolic Compounds from Bergamot Juice

The phenolic compounds in BJ were extracted according to previous reports, with some modifications [26,27]. Briefly, 50 mg of freeze-dried BJ were mixed with 1 mL of 80% aqueous methanol acidified with formic acid (1%). The mixture was vortexed and sonicated for 30 min. The mixture was centrifuged at 10,480× *g* for 10 min at room temperature and the supernatant was collected. The pellet was then extracted with 0.5 mL of dimethyl sulfoxide and followed the procedures described above, after which it was centrifuged. The supernatant and the pellet extracted were filtered through a 0.22 µm nylon membrane before UHPLC-MS analysis. Each sample was extracted in triplicate.

Samples from both the supernatant and pellet were analyzed using an Accela UHPLC 1250 equipped with a linear ion trap-mass spectrometer (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-ESI probe (H-ESI-II; Thermo Fisher Scientific Inc.). Separations were performed using a XSELECTED HSS T3 (50 × 2.1 mm), 2.5 µm particle size (Waters, Milford, MA, USA). The volume injected was 5 µL and the column oven was set to 30 °C. Elution was performed at a flow rate of 0.2 mL/min. The gradient started with 95% of 0.1% aqueous formic acid, isocratic conditions maintained for 3 min and then a 9-min linear gradient from 5 to 40% acetonitrile with 0.1% formic acid was applied. From 12 to 13 min the acidified acetonitrile was increased to 80%, followed by 3 min of 80% acetonitrile and 5 min at the start conditions to re-equilibrate the column.

The mass spectrometer was operated in negative mode. Capillary temperature was equal to 275 °C and the source heater temperature was set to 200 °C. The sheath gas flow was 40 units, while the auxiliary gas was set to 5 units. The source voltage was 4 kV. The capillary voltage and tube lens were −42 and −118 V, respectively. Analyses were carried out using full scan mode, data-dependent MS^3 scanning from m/z 100 to 1500, with collision induced dissociation (CID) equal to 30 (arbitrary units). Pure helium gas was used for CID. Specific MS^2 and MS^3 analyses were carried out to unambiguously identify the flavanones presented in BJ supernatant and pellet. Identification was also performed by comparison with standards, when available and literature. Quantification was performed

with calibration curves of pure commercial standards, when available, in selected ion monitoring (SIM) mode by selecting the relative base peak at the corresponding mass to charge ratio (m/z). Naringenin and eriodyctiol derivatives were quantified as narirutin equivalents, hesperetin derivatives as hesperidin equivalents and isosakuranetin derivatives as didymin equivalents. Data processing was performed using Xcalibur software (Thermo Scientific Inc., San Jose, CA, USA).

2.5. Extraction and UHPLC-MS Analysis of Phenolic Compounds in Urine and Plasma Samples

Urine samples were diluted 1:1 (v/v) adding acidified water (0.1% formic acid v/v), centrifuged at 14,000 rpm for 10 min and filtered through a 0.45 μm nylon filter before UHPLC-MS analysis. Plasma samples were extracted following the method reported by Ludwig et al. [28]. Briefly, 400 μL of plasma were added to 1 mL of 2% formic acid in acetonitrile. Samples were vortexed for 1 min, centrifuged at 14,000 rpm for 10 min and supernatants were then dried under a rotary-vacuum evaporation (Thermo Fisher Scientific Inc.). The pellet was resuspended with 100 μL of methanol:water:formic acid (50:50:0.1, $v/v/v$) and centrifuged at 14,000 rpm for 5 min prior to UHPLC-MS analysis.

Processed biological fluids were analyzed using the Accela UHPLC 1250 with a LTQ XL linear ion trap-mass spectrometer fitted with a heated-ESI probe (Thermo Scientific Inc.). Separations were carried out by means of an Acquity UPLC HSS T3 column (100 × 2.1 mm, 1.8 μm particle size, Waters, Milford, MA, USA). For UHPLC, mobile phase A was acetonitrile containing 0.1% formic acid and mobile phase B was 0.1% formic acid in water. The gradient started with 15% A, isocratic conditions were maintained for 0.5 min and reached 60% A after 6.5 min. From 7 to 8 min, the acidified acetonitrile increased to 80% and was kept at 80% for 2 min. The starting gradient was then immediately reestablished and maintained for 4 min to re-equilibrate the column. The flow rate was 0.4 mL/min, the injection volume was 5 μL and the column temperature was set at 40 °C.

The MS was operated in negative ionization mode with a capillary temperature of 275 °C, with the source temperature of 50 °C. The sheath gas flow was 50 units and the auxiliary gas set to 3 units. The source voltage was 3.7 kV. The capillary and tube lens voltage were −33 and −88 V, respectively. Helium gas was used for CID. A preliminary analysis was performed in full scan, data-dependent MS^3 mode, scanning from a mass to charge (m/z) of 100 to 1000, with CID of 35 (arbitrary units), to perform an assessment of the main flavanone phase II metabolites present in the samples. On the basis of the obtained information, a second preliminary analysis was carried out in full MS^2 and MS^3 mode to confirm the identification of the detected metabolites. Last, quantification of all flavanone metabolites was achieved using the single reaction monitoring (SRM) mode. Data processing was performed using Xcalibur software (Thermo Scientific). Quantification was performed with calibration curves of standards, when available. Eriodyctiol glucuronides were quantified as naringenin-7-*O*-glucuronide equivalents, while all sulfate conjugates as quercetin-3′-*O*-sulfate equivalents.

2.6. Cell Culture and Conditions

MACs were isolated and cultured as previously described [29–31]. Briefly, peripheral blood mononuclear cells were isolated by Lymphoprep (Euroclone, Milan, Italy) density gradient centrifugation from healthy volunteers' buffy-coats. A total of 10^7 cells/well were seeded into fibronectin-coated six-well plates and cultured in endothelial cell growth medium-2 (EGM-2) with supplements (Lonza, Milan, Italy) at 37 °C in a humidified 5% CO_2 incubator for 7 days. On day 7, adherent cells displaying a spindle-shaped morphology were considered MACs.

In order to assess a potential beneficial effect of bergamot-derived flavanone metabolites on lipotoxicity, MACs were incubated with physiological concentrations (100 μM) of SA according to our previous study [11]. Stearate stock solution was prepared by dissolving SA in 0.1 M NaOH at 72 °C for 30 min, then 5 mM stearate was complexed to 10% bovine serum albumin (BSA) (FFA:BSA molar ratio = 3.3:1) as reported in literature [32].

SA-treated MACs were incubated in the presence/absence of those molecules identified in the bioavailability study (naringenin-7-*O*-glucuronide (N7G), naringenin-4′-*O*-glucuronide (N4G),

hesperetin-7-*O*-glucuronide (H7G) and hesperetin-3′-*O*-glucuronide (H3G)) at the physiological concentration of 1 μM [33], in line with data recorded herein. Vehicle (DMSO 0.1%) was used as control.

2.7. Pro-Inflammatory Marker Gene Expression

Quantitative PCR (qPCR) assays were used to assess pro-inflammatory marker gene expression in MACs pre-incubated with N4G, N7G, H3G and H7G for 16 h and then exposed to SA for 3 h. Cells were lysed with QIAzol lysis reagent and total RNA was extracted using miRNeasy Mini Kit (both from Qiagen Ltd., West Sussex, UK) and quantified by NanoDrop (NanoDrop Technologies, Wilmington, DE, USA). iScript Reverse Transcription Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to obtain cDNA, starting from 250 ng of total RNA. Interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor-α (TNF-α) gene expression was assessed using SsoAdvanced Universal Probes Supermix (Bio-Rad) with TaqMan primers and probes (Applied Biosystems, Carlsbad, CA, USA) on a CFX Connect Real-Time (Bio-Rad), as already reported [30]. Specific thermal cycling conditions were used: 98 °C for 30 s, followed by 40 amplification cycles (95 °C for 3 s; 60 °C for 20 s). Gene expression values were calculated based on the ΔΔCt method using GAPDH as reference gene. Eight independent experiments were performed and samples were analyzed in triplicate.

2.8. Apoptosis Assessment

The potential effects of the tested metabolites on SA-induced apoptosis were assessed by Caspase-Glo 3/7 assay, according to manufacturer's instructions (Promega Corporation, Madison, WI, USA). MACs were cultured in 96-well culture plates (2.5×10^5 cells/well) and pre-incubated with the tested metabolites 16 h before being exposed to stearate for 24 h. Cells were then incubated with 100 μL of Caspase-Glo 3/7 reagent at 37 °C for 30 min and luminescence was measured by Cary Eclipse fluorescence spectrophotometer (Varian/Agilent, Santa Clara, CA, USA). Fold increase in caspase activity was normalized to the activity obtained from stearate-treated cells.

2.9. Statistical Analysis

All data are presented as mean ± SEM (standard error of the mean), except for the phenolic composition of the BJ, expressed as mean ± SD (standard deviation). Differences between groups were identified using one-way ANOVA followed by Dunnet's post-hoc test (with SA as reference condition). Statistical significance was set at $p < 0.05$ (two-sided). Data analysis was performed using SPSS version 24.0 (SPSS Inc./IBM, Chicago, IL, USA).

3. Results

3.1. Flavanone Composition of the Bergamot Juice

A total of 16 flavanones were identified and quantified in the BJ (Table 1). Five compounds corresponded to naringenin derivatives (compounds **3**, **5**, **8**, **9** and **13**), four different derivatives were found for both eriodyctiol (**1**, **2**, **6** and **16**) and hesperitin (**4**, **7**, **10** and **15**) and three isosakuranetin compounds (**11**, **12** and **14**) were identified. Most of the compounds detected were rutinosides or neohesperidosides of the aforementioned aglycones, while four compounds were 3-hydroxy-3-methylglutaryl (HMG) glycosides. Three compounds were identified by comparison with their commercial reference standards, while the remaining 13 flavanones were tentatively identified by interpreting and comparing their mass spectra, obtained from MS^2 and MS^3 experiments, with data from the literature [34–37]. A new HMG flavanone (compound **12**) was tentatively identified for the first time, to the best of our knowledge, in bergamot juice. This flavanone, isosakuranetin-7-*O*-neohesperidoside-6″-*O*-HMG (m/z 737), showed the characteristic fragmentation pattern of HMG derivatives according to Salerno et al. [35]: fragment ions at m/z $[M - H - 62]^-$, $[M - H - 102]^-$ and $[M - H - 144]^-$. It also exhibited a fragment ion at m/z 285, which was identified as isosakuranetin by a further MS^4 experiment attending to its characteristic fragmentation spectra. Some flavones previously reported in BJ [34,35] were also identified in the BJ (data not shown).

Table 1. Flavanone compounds in bergamot juice.

Id.	Compounds	RT (min)	$[M-H]^-$ (*m*/*z*)	MS^2 Ion Fragments (*m*/*z*) [a]	MS^3 Ion Fragments (*m*/*z*) [a]	Ident. [c]	Concentration (μmol/g dw)
1	Eriodictyol-7-*O*-rutinoside (eriocitrin)	9.5	**595**	**287** [b]	151	[34]	2.30 ± 0.22
2	Eriodictyol-7-*O*-neohesperidoside (neoeriocitrin)	9.71	**595**	**459**, 287, 235	235, 357, 441, 271, 151	[34]	7.83 ± 0.85
3	Naringenin-7-*O*-rutinoside (narirutin)	10.19	**579**	**271**	151, 177	Std	1.06 ± 0.05
4	Hesperetin-7-*O*-rutinoside (hesperidin)	10.2	**609**	**301**	286, 242, 283, 257, 125	Std	2.10 ± 0.22
5	Naringenin-7-*O*-neohesperidoside (naringin)	10.4	**579**	**459**, 271, 235	357, 235, 271, 441, 339	[38]	12.46 ± 1.61
6	Eriodictyol-7-*O*-neohesperidoside-6″-*O*-HMG	10.57	**739**	**595**, 637, 677	459, 287	[35]	7.53 ± 0.76
7	Hesperetin-7-*O*-neohesperidoside (neohesperidin)	10.79	**609**	**301**, 343, 489, 447	286, 242, 283, 257, 125	[34]	10.31 ± 0.61
8	Naringenin-7-*O*-neohesperidoside-6″-*O*-HMG (melitidin)	11.13	**723**	**579**, 621, 661	459, 271, 313	[34]	8.25 ± 0.93
9	Naringenin-7-*O*-glucoside-6″-*O*-HMG	11.39	**577**	**271**, 433, 475	151, 177	[35]	0.27 ± 0.03
10	Hesperetin-7-*O*-neohesperidoside-6″-*O*-HMG (brutieridin)	11.43	**753**	**609**, 651, 691	301, 343, 489	[34]	19.70 ± 2.04
11	Isosakuranetin-7-*O*-rutinoside (didymin)	12.25	**593**	**285**, 327, 473	270, 243, 164, 241, 151	Std	0.26 ± 0.02
12	Isosakuranetin-7-*O*-neohesperidoside-6″-*O*-HMG (parmigin)	12.75	**737**	**593**, 635, 675	**285** (MS^4: 270, 243), 327, 473	-	0.99 ± 0.03
13	Naringenin	13.32	**271**	151, 177		Std	traces
14	Isosakuranetin	13.57	**285**	270, 243		Std	traces
15	Hesperetin	13.78	**301**	286, 242		Std	traces
16	Eriodyctiol	13.82	**287**	151		[38]	traces

[a] Fragment ions are listed in order of relative abundance; [b] MS ions in bold were those subjected to successive MS fragmentation; [c] Ident., identification mode: [Reference] or Std (standard, compound identified by comparison of its retention time and MS data with that of a reference compound). Mean (n = 3) ± SD. RT, retention time.

The main flavanones in BJ were brutieridin, naringin, neohesperidin, melitidin, neoeriocitrin and eriodictyol-7-*O*-neohesperidoside-6″-*O*-HMG (Table 1). Small quantities of the other glycosides identified were also detected. With regard to the amount of specific flavanone aglycones, hesperetin derivatives accounted for the 44% of the flavanone composition, naringenin derivatives comprised the 30% and eriodyctiol derivatives the 24%, while isosakuranetin derivatives made up the remaining 2%.

3.2. Flavanone Metabolites in Plasma and Urine Samples

Up to 12 structurally related flavanone metabolites were identified in plasma and urine samples after BJ consumption. Their UHPLC retention times and mass spectra, as well as their occurrence in plasma and urine, are presented in Table 2. Some of these metabolites were identified by comparison with commercially available reference compounds, while the criteria for the identification of the rest of the compounds were based on previous studies [39–41]. Glucuronide and sulfate phase II metabolites were identified through the loss of the conjugation moiety (m/z 176 and 80 for glucuronides and sulfates, respectively). Successive MS fragmentation of the aglycone was performed to confirm the identification of the metabolites through their characteristic fragmentation patterns [38]. Five metabolites were hesperetin conjugates (compounds **M1**, **M2**, **M7**, **M8** and **M12**), 4 naringenin conjugates (**M4**, **M5**, **M9** and **M11**) and 3 eriodictyol derivatives (**M3**, **M6** and **M10**). No isosakuranetin conjugates were found.

Table 2. Flavanone metabolites in plasma and urine collected after consumption of bergamot juice (BJ).

Id.	Compounds	RT (min)	$[M-H]^-$ (m/z)	MS^2 Ion Fragments (m/z) [a]	MS^3 Ion Fragments (m/z) [a]	MS^4 Ion Fragments (m/z) [a]	Location [c]
M1	Hesperitin-*O*-glucuronide-sulfate	3.32	**557**	**381** [b], 447	**301**	286, 242, 199, 283	P
M2	Hesperitin-*O*-glucuronide-sulfate	3.51	**557**	**381**, 447	**301**, 229	286, 242, 199, 283	P, U
M3	Eriodictyol-*O*-glucuronide	4.04	**463**	**287**, 175	151		P, U
M4	Naringenin-7-*O*-glucuronide	4.09	**447**	**271**, 175	151, 177		P, U
M5	Naringenin-4′-*O*-glucuronide	4.23	**447**	**271**, 175	151, 177		P, U
M6	Eriodictyol-*O*-glucuronide	4.23	**463**	**287**, 175	151, 269		P, U
M7	Hesperitin-7-*O*-glucuronide	4.45	**477**	**301**, 175	286, 243, 283		P, U
M8	Hesperitin-3′-*O*-glucuronide	4.63	**477**	**301**, 175	286, 243, 283		P, U
M9	Naringenin-sulfate	4.68	**351**	**271**	151, 177		P
M10	Eriodictyol-sulfate	4.78	**367**	**287**	151		P, U
M11	Naringenin-sulfate	4.82	**351**	**271**	151, 177, 165		P
M12	Hesperetin-sulfate	4.9	**381**	**301**	286, 243, 283, 199		P, U

[a] Fragment ions are listed in order of relative abundance; [b] MS ions in bold were those subjected to successive MS fragmentation; [c] P, plasma; U, urine. RT, retention time.

Most of the flavanone phase II conjugates were recorded in both biological fluids. They were not recorded at baseline, while they were detected at both 1 h and 4 h in the plasma samples of all the volunteers. The majority of them were also detected in urine at 2 h and 6 h, accounting for the absorption profile observed in plasma. The main flavanone metabolites in plasma were sulfate conjugates of hesperetin and eriodyctiol, followed by glucuronides of hesperetin and naringenin (Table 3). Plasma concentrations for these compounds ranged from 0.11 to 3.54 μM, while the rest of the compounds presented concentrations below 0.1 μM. Despite the reduced number of subjects considered, a certain degree of inter-individual variability was observed, being notably higher at 4 h than at 1 h (mean variability of 23% and 69% at 1 h and 4 h, respectively).

3.3. Effects of Selected Flavanone Metabolites on the Gene Expression of Inflammation Markers

On the basis of the metabolites found in plasma of healthy volunteers and considering the limited commercial availability of phase II conjugates of flavanones, four glucuronides (two isomers of hesperetin and two of naringenin) were tested on MACs exposed to SA (Figure 1). The concentrations were selected taking into account the concentrations found in the study in humans and previous literature reporting on the concentration of these metabolites after consumption of other citrus fruit juices [33].

Table 3. Concentration of flavanone metabolites in plasma after consumption of bergamot juice [a].

Id.	Compounds	1 h		4 h	
		Mean ± SEM	CV	Mean ± SEM	CV
M1	Hesperitin-*O*-glucuronide-sulfate	0.021 ± 0.004	19%	0.019 ± 0.006	31%
M2	Hesperitin-*O*-glucuronide-sulfate	0.033 ± 0.008	24%	0.094 ± 0.093	98%
M3	Eriodictyol-*O*-glucuronide	0.096 ± 0.010	10%	0.085 ± 0.022	26%
M4	Naringenin-7-*O*-glucuronide	0.169 ± 0.032	19%	0.163 ± 0.042	26%
M5	Naringenin-4′-*O*-glucuronide	0.183 ± 0.017	9%	0.194 ± 0.050	26%
M6	Eriodictyol-*O*-glucuronide	0.095 ± 0.016	16%	0.087 ± 0.045	52%
M7	Hesperitin-7-*O*-glucuronide	0.112 ± 0.011	10%	0.162 ± 0.091	56%
M8	Hesperitin-3′-*O*-glucuronide	0.177 ± 0.020	11%	0.411 ± 0.355	86%
M9	Naringenin-sulfate	0.085 ± 0.050	59%	0.100 ± 0.091	91%
M10	Eriodictyol-sulfate	0.620 ± 0.195	31%	0.960 ± 0.935	97%
M11	Naringenin-sulfate	0.059 ± 0.016	28%	0.058 ± 0.059	102%
M12	Hesperetin-sulfate	0.451 ± 0.163	36%	3.542 ± 4.562	129%

[a] Data expressed in μM (n = 3). CV, coefficient of variation.

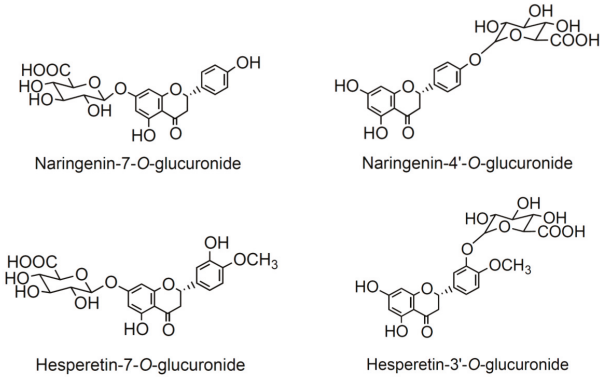

Figure 1. Molecular structures of tested compounds.

Gene expression of pro-inflammatory markers was assessed to evaluate whether H3G, H7G, N4G and N7G were effective in blunting lipotoxic damage in MACs. As expected, gene expression of IL-1β, IL-6, IL-8 and TNF-α increased following stearate exposure (100 μM, 3 h) (Figure 2). Of note, H7G curbed IL-1β (by 37 ± 6%; $p < 0.05$) and TNF-α (−32 ± 7%; $p < 0.05$) gene expression. Exposure to N4G mitigated mRNA expression of IL-8 and TNF-α (both by 34 ± 4%; $p < 0.05$) in MACs following SA exposure. Neither H3G nor N7G had any significant effect on the gene expression of the aforementioned pro-inflammatory markers.

3.4. Effects of Flavanone Metabolites on Cell Apoptosis

Pilot experiments confirmed that none of the tested molecules induced cytotoxic effects in MACs at 1 μM concentration (data not shown). The effect of H3G, H7G, N4G and N7G in curbing caspase 3 and 7 activation in SA-treated MACs was evaluated in order to detect whether bergamot-derived phenol metabolites may also have anti-lipoapoptotic properties.

No effects in reducing stearate-induced activation of caspases 3 and 7, compared to vehicle treated cells, was shown following metabolite addition in culture at the physiological concentration tested (Figure 3).

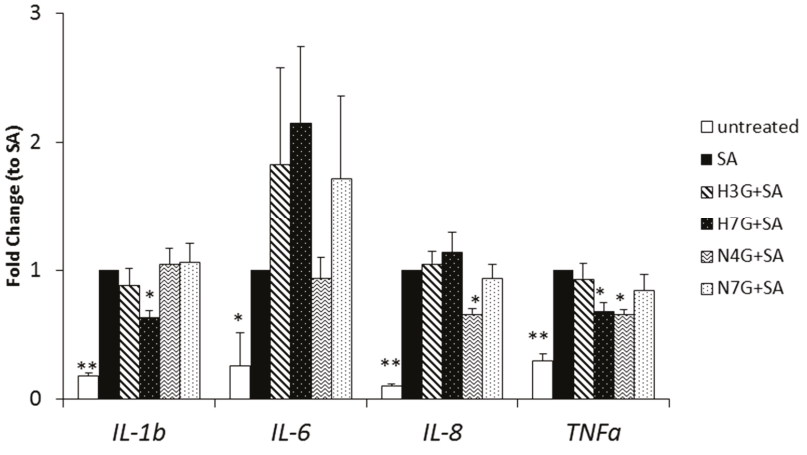

Figure 2. Flavanone metabolites reduce stearate-induced inflammation in MACs. Pro-inflammatory marker (IL-1β, IL-6, IL-8 and TNF-α) gene expression in MACs pre-incubated with 1 μM of naringenin-7-*O*-glucuronide (N7G), naringenin-4′-*O*-glucuronide (N4G), hesperetin-7-*O*-glucuronide (H7G) and hesperetin-3′-*O*-glucuronide (H3G) for 16 h and then exposed to stearate (SA, 100 μM) for 3 h is shown. Vehicle (DMSO 0.1%) was used as control (untreated). Real time PCR data are normalized to *GAPDH* housekeeping gene and expressed as fold induction of SA culture condition. The results represent means ± SEM (* $p < 0.05$ vs. stearate; ** $p < 0.01$ vs. stearate; $n = 8$).

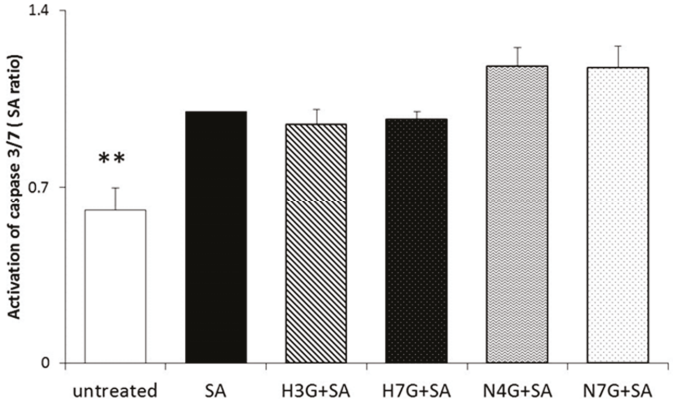

Figure 3. Effects of flavanone metabolites on apoptosis. Caspase 3/7 activation in myeloid angiogenic cells (MACs) pre-incubated with naringenin-7-*O*-glucuronide (N7G), naringenin-4′-*O*-glucuronide (N4G), hesperetin-7-*O*-glucuronide (H7G) and hesperetin-3′-*O*-glucuronide (H3G) at 1 μM for 16 h and then exposed to stearate (SA, 100 μM) for 24 h. Vehicle (DMSO 0.1%) was used as control (untreated) and results are expressed as means ± SEM (** $p < 0.01$ vs. stearate; $n = 8$).

4. Discussion

This study presents a comprehensive approach focused on exploring the bioactivity of the metabolites in circulation after consumption of sources of phenolic compounds. As required to fully pinpoint the responsible bioactive(s) and mechanisms of action in the extremely complex physiological scenario, the study was conducted by applying integrated multidisciplinary lenses: first, a detailed characterization of the food matrix; second, the study in humans of the characteristic transformations

of the compounds found in food; and third, the study of the biological activity of the compounds in circulation, tested at physiologically achievable concentrations.

The characterization of the (poly)phenolic fingerprint of BJ had been addressed in recent years [34,35]. It is composed mainly of flavanones and relevant amounts of flavones. However, among all its compounds, it was mainly the specific composition of bergamot in 3-hydroxy-3-methylglutaryl-glycoside flavonoids to attract the attention of the research community during the last decade [20,36,42]. Interestingly, despite the accurate works carried out in the past, it was possible to identify, for the first time, a new HMG flavanone, isosakuranetin-7-*O*-neohesperidoside-6″-*O*-HMG (compound **12**). Although additional analytical techniques are required to fully confirm the structure of this molecule (i.e., NMR), we proposed it to be named "parmigin." This is a point worth mentioning since it extends the knowledge on the phenolic composition of bergamot and citrus plant biology. On the contrary, a compound previously reported in bergamot products at high concentrations, isosakuranetin-7-*O*-neohesperidoside (poncirin) [34,43], was not detected in the present work; although the flavone luteolin-rutinoside/neohesperidoside, which shares a similar fragmentation pattern with poncirin and has been previously reported in bergamot [35], was detected. This could be due to differences in the methodological identification of this compound or to some factors affecting the phenolic composition of citrus products like juice processing or pre-harvest conditions [44].

The bioavailability study accounted for the extensive presence of phase II conjugated flavanone metabolites 1 and 4 h after consumption of BJ. The occurrence of these metabolites in circulation is related to the cleavage of the glycoside units of the compounds present in the juice at intestinal level and the subsequent conjugation of the flavanone aglycones with glucuronide and sulfate moieties by phase II enzymes [39,41,45]. So far, no studies on the bioavailability of BJ flavanones had been conducted. This study has allowed to describe the absorption profile of BJ flavanones during the first few hours after BJ consumption. The profile of flavanone metabolites in plasma matched the pharmacokinetic profiles previously reported for these compounds [39,41,44–47]. With respect to the absorption and metabolism of the characteristic HMG-containing flavanones of bergamot, they were not found in plasma or urine samples despite they were selectively targeted. The lack in circulation of these compounds may indicate that they undergo the same transformations of glycosylated flavanones, circulating thus as phase II conjugates. Nevertheless, their appearance at time points beyond 4–6 h cannot be ruled out, although it does not seem plausible considering the metabolic steps undergone by flavanones with different glycoside moieties [45,48,49]. Last, the inter-individual variability observed in the plasmatic concentrations of flavanone conjugates is in line with a recent study [50]. Nevertheless, data on inter-individual variability should be intended to be of preliminary nature, considering the low number of subjects involved in this study, enough to identify the metabolites and concentrations to be tested but not to properly address this topic.

Substantial clinical evidence supports the beneficial vascular effects of flavanones. Randomized-controlled studies showed that hesperidin (500 mg/day for 3 weeks) [51] and flavanone-rich citrus (orange) beverage [52,53] intake was associated with an improvement in endothelial function in healthy subjects [52,53] and in individuals with metabolic syndrome [51]. Of note, in one of these studies [52], the improvement in flow mediated dilation coincided with the peak of naringenin/hesperetin metabolites in circulation, supporting a causal relationship. Some results have also been published reporting the effects of BJ and its flavanones on the CV system [20,34,42,54]. BJ has been reported to exert anti-inflammatory activities [21], as recently demonstrated in an experimental model of bowel disease via modulation of several pathways [55].

In mechanistic studies, BJ reduced LPS-induced pro-inflammatory response (IL-1β, IL-6 and TNF-α) in THP-1 macrophages by decreasing NF-kB activation [23]. The same authors reported a similar anti-inflammatory effect on THP-1 exposed to amyloid-beta, associated with up-regulation of ERK1/2 and JNK MAPK [22]. However, the above cited works are fraught with methodological concerns as cultured cells were either exposed to flavonoid levels far from those achievable in the bloodstream, or to BJ itself, which obviously represents a non-physiological condition with evident

misleading and paradoxical results. A methodological strength of the present study is that the cell experiments were carried out by treating cells with the metabolites identified in circulation following BJ intake in humans. Moreover, the concentrations tested, although slightly higher than those registered at 4 h, are in line with concentrations achievable after citrus juice consumption or that could even be achievable at longer time points [33,45].

To date, there is a paucity of studies investigating the effects of phase II flavonoid metabolites at physiological concentrations on the mechanisms underlying their possible benefits on vascular biology. Physiological concentrations of naringenin and hesperetin glucuronides have been reported to reduce migration and inflammation in human aortic endothelial cells [56], monocyte adhesion to umbilical vein endothelial cells activated by TNF-α [57] and gene expression in differently activated human macrophages [58]. This study expands these findings demonstrating that these metabolites also reduce pro-inflammatory cytokine expression (i.e., IL-1β, IL-8 and TNF-α) in key primary cells mediating vascular reparative processes. In particular, our novel findings show that some BJ flavanone circulating derivatives (naringenin-4′-*O*-glucuronide and hesperetin-7-*O*-glucuronide) improve MAC function in terms of inflammatory cytokine activation under lipotoxic conditions. However, these compounds were not effective in curbing stearate-induced lipoapoptosis at the tested concentrations. A potential anti-apoptotic/pro-survival effect of these compounds at higher metabolite levels or in a concomitant presence cannot be excluded. In general, these results are particularly significant when considering (a) the variety of conditions and diseases associated to increased SA levels and thus lipotoxicity, such as IR, obesity and diabetes [5]; (b) the growing evidence of the role of dietary compounds, including citrus polyphenols, in the prevention/treatment of metabolic and (cardio)vascular diseases [14,59]; and (c) the pivotal role played by MACs in the maintenance of vascular integrity, emphasized by their role in the prediction of CV events and mortality in humans [3]. Nevertheless, despite the interest of these novel findings, whether bergamot consumption can display anti-atherogenic actions by improving endothelial regenerative capacity when challenged by a lipotoxic clue is a question that should be answered with further in vivo studies. In the light of these results, not only bergamot but also other citrus fruits might exert this bioactivity.

The observation that only N4G and H7G (and not N7G and H3G) modulated SA-induced inflammation suggests that the biological action of naringenin and hesperetin glucuronide metabolites might depend on the position of the conjugation, as already hypothesized [56,60]. Our results are in accordance with the work by Yamamoto and colleagues in which H7G but not H3G, decreased H_2O_2-induced pro-inflammatory markers (intracellular adhesion molecule-1 and monocyte chemoattractant protein-1) gene expression in endothelial cells [60]. The different activity on the expression of selected genes in human macrophages of naringenin metabolites depending on the glucuronide position had also been previously reported [58].

In conclusion, these results demonstrated that some flavanone metabolites, derived from the metabolism of BJ phenolics in humans, ameliorated SA-induced inflammation in MACs. To better understand the prospects of bergamot cardiovascular protection, further intervention studies are required. Future bioavailability studies should assess longer periods of time (up to 24/48 h), evaluate the production of colonic metabolites and take into account inter-individual differences among volunteers. Randomized controlled studies with bergamot juice/extract on recognized markers of CV health are also needed. Lastly, the underlying molecular mechanism behind the bioactivity observed remains uninvestigated and future studies on this regard should be performed.

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Evaluation of the Cytotoxicity and Genotoxicity of Flavonolignans in Different Cellular Models

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Abstract: Flavonolignans are the main components of silymarin, which represents 1.5–3% of the dry fruit weight of Milk thistle (*Silybum marianum* L. Gaernt.). In ancient Greece and Romania, physicians and herbalists used the *Silybum marianum* to treat a range of liver diseases. Besides their hepatoprotective action, silymarin flavonolignans have many other healthy properties, such as anti-platelet and anti-inflammatory actions. The aim of this study was to evaluate the toxic effect of flavonolignans on blood platelets, peripheral blood mononuclear cells (PBMCs) and human lung cancer cell line—A549—using different molecular techniques. We established that three major flavonolignans: silybin, silychristin and silydianin, in concentrations of up to 100 μM, have neither a cytotoxic nor genotoxic effect on blood platelets, PMBCs and A549. We also saw that silybin and silychristin have a protective effect on cellular mitochondria, observed as a reduction of spontaneous mitochondrial DNA (mtDNA) damage in A549, measured as mtDNA copies, and mtDNA lesions in *ND1* and *ND5* genes. Additionally, we observed that flavonolignans increase the blood platelets' mitochondrial membrane potential and reduce the generation of reactive oxygen species in blood platelets. Our current findings show for the first time that the three major flavonolignans, silybin, silychristin and silydianin, do not have any cytotoxicity and genotoxicity in various cellular models, and that they actually protect cellular mitochondria. This proves that the antiplatelet and anti-inflammatory effect of these compounds is part of our molecular health mechanisms.

Keywords: flavonolignans; silybin; silychristin; silydianin; blood platelets; mitochondria; ROS; cytotoxicity; genotoxicity

1. Introduction

Milk thistle (*Silybum marianum* L. Gaernt.), sometimes called "wild artichoke", is a medicinal plant that has been used for thousands of years as a remedy for a variety of ailments [1]. There is a lot of epidemiological evidences that plant secondary metabolites have various health activities—protected against development of cancers, cardiovascular diseases, diabetes, osteoporosis as well as neurodegenerative diseases [2]. In ancient Greece and Romania, physicians and herbalists used *Silybum marianum* to treat a range of liver and gallbladder disorders, including hepatitis, cirrhosis and jaundice, as well as to protect the liver against poisoning from chemical and environmental toxins [3]. By the 19th century, American Eclectic physicians used milk thistle for varicose veins and liver, spleen and kidney disorders, while in the mid-19th century, the German physician Rademacher used

the milk thistle fruit for treatment of liver diseases [4]. Needless to say, its hepatoprotective action has been proven by many studies [5–10].

Silymarin represents 1.5–3% of the fruit's dry weight and is an isomeric mixture of flavonoid complexes—flavonolignans. These compounds possess a unique chemical structure, which is composed of two main units. The first is based on a taxifolin, which is a flavanonol group in flavonoids. The second is a phenylpropanoid unit [11,12]. The main representatives of this group that are present in silymarin are silybin, isosilybin, silychristin, isosilychristin silydianin and silymonin [3,13–17]. The main silymarin flavonolignans are silybin, silychristin and silydianin, which form approximately 90% of the extract's composition. The silybin content is approximately 50% to 70%; silychristin, about 20%, and silydianin, about 10% of silymarin composition [3].

Besides their hepatoprotective action, silymarin flavonolignans have many other healthy properties [18]. It has been established that these compounds have strong antioxidative properties and modulate a variety cell-signaling pathways, resulting in the reduction of pro-inflammatory mediators [19]. There is a strong body of evidence that silymarin is also a potential anticancer and chemopreventive agent [20–25].

Research performed by our team demonstrated that three major silymarin flavonolignans—silybin, silychristin and silydianin—are able to reduce blood platelet activation in different physiological pathways. These compounds were shown to reduce ADP-induced blood platelet activation and aggregation in whole blood samples [26], as well as collagen-induced blood platelets' activation, adhesion, aggregation and secretion of Platelet Factor 4 (PF-4) [27]. We also determined the effect of these flavonolignans on arachidonic acid pathway blood platelets, and showed that the compounds that we tested decrease platelet aggregation level—both thromboxane A2 and malondialdehyde formation, as well as inhibiting cyclooxygenase activity [28]. The other positive effect observed in our previous study was a reduction in cross-talk between hemostasis and the immunological system. We observed that silybin and silychristin inhibit the IL-1β-induced formation of blood platelet-leukocyte aggregates in whole blood samples in a dose-dependent manner, as well as leucocyte production of the pro-inflammatory cytokines-IL-2, TNF, INF-α, and INF-γ. Additionally, these two flavonolignans abolished the IL-1β-induced expression of mRNA for IFN-γ and TNF in leucocytes [29].

Based on the results obtained in our previous studies, and to confirm that all the observed positive effects are related to healthy mechanisms of the action of flavonolignans, we aimed to evaluate the toxic effect of silybin, silychristin and silydianin on blood platelets, peripheral blood mononuclear cell (PBMC) and human lung cancer cell line—A549—using different molecular techniques.

2. Materials and Methods

2.1. Reagents

Dimethyl sulfoxide (DMSO), 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), glucose, Tris, NADH, sodium pyruvate, Histopaque®-1077, RPMI 1640 and DMEM mediums as well as the flavonolignans (silybin, silychristin and silydianin), were all obtained from the Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Trypan blue dye and cell counter slides were sourced from Bio-Rad (Hercules, California, USA). Both the JC-1 Dye and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA Dye), came from Thermo Fisher Scientific (Waltham, MA, USA). All other chemicals were reagent grade or the highest-quality available.

2.2. Blood Samples

Blood samples collected from 12 different healthy donors were purchased from the Regional Centre for Transfusion Medicine in Lodz (Lodz, Poland). All samples were drawn in the morning, from fasting donors. All donors were checked by a doctor and found to have no cardiovascular disorders, allergies, lipid or carbohydrate metabolism disorders, nor were they being treated with any

drugs [30]. Our analysis of the blood samples was performed under the guidelines of the Helsinki Declaration for Human Research, and approved by the Committee on the Ethics of Research in Human Experimentation at the University of Lodz (Resolution No. 16/KBBN-UŁ/II/2016).

2.3. Blood Platelets' Isolation and Sample Preparation

The blood was centrifuged (200× *g*, 10 min, room temperature—RT) to isolate the platelet rich plasma (PRP). Blood platelets were isolated from PRP using BSA–Sepharose 2B gel filtration, according to Walkowiak [31]. After isolation, the platelets were suspended in a modified Tyrode's Ca^{2+}/Mg^{2+} free buffer (127 mM NaCl, 2.7 mM KCl, 0.5 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5 mM HEPES, 5.6 mM glucose, pH 7.4). Platelet suspensions (2×10^8 platelets/mL) were pre-incubated with the silybin, silychristin and silydianin in 3 concentrations (10–50–100 μM) for 30 min at 37 °C. All tested compounds were initially dissolved in 20% DMSO to a preliminary concentration of 20 mM. Other solutions of the compounds used were also performed in 20% DMSO (prepared in 50 mM TBS, pH 7.4). The final DMSO concentration of all samples was 0.1%. In control samples the same volume of solvent was added (20% DMSO prepared in 50 mM TBS, pH 7.4), with the probes warmed for 30 min at 37 °C.

2.4. Blood Platelets' Mitochondrial Membrane Potential (MMP)

To measure MMP the fluorescent dye JC-1 (5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarb ocyanine iodide) was used [32]. The ratio of red to green fluorescence of JC-1 is dependent only on membrane potential, and is not influenced by mitochondrial size, shape, or density. Blood platelet samples were pre-incubated with 5 μM JC-1 (prepared in Tyrode's Ca^{2+}/Mg^{2+} free buffer), at 37 °C for 30 min. The fluorescence was measured on a Bio-Tek Synergy HT Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA), with filter pairs of 530 nm/590 nm and 485 nm/538 nm. Results are shown herein as a ratio of fluorescence, measured at 530 nm/590 nm to that measured at 485 nm/538 nm (aggregates to monomer fluorescence).

2.5. Measurement of Intracellular Reactive Oxygen Species (ROS) Levels

The relative level of intracellular ROS in blood platelets was measured using the redox-sensitive fluorescent dye-DCFH-DA [33]. Blood platelet samples were pre-incubated with 5 μM of DCFH-DA (prepared in Tyrode's Ca^{2+}/Mg^{2+} free buffer), at 37 °C for 45 min. Fluorescence was measured at a 480 nm excitation wavelength and an emission wavelength of 510 nm, using a Bio-Tek Synergy HT Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA), and expressed as a percentage of the control (blood platelets without tested compounds).

2.6. Generation of Superoxide Anion Radicals ($O_2^-\bullet$)

Generation of superoxide anion radicals ($O_2^-\bullet$) in blood platelets was measured using the cytochrome C reduction method, as described earlier [34,35]. For that, 250 μL of cytochrome C (160 μM) prepared in Ca^{2+}/Mg^{2+} free Tyrode's buffer was added to an equal volume of platelet suspensions in the same buffer. After incubation (30 min at 37 °C), the platelets were sedimented by centrifugation at 2000× *g* for 5 min, and the supernatants (200 μL) were added to the microplatelet wells. Reduction of cytochrome C was measured spectrophotometrically at 550 nm. To calculate the molar concentration of $O_2^-\bullet$, an extinction coefficient for cytochrome C of 18,700 $M^{-1}\cdot cm^{-1}$ was used.

2.7. Lactic Dehydrogenase Releasing from Blood Platelets

Blood platelet viability and the cytotoxic effects of tested flavonolignans were determined by measuring the activity of lactic dehydrogenase—LDH (cell lysis marker)—in an extracellular medium (PPP) using the spectrophotometric method described by Wróblewski and LaDue [36]. The PRP samples were pre-incubated with the silybin, silychristin and silydianin in 3 concentrations (10–50–100 μM) for 30 min at 37 °C. All tested compounds were initially dissolved in 20% DMSO (prepared in 50 mM TBS,

pH 7.4), to a preliminary concentration of 20 mM. After that, the samples were centrifuged (1000× *g*, 10 min, RT). Into the microplate (Corning® Brand 96-Well UV Plates) reaction wells, 270 µL of 0.1 M phosphate buffer (pH 7.4), 10 µL of the obtained PPP, and 10 µL of NADH were then all added. After 20 min, 10 µL of sodium pyruvate was added to all reaction wells using a multichannel pipette, thus all the reactions began simultaneously. The absorbance measurements were performed in a 96-well microplate reader (SPECTROstarNano, BMG Labtech, Ortenberg, Germany), at ambient temperature. The absorbance values were monitored every 60 s for 10 min. Lactic dehydrogenase activity was determined by a decrease of absorbance at 340 nm, and expressed as a percentage of the control (PPP obtained from PRP without tested compounds).

2.8. Peripheral Blood Mononuclear Cell Viability Assay

A PBMC fraction was isolated from human blood, obtained and collected as described above, with a density gradient centrifugation method using a Histopaque®-1077 (a sterile solution of polysucrose, 57 g/L, and sodium diatrizoate, 90 g/L, with a density of 1.077 g/mL). The blood was carefully layered (in a volume ratio of 1:1) onto the Histopaque®-1077, and centrifuged for 30 min (400× *g*, at room temperature). Afterwards, the collected pellet was washed twice with RPMI 1640 (400× *g*, 10 min at room temperature). The obtained fraction of PBMCs was suspended in RPMI 1640 to suspension 1×10^6 PBMCs/mL. The PBMC samples were pre-incubated with the flavonolignans (silybin, silychristin and silydianin), in 3 concentrations (10–50–100 µM) for 1 h at 37 °C. All tested compounds were initially dissolved in 20% DMSO (prepared in 50 mM TBS, pH 7.4) to the preliminary concentration of 20 mM. Cell viability (%) was determined during a spectrofluorimetric analysis, involving the use of propidium iodide as a fluorescent dye. Measurements were conducted using a microchip-type automatic cell counter Adam-MC DigitalBio (NanoEnTek Inc., Seoul, Korea) according to the manufacturer's protocol.

2.9. Cell Cultures

The cytotoxicity and genotoxicity experiments were performed on a human lung cancer cell line—A549 (CCL-185; ATCC) cell line, obtained from American Type Culture Collection (ATCC™, Manassas, VA, USA). The cell line was placed in a humidified incubator with a 5% CO_2 atmosphere at 37 °C in a DMEM medium, supplemented with 10% (*v*/*v*) heat-inactivated Fetal Bovine Serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cell culture reagents were obtained from Lonza (Basel, Switzerland). For cytotoxicity and genotoxicity, cells were seeded at 3×10^6 cells per well and were left overnight before treatment. The following day, the cell samples were incubated with flavonolignans (silybin, silychristin and silydianin), in 3 concentrations (10–50–100 µM) for 24 h.

2.10. Cell Viability Determination

The viability of A549 cells treated with different concentrations of flavonolignans was estimated using a Bio-Rad TC20 automated cell counter (Hercules, CA, USA), using trypan blue dye (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. Cell viability was expressed as a percentage relative to the untreated (control) cells, defined as 100%.

2.11. DNA Extraction from Cell Cultures

Treated cell suspensions were collected by centrifugation (400× *g*, 12 min) and total genomic DNA (nuclear and mitochondrial) was isolated using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The DNA concentrations were determined by spectrophotometric measurement of absorbance at 260 nm, and the purities were calculated at a ratio of A260/A280 using a Bio-Tek Synergy HT Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA). The purified DNA was stored at −32 °C until further analysis.

2.12. Mitochondrial DNA Copy Number Quantification

The relative number of copies of human mitochondrial DNA using nuclear DNA (nDNA) content as a standard was assessed by quantitative real-time PCR (qRT-PCR). To this quantification two primer pairs for detecting mtDNA (*ND1*, *ND5*), and two primer pairs for detecting nDNA (SLCO2B1, SERPINA1), were selected. All primers were designed with the help of the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) and synthesized by Sigma-Aldrich (St. Louis, MO, USA). Complete nucleotide sequences for each gene were taken from the ENSEMBL database (https://ensembl.org/). Mitochondrial *ND1* (124 bp fragment size), and *ND5* genes (124 bp fragment size), were amplified using the primer pair (forward primer 5′-CCTAAAACCCGCCACATCTA-3′ and reverse primer 5′-GCCTAGGTTGAGGTTGACCA-3′; forward primer 5′-AGGCGCTATCACCACTCTGT-3′ and reverse primer 5′-TTGGTTGATGCCGATTGTAA-3′), respectively. For determination of the amount of nuclear DNA, the SLCO2B1 (135 bp fragment size) and SERPINA1 (148 bp fragment size) genes were used as a reference: forward primer 5′-TGCAGCTTCCTCTTCACAGA-3′ and reverse primer5′-CTCAGCCCCAAGTATCTCCA-3′; forward primer 5′-GATCCCAGCCAGTGGACTTA-3′ and reverse primer: 5′-CCTGAAGCTGAGGAGACAGG-3′.

qRT-PCR was amplified using a CFX96 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). A reaction mix of 10 µL contained 1 × RT PCR Mix SYBR A (A&A Biotechnology, Gdynia, Poland), 250 nM of each primer, and 1 µL (5 ng) of isolated genomic DNA. The RT-PCR conditions were as follows: 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s, 65 °C for 30 s, and 72 °C for 15 s, with plate reading at this step. Each reaction was performed in duplicate and included a negative control (without template DNA). The cycle threshold (Ct) values were calculated automatically and analyzed using the CFX Manager™ Software (version 3.1). The relative mtDNA copy number was calculated using the formula $2^{\Delta Ct1 \text{ and } \Delta Ct2}$, where ΔCt1 = Ct for *SLCO2B1*−Ct for *ND1*; ΔCt2 = Ct for *SERPINA1*−Ct for *ND5*.

2.13. Determination of Mitochondrial and Nuclear DNA Damage—Semi-Long Run qRT-PCR (SLR-qRT-PCR)

To assess the mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) damage, the semi-long run quantitative RT-PCR (SLR-qRT-PCR) was used [37]. To measure the levels of the DNA lesions in the tested region of the mitochondrial or nuclear genome, two fragments of different lengths were used, i.e., long and small fragments, located in the same mitochondrial/nuclear genomic region. The sequence of all primers used in this study are listed in Table S1 in supplementary material.

SLR-qRT-PCR amplification was performed using a CFX96 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). The SLR-qRT-PCR reaction mix of 10 µL consisted of 1 × RT PCR Mix SYBR A (A&A Biotechnology, Gdynia, Poland), 250 nM of each primer and 1 ng of template DNA. The cycling sequence was as follows: initial denaturation of 3 min at 95 °C followed by up to 40 cycles of 15 s at 95 °C, 30 s at 65 °C, and 15 s at 72 °C (for short amplicons), or 45 s at 72 °C (for long amplicons). The cycle threshold (Ct) values were calculated automatically and the analysis made using CFX Manager™ Software, version 3.1 (Bio-Rad Laboratories, Hercules, CA, USA). DNA damage was calculated as lesion per 10 kb DNA of each region, by including the size of the respective long fragment: Lesion per 10 kb DNA = $(1 - 2^{-(\Delta long - \Delta short)}) \times 10{,}000$ (bp)/size of long fragment (bp), where Δlong and Δshort indicate differences in Ct value between non-treated control and treated samples. DNA isolated from the non-treated cells (controls) was used as the reference, whereas the Ct of the large and small mitochondrial/nuclear fragments was used for DNA damage quantification.

2.14. Isolation of RNA and Reverse Transcription to cDNA

Treated cell suspensions were collected by centrifugation (400× *g*, 12 min). RNAs were then extracted from pelleted cells using a GenElute™ Mammalian Total RNA Miniprep Kit Q-PCR (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's instructions. RNA concentrations

were determined by spectrophotometric measurement of absorbance at 260 nm, and purity was calculated at a ratio of A260/A280 with a Bio-Tek Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

Total RNA (1 µg) was reverse transcribed into cDNA with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Waltham, MA, USA). All steps were performed according to the manufacturer's recommendations.

2.15. Analysis of Apoptotic Gene Expression Level

Expression levels of apoptotic genes were obtained using the following TaqMan probes: Hs00608023_m1 for the *BCL2* gene; Hs00180269_m1 for the *BAX (BCL2 associated X)* gene; Hs00559441_m1 for the *APAF1* (*apoptotic peptidase activating factor 1*) gene; Hs00234387_m1 for the *CASP3* (*caspase 3*) gene; Hs01018151_m1 for the *CASP8* (*caspase 8*) gene; Hs00962278_m1 for the *CASP9* (*caspase 9*) gene, and Hs99999901_s1 as an endogenous control (the human *18S rRNA* gene, sourced from Life Technologies, Carlsbad, CA, USA). Real-time PCR analyses were performed using a CFX96 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA), with a TaqMan Universal Master Mix II without UNG (Life Technologies, Carlsbad, CA, USA). All procedures were performed according to the manufacturers' protocols. The C_t values were calculated automatically and the analyses performed using CFX Manager™ Software (version 3.1). Relative expressions of the studied genes were calculated using the equation $2^{-\Delta Ct}$, where ΔCt = Ct target gene − Ct 18S rRNA.

2.16. Data Analysis

The statistical analysis was performed using StatsDirect statistical software V. 2.7.2 (Cheshire, UK). All experimental values presented in this study were expressed as mean ± standard deviation (SD). To analyze the normality of the distribution of results, the Shapiro-Wilk test was used. Next, the results were analyzed for equality of variance using Levene's test. The significance of the differences between the values was analyzed using ANOVA, followed by Tukey's range test for multiple comparisons (for data with normal distribution and equality of variance), and the Kruskal-Wallis test; $p < 0.05$ was accepted as statistically significant [38–41].

3. Results

3.1. Effect of Flavonolignans on Platelets Viability and MMP

In the first step of our study, we determined the effect of flavonolignans on the viability of blood platelets. At first, we checked the effect of flavonolignans on blood platelets' mitochondrial membrane potential. We observed that the tested compounds did not reduce mitochondrial membrane potential in blood platelets (Figure 1). Moreover, in our experiments silychristin and silybin in 50 and 100 µM concentrations ($p < 0.05$ and $p < 0.001$ respectively), were seen to increase the blood platelets mitochondrial membrane potential. The cytotoxicity of flavonolignans on human blood platelets was also evaluated using estimation of LDH activity. None of the flavonolignans we used—silychristin, silybin and silydianin—in any of the tested concentrations (10, 50 and 100 µM) caused damage to blood platelets, as determined by lactate dehydrogenase activity in PPP (Figure 2).

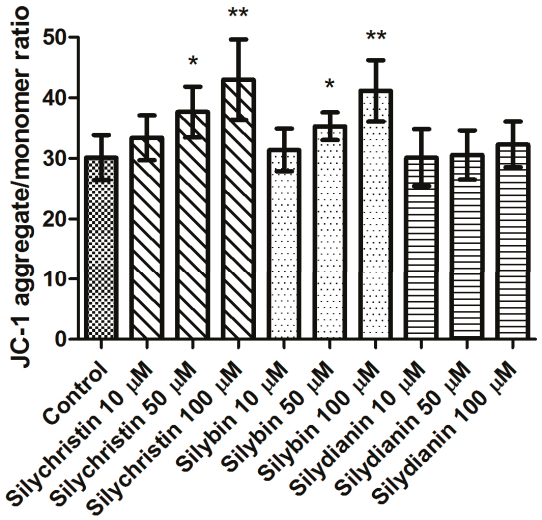

Figure 1. The effect of flavonolignans (silychristin, silybin and silydianin in concentrations of 10, 50 and 100 μM) on blood platelet mitochondrial membrane potential. MMP is expressed as a ratio of 530 nm/590 nm to 485 nm/538 nm (aggregates to monomer) fluorescence, as quantified with a fluorescent plate reader after JC-1 staining. The data represent means of ± standard deviation (SD), $n = 12$, * $p < 0.05$, ** $p < 0.001$.

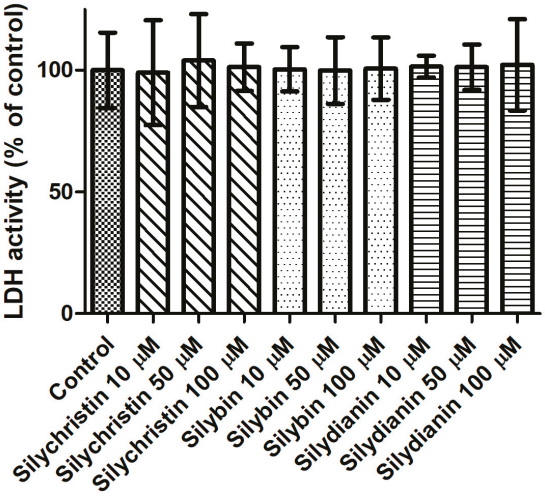

Figure 2. The effect of flavonolignans (silychristin, silybin and silydianin in concentrations of 10, 50 and 100 μM) on lactic dehydrogenase release from blood platelets. The results are expressed as a percentage of LDH activity in the control samples (without tested compounds). The data represent means of ± SD, $n = 12$.

3.2. Effect of Flavonolignans on ROS Generation in Platelets

The next step in our studies of blood platelets was to measure ROS level generation. We observed that incubation with silychristin and silybin, in all tested concentrations, statistically significantly

reduced intracellular ROS levels (Figure 3A) and generation of $O_2^{-}\bullet$ in blood platelets (Figure 3B). In the case of samples treated with silydianin, this effect was observed only for the highest tested concentration (100 μM).

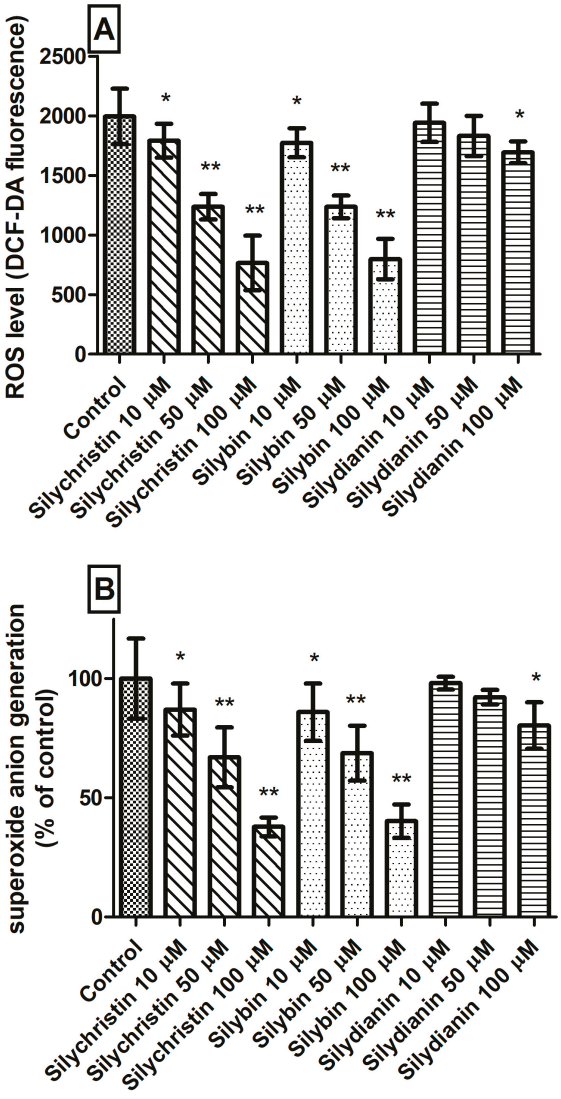

Figure 3. The effect of flavonolignans (silychristin, silybin and silydianin in concentrations of 10, 50 and 100 μM) on generation of reactive oxygen species in blood platelets. (**A**) intracellular ROS production measured as intensity of DCF fluorescence; (**B**) superoxide anion generation measured as cytochrome C reduction and expressed as a percentage value of the control sample. The data represent means of ± SD, $n = 12$. Statistical analysis was performed using Tukey's Range Test, * $p < 0.05$, ** $p < 0.001$.

3.3. Effect of Flavonolignans on PMBCs Viability

Next, we evaluated the potential cytotoxicity of tested flavonolignans using a model of PBMCs after 60 min of incubation. The lack of a toxic effect of flavonolignans was evidenced by a lack of significant differences ($p > 0.05$) in cell viability, observed between PBMCs incubated with flavonolignans (93.3–95.4% viability), and the control (untreated) samples (95.8 viability), regardless of the flavonolignans type and concentration (Figure 4).

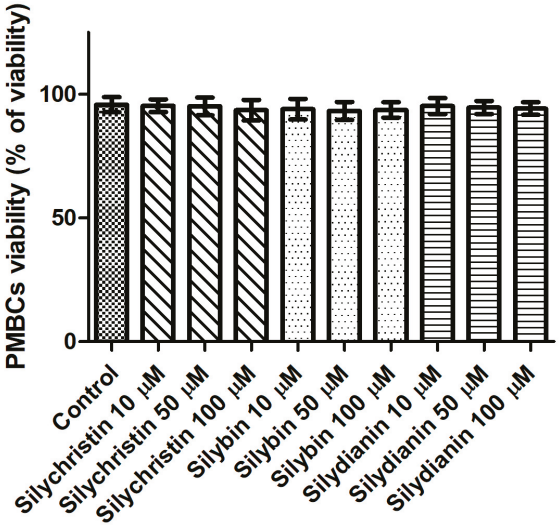

Figure 4. The effect of flavonolignans (silychristin, silybin and silydianin in concentrations of 10, 50 and 100 μM) on PMBCs viability. The data represent the mean of 8 measurements.

3.4. Effect of Flavonolignans on Human Lung Cancer Cell Line A549 Viability

To determine whether flavonolignans can affect cells, an assay was performed for in vitro cytotoxic activities in the standardized cell-culture system. For this assay, the human lung cancer cell line A549 was used. Cell morphology and cell viability were selected as the parameters for determining cytotoxicity. Cells were exposed to flavonolignans for 24 h, in different concentrations from 10 to 100 μM in the examined samples. For cell viability, Trypan blue exclusion assay was used. The result of testing flavonolignans on A549 human lung cancer cell line did not show any effects of cytotoxicity ($p > 0.05$), as shown in Figure 5. No agglutination, vacuolization, separation from the medium or cell membrane lyses were observed. In cultures which had contact with the investigated material samples, single rounded cells were noted at each observation time (Figure S1). After contact with these compounds, none of the cell cultures showed any damage, and the cells had proper morphologies and showed good proliferation in comparison to the control cells. Furthermore, proliferation of cells in the control and test cultures was similar, and the cells formed colonies across the entire surface of the plates.

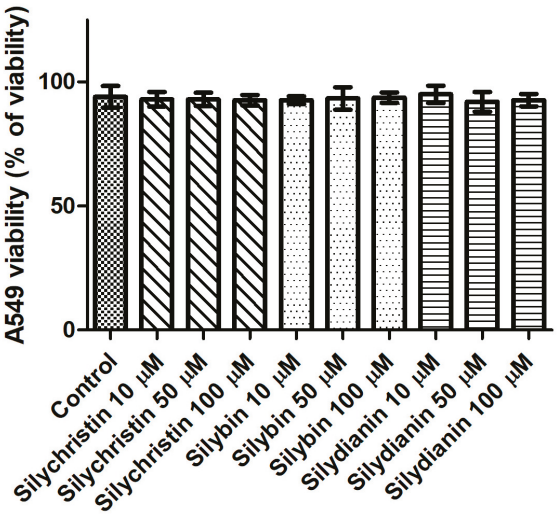

Figure 5. The effect of flavonolignans (silychristin, silybin and silydianin in concentrations of 10, 50 and 100 μM) on an A549 cell line, determined by trypan blue assay. The data represent the mean of 8 measurements.

3.5. Effect of Flavonolignans on mtDNA Copy Number in Human Lung Cancer Cell Line A549

Additionally, in this study we demonstrated that after 24 h of incubation with silydianin, the mtDNA copy number in the A549 cells is at the same level as in the untreated cells (Figure 6). After 24 h of treatment with silychristin and silybin in all tested concentrations, the cells had a higher ($p < 0.05$) mtDNA copy number.

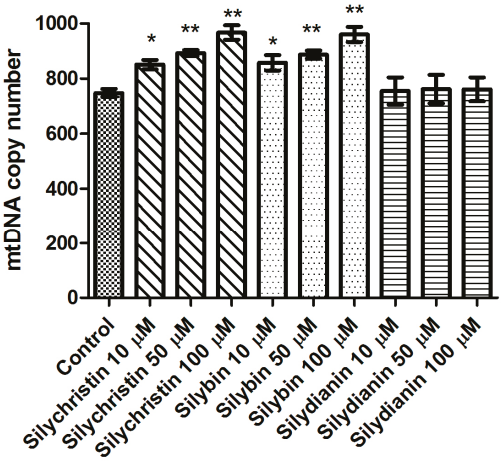

Figure 6. The effect of flavonolignans (silychristin, silybin and silydianin in concentrations of 10, 50 and 100 μM) on mitochondrial DNA copy number in an A549 cell line measured by real-time quantitative PCR. The data represent means of ± SD, $n = 8$. Statistical analysis was performed using the Kruskall-Wallis Test, * $p < 0.05$, ** $p < 0.001$.

3.6. Effect of Flavonolignans on mtDNA and nDNA Damages in Human Lung Cancer Cell Line A549

The next part of our study was to evaluate the genotoxicity of flavonolignans by determination of mtDNA and nuclear DNA damages. We examined the mtDNA damage by SLR-qRT-PCR amplification of DNA isolated from cells exposed to the flavonolignans: silychristin, silybin and silydianin in concentrations of 10, 50 and 100 μM for 24 h. In cell samples treated with silychristin and silybin in concentrations of 50 and 100 μM, we found a decreased level of mtDNA damage in both the *ND1* and *ND5* regions, while any differences were observed in the level of mtDNA damage between silydianin-treated samples and control samples (Figure 7). However, we did not find any differences in the level of nDNA damage in either the *HPRT1* or *TP53* genes between all samples treated with flavonolignans and the A549 control cells (Figure 8).

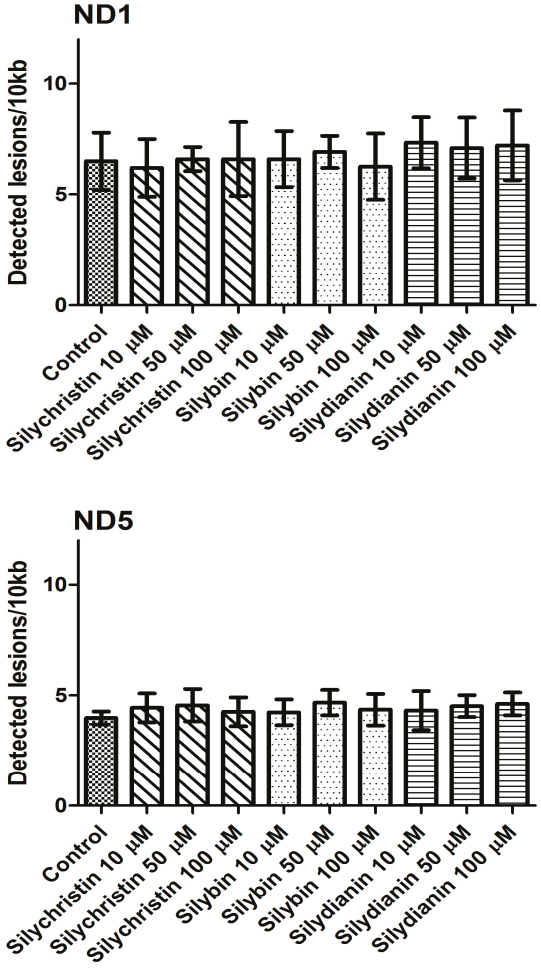

Figure 7. The effect of flavonolignans (silychristin, silybin and silydianin in concentrations of 10, 50 and 100 μM) on mitochondrial DNA (mtDNA) lesion frequency per 10 kb DNA in *ND1* and *ND5* genes, estimated by SLR-qRT-PCR amplification of total DNA from A549. The data represent means of ± SD, $n = 8$.

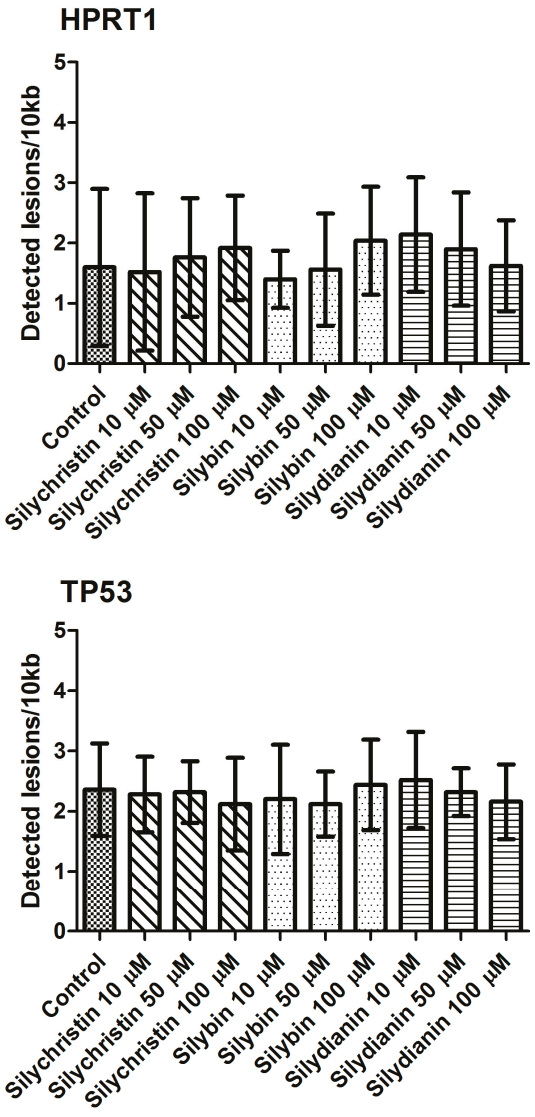

Figure 8. The effect of flavonolignans (silychristin, silybin and silydianin in concentrations of 10, 50 and 100 μM) on nuclear DNA (nDNA) lesion frequency per 10 kb DNA in *HPRT1* and *TP53* genes estimated by SLR-qRT-PCR amplification of total DNA from A549. The data represent means of ± SD, $n = 8$.

3.7. Effect of Flavonolignans on Apoptotic Genes Expression in Human Lung Cancer Cell Line A549

The results presented in Table 1 have shown that none of the tested flavonolignans have an influence on expression of the main apoptotic genes (*CASP3*, *CASP8*, *CASP9*, *BCL-2*, *BAX*, *APAF1*) under any of the tested concentrations.

Table 1. The effect of flavonolignans (silychristin, silybin and silydianin in concentrations of 10, 50 and 100 μM) on pro-apoptotic genes' expression in the A549 cell line. The data represent means of ± SD, $n = 8$.

Gene Expression ($2^{-\Delta Ct}$)	Control	Silychristin (μM)			Silybin (μM)			Silydianin(μM)		
		10	50	100	10	50	100	10	50	100
CASP3	$5.2 \times 10^{-6} \pm 6.5 \times 10^{-7}$	$5.3 \times 10^{-6} \pm 1.7 \times 10^{-7}$	$4.5 \times 10^{-6} \pm 8.5 \times 10^{-7}$	$6.1 \times 10^{-6} \pm 8.2 \times 10^{-7}$	$5.4 \times 10^{-6} \pm 8.9 \times 10^{-7}$	$5.5 \times 10^{-6} \pm 4.9 \times 10^{-7}$	$5.6 \times 10^{-6} \pm 5.7 \times 10^{-7}$	$6.2 \times 10^{-6} \pm 2.2 \times 10^{-6}$	$5.2 \times 10^{-6} \pm 4.5 \times 10^{-7}$	$5.7 \times 10^{-6} \pm 1.1 \times 10^{-7}$
CASP8	$3.8 \times 10^{-6} \pm 7.2 \times 10^{-7}$	$3.4 \times 10^{-6} \pm 6.3 \times 10^{-7}$	$3.3 \times 10^{-6} \pm 3.9 \times 10^{-7}$	$3.4 \times 10^{-6} \pm 4.2 \times 10^{-7}$	$3.2 \times 10^{-6} \pm 4.9 \times 10^{-7}$	$3.7 \times 10^{-6} \pm 8.1 \times 10^{-7}$	$3.5 \times 10^{-6} \pm 5.7 \times 10^{-7}$	$5.7 \times 10^{-6} \pm 1.2 \times 10^{-6}$	$4.6 \times 10^{-6} \pm 7.5 \times 10^{-7}$	$3.7 \times 10^{-6} \pm 2.9 \times 10^{-7}$
CASP9	$5.7 \times 10^{-6} \pm 6.9 \times 10^{-7}$	$5.5 \times 10^{-6} \pm 6.9 \times 10^{-7}$	$5.4 \times 10^{-6} \pm 1.4 \times 10^{-7}$	$5.9 \times 10^{-6} \pm 6.9 \times 10^{-7}$	$5.9 \times 10^{-6} \pm 9.9 \times 10^{-7}$	$5.7 \times 10^{-6} \pm 9.1 \times 10^{-7}$	$5.4 \times 10^{-6} \pm 9.7 \times 10^{-7}$	$5.9 \times 10^{-6} \pm 2.1 \times 10^{-7}$	$5.4 \times 10^{-6} \pm 6.4 \times 10^{-7}$	$5.3 \times 10^{-6} \pm 6.7 \times 10^{-7}$
BCL2	$5.8 \times 10^{-8} \pm 4.9 \times 10^{-9}$	$5.9 \times 10^{-8} \pm 8.4 \times 10^{-9}$	$5.3 \times 10^{-8} \pm 7.7 \times 10^{-9}$	$5.6 \times 10^{-8} \pm 5.9 \times 10^{-9}$	$4.9 \times 10^{-8} \pm 1.1 \times 10^{-8}$	$6.7 \times 10^{-8} \pm 1.0 \times 10^{-8}$	$5.8 \times 10^{-8} \pm 5.5 \times 10^{-9}$	$5.7 \times 10^{-8} \pm 8.9 \times 10^{-9}$	$6.3 \times 10^{-8} \pm 1.1 \times 10^{-8}$	$6.1 \times 10^{-8} \pm 5.7 \times 10^{-9}$
BAX	$5.6 \times 10^{-5} \pm 3.1 \times 10^{-6}$	$5.2 \times 10^{-5} \pm 4.3 \times 10^{-6}$	$5.5 \times 10^{-5} \pm 1.7 \times 10^{-6}$	$5.4 \times 10^{-5} \pm 3.4 \times 10^{-6}$	$5.4 \times 10^{-5} \pm 9.8 \times 10^{-6}$	$5.5 \times 10^{-5} \pm 1.1 \times 10^{-6}$	$5.5 \times 10^{-5} \pm 8.0 \times 10^{-6}$	$5.9 \times 10^{-5} \pm 9.1 \times 10^{-6}$	$5.6 \times 10^{-5} \pm 5.7 \times 10^{-6}$	$5.7 \times 10^{-5} \pm 3.3 \times 10^{-6}$
APAF	$3.5 \times 10^{-6} \pm 9.6 \times 10^{-7}$	$3.4 \times 10^{-6} \pm 2.3 \times 10^{-7}$	$3.8 \times 10^{-6} \pm 8.9 \times 10^{-7}$	$3.4 \times 10^{-6} \pm 2.3 \times 10^{-7}$	$3.9 \times 10^{-6} \pm 7.8 \times 10^{-7}$	$3.7 \times 10^{-6} \pm 5.9 \times 10^{-7}$	$3.6 \times 10^{-6} \pm 8.7 \times 10^{-7}$	$3.3 \times 10^{-6} \pm 4.5 \times 10^{-6}$	$3.9 \times 10^{-6} \pm 6.0 \times 10^{-7}$	$3.5 \times 10^{-6} \pm 5.7 \times 10^{-7}$

4. Discussion

Blood platelets are the smallest un-nucleated blood cells that play a significant role in maintaining hemostasis. The average life span of the blood platelets is from 7 to 10 days, after which they are removed from the bloodstream through the reticulo-endothelial system in the spleen, liver and bone marrow [42,43]. During platelet activation $O_2^{-}\bullet$ and other ROS are produced, and are involved in regulation of platelet activities [44]. The $O_2^{-}\bullet$ is central to ROS chemistry, because it can be converted into other physiologically relevant ROS by enzymatic or non-enzymatic reactions. ROS production in activated platelets is dependent on enzymatic cascade of arachidonic acid metabolized via cyclooxygenase (COX) or 12-lipoxygenase, the glutathione (GSH) cycle and metabolism of phosphoinositides [45,46]. ROS are generated in platelets mostly by activation of NAD(P)H oxidase [47] and xanthine oxidase [48].

Blood platelets are very sensitive cells that undergo spontaneous activation after collection from the blood flow. For this reason, these materials need to be very quickly analyzed. Blood platelets after collection without any agonist generate ROS and undergo apoptosis within a few hours. In our analysis, we demonstrated not only that the tested flavonolignans are not toxic to blood platelets, but may also protect the cells against apoptosis and ROS generation. We showed reduction of $O_2^{-}\bullet$ generation by platelets treated with all tested flavonolignans (Figure 3B). As part of our current research, we performed studies with DCFH-DA dye. The DCFH-DA enters through the cell membrane and is enzymatically hydrolyzed with intracellular esterases to non-fluorescent DCFH, which is then oxidized to highly fluorescent dichlorofluorescin (DCF) in the presence of intracellular ROS. That analysis also shows that tested compounds reduce the intracellular ROS level in blood platelets (Figure 3A).

The studies performed by our research team in recent years [26–28] have shown that flavonolignans, mainly silychristin and silybin, have strong anti-platelet properties, observable as a reduction of blood platelet activation to physiological agonists such as ADP and collagen, as well as a reduction of the arachidonic acid pathway. In the current study, we documented these effects as being unrelated to flavonolignan toxicity in platelets. Additionally, we demonstrated polarization of the mitochondrial membrane of silychristin- and silybin-enhanced platelets (Figure 1). The mitochondrial membrane potential ($\Delta\Psi m$) generated by proton pumps is an essential component in the process of energy storage during oxidative phosphorylation. The decrease of $\Delta\Psi m$ may be signal of loss of cell viability and be a cause of various pathologies [49]. Mitochondria are the primary cellular consumers of oxygen and contain numerous redox enzymes capable of transferring single electrons to oxygen, generating the $O_2^{-}\bullet$. Furthermore, mitochondrial insults, including oxidative damage itself, can cause an imbalance between ROS production and removal, resulting in net ROS production [50]. It has previously been proven that silymarin has a protective effect on mitochondrial structure and function. Rolo et al. [51] showed that silybin supplementation optimizes the mitochondria electron-transport chain, decreasing electron leakage and ROS formation and directly reducing the activities of ROS-producing enzymes. The results obtained in our current study also suggest a protective effect of silychristin and silybin on spontaneous platelet mitochondrial damage, and on stabilization of the mitochondrial membrane.

There are many studies presenting flavonolignans as having strong anti-inflammatory properties [52–54], resulting in significantly reduced production of pro-inflammatory cytokines. In our previous study, we also documented the anti-inflammatory properties of flavonolignans on IL-1β-induced human blood samples [29]. For this reason, we decided to verify the toxic effect of flavonolignans on blood platelets' peripheral blood mononuclear cell. The results obtained in this study confirm that the observed anti-inflammatory properties of flavonolignans are not associated with its cytotoxicity against white blood cells. We showed that these compounds have no influence on the viability of PMBCs (Figure 4).

There are also many studies that demonstrate that in selected cell lines, flavonolignans cause cell cycle arrest (G1 and G2-M arrest) and can induce apoptosis. These effects are exerted mainly through the inhibition of growth factor receptor-mediated mitogenic and cell survival signaling, particularly in

the activation of tyrosine kinases [24,55]. Li et al. [56] showed that silybin induces apoptotic cell death in human malignant melanoma A375-S2 cells by increasing the expression of Fas-associated proteins with a death domain (FADD)—a downstream molecule of the death receptor pathway followed by cleavage of procaspase-8 that then induces apoptosis. It has also been demonstrated that silymarin inhibits the growth and survival of human umbilical vein endothelial cells (HUVECs), by inhibiting capillary tube formation, and induces cell cycle arrest and apoptosis while reducing invasion and migration [55]. In all our studies, the aim of which was to demonstrate flavonolignans' health benefits, we used active concentrations (10–50–100 μM) of these compounds. They can be reached in plasma after oral administration of a novel form of Milk thistle extract, delivered in a phytosome-based, self-emulsifying and self-microemulsifying drug delivery system, as well as in the form of nano-emulsions [57–59]. As such, we investigated the cytotoxic and genotoxic effect of flavonolignans in 3 concentrations (10–50–100 μM) on the human lung cancer cell line A549 cell line according to standard protocol. At first, we checked the effect of flavonolignans on cell viability and morphology. We did not observe any changes between control cells and cells treated by flavonolignans. Similar results were observed for examination of nDNA damage by SLR-qRT-PCR amplification of DNA isolated from the A549 exposed to silychristin, silybin and silydianin.

Next, we investigated the effect of flavonolignans on mitochondrial DNA. First, we estimated the number of copies of mitochondrial DNA in the cells. The mtDNA copy number is a critical component of overall mitochondrial health. The mtDNA replication is carried out independently of the cell cycle by the nuclear DNA (nDNA) encoded polymerase γ, the only DNA polymerase found in the mitochondria [60]. Replication of the mtDNA is important for ensuring that cells have a sufficient number of mtDNA copies to meet their specific requirements for the generation of cellular energy through oxidative phosphorylation [61]. The results show that silybin and silychristin increased the number of mtDNA copies, while silydianin had no effect. The oxidative production of ATP required for cellular function also generates reactive oxygen species that damage mitochondrial DNA, and this ultimately results in mitochondrial dysfunction [62]. In the current study, we showed that silybin and silychristin not only have no genotoxic effect on mtDNA, but also decrease the number of mtDNA lesions.

The last parameter evaluated in this study was the expression of genes for proteins involved in apoptosis. Caspases have a proteolytic activity and are able to cleave proteins at aspartic acid residues, although different caspases have different specificities involving recognition of neighboring amino acids. Once caspases are initially activated, there seems to be an irreversible commitment towards cell death. For our analysis we selected two "initiators" (caspase 8 and 9), and one "executioner" (caspase 3). The BCL-2 proteins family is at the center of the apoptotic cascade, and determines cytochrome C release from the mitochondria, via alteration of mitochondrial membrane permeability. These proteins have special significance since they can determine whether the cell commits to apoptosis or aborts the process [63]. For our analysis, we selected one anti-apoptotic protein—Bcl-2, and one pro-apoptotic protein—Bax. Additionally, we chose to analyze APAF-1, which is an evolutionarily conserved component of the apoptosome responsible for activation of caspase 9 in the initiation of apoptosis [64]. However, we did not observe any changes in the tested genes' expression at the mRNA level, between all samples treated with flavonolignans and the control sample of A549 cells.

For the first time in any research, we have presented in our current study's findings that the three major flavonolignans, silybin, silychristin and silydianin, do not have any cytotoxicity and genotoxicity effects in concentrations of up to 100 μM in various cellular models. This proves that the antiplatelet and anti-inflammatory effect of these compounds is related to the molecular health mechanism. Additionally, we demonstrated that silybin and silychristin have very strong positive effects on cell mitochondria, reducing ROS formation as well as protecting them from spontaneous mtDNA damage.

After oral administration, flavonolignans undergoes extensive enterohepatic circulation which eliminated them from organism. About 80% of flavonolignans is excreted as glucuronide and sulfate

conjugates with bile while 8% is excreted in an unchanged form in the urine. However the elimination half-life of flavonolingnans (based on silybin) is approximately 6 h [65], which is enough to time to act as bioactive components. Additionally, oral supplementation of novel forms of silymarin increase bioavailability of flavonolignans to concentrations used in this study. In study performed by Hwang et al. [59], the oral supplementation (equivalent to 140 mg/kg dose of silymarin) of a novel form of silymarin—silymarin/PVP/Tween 80 at a weight ratio of 5/2.5/2.5 result the maximum plasma concentration of silybin at level 44.85 ± 11.42 μg/mL, which correspond to 100 μM (maximum concentration used in this study).

5. Conclusions

Results presented in this study clearly suggests that tested flavonolignans can be considered to be promising nutraceuticals, not only for liver treatment, but also in cardiovascular and inflammatory disorders. Additionally, these compounds have beneficial effects on cell mitochondria.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/9/12/1356/s1, Table S1: Description of semi-long run RT-PCR primers used for mitochondrial and nuclear DNA damage quantification. All primers were designed with the help of the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) and synthesized by Sigma-Aldrich (St. Louis, MO, USA). Complete nucleotide sequences for each gene were taken from the ENSEMBL database (https://ensembl.org/). Figure S1: The absence of morphological changes in the A549 cells cultures in the presence of flavonolignans ((A) silychristin, (B) silybin, (C) silydianin) in a concentration of 100 μM. The figure here presents samples of photos obtained during the viability analysis in the cell counter.

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Anti-Atherogenic Activity of Polyphenol-Rich Extract from Bee Pollen

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Abstract: The aim of this study was to determine the effect of polyphenol-rich ethanol extract of bee pollen (EEP) on atherosclerosis induced by a high-fat diet in ApoE-knockout mice. EEP was given with feed in two doses of 0.1 and 1 g/kg body mass (BM). The studies have been conducted in a period of 16 weeks. The following factors were estimated: total cholesterol (TC), oxidized low density lipoproteins (ox-LDL), asymmetric dimethylarginine (ADMA), angiotensin-converting enzyme (ACE) and angiotensin II (ANG II) in the 5th, 10th, 12th, 14th, and 16th week of the experiment. In the last, i.e., 16th week of the studies the development of coronary artery disease (CAD) was also estimated histopathologically. Supplementing diet with EEP resulted in decreasing TC level. EEP reduced oxidative stress by lowering the levels of ox-LDL, ADMA, ANG II and ACE. EEP protected coronary arteries by significantly limiting the development of atherosclerosis (the dose of 0.1 g/kg BM) or completely preventing its occurrence (the dose of 1 g/kg BM). The obtained results demonstrate that EEP may be useful as a potential anti-atherogenic agent.

Keywords: bee pollen; polyphenols; atherosclerosis; oxidised low density lipoproteins (ox-LDL); asymmetric dimethylarginine (ADMA); angiotensin-converting enzyme (ACE); angiotensin II (ANG II)

1. Introduction

Recently, a lot of studies have focused on pro-health and curative properties of natural products, including bee products, and bee pollen is among these valuable apitherapeutics. It is a product of plant origin, collected and partly processed by bees. It is produced from pollen grains enriched with nectar, honey and honeybee salivary glands secretion. Bee pollen is composed of more than 250 various substances. The type and role of separate compounds in the total mass is diverse, and depend on plant species, the climate zone and season when it was collected [1–3].

Bee pollen is characterized by high nutritious value and different biological activities. Nutritious properties of bee pollen result from the presence of such substances as proteins, amino acids, carbohydrates, lipids (including ω-3 and ω-6 acids), vitamins and bio-elements. Therapeutic and protective effects are related to the content of functional compounds such as polyphenols [4–7].

Polyphenols are the main ingredients that determine antioxidant activity of bee pollen [8,9]. Their content may vary significantly depending on the origin of the material [10–12]. The profile of polyphenolic compounds in bee pollen may serve as an important indicator of the product quality

due to its specificity as well as qualitative and quantitative stability [13,14]. Polyphenols in bee pollen are mainly phenolic acids and flavonoids, and their biological activity is conditioned by their specific chemical structure. The presence of a benzene ring in the particle structure is a common characteristic of the compounds. High antioxidant capacity of polyphenols is closely related to the presence of conjugated double bonds and the number and location of hydroxyl groups in an aromatic ring [15,16]. Since polyphenols can neutralize free radicals, they have the following activities: anti-inflammatory, antiallergenic, antiviral, anti-clotting, anticancer, immunostimulating, hepatoprotective, and they also inhibit specific enzymes. Furthermore, polyphenols have a protective function in cardio-vascular diseases caused by oxidative stress [17–19].

Cardio-vascular diseases, including cardiac artery disease, are a frequent cause of death in the majority of highly developed countries. Atherosclerosis is a specific form of a chronic inflammatory process in the aorta and medium-sized arteries. Pathogenesis of atherosclerosis is caused by various factors. The development of atherosclerosis is modulated by disturbances of lipid metabolism, oxidative stress, fibrinolysis and coagulation in the vascular wall as well as disturbed homeostasis of the renin-angiotensin-aldosterone system, which impairs the function of endothelial cells [20,21].

Taking into consideration various biological activities of bee pollen ingredients, the aim of the current study was to determine the effect of polyphenol-rich EEP on atherosclerosis induced by a high-fat diet in ApoE-knockout mice. The latter constitute an internationally recognized animal model for studying atherosclerosis and hypercholesterolemia.

2. Materials and Methods

2.1. Reagents and Drugs

Xylene, hematoxylin, eosin aqueous solution (1%), anhydrous ethyl alcohol (99.8%), acetone, phosphate buffered saline (PBS), formalin were purchased from POCh (Gliwice, Poland). DPX mounting medium for histology (mixture of Distyrene, a Plasticizer, and Xylene) was purchased from Fluka (Dresden, Germany). Tiopental was purchased from Sandoz (Warsaw, Poland), total cholesterol Test (Cat. No. OSR 6116) was purchased from Beckman Coulter (Praha, Czech Republic), ELISA Kit for mouse oxidized low density lipoprotein (Cat. No. E90527Mu) was purchased from Uscn Life Science Inc. (Wuhan, China), ADMA ELISA Kit for the determination of ADMA (Cat. No. K 3001) was purchased from Immundiagnostik AG (Bensheim, Germany), ELISA Kit for mouse angiotensin I converting enzyme (ACE) (Cat. No. E90004Mu) was purchased from Uscn Life Science Inc. (Wuhan, China), Angiotension II (Human, Rat, Mouse, Porcine, Caniane) EIA Kit (Cat. No. EK-002-12) was purchased from Phoenix Pharmaceutical Inc. (Karlsruhe, Germany).

2.2. Preparation of Ethanol Extract of Bee Pollen (EEP)

The material for the tests were ground pollen loads obtained from ecologically clean harvest areas in the south of Poland. Samples of polyfloral bee pollen were collected from an apiary at Kamianna (GPS N 49°31′527, E 20°56′116), Poland, in the Beskidy Mountains. From May to July, bee pollen samples were collected by beekeepers with the use of pollen traps mounted on selected beehives. Mainly, they were pollens of common dandelion *Taraxacum officinale*, rapeseed *Brassica napus*, European raspberry *Rubus ideaeus*, acacia *Robinia pseudoacacia*, buckwheat *Fagopyrum esculentum*, linden *Tilia mordata*, clover *Trifolium repens*. Ethanol extract was prepared according to a slightly modified method of Almaraz-Abarca et al. [8]. The ethanol extract of bee pollen was prepared by weighing 20 g of ground bee pollen, with accuracy of 0.01 g. Then, the bee pollen sample was extracted 5 times with 50% (*v*/*v*) ethanol aqueous solution, in 200 mL portions, and shaken each time for 60 min at room temperature, in order to macerate the sample. After each extraction, the sample was filtered under reduced pressure with the use of a water pump. The filtrate was collected, and substrate was extracted again with another portion of ethanol aqueous solution. The obtained filtrate was centrifuged at 10,000 rpm for 10 min, and then it was evaporated under reduced pressure in a rotary vacuum

evaporator (UNIPAN-PRO 350P). The evaporated extract was dried in a laboratory incubator at 38 °C to obtain solid mass. Studies of EEP focused on the content of polyphenols and flavonoids, and their antioxidant effect [22].

2.3. Animals and Treatments

The study was conducted on 56 females of $C_{57}BL_6$ ApoE-knockout mice, aged 4 weeks, 25 ± 5 g of body mass. During the experiment, the animals were kept in standard breeding conditions, i.e., in groups of 10 animals in polypropylene cages, in rooms of constant temperature (23 ± 2 °C), and constant air humidity (50–70%), keeping the daytime rhythm in the inflow of light (a 12-h cycle). The high-fat diet (HFD) comprised High Fat Rodent Diet supplemented with 21% lard and 0.15% cholesterol (Special Diets Services, Witham, Essex, UK). The standard diet (SD) was standard feed (Labofeed B) containing 17% protein, 3.5% fat and 38% carbohydrates (Animal Feed Manufacturer "Morawski" in Kcynia). EEP was added to the feed in a dose of 0.1 and 1 g/kg BM, respectively. Two dosing levels of EEP used in the research were calculated based on the daily ingestion of polyphenols in the diet of the inhabitants of Central Europe [23].

Experimental animals were divided into 6 groups according to the following scheme: 5 study groups: HFD, HFD-0.1, HFD-1, SD-0.1, SD-1 (10 animals in each group), and a control group SD (6 animals). Characteristics of the groups: HFD: high-fat diet, HFD-0.1: high-fat diet supplemented with EEP (0.1 g/kg BM), HFD-1: high-fat diet supplemented with EEP (1 g/kg BM), SD-0.1: standard diet supplemented with EEP (0.1 g/kg BM), SD-1: standard diet supplemented with EEP (1 g/kg BM), and SD: standard diet.

The project with the use of animal models was approved by the Local Ethics Committee on Animal Experimentation of the Medical University of Silesia in Katowice.

2.4. Collecting Biological Material for Tests

The material for tests was collected in the fasted state, in the 5th, 10th, 12th, 14th, and 16th week of the experiment, after general anesthesia with a drug called Tiopental in a dose of 20 mg/kg BM, administered by injection.

Blood for analyses was collected by puncturing the tip of the heart with a cannula. Blood was collected to chemically pure test tubes in order to obtain serum. Blood after clotting and clot retraction was centrifuged, and serum was frozen at −80 °C, and stored for further tests.

The collection of material for histopathological tests was as follows: before the material was collected, perfusion was performed by decompression surgery in the 1/3 of descending abdominal aorta. Perfusion was performed with phosphate buffered saline (PBS) until the light pink translucent liquid flew out in the decompressing orifice. Then, perfusion with 10% PBS-buffered formaldehyde was performed for 2 min to obtain preliminary fixing of the heart and trunks of major blood vessels. Next, both, the heart and the brachiocephalic trunk were fixed in 10% PBS-buffered formaldehyde, and kept for further tests.

2.5. Histopathological Tests

Histopathological evaluation of the heart and the brachiocephalic trunk was conducted in the 16th week of the study. The collected material was fixed in a 10% formalin solution for minimum 24 h. After fixing, the tissues were treated with the aqueous solutions of ethyl alcohol. The material was subsequently rinsed with acetone and xylene, respectively. Then, tissues were placed in a xylene and paraffin mixture at a 1:1 ratio and then in liquid paraffin. After solidification, the paraffin was cut into 3–5 micron thick bands. Paraffin bands were treated with xylene, an ethyl alcohol/xylene mixture at a 1:1 ratio and aqueous solutions of ethyl alcohol, and then rinsed in distilled water. The material prepared in this way was stained with the standard hematoxylin-eosin (HE) method, which included staining with alkaline solution of hematoxylin, rinsing in distilled water, staining with acid solution of eosin, and rinsing in distilled water. Later, preparations were treated with aqueous solutions of ethyl

alcohol, ethanol/xylene mixture at 1:1 ratio and rinsed in xylene in order to finally dehydrate and radiate the tissue. Slides were covered with a cover glass with DPX-medium.

Histopathological preparations were evaluated in 40×, 100×, and 200× microscopy objectives magnification using Olympus BX60 microscope equipped with XC50 digital camera and Olympus cellSens Standard software (Olympus Corp., Tokyo, Japan).

2.6. Biochemical Tests

We tested serum obtained in the scheduled study periods, i.e., in the 5th, 10th, 12th, 14th, and 16th week of the experiment for the study groups HFD, HFD-0.1, HFD-1, SD-0.1, SD-1, and in the 1st, 10th, and 16th week for the control group (SD). The levels of ox-LDL, ADMA, ACE, ANG II were determined with Asys HiTech UVM 340 microplate reader and Micro Win 4.35.

2.6.1. Determination of Total Cholesterol (TC)

Total cholesterol serum level was determined with an enzymatic method. A reagent set from Beckman Coulter (Cat. No. OSR 6116) was used for the determination, which was conducted according to the manufacturer's instruction for Beckman Coulter AU 680 analyzer. Total cholesterol level was given in mg/dL.

2.6.2. Determination of Oxidized Low Density Lipoprotein (Ox-LDL) Concentration

The concentration of ox-LDL was determined with a sandwich ELISA using Mouse Oxidized Low Density Lipoprotein ELISA Kit. The analysis was conducted according to the manufacturer's instruction.

The microtiter plate was pre-coated with an antibody specific to ox-LDL. Samples diluted 10 times or standards were then added to the appropriate microtiter plate wells, and incubated for 2 h (37 °C). Then, the liquid was removed and a biotin-conjugated polyclonal antibody specific for ox-LDL was added. The plate was incubated for 1 h at 37 °C. Then the plate was washed, and avidin conjugated to horseradish peroxidase (HRP) was added to each microplate well, and the plate was incubated for 1 h (37 °C). Next, a 3,3′,5,5′-tetramethyl-benzidine (TMB) substrate solution was added to each well and incubated for 30 min (37 °C). Only those wells that contained ox-LDL, biotin-conjugated antibody and enzyme-conjugated avidin exhibited a change in color. The enzyme-substrate reaction was terminated by the addition of a sulfuric acid solution, and the color change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of ox-LDL (ng/mL) in the samples was then determined by comparing the optical density (O.D.) of the samples to the standard curve.

2.6.3. Determination of Asymmetric Dimethylarginine (ADMA) Concentration

The concentration of ADMA was determined with ADMA ELISA Kit for the determination of ADMA. The assay was conducted according to the manufacture's instruction.

This assay is based on the method of competitive enzyme-linked immunoassays. The sample preparation includes the addition of a derivatization-reagent for ADMA coupling. Afterwards, the treated samples and the polyclonal ADMA-antiserum are incubated in wells of microplate coated with ADMA-derivative (tracer) for 18 h at 4–8 °C. During the incubation period, the target ADMA in the sample competes with the tracer immobilized on the wall of the microtiter wells for the binding of the polyclonal antibodies. The ADMA in the sample displaces the antibodies out of the binding to the tracer. Therefore the concentration of the tracer-bound antibody is inversely proportional to the ADMA concentration in the sample. During the second incubation step (for 1 h at 18–26 °C), a peroxidase-conjugated antibody is added to each microtiter well to detect the anti-ADMA antibodies. After washing away the unbound components, 3,3′,5,5′-tetramethyl-benzidine (TMB) is added as a substrate for peroxidase and then incubated for 10 min at 18–26 °C. Finally, the enzymatic reaction is terminated by acidic stop solution. The color changes from blue to yellow and the absorbance is measured in the photometer at 450 nm. The intensity of the yellow color is inversely proportional to the ADMA concentration in the sample; this means high ADMA concentration in the sample reduces

the concentration of tracer-bound antibodies and lowers the photometric signal. The concentration of ADMA (μmol/L) in the samples is then determined by comparing the O.D. of the samples to the standard curve.

2.6.4. Determination of Angiotensin-Converting Enzyme (ACE) Concentration

The concentration of ACE was determined with a sandwich ELISA using mouse angiotensin I converting enzyme (ACE) ELISA Kit. The assay was conducted according to the manufacturer's instruction.

The microtiter plate in this kit was pre-coated with an antibody specific to ACE. Samples diluted 10 times or standards were added to the appropriate microtiter plate wells and incubated for 2 h (37 °C). Then the liquid was removed and a biotin-conjugated polyclonal antibody specific for ACE was added. The plate was incubated for 1 h at 37 °C. Then the plate was washed, and avidin conjugated to horseradish peroxidase (HRP) was added to each microplate well, and incubated for 1 h (37 °C). Then a 3,3′,5,5′-tetramethyl-benzidine (TMB) substrate solution was added to each well and incubated for 15 min (37 °C). Only those wells that contained ACE, biotin-conjugated antibody and enzyme-conjugated avidin would exhibit a change in color. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of ACE (ng/mL) in the samples was then determined by comparing the O.D. of the samples to the standard curve.

2.6.5. Determination of Angiotensin II (ANG II) Concentration

The concentration of ANG II was determined with Angiotension II (Human, Rat, Mouse, Porcine, Caniane) EIA Kit. The assay was carried out according to the manufacturer's instruction.

The microtiter plate in this kit was pre-coated with an antibody specific to ANG II. Samples diluted 10 times or standards were then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for ANG II and incubated for 2 h (20–23 °C). Then streptavidin conjugated to Horseradish Peroxidase (SA-HRP) was added to each microplate well and incubated 1 for hour (20–23 °C). Next a 3,3′,5,5′-tetramethyl-benzidine (TMB) substrate solution was added to each well and incubated for 1 h (20–23 °C). Only those wells that contained ANG II, biotin-conjugated antibody and enzyme-conjugated streptavidin would exhibit a colour change. The enzyme-substrate reaction was terminated by the addition of a hydrochloric acid solution, and the colour change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of ANG II (ng/mL) in the samples was then determined by comparing the O.D. of the samples to the standard curve.

2.7. Statistical Analysis

The obtained results in particular groups were given as the mean ± standard deviation (±SD), and checked for normal distribution and homogeneity of these groups. The Shapiro-Wilk test was performed for normality of the obtained results. Homogeneity of variance was evaluated with the Levene's test with two variables. The comparison of homogenous groups and the effect of parameters (mouse model, diet type, supplement dose) on group differentiation were analyzed with ANOVA and the Least Significant Differences (LSD) test.

The differences were considered to be statistically significant when a significance level was less than 0.05 ($p \leq 0.05$). The calculations were performed with Statistica 10.0 (Polish version), and Microsoft Excel.

3. Results and Discussion

3.1. Histopathological Tests of Arteries

In our study, we evaluated the anti-atherogenic effect of polyphenol-rich extract of bee pollen on the development of atherosclerosis induced by a high-fat diet in ApoE-knockout mice. We showed that supplementation of a high-fat diet with EEP in a dose of 1 g/kg BM protected heart arteries from

development of atherosclerotic plaque. Histopathological presentation did not reveal any proliferative changes which would be evidence of atherosclerosis development (Figure 1C). Supplementing a high-fat diet with EEP in a dose of 0.1 g/kg BM significantly limited the growth of atherosclerotic plaque (Figure 1B), while severe atherosclerotic changes occurred in mice on a high-fat diet without supplementation. Near the aortic arch, atherosclerotic plaque almost completely filled the vascular lumen (Figure 1A). In mice on a standard diet, the control group SD (Figure 1F), and in the mice on a standard diet supplemented with EEP in a dose of 0.1 g/kg BM (Figure 1D), and 1 g/kg BM (Figure 1E) atherosclerotic characteristics were not observed.

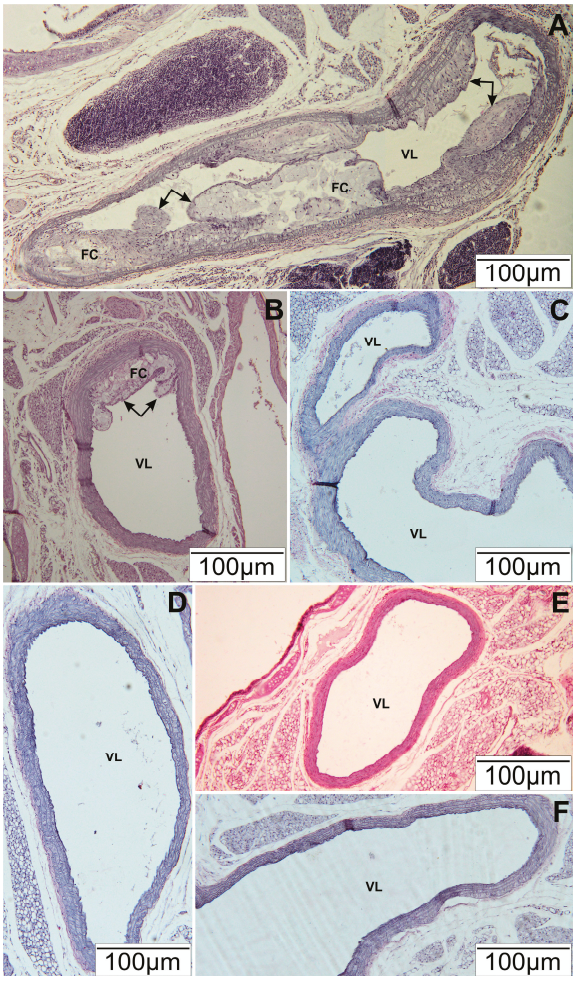

Figure 1. Representative section of the aortic arch of ApoE-knockout mice. Slides stained Hematoxylin and Eosin. (**A**) HFD—high-fat diet. Arrows—atherosclerotic plaque, FC—foam cells, VL—vascular lumen; (**B**) HFD-0.1—high-fat diet supplemented with EEP 0.1 g/kg BM. Arrows—atherosclerotic plaque, FC—foam cells, VL—vascular lumen; (**C**) HFD-1—high-fat diet supplemented with EEP 1 g/kg BM. VL—vascular lumen; (**D**) SD-0.1—standard diet supplemented with EEP 0.1 g/kg BM. VL—vascular lumen; (**E**) SD-1—standard diet supplemented with EEP 1 g/kg BM; (**F**) SD—standard diet. VL—vascular lumen. Scale Bar: 100 μm.

The mechanism of inhibitory capacity of EEP, as far as atherosclerosis is concerned, is difficult to explain. Our study has led to a conclusion that the mechanism is related to a significant decrease in total cholesterol, oxidatively modified pro-atherogenic ox-LDL molecules, and the decrease of ADMA and ANG II level. To the best of our knowledge, the current study is the first report on the subject.

EEP used in our study was characterized by a high content of polyphenols (27 mg GAE/g) and flavonoids (20 mg QE/g), which corresponded to a strong antioxidant effect resulting from reduction of DPPH (EC_{50} = 57.5 µmol/g), free radicals, and $ABTS^{\bullet+}$ (TEAC = 0.692 mmol/g). Rutin was a dominant flavonoid, which is a glycoside of quercetin. Other flavonoids present in the extract are: mireycetin > quercetin > isorhamnetin > kaempferol. The main phenolic acids which are present in EEP are: gallic acid > trans-cinnamic acid > 4-hydroxycinnaminic acid > felluric acid > 4-trans-p-coumaric acid > caffeic acid. Detailed characteristics of particular phenolic acid and flavonoid content in EEP used in research were presented in a previously published paper [22]. These compounds are characterized by a strong antioxidant effect reducing oxidative stress, and strongly inhibiting lipid peroxidation, which probably was crucial for preventing the formation of atherosclerotic plaque in ApoE-knockout mice.

In vitro studies showed that quercetin and catechin inhibit platelet aggregation, limit pro-clotting activity and block phosphoinositide cascade [24]. Flavonoids interact with platelet receptors. Quercetin decreases reactivity of platelets by blocking activation that depends on GPVI receptors [25]. Quercetin and catechin inhibit oxidative stress in platelets, and simultaneously limit activation of a fibrogen receptor, and increase NO synthesis [26]. In studies conducted on mice with apolipoprotein E deficiency, it was shown that polyphenol mixture, e.g., catechin, caffeic acid and resveratrol, decreases progression of atherosclerotic changes in the aortic arch. According to authors, the inhibition of endothelin 1 synthesis is one of the mechanisms in which polyphenols affect the balance between vasoconstrictive and vasodilating factors [27]. Hayek et al. [28] obtained significant reduction of atherosclerotic plaque on the surface of the aortic intima-media in ApoE-knockout mice after administration of quercetin.

The anti-atherosclerotic effect of propolis, an important apitherapeutic, was described in the literature. The reduction of early and advanced atherosclerotic changes due to a polyphenol fraction from green, brown, and red propolis was confirmed in in vitro studies conducted on genetically modified mice without LDL receptor (LDLr-/- mice). Polyphenols from red propolis had the strongest effect, and they reduced atherosclerotic changes in the aortic sinus. This was related to improving the lipid profile, reducing the level of pro-inflammatory cytokines, monocyte chemotactic protein-1 (MCP1) and interleukin 6 (IL6), chemokine, and angiogenic factors [29]. Limitation of atherosclerotic progression in the aortic sinus was observed in the case of a polyphenol fraction from Chilean propolis. Authors claim that this can result from the synergic effect of a polyphenol complex present in propolis that involves a significant reduction of the expression of a pro-angiogenic vascular endothelial growth factor A (VEGF-A) [30].

3.2. Effect of EEP on Total Cholesterol (TC)

Improper diet can impair lipid management and contribute to the development of cardio-vascular diseases. Disorders of lipid metabolism, manifested by hypercholesterolemia, are recognized and important factors which increase the risk of atherosclerosis [31].

In the current study, we have evaluated the effect of polyphenol-rich extract of bee pollen (EEP) on total cholesterol level in ApoE-knockout mice on high-fat and standard diets. Total cholesterol level in ApoE-knockout mice is presented in Figure 2.

After five weeks, a high-fat diet in genetically modified animals resulted in an increase of TC to 779 mg/dL (Figure 2A), i.e., by 101% compared with the average level (388 mg/dL, Figure 2B) in the controls. The use of a high-fat diet for 16 weeks further increased the level of TC to 1303 mg/dL (Figure 2A), i.e., by 67% compared with values recorded after five weeks. In this group, the average TC level was the highest and the difference in comparison with all remaining experimental groups was statistically significant ($p < 0.05$, Figure 2B).

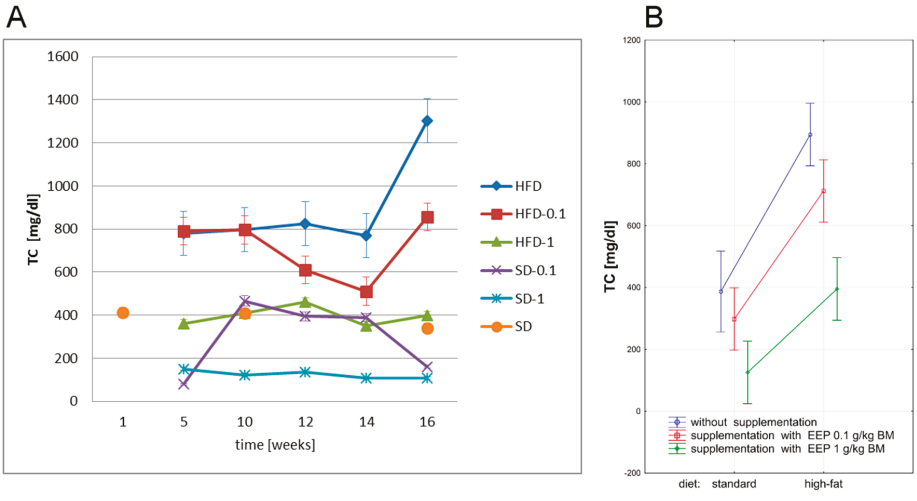

Figure 2. Total cholesterol (TC) level in ApoE-knockout mice. (**A**) TC level in particular weeks of the study; (**B**) Average TC level (n = 10). HFD—high-fat diet; HFD-0.1—high-fat diet supplemented with EEP 0.1 g/kg BM; HFD-1—high-fat diet supplemented with EEP 1 g/kg BM; SD-0.1—standard diet supplemented with EEP 0.1 g/kg BM; SD-1—standard diet supplemented with EEP 1 g/kg BM; SD—standard diet. In HFD-1 group, TC concentration during the total research period corresponded to TC level determined in SD. The lowest TC values were recorded for SD-1.

Supplementing a high-fat diet with EEP in a dose of 0.1 g/kg BM decreased TC level, which reached the value of 856 mg/dL (Figure 2A) in the 16th week, and was 34% lower when compared with an unsupplemented high-fat diet. Supplementation of a high-fat diet with EEP in a dose of 1 g/kg BM stabilized TC level as early as in the 5th week. This parameter level in all experimental periods was similar to the control group level, and the difference was not statistically significant (Figure 2B). In the 16th week, TC level was 398 md/dL (Figure 2A), i.e., 69% lower than in an unsupplemented high-fat diet.

Supplementation of a standard diet with EEP in a dose of 0.1 g/kg did not significantly change TC level ($p > 0.05$), but a dose of 1 g/kg MB resulted in a high decrease in TC level ($p < 0.05$) when compared with the control group (Figure 2A,B).

A significant decrease of TC level due to polyphenol fraction in bee pollen extract (EEP), which was recorded in our study, can be an effect of various mechanisms. It can be a sign of decreased biosynthesis of cholesterol in the liver. It can be a result of increased excretion of cholesterol with bile, or a result of the activation of peroxisome proliferator-activated receptors (PPAR), which regulate adipocyte maturation and lipid storage; therefore, they are regulators of hepatic metabolism of lipids.

It can be concluded from published data that polyphenols from natural products affect lipid metabolism as well as having cardio- and angio-protective effect. During in vitro studies, quercetin activates PPAR-γ receptors by increasing their expression. It also increases the expression of ATP-binding cassette transporter (ABCA1), which plays a key role in reverse cholesterol transport. Quercetin-induced modulation of ABCA1 expression reduces cholesterol build-up in macrophages, and increases cholesterol outflow from macrophages, and therefore reduces formation of foam cells, lowering the risk of atherosclerosis [32]. The lipid level in LDLr-knockout mice is normalized by a polyphenol fraction from different types of propolis. The polyphenol fraction from red propolis had the strongest protective effect. This fraction caused the most significant reduction of triglyceride level, and TC level as well as an increase in HDL. The anti-atherogenic effect was weaker in the case of green and brown propolis. This may be related to different content of main polyphenol

components, in particular, types of propolis. The following were mostly present in red propolis: 3-hydroxy-8,9-dimethoxypterocarpan, medicarpin, daidzein, whereas artepellin C, pinocembrin, kampferol in green propolis, and pinocembrin, caffeic acid phenyl ester, quercetin, galangin in brown propolis [29].

The current study shows that EEP in a dose of 1 g/kg BM significantly decreases TC levels in ApoE-knockout mice on both, high-fat and standard diets. Consequently, it reduces hypercholesterolemia, which is a risk factor for atherosclerosis.

3.3. Effect of EEP on Oxidized Low Density Lipoprotein (Ox-LDL)

Oxidized LDL molecules are one of the main factors responsible for atherosclerosis development. They are formed due to free radical activity during the process of chemical modification, namely, oxidation of low density lipoproteins (LDL). Ox-LDL are highly atherogenic molecules, since they have pro-inflammatory activity and they cause focal inflammation of arterial vascular endothelium [33].

In our study, we have investigated the effect of polyphenol-rich EEP on ox-LDL levels in ApoE-knockout mice on high-fat and standard diets. Ox-LDL level in ApoE-knockout mice is presented in Figure 3.

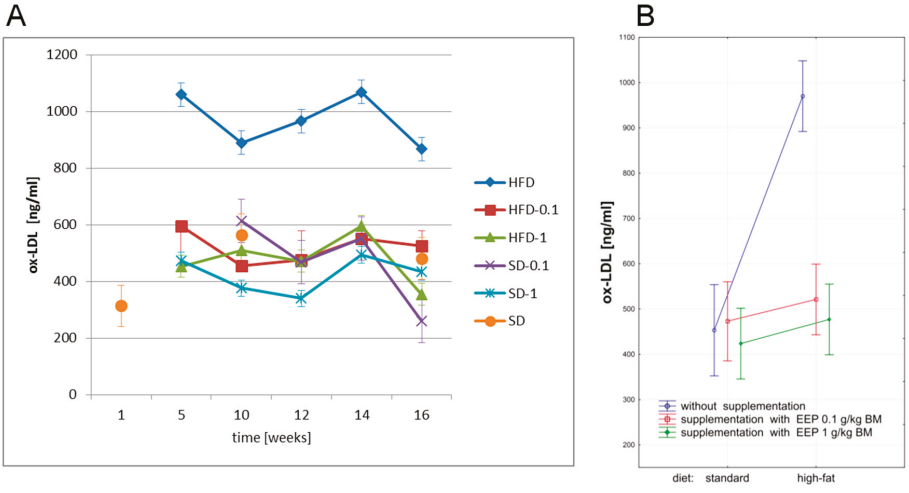

Figure 3. Oxidized low density lipoproteins (ox-LDL) level in ApoE-knockout mice. (**A**) Ox-LDL level in particular weeks of the study; (**B**) Average ox-LDL level (n = 10). HFD—high-fat diet; HFD-0.1—high-fat diet supplemented with EEP 0.1 g/kg BM; HFD-1—high-fat diet supplemented with EEP 1 g/kg BM; SD-0.1—standard diet supplemented with EEP 0.1 g/kg BM; SD-1—standard diet supplemented with EEP 1 g/kg BM; SD—standard diet. The highest values of ox-LDL were recorded in HFD group. In groups HFD-0.1 and HFD-1, the concentration of ox-LDL was much lower than in HFD.

The highest levels of ox-LDL in all experimental periods were recorded in mice on a high-fat diet, whereas the lowest ones were noted when a standard diet was supplemented with EEP at a dose of 1 g/kg BM (Figure 3A,B). Since the fifth week, a high-fat diet resulted in a significant increase of ox-LDL level, which was 867 ng/mL in the 16th week (Figure 3A) and was 91% higher than the average level (453 ng/mL, Figure 3B) in the controls. Supplementing a high-fat diet with EEP as early as in the fifth week resulted in lowering ox-LDL levels, which diminished to 526 ng/mL (Figure 3A), i.e., by 40% in the case of a dose of 0.1 g/kg BM, and to 355 ng/mL (Figure 3A), i.e., by 59% when the dose was 1 g/kg BM compared with an unsupplemented high-fat diet. In Apo-E-knockout mice,

supplementation of a high-fat diet with EEP regardless of the dose, causes statistically significant reduction of average ox-LDL level to a level similar to the one of the controls (Figure 3B).

The literature indicates that polyphenols are inhibitors of oxidative modification of LDL. They play an important role in prevention of many degenerative diseases, including cardio-vascular diseases. Polyphenols inhibit progression of atherosclerosis by reducing oxidative stress, and inflammatory biomarkers of atherosclerosis, as well as by improving lipid profile and insulin sensitivity, and by making endothelial function more efficient [17,34–36].

Loke et al. [37], in their studies on ApoE-knockout mice showed that quercetin reduced hydrogen peroxide and leukotrien B4 in vessels, and P-selectin serum level as well as increased the activity of endothelial nitric oxide synthase (eNOS). Theaflavin (catechin dimer) and epicatechin have a similar but weaker effect. In vitro studies of olive oil polyphenols have shown that they reduce intracellular levels of reactive oxygen species (ROS) and the expression of nuclear factor-κB (NFκB) transcription. Reduction of NFκB expression has been related to lowering metalloproteinase 9 level [36]. Oxidative stress in ApoE-knockout mice is also reduced by cocoa polyphenols. This effect has resulted from reducing expression of vascular cell adhesion molecule-1 (VCAM1) and intercellular adhesion molecule-1 (ICAM1) [38]. Olive oil supplemented with epigallocatechin gallate (EGCG) significantly improves vascular endothelium function in patients with early atherosclerotic dysfunction of endothelium due to the levels of ICAM, monocytes and lymphocytes [39]. Polyphenols inhibit LDL oxidation, reduce thrombocyte aggregation, inhibit the activity of enzymes that mediate the immune cells response to LDL, and reduce TC levels [40].

The presented effect of the polyphenol fraction of bee pollen extract on ox-LDL level in ApoE-knockout mice has been reported for the first time. Any information on the effect of polyphenols from bee pollen on ox-LDL level has not been found in the literature. Our study can lead to the conclusion that a polyphenol fraction from bee pollen significantly reduces ox-LDL level, and it results, most probably, from high antioxidant capacity of EEP. Reduction of proatherogenic ox-LDL level and consequently, limitation of the development of atherosclerotic changes are the signs that ethanol extract of bee pollen is highly efficient at reducing oxidative stress. Therefore, it can be useful as a potential anti-atherogenic substance.

3.4. Effect of EEP on Asymmetric Dimethylarginine (ADMA)

ADMA is an endogenous inhibitor of endothelial nitric oxide synthase (eNOS). ADMA participates in one of the mechanisms limiting bioavailability of nitric oxide (NO), which is an endogenous strongly anti-atherogenic substance [41,42]. Impairment of endothelial functions, which is observed in various disorders, results from high levels of ADMA. This allows us to single out ADMA as an early biochemical marker of endothelial dysfunction in the prophylaxis of cardio-vascular diseases and insulin resistance [42–44].

We have studied the effect of bee pollen extract on ADMA level in ApoE-knockout mice on both, high-fat and standard diets. ADMA level is presented in Figure 4.

After five weeks, a standard diet led to an increases in ADMA level to 0.653 μmol/L (Figure 4A), i.e., by 26%, and up to 0.704 μmol/L (Figure 4A), i.e., by 36% after 16 weeks when compared with an average level (0.519 μmol/L, Figure 4B) in the controls.

Sixteen-week supplementation of a high-fat diet with EEP in a dose of 0.1 g/kg BM did not significantly change this parameter level. When a high-fat diet was supplemented with EEP in a dose of 1 g/kg BM, the lowest ADMA level was recorded in the 14th week (0.431 μmol/L, Figure 4A), while in the 16th week ADMA level was 0.648 μmol/L (Figure 4A), 8% lower than in the unsupplemented group. The lowest levels of this parameter were obtained for a standard diet supplemented with bee pollen extract in a dose of 1 g/kg BM (Figure 4A,B). A high-fat diet in ApoE-knockout mice caused a statistically significant increase of ADMA level. Supplementing a high-fat diet with EEP in a dose of 1 g/kg BM in ApoE-knockout mice resulted in a statistically significant decrease of average ADMA levels (Figure 4B).

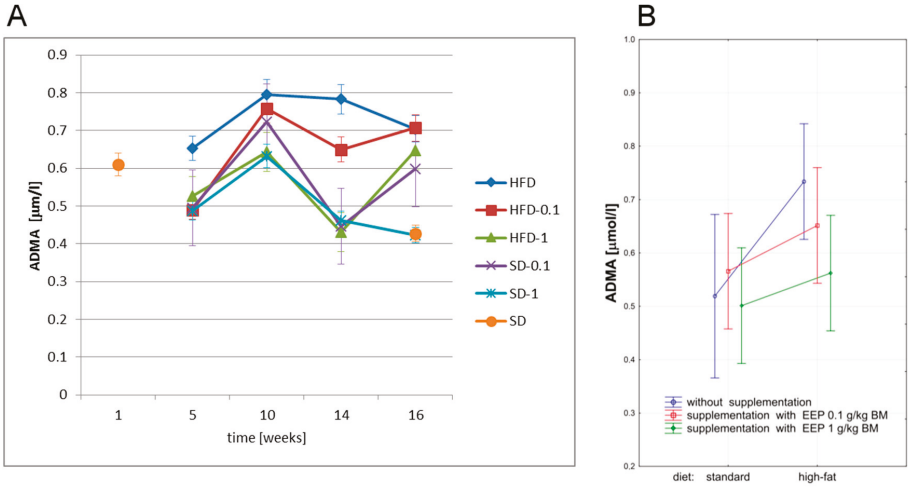

Figure 4. Asymmetric dimethylarginine (ADMA) level in ApoE-knockout mice. (**A**) ADMA level in particular weeks of the study; (**B**) Average ADMA level (n = 8). HFD—high-fat diet; HFD-0.1—high-fat diet supplemented with EEP 0.1 g/kg BM; HFD-1—high-fat diet supplemented with EEP 1 g/kg BM; SD-0.1—standard diet supplemented with EEP 0.1 g/kg BM; SD-1—standard diet supplemented with EEP 1 g/kg BM; SD—standard diet. In HFD-1 group, during the total period of observation, lower ADMA concentration than HFD concentration was recorded.

According to the literature, polyphenols added to diet lower ADMA. Li Volti's et al. [45] in vitro studies showed that supplementation with silibinin—flavonoid belonging to the group of flavonolignans—lowered ADMA serum level in mice, limited endothelial dysfunction, and reduced insulin resistance. A significant decrease of ADMA level, ox-LDL level and C-reactive protein (CRP) level, as a result of polyphenol-rich olive oil consumption, was observed in studies carried out in a group of young women with slight hypertension or hypertension stage 1. A significant decrease of hypertension and improving endothelial function was recorded [46].

There are no published reports on the effect of bee pollen extract on ADMA level. Our results show that a polyphenol fraction from bee pollen reduces ADMA level, and consequently increases NO bioavailability, by limiting endothelial dysfunction and development of atherosclerotic changes. It is very important for prevention of cardio-vascular diseases, because an increase of ADMA level is an early risk factor of vascular endothelium dysfunction, and limiting its excessive synthesis can be used in atherosclerotic prevention.

3.5. Effect of EEP on Angiotensin-Converting Enzyme (ACE) and Angiotensin II (ANG II)

The renin-angiotensin-aldosterone system (RAA) is a key mechanism of physiologic regulation of blood pressure and electrolyte balance. Individual constituents of the system can participate in pathogenesis of hypertension and organ changes related to hypertension such as cardiac and vascular remodeling, atherosclerosis, myocardial fibrosis, and renal fibrosis [47].

ACE belongs to zinc metalloproteinases. It is an enzyme that participates in blood pressure regulation through converting inactive angiotensin I into biologically active angiotensin II, with a vassopresor-like activity [48].

ANG II has a harmful effect on vascular endothelium and cardiac muscle. It causes endothelial dysfunction, increases oxidative stress, and contributes to atherosclerotic plaque rupture. ANG II also accelerates apoptosis of cardiac muscle cells, and increases tissue-specific insulin resistance [49]. By contributing to ox-LDL formation, ANG II can indirectly lead to dysfunctions of endothelial function

related to clotting and fibrinolysis [50]. ANG II can also cause a change in endothelial function from anti-adhesive to pro-adhesive one, thus affecting the expression of endothelial adhesion molecules (ICAM, VCAM) [51].

In our study, we have investigated the effect of EEP on ACE and ANG II level in ApoE-knockout mice on high-fat and standard diets. ACE level is presented in Figure 5.

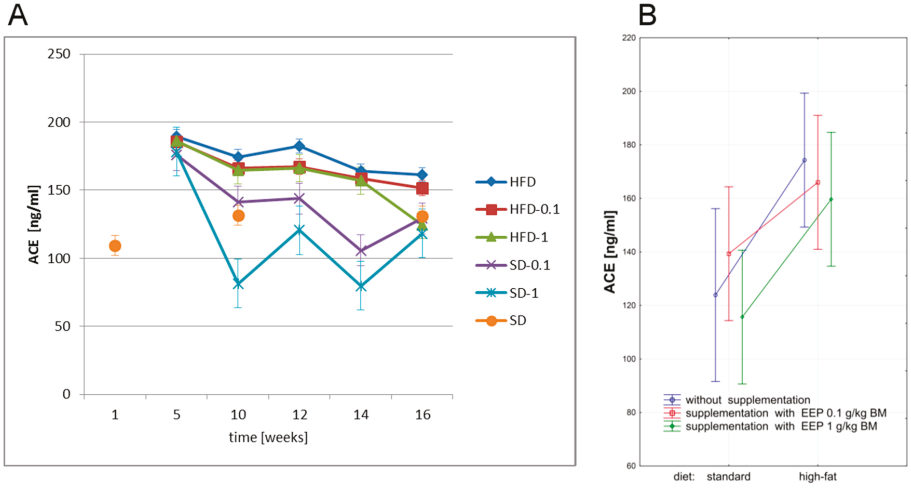

Figure 5. Angiotensin-converting enzyme (ACE) level in ApoE-knockout mice. (**A**) ACE level in particular weeks of the study; (**B**) Average ACE levels (n = 10). HFD—high-fat diet; HFD-0.1—high-fat diet supplemented with EEP 0.1 g/kg BM; HFD-1—high-fat diet supplemented with EEP 1 g/kg BM; SD-0.1—standard diet supplemented with EEP 0.1 g/kg BM; SD-1—standard diet supplemented with EEP 1 g/kg BM; SD—standard diet. In HFD-1, a decrease in ACE concentration was observed in the 16th week, when compared to HFD. The lowest levels of ACE were determined in SD-1.

The highest levels of ACE in particular experimental periods were recorded in animals on a high-fat diet, whereas the lowest ones were observed for a standard diet supplemented with bee pollen extract in a dose of 1 g/kg BM (Figure 5A). After five weeks, a high-fat diet increased ACE level, which was 161 ng/mL (Figure 5A) in the 16th week, i.e., 30% higher than the average level (124 ng/mL, Figure 5B) in the control group. Supplementing a high-fat diet with EEP resulted in lowering this parameter level to 152 ng/mL after 16 weeks, i.e., by 6% for 0.1 g/kg BM, and to 124 ng/mL (Figure 5A) i.e., by 23% for a dose of 1 g/kg BM when compared with the unsupplemented group.

The highest level of ANG II was recorded in mice on a high-fat diet. In the fifth and sixth week, ANG II level was 2.16 ng/mL (Figure 6A), i.e., 95% higher than the average level (1.11 ng/mL, Figure 6B) in the control group. Supplementing a high-fat diet with EEP decreased this parameter as early as in the fifth week, and after 16 weeks ANG II level was reduced to 1.31 ng/mL (Figure 6A), i.e., by 39% for a dose of 0.1 g/kg BM, and to 1.09 ng/mL (Figure 6A), i.e., by 50% for a dose of 1 g/kg BM compared with the unsupplemented group. The lowest ANG II level was recorded in mice on a standard diet supplemented with EEP in a dose of 1 g/kg BM (Figure 6A). Supplementation of a high-fat diet with bee pollen extract, regardless of the dose, caused a statistically significant decrease of ANG II level (Figure 6B).

The published data reveals that polyphenols are hypotensive, which results from their antioxidant capacity. This feature leads to lowering ROS concentration and to weakening ACE activity [19,51,52]. Xu et al. [53], both in studies on rats and in vitro studies showed that genistein had reduced expression and activity of ACE in endothelium and serum. The estrogen receptor activating a

pathologic path of ERK1/2 participated in this activity. Genistein stimulates the synthesis of nitrogen oxide in endothelial cells through a mechanism dependent on cyclic 3′5′-adenosine monophosphate. Taxifolin inhibits ACE activity in the rat aorta [52], while quercetin lowers blood pressure in spontaneously hypertensive rats (SHR), and eliminates all pathological changes in their blood vessels [54]. The antihypertensive effect of polyphenols in SHRs was related to activation of eNOS, and inhibition of metalloproteinase-2 [55]. Lowering blood pressure by polyphenols can also result from modelling the renin-angiotensin-aldosterone system through reducing oxidative stress [56]. Polyphenols from various tea types inhibit ACE activity in in vivo studies [57]. Furthermore, also bee pollen extract has inhibited ACE activity in in vitro studies, which results from its high antioxidant potential [58].

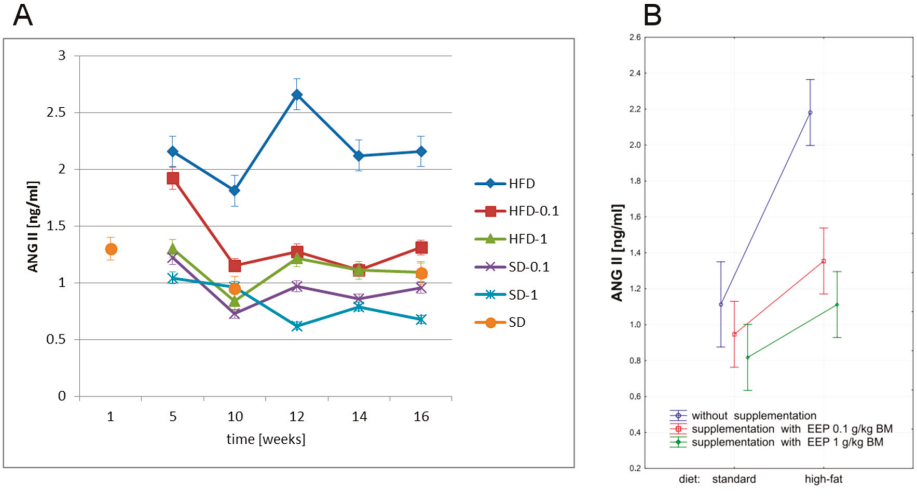

Figure 6. Angiotensin II (ANG II) level in ApoE-knockout mice. (**A**) ANG II level in particular week of the study; (**B**) Average ANG II level (n = 10). HFD—high-fat diet; HFD-0.1—high-fat diet supplemented with EEP 0.1 g/kg BM; HFD-1—high-fat diet supplemented with EEP 1 g/kg BM; SD-0.1—standard diet supplemented with EEP 0.1 g/kg BM; SD-1—standard diet supplemented with EEP 1 g/kg BM; SD—standard diet. The highest ANG II concentration was recorded in HFD. In HFD-0.1 and HFD-1, the concentration of ANG II during the whole research period was lower when compared to HFD.

Based on our study, it can be concluded that supplementing a high-fat diet and a standard diet with EEP decreases angiotensin II level in ApoE-knockout mice. This is related to weakening the activity of ACE. The available literature does not offer any data on the effect of bee pollen on ANG II level; this issue has been presented in our study for the first time. Our study may lead to a conclusion that a polyphenol fraction from bee pollen, due to its high antioxidant capacity, affects the modulation of the renin-angiotensin-aldosterone system; therefore, it improves endothelial function, and consequently it can inhibit the development of atherosclerotic changes.

In spite of the fact that many studies have been conducted, the mechanism of the anti-atherosclerotic effect has not been yet determined. According to the published data, it can be assumed that the main protective effect from the development of atherosclerosis consists improvement of endothelial function, oxidative reduction, LDL modification, decrease of total cholesterol, inhibiting the synthesis of pro-inflammatory cytokines such as TNF-α, IL-6, IL-8, and inhibiting the adhesion of ICAM-1, VCAM-1 molecules as well as the stimulation of eNOS synthase, and intensification of NO synthase [59].

4. Conclusions

Supplementing a high-fat diet with polyphenol-rich EEP modulates lipid profile in ApoE-knockout mice by lowering total cholesterol level. EEP is protective for cardiac arteries since it significantly limits the development of atherosclerotic changes (dose of 0.1 g/kg BM) or prevents their development (dose of 1 g/kg BM). This results from lowering the level of strongly pro-atherosclerotic ox-LDL. Due to high antioxidant activity, EEP efficiently reduces oxidative stress, and is hence an inhibitor of LDL oxidation. The protective effect of EEP results from lowering ADMA level, which increases NO bioavailability, limits endothelial dysfunction, and prevents the development of atherosclerotic changes. An increase in ADMA level is an early risk factor of endothelium dysfunction, and limiting its excessive synthesis is crucial and can be used in prophylaxis of atherosclerosis as well as other cardio-vascular diseases. EEP prevents and inhibits the development of atherosclerotic changes in ApoE-knockout mice through inhibiting the activity of ACE and lowering ANG II level. Consequently, it affects modulation of the renin-angiotensin-aldosterone system, and can limit endothelial dysfunction in this way. Further studies should aim at comprehensive explanation of both, cellular and genetic mechanisms of a cardioprotective effect of polyphenols, including their stability, absorption, distribution, and biotransformation.

To the best of our knowledge, we have been the first to prove that in experimental conditions, EEP efficiently reduces oxidative stress through limiting atherosclerotic changes or eliminating their development. Hence, EEP can be useful as a potential anti-atherogenic agent.

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Effect of an Extract from *Aronia melanocarpa* L. Berries on the Body Status of Zinc and Copper under Chronic Exposure to Cadmium: An In Vivo Experimental Study

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Abstract: In an experimental model of low-level and moderate environmental human exposure to cadmium (Cd), it was investigated whether the consumption of a polyphenol-rich *Aronia melanocarpa* L. berries (chokeberries) extract (AE) may influence the body status of zinc (Zn) and copper (Cu). The bioelements' apparent absorption, body retention, serum and tissue concentrations, total pool in internal organs, excretion, and the degree of binding to metallothionein were evaluated in female rats administered 0.1% aqueous AE or/and Cd in their diet (1 and 5 mg/kg) for 3–24 months. The consumption of AE alone had no influence on the body status of Zn and Cu. The extract administration at both levels of Cd treatment significantly (completely or partially) protected against most of the changes in the metabolism of Zn and Cu caused by this xenobiotic; however, it increased or decreased some of the Cd-unchanged indices of their body status. Based on the findings, it seems that rational amounts of chokeberry products may be included in the daily diet without the risk of destroying Zn and Cu metabolisms; however, their potential prophylactic use under exposure to Cd needs further study to exclude any unfavourable impact of these essential elements on the metabolism.

Keywords: apparent absorption; *Aronia melanocarpa* berries; body status; cadmium; copper; excretion; metabolism; metallothionein; polyphenols; tissue concentrations; zinc

1. Introduction

In recent years, there has been growing interest in the beneficial impact on human health of substances naturally occurring in plants which could be included in the diet as functional food, such as polyphenolic compounds [1–4]. One of the richest sources of polyphenols is *Aronia melanocarpa* L. (*A. melanocarpa*, (*Michx.*) Elliott, *Rosaceae*) berries (chokeberries) [1]. Among the polyphenolic compounds present in these fruits are anthocyanins (cyanidin derivatives), proanthocyanidins, phenolic acids (chlorogenic acid and neochlorogenic acid), and quercetin and its derivatives [1,5]. *Aronia* berries are also rich in other bioactive compounds such as fiber, tannins, pectin, vitamins, minerals, and organic acids [1,6]. Chokeberries are considered to be useful in the protection and treatment of civilisation diseases [1–4]. Moreover, recent findings from studies in animal models [5,7–12] show that polyphenolic compounds, including those present in *A. melanocarpa* berries, may be a promising preventive/therapeutic strategy for xenobiotics, including toxic heavy metals such as cadmium (Cd).

Cd is an environmental pollutant harmful to human health, to which the general population is exposed in everyday life, mainly via food [13–15]. Epidemiological studies indicate that chronic,

even relatively low-level exposure to this heavy metal, occurring nowadays in industrialised countries, may contribute to the development of numerous unfavourable health outcomes, mainly including renal dysfunction, osteoporosis, cardiovascular system disorders, liver damage, age-related macular degeneration, hearing loss, and cancers [16–21]. Moreover, epidemiological evidence shows that there is no safe level of exposure to Cd and it is forecast that the general population's exposure to this xenobiotic will increase [13].

Taking the above into account, there has been growing interest in the search for effective ways of prevention and treatment of unfavourable health outcomes caused by Cd [8,22–27]. In this regard, scientists and nutritionists have been especially focused on dietary products rich in bioelements, vitamins, and polyphenolic compounds [8,22–27]. Previously, we revealed that supplementation with zinc (Zn), under moderate and relatively high repeated exposure to Cd, helped decrease the body burden of this toxic metal and protected against some effects of its toxic action [22–27]. Nowadays, we are particularly interested in the possibility of using chokeberries in the protection against negative health effects of low-level and moderate chronic exposure to this heavy metal [1,5,7–11]. Using a female rat model of low-level and moderate environmental lifetime human exposure to Cd (1 and 5 mg Cd/kg diets, respectively), we revealed that the consumption of 0.1% aqueous extract from *Aronia* berries (AE) decreased the gastrointestinal absorption and body burden of this toxic metal and increased its urinary excretion [7]. Moreover, the extract also protected against oxidative stress and oxidative changes in the serum and bone tissue [9], bone metabolism disorders and worsening of bone biomechanical properties [5,10], as well as damage to the liver caused by Cd [11] (data in preparation for publication). The beneficial impact of AE may be explained by its antioxidative action and the ability of polyphenols rich in hydroxyl (–OH) groups to complex Cd ions (Cd^{2+}) [7–9,28]. It is also possible that fiber present in chokeberries may bind Cd ions in the gastrointestinal tract, thus preventing their absorption [6].

Taking into account the above findings, products containing *Aronia* berries seem to be very promising agents for use in the protection against toxic action of Cd; however, their possible prophylactic use needs further studies that allow the evaluation of a wide spectrum of aspects of influence, including the exclusion of any adverse outcomes of their prolonged consumption. Generally, there is no evidence of serious adverse health outcomes of long-term enhanced consumption of polyphenol-rich products in humans. However, it has been noticed that such products may cause antinutritional effects and disorders in bioelements metabolism, as well as affect drug bioavailability and pharmacokinetics [29,30]. It seems crucial to take into account the risk of disorders in the metabolism of necessary elements in the case of prolonged consumption of polyphenol-rich products because of polyphenolic compounds' ability to bind divalent metals, including bioelements [28,31,32]. Under the conditions of simultaneous exposure to Cd, this is especially important for bioelements interacting with toxic heavy metals, such as Zn and copper (Cu) [22,24,33–38].

It is well known that some effects of Cd toxicity result from destroying the metabolisms and biological functions of Zn and Cu [22,24,33–36,38,39], and that the intake and body status of these bioelements have an influence on the body burden of Cd and its toxicity [22–27]. Owing to the presence in chokeberries of compounds capable of binding Zn and Cu [1,6,31,32], and taking into account that changes in the erythrocyte concentrations of both bioelements were noticed in people consuming *Aronia* anthocyanins [29], we found it necessary to investigate whether and how the prolonged consumption of AE, demonstrated by us to have a protective impact under exposure to Cd [5,7,9–11], may modify the body status of these elements. Zn and Cu are among the necessary elements playing a key role in the proper functioning of the organism [40,41], and both their deficiency and excessive concentrations in biological fluids and tissues are dangerous for health [41,42].

Taking into account the lower body burden of Cd due to AE consumption [7] and the available data on Cd interactions with Zn and Cu [22,24,33–38], we hypothesised that the administration of the extract could, at least partially, prevent Cd-induced disorders in the body status of Zn and/or Cu. However, because AE ingredients may potentially form complexes with Zn and Cu [31,32] and

influence the metabolism of these bioelements [29], it cannot be ruled out that the consumption of the extract may have no impact on the body status of these elements or even lead to their deficiency in the organism. Thus, the aim of the present study was to investigate the influence of AE intake on the body status of Zn and Cu in the experimental model of exposure to Cd that we used previously [5,7,9–11]. For this purpose, the apparent absorption, retention in the body, and excretion, as well as the serum and tissue concentrations, and the total pool in internal organs were evaluated for both bioelements. The concentration of metallothionein (MT) in the liver, kidney, and duodenal tissue was also determined. Also, the degree of Zn, Cu, and Cd binding to this protein was evaluated, because polyphenols may induce the biosynthesis of MT [43], which plays a key role in the accumulation and detoxification of Cd, as well as in the regulation of Zn and Cu metabolisms [22,33,43–45], and due to the fact that this protein is involved in Cd-induced irregularities of the metabolism of both bioelements [22,33]. To the best of our knowledge, such research has not been carried out until now.

2. Materials and Methods

2.1. Chemicals

Cadmium chloride ($CdCl_2 \times 2\frac{1}{2}\ H_2O$) and sodium chloride (NaCl) were purchased from POCh (Gliwice, Poland), while Morbital and heparin were obtained from Biowet (Pulawy, Poland) and Biochemie GmbH (Kundl, Austria), respectively. Trace-pure 65% nitric acid (HNO_3; Merck, Darmstadt, Germany) and 30% hydrochloric acid (HCl; Merck), as well as stocks of standard solutions of Zn, Cu, and Cd (Sigma, St. Louis, MO, USA) assigned for atomic absorption spectrometry (AAS method) were used. The mixture of palladium and magnesium (as nitrates; Merck) was used as a matrix modifier in Cd analysis. In order to check the analytical quality of metals measurement, the following certified materials were used: Trace Elements Serum L-1 LOT (No. 0903106; Sero, Billingstad, Norway), Trace Elements Urine L-2 LOT (No. 1011645; Sero, Billingstad, Norway), Standard Reference Material Bovine Liver (No. 1577b; National Institute of Standards and Technology, Gaithersburg, MD, USA), Certified Reference Material BCR Pig Kidney (BCR-186; Institute for Reference Materials and Measurements, Geel, Belgium), and Standard Reference Bone Ash (No. 1400; National Institute of Standards and Technology, Gaithersburg, MD, USA). The diagnostic ELISA kit for MT determination was obtained from MyBioSource (San Diego, CA, USA). Ultra-pure water received from a two-way water purification MAXIMA system (ELGA, Bucks, UK) was used in all of the measurements.

2.2. Experimental Animals

A total of 192 young (3 to 4 weeks old) female Wistar rats (Crl: WI (Han)) purchased from the certified Laboratory Animal House (Brwinów, Poland) were used. Throughout the experiment, the animals were housed in controlled conventional conditions (temperature 22 ± 2 °C, relative humidity 50 ± 10%, 12/12 h light-dark cycle). They were maintained in stainless-steel cages, four animals in each, and had free access to drinking water and food during the whole experiment. Throughout the first 3 months of the study, all rats received the Labofeed H diet (breeding diet ensuring the proper growth and development of young animals; Label Food 'Morawski', Kcynia, Poland), following which they were fed with the Labofeed B diet (maintenance diet). According to the manufacturer, the Labofeed H diet contained 210 mg Zn/kg and 33 mg Cu/kg, whereas the Labofeed B diet contained 150 mg Zn/kg and 25 mg Cu/kg. The concentrations of Zn and Cu determined (using the AAS method) in our laboratory in the Labofeed H diet reached 202.1 ± 2.44 mg/kg (mean ± standard deviation—SD) and 29.62 ± 0.51 mg/kg, respectively, while in the Labofeed B diet they were 143.3 ± 2.6 mg/kg and 22.91 ± 1.37 mg/kg, respectively.

2.3. Experimental Protocol

The research protocol was approved by the Local Ethics Committee for Animal Experiments in Bialystok, Poland (approval No. 60/2009 of 21 September 2009 and approval No. 80/2015 of 9 June 2015) and performed according to the ethical principles and institutional guidelines, as well as the international Guide for the Use of Animals in Biomedical Research.

The experimental model has been described in detail in our previous reports [5,7,9,10]. In brief, the rats were randomly divided into six groups (32 animals each) receiving Cd and/or AE or not, as described in Table 1. One group received 0.1% AE alone, two groups were given Cd alone in the Labofeed diets containing 1 and 5 mg Cd/kg, and the next two groups received AE during the whole course of Cd exposure (for up to 24 months). The control group drank redistilled water without AE addition and was fed with the standard Labofeed diets without Cd (Table 1). The daily intake of Cd at particular levels of treatment and the intake of AE throughout the experiment did not differ regardless of whether they were administered alone or in conjunction (Table 1).

Table 1. Experimental model.

Group	Administration		Range of the Daily Intake During the 24-Month Administration	
	AE [1]	Cd (1 or 5 mg Cd/kg) [2]	AE (PF) (mg/kg b.w.) [3]	Cd (μg/kg/b.w.) [4]
Control	–	-		2.30–4.98
AE	+	-	67.4–146.6 (44.3–96.4)	2.25–4.95
Cd_1	–	+(1 mg Cd/kg)		39.2–83.8
Cd_1 + AE	+	+(1 mg Cd/kg)	67.2–154.7 (44.2–101.7)	37.5–84.9
Cd_5	–	+(5 mg Cd/kg)		210.1–403.2
Cd_5 + AE	+	+(5 mg Cd/kg)	63.1–150.3 (41.5–98.8)	200.2–401.9

[1] AE (extract from the berries of *Aronia melanocarpa*) was administered as the only drinking fluid, in the form of 0.1% aqueous solution prepared by dissolving in redistilled water the powdered *Aronia* extract (Adamed Consumer Healthcare, Tuszyn, Poland; Certificate KJ 4/2010, Butch No. M100703), containing, according to the manufacturer, 65.74% of polyphenols (including 18.65% of anthocyanins). The powdered extract contained total polyphenols—612.40 ± 3.33 mg/g, total anthocyanins—202.28 ± 1.28 mg/g (cyanidin 3-*O*-β-galactoside—80.07 ± 1.05 mg/g, cyanidin 3-*O*-α-arabinoside—33.21 ± 0.01 mg/g, cyanidin 3-*O*-β-glucoside—3.68 ± 0.01 mg/g), total proanthocyanidins—129.87 ± 1.12 mg/g, total phenolic acids—110.92 ± 0.89 mg/g (chlorogenic acid—68.32 ± 0.08 mg/g), and total flavonoids—21.94 ± 0.98 mg/g [5]. The concentrations of zinc (Zn) and copper (Cu) in 0.1% AE reached 1.39 ± 0.04 μg/L and 0.803 ± 0.065 μg/L, respectively, whereas the cadmium (Cd) concentration was below the limit of detection (<0.05 μg/L [7]). [2] Cd diets were prepared by the addition, at their production stage, of appropriate amounts of cadmium chloride ($CdCl_2 \times 2\frac{1}{2}\ H_2O$) into the ingredients of the standard Labofeed H and B diets to achieve the concentrations of 1 and 5 mg Cd/kg. The concentration of Cd determined in our laboratory in the Labofeed H and B diets did not differ (reached by mean 1.09 ± 0.13 mg/kg and 4.92 ± 0.53 mg/kg) [7] and agreed with the values certified by the producer. [3] Data represent the range of the daily intake of AE and polyphenols (PF) throughout the 24-month study. Polyphenols intake was calculated assuming that the commercial extract contained 65.74% of these compounds (certified value). The intake of AE and polyphenols in the control group, Cd_1 group, and Cd_5 group was recognised to be 0. Detailed data on polyphenols intake in particular experimental groups have already been published [7]. [4] Data represent the range of the daily Cd intake throughout the 24-month study. Cd intake in the control group and AE group was calculated based on this metal concentration determined in the standard diet (0.0584 ± 0.0049 mg/kg) [7], while this metal intake in the groups exposed to Cd was calculated based on its concentration in the diet declared by the manufacturer (1 or 5 mg Cd/kg). Detailed data on Cd intake in particular groups have already been published [7]. "–" AE and/or Cd were not administered; "+" AE and/or Cd were administered.

Because the diet, being the main source of the general population's exposure to Cd [13], is also a source of bioelements and various biologically active compounds, including polyphenols, toxic (Cd) and essential (Zn and Cu) metals and polyphenolic compounds can interact with one another in the gastrointestinal tract, thus affecting their own bioavailability and absorption, and thus also the body status [29,43,46,47]. That is why, in our experimental model, Cd was administered in the diet at concentrations that simulated the conditions reflecting the general population's exposure [14,16–20,48,49]. The measurements of Cd concentration in the blood and urine of the rats receiving the diets containing 1 and 5 mg Cd/kg (0.113–0.324 μg/L and 0.085–0.354 μg/g creatinine, and 0.584–1.332 μg/L and 0.284–0.820 μg/g creatinine, respectively) [7] confirmed that the used

experimental model corresponds well with human environmental exposure to this toxic metal in industrialised countries [20,48,49]. Since women are characterised by higher gastrointestinal absorption of Cd and are more susceptible to its toxicity [50], the study was performed in a female rat model.

During the experiment, there were no statistically significant differences in the consumption of food and drinking water, or body weight gain among the experimental groups [7]. Moreover, no unfavourable health outcomes were observed; however, three animals died between the 18th and 24th month of the experiment (one case of death in each of the AE, Cd_1, and Cd_5 groups) [7].

In the last week of the 3rd, 10th, 17th, and 24th month of the experiment, a 5-day balance study was performed. For this purpose, the rats (eight animals of each group at each time point, except for seven animals in some groups after 24 months) were placed individually in metabolic cages and 24-hour urine and faeces were collected on 5 consecutive days. During this time, the animals had free access to food and drinking water (with or without Cd and AE, depending on the experimental group), the consumption of which was monitored. The urine and faeces were removed from the metabolic cages every 24 h and stored for further analysis. The urine was centrifuged (MPW-350R centrifugator, Medical Instruments, Warsaw, Poland) immediately after collection and its volume was recorded. After the balance study, the rats were deprived of food overnight and then they were subjected to anaesthesia (Morbital, 30 mg/kg b.w., intraperitoneally). The whole blood was taken by cardiac puncture with and without anticoagulant (heparin). Stomach, duodenum, liver, kidneys, spleen, heart, brain, femur, and femoral muscle were dissected. The content of the stomach and duodenum was immediately removed by multiple rinsing with ice-cold physiological saline (0.9% NaCl). The dissected soft tissues, after rinsing with ice-cold 0.9% NaCl, were gently dried on the filter paper, whereas the femurs were cleaned of all adherent soft tissues. Next, they were weighed with an analytical balance (OHAUS®, Nanikon, Switzerland; accuracy to 0.0001 g). The biological material which was not used immediately was stored frozen at −70 °C until assayed.

2.4. Determination of Zn and Cu in Biological Fluids, Tissues, and Faeces

The 5-day faeces, after drying to constant weight, was crumbled, and three representative samples for each rat were collected for analysis. Known-weight slices of the liver, kidney (left), spleen, heart, brain, femoral muscle, stomach, and duodenum, as well as the samples of faeces were wet-digested with a mixture of trace-pure concentrated HNO_3 and HCl using a microwave system (Multiwave, Anton Paar GmbH, Graz, Austria) as reported [7]. Next, the excess of acids was evaporated by slightly warming up the samples and the preparations were diluted with ultra-pure water. Bone slices (0.1–0.2 g) obtained from the distal epiphysis (trabecular bone region) and diaphysis (compact bone region) of the femur (left), after rinsing with ultra-pure water (to eliminate the removable bone marrow), drying (to receive dry bone weight), and ashing, were wet-digested with trace-pure 65% HNO_3, and then diluted with ultra-pure water [7]. The samples of the serum and 24-h urine (representative samples from the 5-day balance study) were diluted with 0.5% HNO_3.

The concentrations of Zn and Cu in such preparations of the serum, urine, faeces, soft tissues, and bone tissue, except for Cu in the bone tissue, were determined (after appropriate dilution if necessary) by the flame AAS method (F AAS; atomisation in an air–acetylene burner). The concentration of Cu in the bone preparations was measured by the flameless AAS method with electrothermal atomisation in a graphite furnace (GF AAS). An atomic absorption spectrophotometer model Z-5000 (Hitachi, Tokyo, Japan) equipped with cathode lamps of Zn and Cu (resonance lines of 213.8 nm and 324.8 nm, respectively; Photron, Narre Warren, Australia) was used. The limit of detection of Zn and Cu for the F AAS method was 8.9 μg/L and 21 μg/L, respectively, while the limit of Cu detection for the GF AAS method was 0.355 μg/L. Zn and Cu concentrations in the soft tissues are expressed as per gram of wet tissue weight, while in the bone tissue they are expressed as per gram of dry bone weight.

In order to check the analytical quality of Zn and Cu measurements, the concentrations of both metals in the certified reference serum, liver, kidney, bone, and urine were measured. The concentrations determined in our laboratory agreed exactly with the certified values, and the

recovery ranged from 95% to 103% for Zn and from 96% to 105% for Cu, whereas the precision of measurements, expressed as a coefficient of variation (CV), was <3.3% for Zn and <9.4% (<7.9% for the GF AAS and <9.4% for the F AAS) for Cu (detailed results are presented in Table S1).

2.5. Estimation of the Bioavailability and Retention of Zn and Cu in the Body

The bioavailability of Zn and Cu was evaluated based on their apparent absorption expressed as the absorption index (Abs_{Zn} and Abs_{Cu} (%)) calculated from the following equation: $Abs_{Me} = (I_{Me} - FE_{Me})/I_{Me} \times 100\%$, where Me means Zn or Cu, I_{Me} is the mean daily intake of Zn or Cu via food during the 5-day balance study, and FE_{Me} is the mean amount of Zn or Cu_excreted daily with faeces during the 5-day study [51].

The mean daily body retention of Zn or Cu (R_{Me} (%)) was calculated as the difference between the mean daily intake of a particular bioelement (I_{Me}) during the 5-day balance study and the mean amount of the element excreted daily with faeces (FE_{Me}) and urine (UE_{Me}) during this time, according to the following formula: $R_{Me} = [I_{Me} - (FE_{Me} + UE_{Me})]/I_{Me} \times 100\%$ [51].

2.6. Calculation of the Total Pool of Zn and Cu in Internal Organs

The total pool of Zn or Cu in internal organs was calculated as the sum of the content of a particular element in organs such as liver, spleen, heart, brain, and both kidneys. Because the concentrations of Zn and Cu were determined only in the left kidney, to evaluate the total content of these bioelements in both kidneys it was assumed that their concentrations in the right kidney were the same as those in the left one. Zn and Cu concentrations in the right kidney were calculated by the multiplication of these metal concentrations in the left organ and the weight of the right organ.

The total content of a bioelement in particular internal organs, and especially its total pool in these organs, are better parameters to reflect the body status of the bioelement than its content or concentration in particular organs. Differences in the weight of internal organs may mask changes in the element concentration in these organs, especially in the case of a very slight change. The estimation of the total pool of a bioelement in internal organs may allow the detection of a slight change (shortage or growth) of the bioelement status in the body, which does not yet result in a decrease in this element's content or concentration in particular organs.

2.7. Determination of Cd in the Duodenum

Cd concentration in the wet-digested slices of the duodenal tissue (prepared as described in Section 2.4) was determined using the GF AAS method (detection limit—0.018 μg Cd/L; Hitachi Z-5000 spectrophotometer), as previously reported for this metal assay in other tissues and biological fluids of these rats [7]. Cd concentration determined by us in the certified reference soft tissues (liver—0.513 ± 0.024 μg/g and kidney—2.66 ± 0.14 μg/g; mean ± SD) agreed exactly with the certified values (0.50 ± 0.03 μg/g, 2.71 ± 0.15 μg/g, respectively). The recovery of Cd was 103% and 98%, respectively, and the CV was <4.7%.

2.8. Determination of MT Concentration in the Liver, Kidney, and Duodenum, as Well as Estimation of the Degree of Zn, Cu, and Cd Binding to This Protein

The concentration of MT in the aliquots of 10% homogenates of the liver and kidney, and 20% homogenates of the duodenal tissue (prepared in 0.9% NaCl) was measured colourimetrically using a Rat Metallothionein ELISA Kit. The CV for MT measurements in the kidney, liver, and duodenum was <7.6%, 7.4%, and 5.5%, respectively.

In order to be stable, MT needs to have all metal-binding sites saturated (1 mole of MT needs to contain 7 moles of divalent metals Zn and/or Cd, and 10–12 moles of Cu in +1 valence state) [33,37]. Thus, an increase in the concentrations of Cd and MT in the kidney, liver, and duodenum may be accompanied with a simultaneous increase in the concentrations of Zn and Cu, as well as changes of the pools of these bioelements bound and unbound to MT [22,37]. A decrease in the non-MT-bound pool of

Zn or Cu in the duodenal tissue or internal organs may result in hampered gastrointestinal absorption of these bioelements or their insufficient availability to ensure the proper course of physiological processes dependent on them. That is why, studying the impact of the administration of AE on the body status of Zn and Cu in the case of exposure to Cd, it was very important to evaluate the pools of MT-bound and MT-unbound elements in the kidney, liver, and duodenum.

Based on the concentrations of Zn, Cu, Cd, and MT in the kidney, liver, and duodenum, the ratio of actual metal concentration (Me) to the theoretical maximum concentration of MT-bound metal (Me-MT) was calculated according to the following formula: Me/Me-MT = Zn/(MT × 7) + Cd/(MT × 7) + Cu/(MT × 12), where Zn, Cu, Cd, and MT are the concentrations (nmol/g wet tissue) of particular metals and MT [22,37]. To estimate the amount of theoretical maximum MT saturation with metals, MT concentration was multiplied by 7 in the case of Zn and Cd (7 moles of divalent metals, including Zn and Cd, can bind to 1 mole of MT) and by 12 in the case of Cu (10–12 moles of Cu(I) can bind to 1 mole of MT) [22,37]. The values of the ratios of Zn/(MT × 7), Cu/(MT × 12), and Cd/(MT × 7) reflect MT saturation with Zn, Cu, and Cd, respectively, in addition to the pool of MT-unbound particular elements, whereas the ratio of Me/Me-MT reflects this protein saturation with all these metals (and the pool of non-MT bound metals). Theoretically, if the value of the Me/Me-MT ratio is lower than 1, all metals can be bound by MT. When the ratio is higher than 1, there exists the pool of non-MT-bound metals because MT cannot bind them further due to the saturation of its all metal-binding sites. The pool of non-MT-bound metals increases together with the growing ratios of Zn/(MT × 7), Cu/(MT × 12), Cd/(MT × 7), and Me/Me-MT. An increase in the Zn/(MT × 7), Cu/(MT × 12), Cd/(MT × 7), or Me/Me-MT ratio compared to the control group or any other experimental group indicates a rise in the pool of MT-unbound metals (Zn, Cu, Cd, or all of the metals, respectively), while a decrease in these ratios reflects a drop in the pool of non-MT-bound metals. For the calculation of MT saturation with Cd, the concentration of this metal in the liver and kidney previously determined in these animals (Table S2) [7] was used. Only non-MT-bound Cd present in tissues may exert toxic action [22,33,37].

2.9. Statistical Analysis

The data are expressed as a mean ± standard error (SE) for eight rats after 3, 10, 17, and 24 months, except for seven animals in groups AE, Cd_1, and Cd_5 after 24 months and eight to 32 animals in the case of the daily intake of Zn and Cu presented in Table 2.

A one-way analysis of variance (ANOVA) was applied to determine whether there were statistically significant ($p < 0.05$) differences among the six experimental groups, and then Duncan's multiple range post hoc test was performed for comparison between individual groups and to determine which two means differed ($p < 0.05$). In tables and figures, statistically significant differences in relation to the control group, the respective group receiving Cd alone (Cd_1 + AE vs. Cd_1 and Cd_5 + AE vs. Cd_5), and the respective group exposed to the 1 mg Cd/kg diet alone or with AE (Cd_5 vs. Cd_1 and Cd_5 + AE vs. Cd_1 + AE) are marked. In the case when the Duncan's multiple range test revealed any influence of the co-administration of Cd and AE on the investigated parameter, a two-way analysis of variance (ANOVA/MANOVA, test F) was conducted so as to discern a possible interactive and independent impact of Cd and AE on this parameter. F values with $p < 0.05$ were recognised as statistically significant. All of the calculations were performed using the Statistica package (StatSoft, Tulsa, OK, USA).

Table 2. The intake of zinc (Zn) and copper (Cu) with diet in particular experimental groups [1,2].

Group	Experiment Duration			
	3 Months	**10 Months**	**17 Months**	**24 Months**
	Zn Intake (mg/kg b.w./24 h)			
Control	16.596 ± 0.097	6.931 ± 0.201 **	6.345 ± 0.064 **	7.149 ± 0.069 **
AE	16.710 ± 0.265	7.037 ± 0.063 **	6.191 ± 0.117 **	7.138 ± 0.126 **
Cd_1	16.282 ± 0.336	7.001 ± 0.015 **	6.361 ± 0.027 **	7.290 ± 0.143 **
Cd_1 + AE	16.656 ± 0.039	7.060 ± 0.159 **	6.231 ± 0.036 **	7.408 ± 0.219 **
Cd_5	16.175 ± 0.213	7.043 ± 0.076 **	6.176 ± 0.041 **	7.612 ± 0.259 **
Cd_5 + AE	16.415 ± 0.144	7.191 ± 0.054 **	6.227 ± 0.019 **	7.557 ± 0.241 **
	Cu Intake (mg/kg b.w./24 h)			
Control	2.678 ± 0.038	1.155 ± 0.033 **	1.026 ± 0.006 **	1.194 ± 0.004 **
AE	2.626 ± 0.042	1.186 ± 0.012 **	1.032 ± 0.019 **	1.190 ± 0.021 **
Cd_1	2.638 ± 0.021	1.159 ± 0.003 **	1.032 ± 0.001 **	1.215 ± 0.024 **
Cd_1 + AE	2.702 ± 0.032	1.177 ± 0.026 **	1.004 ± 0.019 **	1.235 ± 0.036 **
Cd_5	2.616 ± 0.011	1.174 ± 0.013 **	1.026 ± 0.006 **	1.269 ± 0.043 **
Cd_5 + AE	2.614 ± 0.008	1.198 ± 0.009 **	1.019 ± 0.008 **	1.260 ± 0.040 **

[1] The intake of Zn and Cu was calculated based on these bioelements' concentrations in the Labofeed diets declared by the manufacturer. The Labofeed H diet (administered throughout the first 3 months of the study) contained 210 mg Zn/kg and 33 mg Cu/kg, whereas the Labofeed B diet (used thereafter), contained 150 mg Zn/kg and 25 mg Cu/kg. [2] Data represent mean ± SE intake of Zn and Cu for 32, 24, 16, and eight rats during 3, 10, 17, and 24 months of the experiment, respectively. ** $p < 0.01$ (ANOVA, Duncan's multiple range test) compared to the intake during the first 3 months.

3. Results

3.1. Daily Intake of Zn and Cu

The mean daily intake of Zn via the Labofeed diet in the control group during the 5-day balance study in the last week of the 3rd, 10th, 17th, and 24th month reached 5.182 ± 0.073 mg/24 h, 2.994 ± 0.087 mg/24 h, 3.356 ± 0.061 mg/24 h, and 4.196 ± 0.071 mg/24 h, respectively, whereas the intake of Cu reached 0.814 ± 0.011 mg/24 h, 0.499 ± 0.015 mg/24 h, 0.559 ± 0.010 mg/24 h, and 0.699 ± 0.012 mg/24 h, respectively (Table S3). Because the content of Zn and Cu in the Labofeed H diet administered during the first 3 months was higher than that in the Labofeed B diet (used from the beginning of the 4th month), the mean daily intake of these elements in all groups during this period of the experiment (Table 2), including the 5-day balance study in the last week of the 3rd month (Table S3), was higher than thereafter (2.1–2.7 times and 17–73%, respectively, for Zn, and 2.1–2.7 times and 10–63%, respectively, for Cu). The daily intake of Zn and Cu via the Labofeed diet at particular time points did not differ between the experimental groups (Table 2 and Table S3).

The Labofeed diet was the main source of Zn and Cu for the experimental animals. Because of very low concentrations of Zn (1.39 ± 0.04 μg/L) and Cu (0.803 ± 0.065 μg/L) in the 0.1% AE, the daily intake of these bioelements via the extract consumption was negligible compared to their intake with the diet. The mean intake of Zn via AE consumption throughout the 24-month study ranged from 0.057 to 0.060 μg/24 h (0.19–0.20 μg/kg b.w.), whereas the intake of Cu reached 0.033–0.035 μg/24 h (0.11–0.12 μg/kg b.w.), regardless of whether the extract was administered alone or with Cd.

The above data show that the total daily intake of Zn and Cu from all sources during particular experimental periods did not differ between the experimental groups, confirming the usefulness of the experimental model to estimate the influence of AE and/or Cd consumption on the body status of these bioelements.

3.2. Effect of AE on the Body Status of Zn under Exposure to Cd

3.2.1. Zn Apparent Absorption, Retention in the Body, and Its Faecal and Urinary Excretion

Because of the low daily urinary excretion of Zn (UE_{Zn}; Figure 1), the body retention of this bioelement (Ret_{Zn}) in some experimental groups reached almost the same values as the Abs_{Zn} (Figure 1), and thus it is presented in Figure S1 (the same refers to Cu).

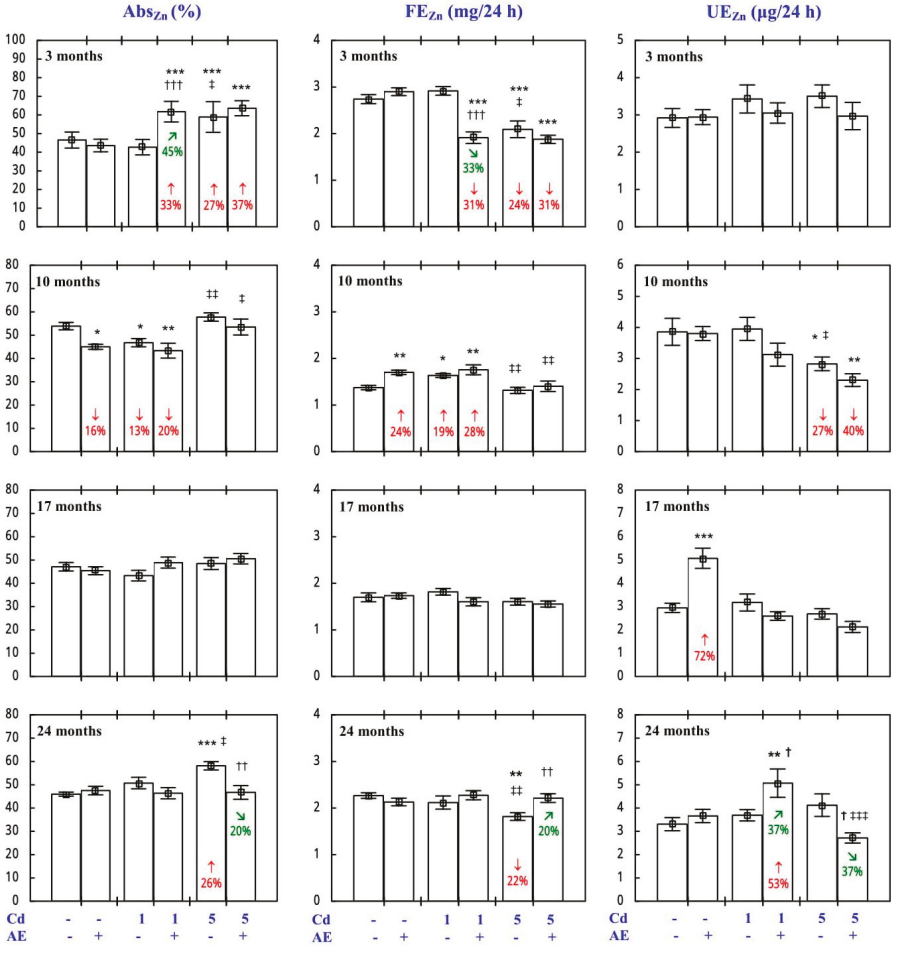

Figure 1. The apparent absorption of zinc (Abs_{Zn}) and its daily faecal (FE_{Zn}) and urinary excretion (UE_{Zn}) in particular experimental groups. The rats received cadmium (Cd) in their diet at the concentration of 0, 1, and 5 mg/kg and/or 0.1% extract from the berries of *Aronia melanocarpa* (AE; "+", received; "-", not received). Data represent mean ± SE for eight rats (except for seven animals in the AE, Cd_1, and Cd_5 groups after 24 months). Statistically significant differences (ANOVA, Duncan's multiple range test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. respective group receiving Cd alone; ‡ $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$ vs. respective group receiving the 1 mg Cd/kg diet (alone or with AE) are marked. Numerical values in bars indicate percentage changes compared to the control group (↓, decrease; ↑, increase) or the respective group receiving Cd alone (↘, decrease; ↗, increase).

The Abs_{Zn} and Ret_{Zn} in the control animals reached 46–54% (Figure 1 and Figure S1). The administration of AE alone had no impact on the Abs_{Zn} and Ret_{Zn}, and Zn excretion (FE_{Zn} and UE_{Zn}), with only a few exceptions (Figure 1 and Figure S1; Supplementary Material—Zinc 1).

In the rats exposed to the 1 mg Cd/kg diet, the only change in the Abs_{Zn} and Ret_{Zn} was their decrease after 10 months, whereas under exposure to the 5 mg Cd/kg diet these variables increased after 3 and 24 months (Figure 1 and Figure S1). The FE_{Zn} was affected at the same time points as the Abs_{Zn} and Ret_{Zn}, but in the opposite direction. The only Cd-induced change in the UE_{Zn} was its decrease after 10 months of the application of the 5 mg Cd/kg diet (Figure 1).

The consumption of AE during the treatment with Cd modified this heavy metal influence on the Abs_{Zn}, Ret_{Zn}, FE_{Zn}, and UE_{Zn} (Figure 1 and Figure S1; Supplementary Material—Zinc 1). The impact depended on the duration of the co-administration of *Aronia* extract and Cd and the level of exposure to this xenobiotic (Figure 1 and Figure S1). The use of the extract for 24 months completely prevented the Cd-induced increase in the Abs_{Zn} and Ret_{Zn}, as well as the decrease in the FE_{Zn} (Figure 1). The ANOVA/MANOVA analysis (Table S4) revealed that the modifying effect of AE consumption during the exposure to Cd on the Abs_{Zn}, Ret_{Zn}, FE_{Zn}, and UE_{Zn}, including its protective action against Cd impact on the values of these variables, was the result of independent action of the extract ingredients ($F = 5.88–27.0$, $p < 0.05–0.001$) and/or their interaction with this heavy metal ($F = 7.45–48.7$, $p < 0.05–0.001$). However, in the case of the Ret_{Zn} in the Cd_5 + AE group after 24 months, in spite of the total protection offered by AE against the Cd-induced increase in the value of this variable, neither a statistically significant independent impact of AE nor its interaction with Cd were observed (Table S4).

3.2.2. Zn Concentration in the Serum and Tissues

The administration of AE alone for up to 24 months had no impact on Zn concentration in the serum, soft tissues, and bone tissue (Figures 2–6), with a few exceptions (Supplementary Material—Zinc 2).

As is presented in detail in Figures 2–6, exposure to Cd alone influenced (increased or decreased) Zn concentration in the serum, soft tissues, and bone tissue depending on the level and duration of treatment. In the rats fed with the 1 mg Cd/kg diet alone, the most important change was an increase in Zn concentration in the liver and kidney with a simultaneous decrease in its concentration in the spleen after 24 months (Figure 2). Exposure to the 5 mg Cd/kg diet alone resulted in considerably varied transitional changes in serum and tissue Zn concentration. The kidney and spleen concentration of this bioelement only increased after 17 months, while its liver concentration was unaffected throughout the study (Figure 2). The bone tissue concentration of this bioelement at both levels of treatment with Cd first decreased after 10 months; however, the change was transitional (Figure 4).

The consumption of AE by the rats fed with diets containing 1 and 5 mg Cd/kg modified the Zn concentration in the serum and tissues (Figures 2–6—Zinc 3). The administration of the extract to the animals intoxicated with the 1 mg Cd/kg diet entirely prevented Cd-induced changes in liver, kidney, and spleen concentration after 24 months (Figure 2), as well as it prevented a decrease in Zn concentration in the bone tissue at the femoral diaphysis after 10 months of the experiment (Figure 4). The extract consumption under exposure to the 5 mg Cd/kg diet completely prevented a Cd-induced decrease in Zn concentration in the spleen after 3 months (Figure 2). Moreover, the administration of the extract under moderate Cd exposure entirely prevented a Cd-induced increase in Zn concentration in the serum (Figure 6) and its decrease in the femoral muscle and duodenum after 3 months (Figures 4 and 5), in addition to an increase in its concentration in the brain after 10 months (Figure 3). Furthermore, the consumption of the extract for 3 and 10 months partially prevented the decrease in the stomach (Figure 3) and bone tissue (Figure 4) concentration of this bioelement, respectively. As is evident from the data presented in Figures 2–5, the administration of AE under moderate exposure to Cd did not protect against all changes in tissue Zn concentrations. The ANOVA/MANOVA

analysis (Tables S5 and S6) revealed that the impact of AE administration to the rats fed with the diets containing 1 and 5 mg Cd/kg on Zn concentration in the serum and tissues, including its protective influence against Cd impact on this bioelement concentration, was the result of independent action of the extract ingredients (F = 4.59–15.2, $p < 0.05$–0.001) and/or their interaction with Cd (F = 4.55–34.7, $p < 0.05$–0.001). However, in some cases, the two-way analysis of variance revealed the lack of a statistically significant independent effect of AE and its interaction with Cd (Tables S5 and S6) on serum and tissue Zn concentration, in spite of the evident impact of the extract administration under Cd exposure noticed on the basis of the findings of one-way analysis of variance (Duncan's multiple range test), presented in Figures 2–6.

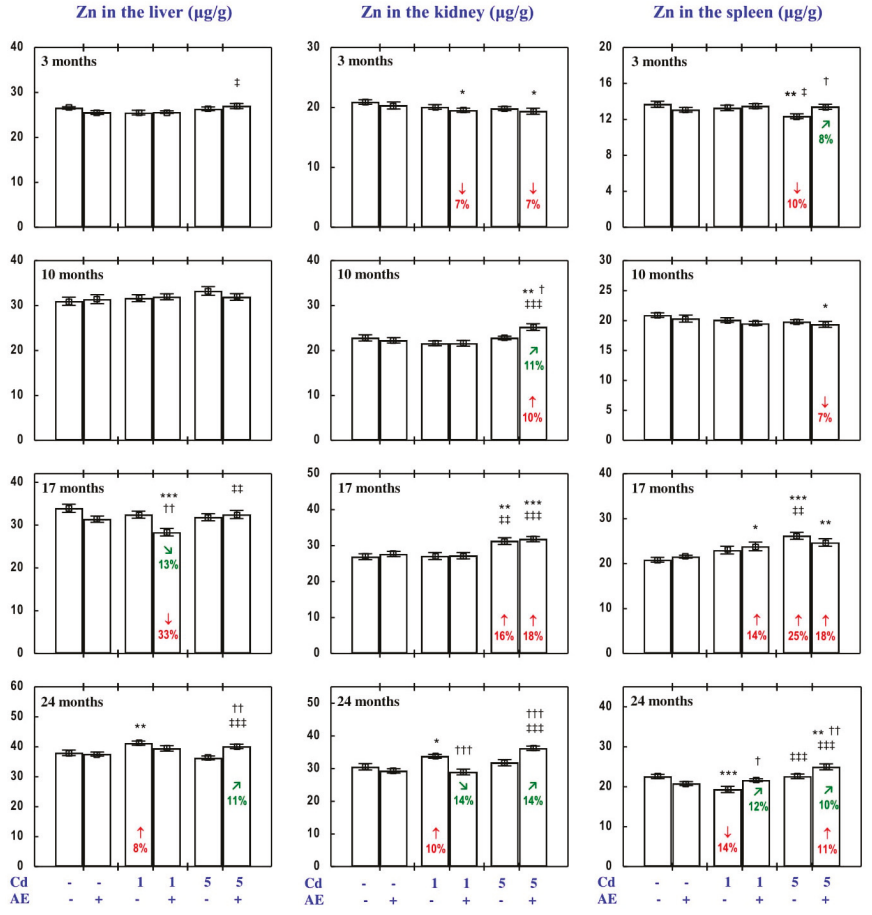

Figure 2. Zinc (Zn) concentration in the liver, kidney, and spleen in particular experimental groups. The rats received cadmium (Cd) in their diet at the concentration of 0, 1, and 5 mg/kg and/or 0.1% extract from the berries of *Aronia melanocarpa* (AE; "+", received; "-", not received). Data represent mean ± SE for eight rats (except for seven animals in the AE, Cd_1, and Cd_5 groups after 24 months). Statistically significant differences (ANOVA, Duncan's multiple range test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. respective group receiving Cd alone; ‡ $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$ vs. respective group receiving the 1 mg Cd/kg diet (alone or with AE) are marked. Numerical values in bars indicate percentage changes compared to the control group (↓, decrease; ↑, increase) or the respective group receiving Cd alone (↘, decrease; ↗, increase).

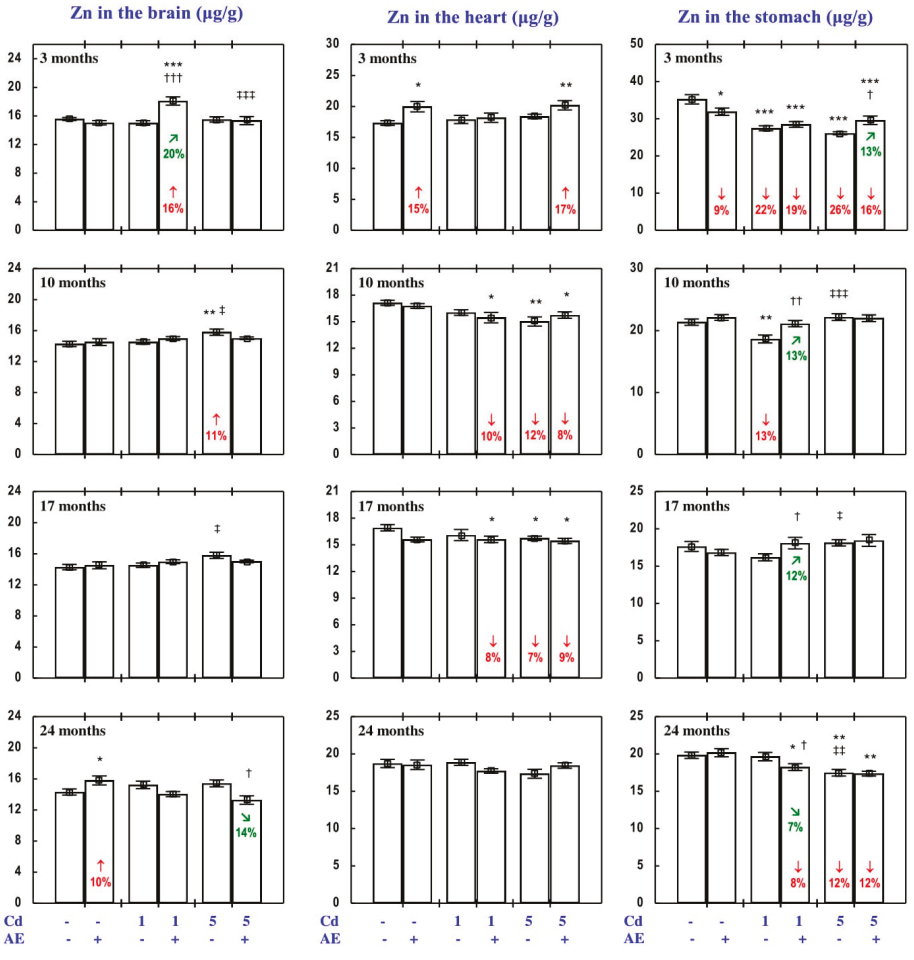

Figure 3. Zinc (Zn) concentration in the brain, heart, and stomach in particular experimental groups. The rats received cadmium (Cd) in their diet at the concentration of 0, 1, and 5 mg/kg and/or 0.1% extract from the berries of *Aronia melanocarpa* (AE; "+", received; "-", not received). Data represent mean ± SE for eight rats (except for seven animals in the AE, Cd_1, and Cd_5 groups after 24 months). Statistically significant differences (ANOVA, Duncan's multiple range test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. respective group receiving Cd alone; ‡ $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$ vs. respective group receiving the 1 mg Cd/kg diet (alone or with AE) are marked. Numerical values in bars indicate percentage changes compared to the control group (↓, decrease; ↑, increase) or the respective group receiving Cd alone (↘, decrease; ↗, increase).

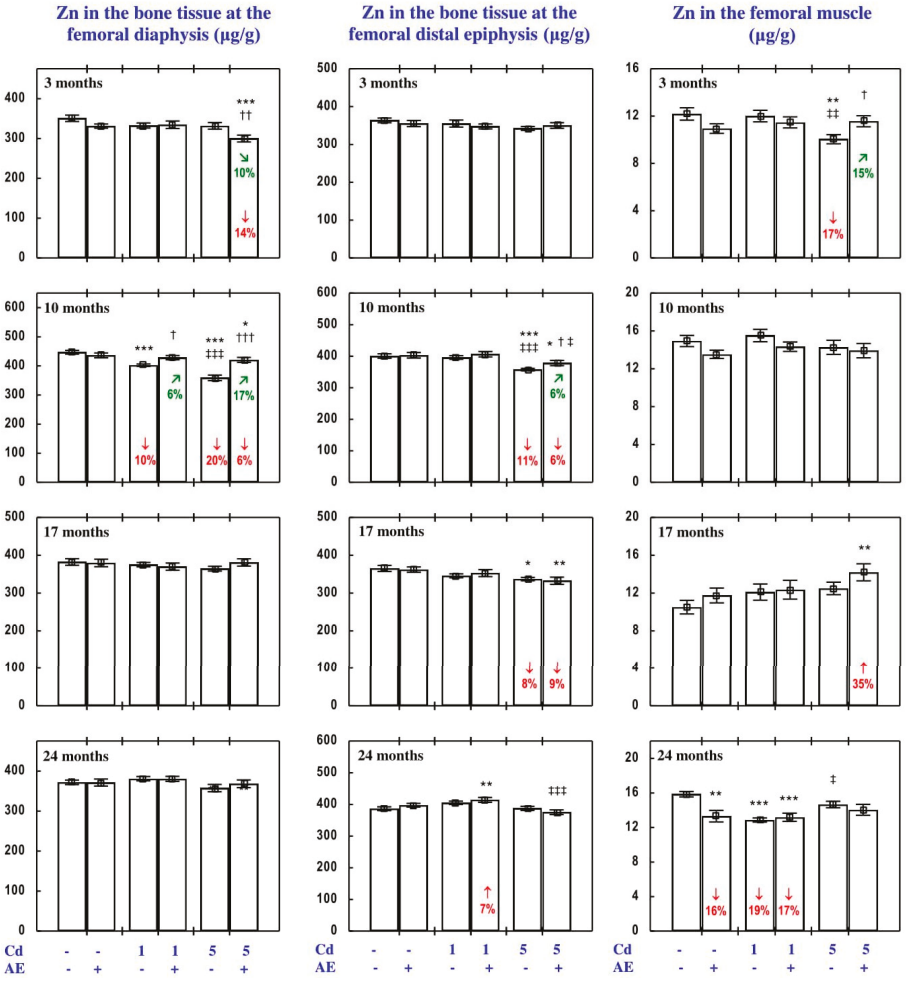

Figure 4. Zinc (Zn) concentration in the bone tissue and femoral muscle in particular experimental groups. The rats received cadmium (Cd) in their diet at the concentration of 0, 1, and 5 mg/kg and/or 0.1% extract from the berries of *Aronia melanocarpa* (AE; "+", received; "-", not received). Data represent mean ± SE for eight rats (except for seven animals in the AE, Cd_1, and Cd_5 groups after 24 months). Statistically significant differences (ANOVA, Duncan's multiple range test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. respective group receiving Cd alone; ‡ $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$ vs. respective group receiving the 1 mg Cd/kg diet (alone or with AE) are marked. Numerical values in bars indicate percentage changes compared to the control group (↓, decrease; ↑, increase) or the respective group receiving Cd alone (↘, decrease; ↗, increase).

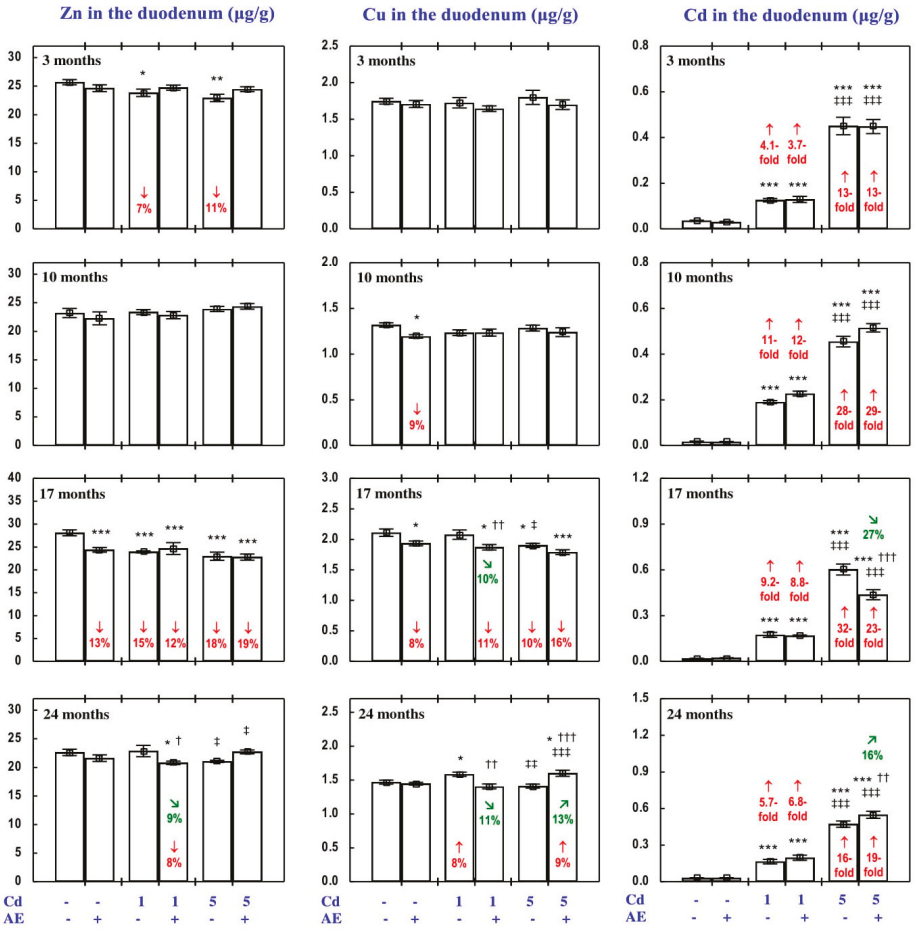

Figure 5. Zinc (Zn), copper (Cu), and cadmium (Cd) concentrations in the duodenum in particular experimental groups. The rats received Cd in their diet at the concentration of 0, 1, and 5 mg/kg and/or 0.1% extract from the berries of *Aronia melanocarpa* (AE; "+", received; "-", not received). Data represent mean ± SE for eight rats (except for seven animals in the AE, Cd_1, and Cd_5 groups after 24 months). Statistically significant differences (ANOVA, Duncan's multiple range test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. respective group receiving Cd alone; ‡ $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$ vs. respective group receiving the 1 mg Cd/kg diet (alone or with AE) are marked. Numerical values in bars (or above the bars) indicate percentage changes or factors of changes compared to the control group (↓, decrease; ↑, increase) or the respective group receiving Cd alone (↘, decrease; ↗, increase).

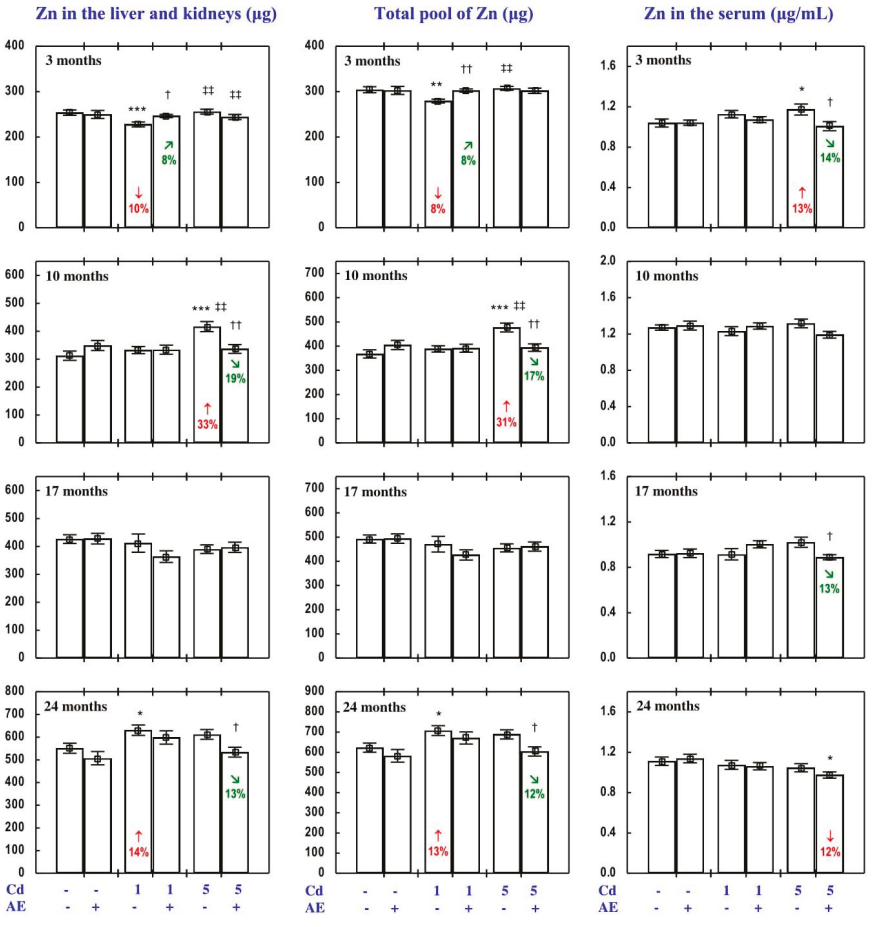

Figure 6. Zinc (Zn) content in the liver and kidneys and its total pool in internal organs, as well as the serum concentration of this element in particular experimental groups. The rats received cadmium (Cd) in their diet at the concentration of 0, 1, and 5 mg/kg and/or 0.1% extract from the berries of *Aronia melanocarpa* (AE; "+", received; "−", not received). Data represent mean ± SE for eight rats (except for seven animals in the AE, Cd_1, and Cd_5 groups after 24 months). Statistically significant differences (ANOVA, Duncan's multiple range test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; † $p < 0.05$, †† $p < 0.01$ vs. respective group receiving Cd alone; ‡‡ $p < 0.01$ vs. respective group receiving the 1 mg Cd/kg diet (alone or with AE) are marked. Numerical values in bars indicate percentage changes compared to the control group (↓, decrease; ↑, increase) or the respective group receiving Cd alone (↘, decrease; ↗, increase).

3.2.3. Zn Content in Internal Organs

The administration of AE alone for up to 24 months had no impact on Zn content in particular internal organs (liver, kidney, heart, spleen, and brain). The total content of this bioelement in the liver and kidneys, as well as its total pool in internal organs did not change, either, as a result of AE consumption (Figure 6, Figures S2 and S3).

The impact of low and moderate exposure to Cd on the total pool of Zn mainly involved changes in the content of this bioelement in the liver and kidneys (Figure 6 and Figure S2). The 3-month

low-level exposure to Cd resulted in a decrease in the total pool of Zn in internal organs (by 8%) connected with a decrease (by 10%) in the total content of this bioelement in the liver and kidneys (resulting from lower Zn content in the liver). The application of the 1 mg Cd/kg diet for 10 and 17 months had no impact on Zn content in particular internal organs and thus its total pool in these organs (Figure 6, Figures S2 and S3). Twenty-four-month intoxication led to an increase in the total pool of Zn in internal organs (by 13%), which was connected with increased (by 14%) total Zn content in the liver and kidneys (determined by a 7% increase in Zn content in the kidneys) and in the heart (determined by a 12% increase; Figure 6 and Figure S2). Ten months of feeding with the 5 mg Cd/kg diet increased (by 31%) the total pool of Zn in internal organs, and the change resulted from its enhanced content in the liver, kidneys, and brain (Figure 6, Figures S2 and S3). The content of Zn in the kidneys also increased after 17 and 24 months of moderate exposure (by 17% and 5%, respectively; Figure S2); however, the total pool of this bioelement in internal organs was unchanged (Figure 6).

The administration of AE under exposure to the 1 and 5 mg Cd/kg diets modified Zn content in particular internal organs and its total pool in these organs (Figure 6, Figures S2 and S3). The extract intake, when treating with the 1 mg Cd/kg diet, completely prevented the abovementioned changes in the total pool of Zn in internal organs and the sum of this bioelement content in the liver and kidneys, as well as in the content of Zn in the liver (after 3 months) and kidney and heart (after 24 months; Figure 6 and Figure S2). The 10-month administration of AE to the animals fed with the diet containing 5 mg Cd/kg entirely prevented the Cd-induced increase in the total pool of Zn in internal organs, the sum of its content in the liver and kidneys, and its content in the liver and brain (Figure 6, Figures S2 and S3). The administration of AE under exposure to the 1 and/or 5 mg Cd/kg diet changed (increased or decreased) the Cd-unaffected content of Zn in some internal organs (liver, heart, spleen, and brain) in comparison to the control/or relevant Cd group (Figure 6, Figures S2 and S3; Supplementary Material—Zinc 3). The ANOVA/MANOVA analysis (Table S7) revealed that the effect of the extract co-administration on the content of Zn in internal organs, including its protective impact against the influence of Cd on this bioelement content in internal organs, was the result of its independent influence ($F = 5.00–9.11$, $p < 0.05–0.01$) and/or interaction with Cd ($F = 5.31–12.6$, $p < 0.05–0.01$). However, in some cases the analysis revealed the lack of a statistically significant independent effect of AE and its interaction with Cd (Table S7) on the content of Zn in particular organs and its total pool in internal organs, in spite of the evident impact of the AE consumption under Cd exposure noticed on the basis of the results of one-way analysis of variance (Duncan's multiple range test) presented in Figure 6, Figures S2 and S3.

3.3. Effect of AE on the Body Status of Cu under Exposure to Cd

3.3.1. Cu Apparent Absorption, Retention in the Body, and Its Faecal and Urinary Excretion

The apparent absorption and body retention of Cu (Abs_{Cu} and Ret_{Cu}, respectively) in the control animals reached 44–57% (Figure 7 and Figure S1). The administration of AE alone had no impact on the Abs_{Cu} and Ret_{Cu} or urinary and faecal excretion of this bioelement (UE_{Cu} and FE_{Cu}, respectively; Figure 7 and Figure S1), with some exceptions (Supplementary Material—Copper 1).

In the rats exposed to the 1 mg Cd/kg diet alone for 3 and 24 months, the Abs_{Cu} and Ret_{Cu} were lower, while the FE_{Cu} was higher compared to the control group (Figure 7 and Figure S1). Three months of exposure to the 5 mg Cd/kg diet decreased the Abs_{Cu} and Ret_{Cu}, and increased the FE_{Cu} (Figure 7 and Figure S1). Low-level exposure to Cd had no impact on the UE_{Cu}, whereas after the 24-month moderate exposure the value of this parameter increased (Figure 7). The consumption of AE by the animals fed with the diets containing Cd modified its influence on the Abs_{Cu}, Ret_{Cu}, FE_{Cu}, and UE_{Cu} (Figure 7 and Figure S1). The administration of AE completely prevented the decrease in the Abs_{Cu} and Ret_{Cu} as well as the increase in the FE_{Cu} caused by 3 and 24 months of exposure to the 1 mg Cd/kg diet (Figure 7 and Figure S1). The consumption of AE by the rats fed with the 5 mg Cd/kg diet completely prevented all Cd-caused changes in the Abs_{Cu}, Ret_{Cu}, FE_{Cu}, and UE_{Cu} (Figure 7 and Figure S1).

The ANOVA/MANOVA analysis (Table S8) revealed that the influence of co-administration of the extract and Cd on the Abs_{Cu}, Ret_{Cu}, FE_{Cu}, and UE_{Cu}, including its protection against the unfavourable effects of Cd, was the result of independent action of the extract ($F = 4.72–29.0$, $p < 0.05–0.001$) and/or interaction of its ingredients with the toxic metal ($F = 4.57–14.8$, $p < 0.05–0.001$). However, at some time points, two-way analysis of variance revealed the lack of a statistically significant independent effect of AE and its interaction with Cd (Table S8) on the Abs_{Cu}, Ret_{Cu}, FE_{Cu}, and UE_{Cu}, in spite of the evident impact of administration of the extract under Cd exposure observed on the basis of the findings of one-way analysis of variance (Duncan's multiple range test), presented in Figure 7 and Figure S1.

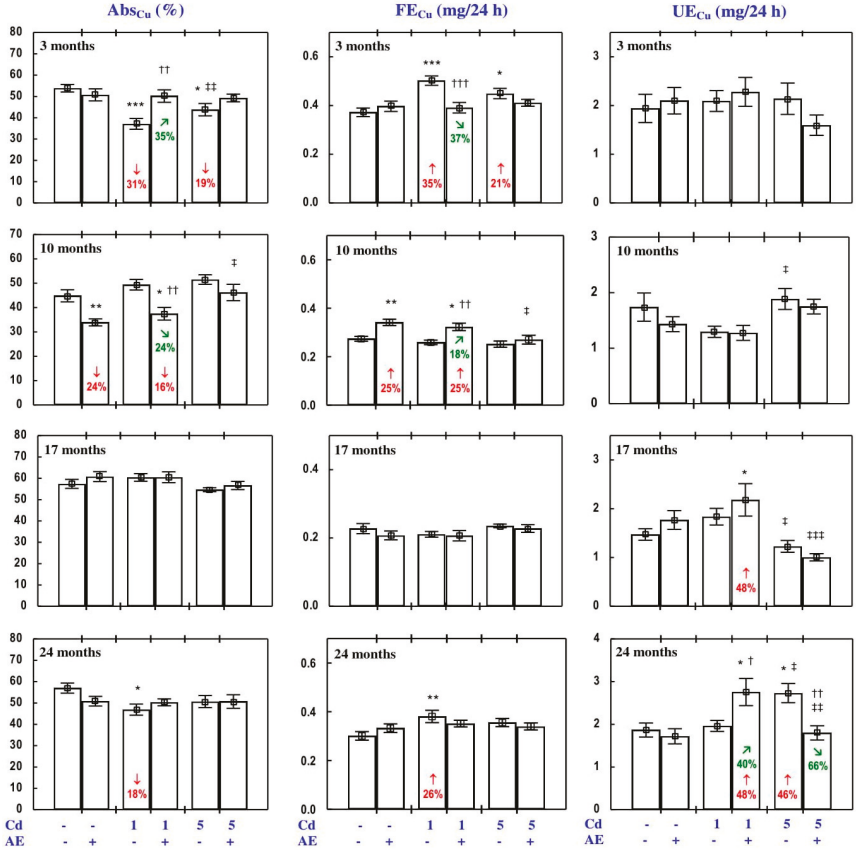

Figure 7. The apparent absorption of copper (Abs_{Cu}) and its daily faecal (FE_{Cu}) and urinary excretion (UE_{Cu}) in particular experimental groups. The rats received cadmium (Cd) in their diet at the concentration of 0, 1, and 5 mg/kg and/or 0.1% extract from the berries of *Aronia melanocarpa* (AE; "+", received; "-", not received). Data represent mean ± SE for eight rats (except for seven animals in the AE, Cd_1, and Cd_5 groups after 24 months). Statistically significant differences (ANOVA, Duncan's multiple range test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. respective group receiving Cd alone; ‡ $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$ vs. respective group receiving the 1 mg Cd/kg diet (alone or with AE) are marked. Numerical values in bars indicate percentage changes compared to the control group (↓, decrease; ↑, increase) or the respective group receiving Cd alone (↘, decrease; ↗, increase).

3.3.2. Cu Concentration in the Serum and Tissues

The administration of AE alone for up to 24 months had no impact on Cu concentration in the serum, soft tissues, and bone tissue (Figures 5 and 8–11), with a few exceptions (Supplementary Material—Copper 2).

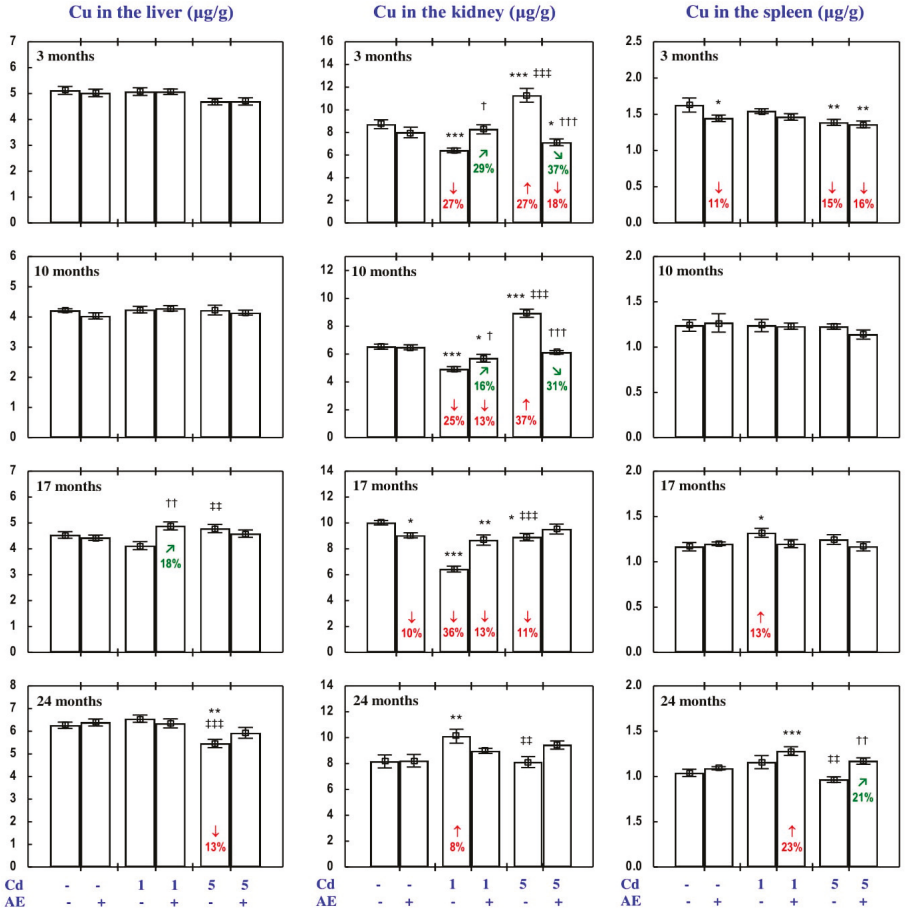

Figure 8. Copper (Cu) concentration in the liver, kidney, and spleen in particular experimental groups. The rats received cadmium (Cd) in their diet at the concentration of 0, 1, and 5 mg/kg and/or 0.1% extract from the berries of *Aronia melanocarpa* (AE; "+", received; "-", not received). Data represent mean ± SE for eight rats (except for seven animals in the AE, Cd_1, and Cd_5 groups after 24 months). Statistically significant differences (ANOVA, Duncan's multiple range test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. respective group receiving Cd alone; ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$ vs. respective group receiving the 1 mg Cd/kg diet (alone or with AE) are marked. Numerical values in bars indicate percentage changes or factors of changes compared to the control group (↓, decrease; ↑, increase) or the respective group receiving Cd alone (↘, decrease; ↗, increase).

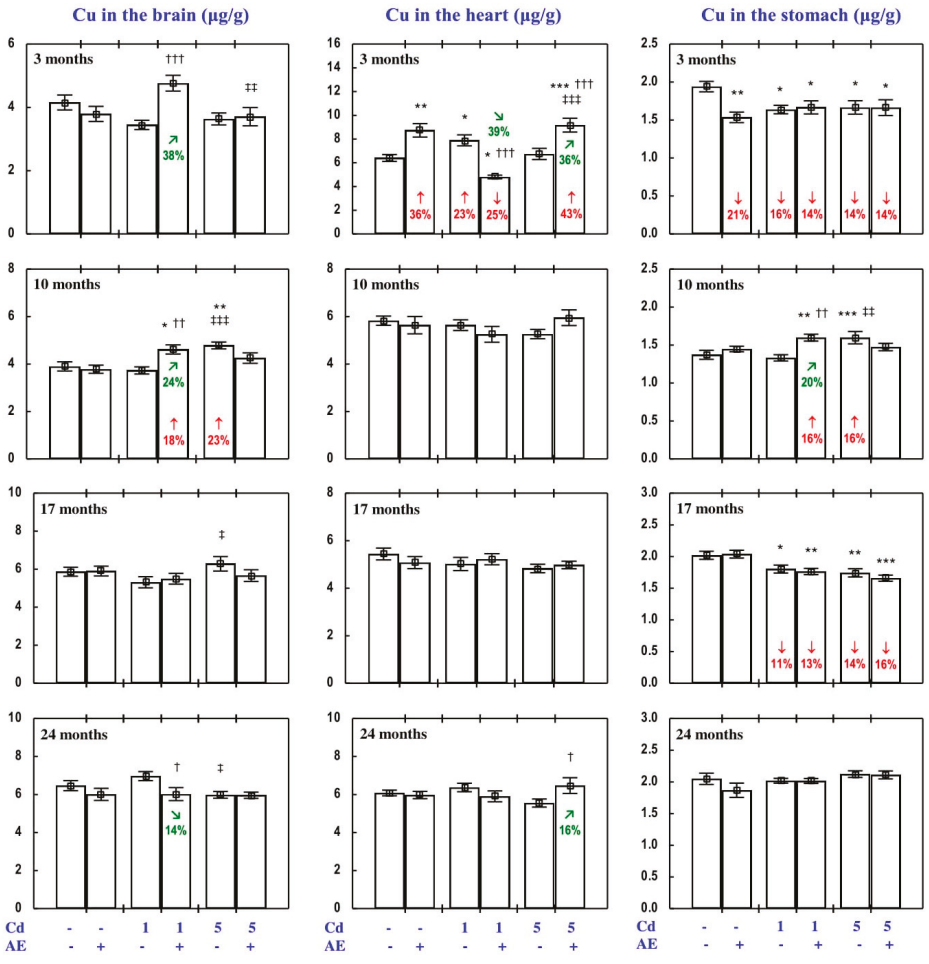

Figure 9. Copper (Cu) concentration in the brain, heart, and stomach in particular experimental groups. The rats received cadmium (Cd) in their diet at the concentration of 0, 1, and 5 mg/kg and/or 0.1% extract from the berries of *Aronia melanocarpa* (AE; "+", received; "-", not received). Data represent mean ± SE for eight rats (except for seven animals in the AE, Cd_1, and Cd_5 groups after 24 months). Statistically significant differences (ANOVA, Duncan's multiple range test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. respective group receiving Cd alone; ‡ $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$ vs. respective group receiving the 1 mg Cd/kg diet (alone or with AE) are marked. Numerical values in bars (or above the bars) indicate percentage changes compared to the control group (↓, decrease; ↑, increase) or the respective group receiving Cd alone (↘, decrease; ↗, increase).

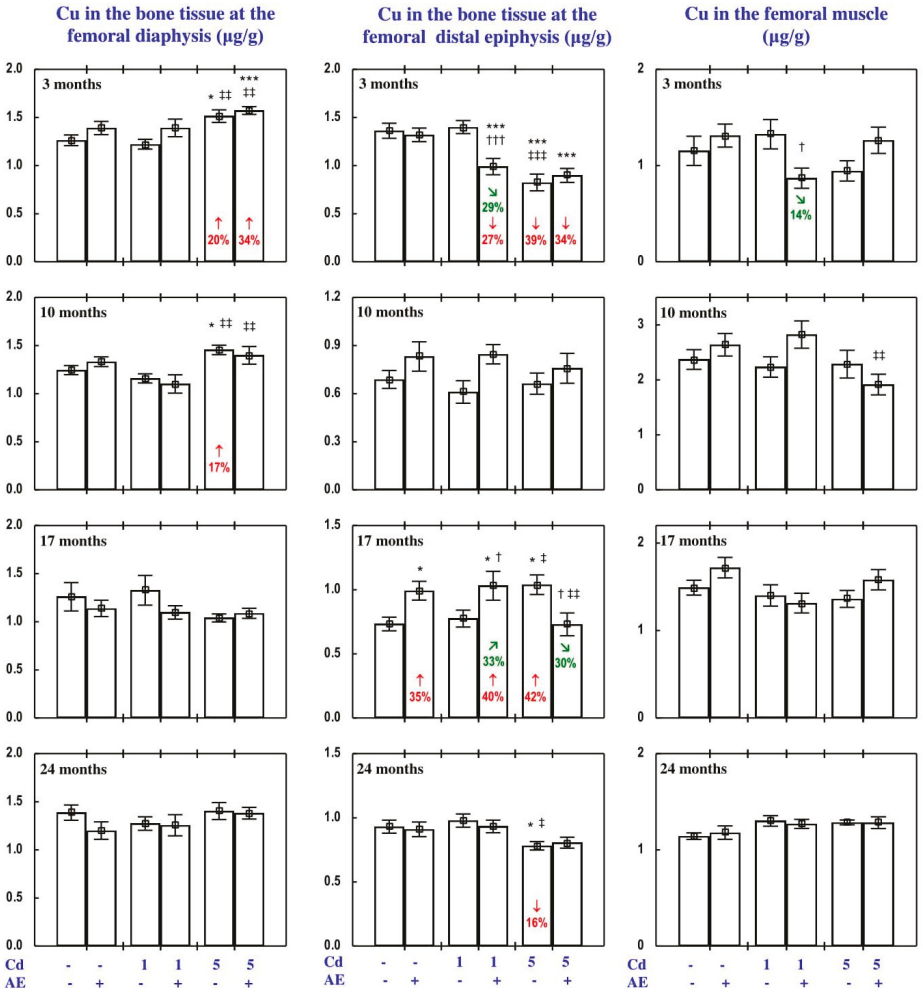

Figure 10. Copper (Cu) concentration in the bone tissue and femoral muscle in particular experimental groups. The rats received cadmium (Cd) in their diet at the concentration of 0, 1, and 5 mg/kg and/or 0.1% extract from the berries of *Aronia melanocarpa* (AE; "+", received; "-", not received). Data represent mean ± SE for eight rats (except for seven animals in the AE, Cd_1 and Cd_5 groups after 24 months). Statistically significant differences (ANOVA, Duncan's multiple range test): * $p < 0.05$, *** $p < 0.001$ vs. control group; † $p < 0.05$, ††† $p < 0.001$ vs. respective group receiving Cd alone; ‡ $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$ vs. respective group receiving the 1 mg Cd/kg diet (alone or with AE) are marked. Numerical values in bars indicate percentage changes compared to the control group (↓, decrease; ↑, increase) or the respective group receiving Cd alone (↘, decrease; ↗, increase).

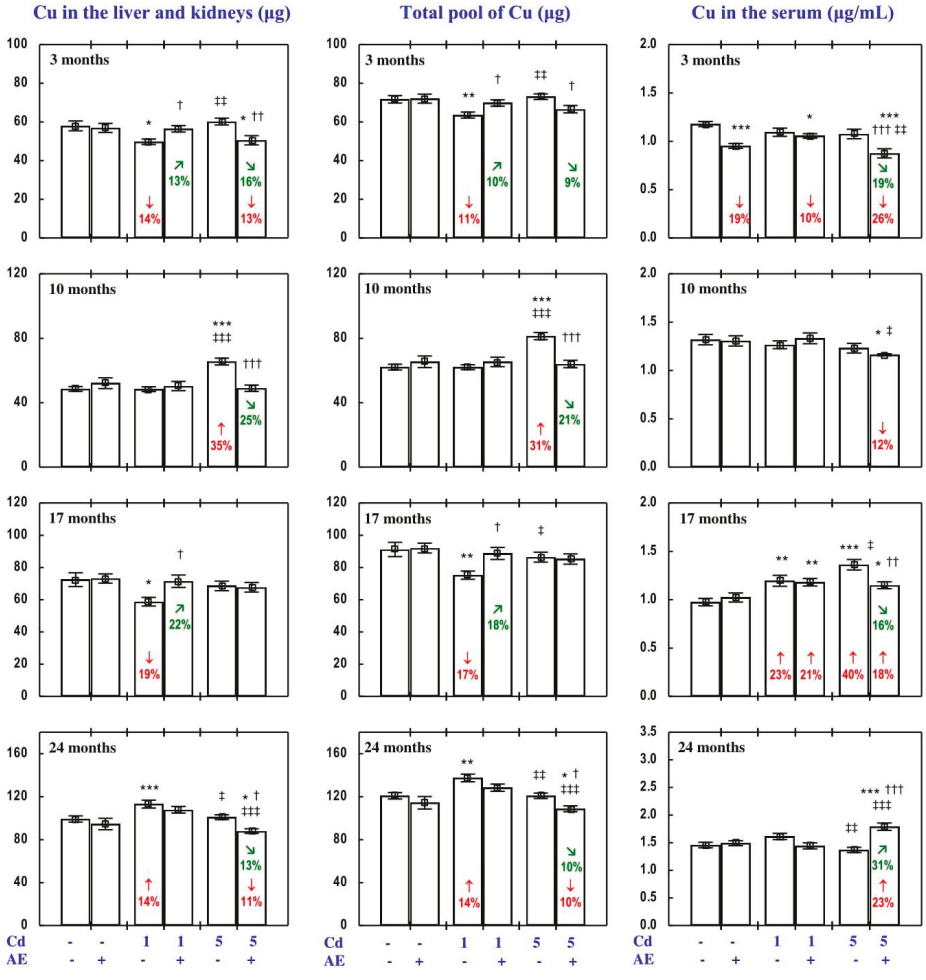

Figure 11. Copper (Cu) content in the liver and kidneys and its total pool in internal organs, as well as the serum concentration of this element in particular experimental groups. The rats received cadmium (Cd) in their diet at the concentration of 0, 1, and 5 mg/kg and/or 0.1% extract from the berries of *Aronia melanocarpa* (AE; "+", received; "-", not received). Data represent mean ± SE for eight rats (except for seven animals in the AE, Cd_1 and Cd_5 groups after 24 months). Statistically significant differences (ANOVA, Duncan's multiple range test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. respective group receiving Cd alone; ‡ $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$ vs. respective group receiving the 1 mg Cd/kg diet (alone or with AE) are marked. Numerical values in bars indicate percentage changes compared to the control group (↓, decrease; ↑, increase) or the respective group receiving Cd alone (↘, decrease; ↗, increase).

As is presented in detail in Figures 5 and 8–11, exposure to the 1 and 5 mg Cd/kg diets alone influenced (increased or decreased) Cu concentration in the serum, soft tissues, and bone tissue depending on the level of the treatment and its duration (Figures 5 and 8–11). In the rats fed with the 1 mg Cd/kg diet alone, the most important change was a decrease in the kidney concentration of Cu after 3, 10, and 17 months, and its increase after 24 months (Figure 8). Exposure to the 5 mg Cd/kg diet alone resulted in very varied transitional changes in tissue Cu concentrations (Figures 5 and 8–11).

Three months of moderate exposure to Cd decreased and increased the concentration of this bioelement in the spleen and kidney, respectively (Figure 8). Moreover, the kidney concentration of Cu increased after 10 months of the treatment and decreased after 17 months (Figure 8). Exposure to the 5 mg Cd/kg diet alone only decreased Cu concentration in the liver after 24 months (Figure 8). Pronounced changes of Cu concentration were also evident in the bone tissue; they depended on the duration of exposure and the kind of the bone tissue (Figure 10). Moderate Cd treatment increased Cu concentration in the bone tissue at the femoral diaphysis after 3 and 10 months, while its concentration in the bone tissue at the femoral distal epiphysis decreased after 3 and 24 months and increased after 17 months (Figure 10).

The administration of AE under exposure to the 1 and 5 mg Cd/kg diets modified Cu concentration in the serum and tissues (Figures 5 and 8–11). The administration of the extract to the rats fed with the 1 mg Cd/kg diet for 3 and 10 months prevented (entirely and partially, respectively) against the Cd-induced decrease in kidney Cu concentration, but it had no protective impact against its decrease after 17 months (however, Cu concentration in the Cd_1 + AE group was lower by 13% than that in the control group, whereas in the Cd_1 group it decreased by 36%), and after 24 months it completely prevented the Cd-caused increase in the concentration of this bioelement in the kidney and duodenum (Figures 5 and 8). AE administration during the 3 months of exposure to the 1 mg Cd/kg diet decreased the Cd-enhanced Cu concentration in the heart compared to the control group and Cd_1 group (Figure 9). The administration of AE under the treatment with the 5 mg Cd/kg diet completely prevented the Cd-induced increase in Cu concentration in the kidney after 3 and 10 months as well as the decrease in this bioelement concentration in the kidney after 17 months; however, the kidney Cu concentration in the Cd_5 + AE group after 3 months was lower than that in the control group (Figure 8). Moreover, the intake of the extract under the moderate Cd treatment completely prevented the Cd-induced increase in Cu concentration in the brain and stomach after 10 months (Figure 9), and in bone tissue at the femoral diaphysis and distal epiphysis after 10 and 17 months, respectively (Figure 10), as well as the decrease in Cu concentration in the liver (Figure 8) and bone tissue at the femoral distal epiphysis caused by 24 months of exposure (Figure 10). As is evident from the data presented in Figures 5 and 8–11, the administration of AE under the moderate exposure to Cd did not protect against all changes in Cu concentrations, but the concentration of this bioelement was modified (decreased or increased) in the serum and various tissues that were unchanged by this heavy metal (Supplementary Material—Copper 2). According to the results of the ANOVA/MANOVA analysis (Tables S9 and S10), the modifying impact of AE consumption on Cu concentration in the serum and tissues under the low-level and moderate exposure to Cd was the result of independent action of the extract ($F = 4.62–48.3$, $p < 0.05–0.001$) and/or interaction of its ingredients with this toxic metal ($F = 5.28–46.0$, $p < 0.05–0.001$). However, at some time points, the two-way analysis of variance revealed the lack of a statistically significant independent effect of AE and its interaction with Cd (Tables S9 and S10) on serum and tissue Cu concentrations, in spite of the evident impact of the administration of the extract under Cd treatment observed on the basis of the findings of one-way analysis of variance (Duncan's multiple range test), presented in Figures 5 and 8–11.

3.3.3. Cu Content in Internal Organs

The administration of AE alone for up to 24 months had no impact on Cu content in particular internal organs (liver, kidney, heart, spleen, brain), nor on the total content of this bioelement in the liver and kidneys, nor its total pool in internal organs (Figure 11, Figures S4 and S5).

The impact of low and moderate exposure to Cd on the total pool of Cu mainly involved changes in the content of this bioelement in the liver and kidneys (Figure 11 and Figure S4). Low-level exposure to Cd decreased the total pool of Cu in internal organs after 3 and 17 months (Figure 11), which resulted from a decrease in its content in the kidney (by 29% and 34%, respectively) and thus also the sum of its content in the liver and kidneys (Figure 11 and Figure S4). Twenty-four months of feeding with the 1 mg Cd/kg diet increased the total pool of this bioelement in internal organs, which was connected with increased Cu content in the liver and kidneys in total, as well as in the

heart (Figure 11 and Figure S4). Moreover, low-level exposure to Cd decreased (by 44%) the kidney content of Cu after 10 months, but in spite of that its total pool in internal organs was unchanged (Figure 11 and Figure S4). Moderate exposure to Cd for 3 months increased Cu content in the kidney (by 27%), but this change had no impact on the total pool of this bioelement in internal organs. Ten months of exposure to the 5 mg Cd/kg diet resulted in an increase in Cu content in the liver, kidney, and brain, as well as the sum of its content in the liver and kidneys and the total pool of this bioelement in internal organs (Figure 11 and Figure S4). Seventeen and 24 months of moderate treatment with Cd had no influence on the content of Cu in any of the estimated internal organs, nor on the total content of this bioelement in the liver and kidneys, nor its total pool in internal organs (Figure 11, Figures S4 and S5).

The administration of AE during the exposure to the 1 mg Cd/kg diet entirely prevented the decrease in the sum of Cu content in the liver and kidneys and the total pool of this bioelement in internal organs after 3 and 17 months induced by this toxic metal, as well as the increase in these contents after 24 months (Figure 11). The extract consumption completely prevented the decrease in Cu content in the kidney caused by 3 and 10 months of low-level exposure to Cd, as well as the increase in heart Cu content after 24 months (Figure S4). The administration of AE accompanying feeding with the diet containing 5 mg Cd/kg completely prevented this toxic metal-induced increase in the content of Cu in the liver and kidney alone, the total content of Cu in both organs together, and its total pool in internal organs after 10 months. Moreover, the extract consumption changed (increased or decreased) the content of Cu in some internal organs that were unaffected by Cd (Supplementary Material—Copper 3). The ANOVA/MANOVA analysis (Table S11) revealed that the above influence of *Aronia* extract administration under the low-level and moderate treatment with Cd on the content of Cu in internal organs was the result of independent action of the extract ($F = 4.34–49.1$, $p < 0.05–0.001$) and/or interaction of its ingredients with this xenobiotic ($F = 6.77–54.1$, $p < 0.05–0.001$). However, in some cases, two-way analysis of variance revealed the lack of a statistically significant independent effect of AE and its interaction with Cd (Table S11) on Cu content in organs, in spite of the clear impact of the extract administration under Cd treatment recognised on the basis of the findings of one-way analysis of variance (Duncan's multiple range test) presented in Figure 11, Figures S4 and S5.

3.4. Effect of AE on MT Concentration in the Liver, Kidney, and Duodenum, and the Degree of Zn, Cu, and Cd Binding to This Protein

3.4.1. MT Concentration

The administration of AE alone for up to 24 months had no impact on MT concentration in the liver, kidney, and duodenal tissue (Figure 12).

In the rats fed with the diet containing 1 and 5 mg Cd/kg, the concentration of MT in the liver, kidney, and duodenum at all evaluated time points was markedly higher (from 23% to 2.9-fold) compared to the control group (Figure 12). MT concentration in the liver and kidney was higher than that in the duodenal tissue.

In the animals receiving AE alongside treatment with the 1 and 5 mg Cd/kg diets, the concentration of MT in the liver, kidney, and duodenum at each time point was markedly lower compared to the respective group exposed to Cd alone, and in some of the cases it did not differ from the control group, except for the lack of influence of the extract on this protein's concentration in the kidney in the Cd_5 + AE group after 10 months of the experiment (Figure 12). This indicates that the extract consumption completely or partially prevented the heavy metal-induced increase in the concentration of MT in these tissues. According to the results of ANOVA/MANOVA analysis (Tables S12–S14), the impact of AE on MT concentration in the liver, kidney, and duodenum described above was the result of independent action of the extract ($F = 7.2–54.8$, $p < 0.05–0.001$) and/or interaction of its ingredients with Cd ($F = 4.86–115.2$, $p < 0.05–0.001$), except for the protein's concentration in the duodenum in the Cd_1 + AE group after 10 months of the experiment. Although MT concentration in the duodenal tissue in this group was lower compared to the Cd_1 group and higher than that in the

control group (Figure 12), the ANOVA/MANOVA revealed that neither AE alone nor its interaction with Cd had a significant effect on this protein's concentration (Table S14).

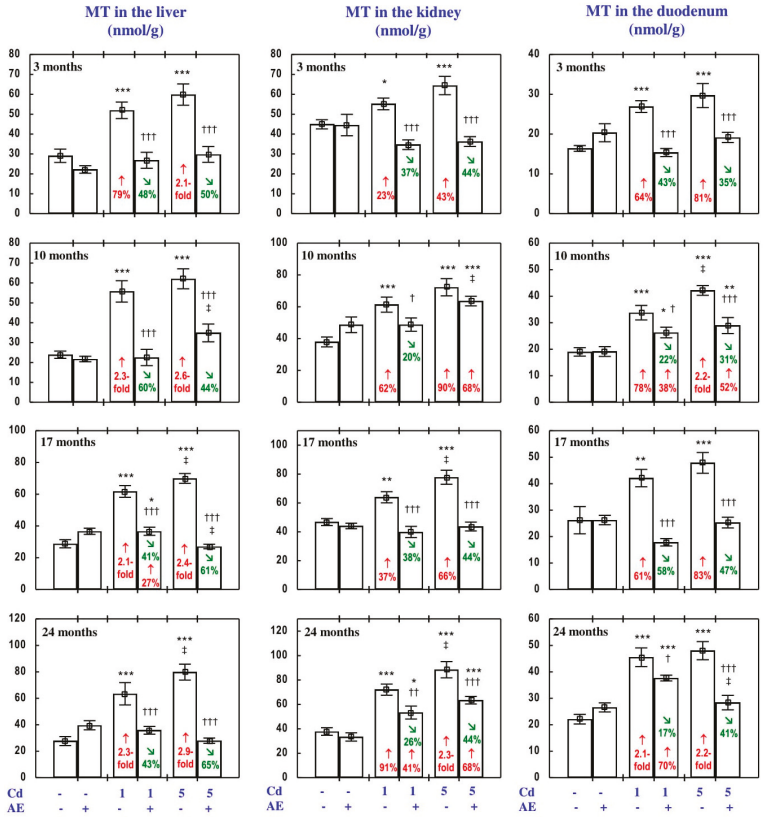

Figure 12. Metallothionein (MT) concentration in the liver, kidney, and duodenum in particular experimental groups. The rats received cadmium (Cd) in their diet at the concentration of 0, 1, and 5 mg/kg and/or 0.1% extract from the berries of *Aronia melanocarpa* (AE; "+", received; "-", not received). Data represent mean ± SE for eight rats (except for seven animals in the AE, Cd_1, and Cd_5 groups after 24 months). Statistically significant differences (ANOVA, Duncan's multiple range test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. respective group receiving Cd alone; ‡ $p < 0.05$ vs. respective group receiving the 1 mg Cd/kg diet (alone or with AE) are marked. Numerical values in bars indicate percentage changes or factors of changes compared to the control group (↑, increase) or the respective group receiving Cd alone (↘, decrease).

3.4.2. The Degree of Zn, Cu, and Cd Binding to MT

The administration of AE alone decreased the pool of non-MT-bound Zn and Cu (Zn/(MT × 7) and Cu/(MT × 12), respectively) and the total pool of MT-unbound Zn, Cu, and Cd (Me/Me-MT) in the liver after 17 and 24 months (Table 3) and in the kidney after 10 months (Table 4), as well as Me/Me-MT in the duodenum after 10 months and the duodenal ratios of Zn/(MT × 7) and Cu/(MT × 12) after 17 and 24 months, compared to the control group (Table 5). The administration of the extract alone throughout the whole experimental period had no impact on MT saturation with Cd (Cd/(MT × 7); Tables 3–5).

Table 3. Effect of the extract from the berries of *Aronia melanocarpa* (AE) and/or cadmium (Cd) on the degree of zinc (Zn), copper (Cu), and Cd binding to metallothionein (MT) in the liver [1,2,3].

Metals Binding to MT in the Liver	Effect of AE Alone	1 mg Cd/kg Diet + AE			5 mg Cd/kg Diet + AE		
		Effect of Cd Alone	Cd + AE		Effect of Cd Alone	Cd + AE	
			Effect of Cd + AE	Effect of AE		Effect of Cd + AE	Effect of AE
3 months							
Zn/(MT × 7)	↔	↓ 48%	↔	↗ 2.1-fold	↓ 53%	↔	↗ 2.2-fold
Cu/(MT × 12)	↔	↓ 47%	↔	↗ 2.1-fold	↓ 57%	↔	↗ 2.2-fold
Cd/(MT × 7)	↔	↔	↔	↔	↑ 11.6-fold	↑ 21.3-fold	↗ 84%
Me/(Me-MT)	↔	↓ 48%	↔	↗ 2.1-fold	↓ 53%	↔	↗ 2.2-fold
10 months							
Zn/(MT × 7)	↔	↓ 55%	↔	↗ 2.7-fold	↓ 58%	↔	↗ 81%
Cu/(MT × 12)	↔	↓ 55%	↔	↗ 2.8-fold	↓ 61%	↓ 29%	↗ 85%
Cd/(MT × 7)	↔	↔	↑ 9.4-fold	↗ 2.4-fold	↑ 25.8-fold	↑ 42.4-fold	↗ 64%
Me/(Me-MT)	↔	↓ 55%	↔	↗ 2.7-fold	↓ 57%	↔	↗ 81%
17 months							
Zn/(MT × 7)	↓ 30%	↓ 57%	↓ 36%	↔	↓ 63%	↔	↗ 2.6-fold
Cu/(MT × 12)	↓ 27%	↓ 60%	↓ 18%	↗ 2.0-fold	↓ 58%	↔	↗ 2.5-fold
Cd/(MT × 7)	↔	↔	↔	↔	↑ 68.7-fold	↑ 136-fold	↗ 98%
Me/(Me-MT)	↓ 30%	↓ 57%	↓ 35%	↗ 52%	↓ 61%	↔	↗ 2.6-fold
24 months							
Zn/(MT × 7)	↓ 34%	↓ 52%	↓ 24%	↗ 57%	↓ 69%	↔	↗ 3.1-fold
Cu/(MT × 12)	↓ 32%	↓ 54%	↓ 26%	↗ 59%	↓ 71%	↔	↗ 3.1-fold
Cd/(MT × 7)	↔	↔	↔	↔	↑ 66-fold	↑ 165-fold	↗ 2.5-fold
Me/(Me-MT)	↓ 34%	↓ 52%	↓ 24%	↗ 57%	↓ 68%	↔	↗ 3.1-fold

[1] The rats received the 0.1% aqueous AE or not and Cd in their diet at the concentration of 0, 1, and 5 mg/kg. [2] In this table, only statistically significant ($p < 0.05$) changes compared to the control group (↓, percentage decrease; ↑, percentage increase or a factor of increase) and the respective group that received Cd alone (↗, percentage increase or a factor of increase) are indicated. ↔, without change ($p > 0.05$) compared to the control group; ↔, without change ($p > 0.05$) compared to the respective group treated with Cd alone. The Zn/(MT × 7) in the liver of the control group reached 2.182 ± 0.222, 2.927 ± 0.166, 2.749 ± 0.259, and 3.334 ± 0.403, after 3, 10, 17, and 24 months, respectively, whereas the Cu/(MT × 12) was 0.249 ± 0.026, 0.238 ± 0.017, 0.218 ± 0.023, and 0.324 ± 0.036, respectively. The Cd/(MT × 7) reached 0.0017 ± 0.0002, 0.0013 ± 0.00005, 0.0006 ± 0.00006, and 0.0007 ± 0.0001, while the Me/(Me-MT) was 1.297 ± 0.062, 2.888 ± 0.214, 3.044 ± 0.442, and 2.468 ± 0.243 after 3, 10, 17, and 24 months, respectively. [3] An increase in the Zn/(MT × 7), Cu/(MT × 12), Cd/(MT × 7), or Me/Me-MT ratios compared to the control group or any other experimental group indicates a rise in the pool of MT-unbound metals (Zn, Cu, Cd, or all of the metals, respectively), while a decrease in these ratios reflects a drop in the pool of non-MT-bound metals. Zn/(MT × 7), the pool of MT-unbound Zn; Cu/(MT × 12), the pool of MT-unbound Cu; Cd/(MT × 7), the pool of MT-unbound Cd; Me/(Me-MT), the pool of MT-unbound metals (Zn, Cu, and Cd).

Table 4. Effect of the extract from the berries of *Aronia melanocarpa* (AE) and/or cadmium (Cd) on the degree of zinc (Zn), copper (Cu), and Cd binding to metallothionein (MT) in the kidney [1,2,3].

Metals Binding to MT in the Kidney	Effect of AE Alone	1 mg Cd/kg Diet + AE			5 mg Cd/kg Diet + AE		
		Effect of Cd Alone	Cd + AE		Effect of Cd Alone	Cd + AE	
			Effect of Cd + AE	Effect of AE		Effect of Cd + AE	Effect of AE
3 months							
Zn/(MT × 7)	↔	↔	↔	↗ 57%	↔	↓ 33%	↗ 73%
Cu/(MT × 12)	↔	↓ 40%	↔	↗ 2.1-fold	↔	↔	↔
Cd/(MT × 7)	↔	↑ 7.4-fold	↑ 8.5-fold	↔	↑ 25.2-fold	↑ 39.1-fold	↗ 55%
Me/(Me-MT)	↔	↔	↔	↗ 65%	↔	↔	↗ 56%
10 months							
Zn/(MT × 7)	↓ 22%	↓ 41%	↓ 24%	↗ 29%	↓ 47%	↓ 35%	↔
Cu/(MT × 12)	↓ 20%	↓ 53%	↓ 29%	↗ 50%	↓ 28%	↓ 45%	↔
Cd/(MT × 7)	↔	↑ 13.3-fold	↑ 33.6-fold	↔	↑ 47.9-fold	↑ 51.3-fold	↔
Me/(Me-MT)	↓ 20%	↓ 42%	↓ 23%	↗ 32%	↓ 39%	↓ 31%	↔
17 months							
Zn/(MT × 7)	↔	↓ 26%	↓ 24%	↗ 67%	↑ 48%	↑ 29%	↘ 13%
Cu/(MT × 12)	↔	↓ 53%	↔	↗ 2.2-fold	↓ 46%	↔	↗ 91%
Cd/(MT × 7)	↔	↔	↑ 33.6-fold	↔	↑ 139-fold	↑ 223-fold	↗ 61%
Me/(Me-MT)	↔	↓ 29%	↑ 23%	↗ 26%	↓ 21%	↑ 42%	↗ 81%
24 months							
Zn/(MT × 7)	↔	↓ 42%	↓ 29%	↔	↓ 56%	↓ 30%	↗ 90%
Cu/(MT × 12)	↔	↓ 34%	↔	↔	↓ 58%	↓ 32%	↗ 62%
Cd/(MT × 7)	↔	↑ 12.1-fold	↑ 17.1-fold	↔	↑ 41.1-fold	↑ 51.3-fold	↗ 25%
Me/(Me-MT)	↔	↓ 39%	↓ 26%	↔	↓ 50%	↓ 24%	↗ 54%

[1] The rats received the 0.1% aqueous AE or not and Cd in their diet at the concentration of 0, 1, and 5 mg/kg. [2] In this table, only statistically significant ($p < 0.05$) changes compared to the control group (↓, percentage decrease; ↑, percentage increase or a factor of increase) and the respective group that received Cd alone (↘, percentage decrease; ↗, percentage increase or a factor of increase) are indicated. ↔, without change ($p > 0.05$) compared to the control group; ↔, without change ($p > 0.05$) compared to the respective group treated with Cd alone. The Zn/(MT × 7) in the kidney of the control group reached 1.039 ± 0.05, 1.364 ± 0.08, 1.286 ± 0.069, and 1.830 ± 0.106, after 3, 10, 17, and 24 months, respectively, whereas the Cu/(MT × 12) was 0.257 ± 0.016, 0.232 ± 0.016, 0.284 ± 0.015, and 0.289 ± 0.022. The Cd/(MT × 7) reached 0.001 ± 0.0001, 0.002 ± 0.0002, 0.001 ± 0.0001, and 0.003 ± 0.0003, while the Me/(Me-MT) was 1.297 ± 0.062, 1.598 ± 0.095, 1.571 ± 0.083, and 2.122 ± 0.125 after 3, 10, 17, and 24 months, respectively. [3] An increase in the Zn/(MT × 7), Cu/(MT × 12), Cd/(MT × 7), or Me/Me-MT ratios compared to the control group or any other experimental group indicates a rise in the pool of MT-unbound metals (Zn, Cu, Cd, or all of the metals, respectively), while a decrease in these ratios reflects a drop in the pool of non-MT-bound metals. Zn/(MT × 7), the pool of MT-unbound Zn; Cu/(MT × 12), the pool of the MT-unbound Cu; Cd/(MT × 7), the pool of the MT-unbound Cd; Me/(Me-MT), the pool of MT-unbound metals (Zn, Cu, and Cd).

Table 5. Effect of the extract from the berries of *Aronia melanocarpa* (AE) and/or cadmium (Cd) on the degree of zinc (Zn), copper (Cu), and Cd binding to metallothionein (MT) in the duodenum [1,2,3].

Metals Binding to MT in the Duodenum	Effect of AE Alone	1 mg Cd/kg Diet + AE			5 mg Cd/kg Diet + AE		
		Effect of Cd Alone	Cd + AE		Effect of Cd Alone	Cd + AE	
			Effect of Cd + AE	Effect of AE		Effect of Cd + AE	Effect of AE
3 months							
Zn/(MT × 7)	↔	↑ 64%	↔	↗ 81%	↑ 81%	↔	↗ 62%
Cu/(MT × 12)	↔	↓ 39%	↔	↗ 66%	↓ 41%	↔	↗ 42%
Cd/(MT × 7)	↔	↑ 2.1-fold	↑ 3.8-fold	↗ 78%	↑ 7.2-fold	↑ 10.7-fold	↗ 50%
Me/(Me-MT)	↔	↔	↔	↗ 65%	↔	↔	↗ 56%
10 months							
Zn/(MT × 7)	↔	↓ 43%	↓ 28%	↔	↓ 55%	↓ 26%	↗ 64%
Cu/(MT × 12)	↔	↓ 47%	↓ 32%	↔	↓ 58%	↓ 34%	↗ 57%
Cd/(MT × 7)	↔	↑ 6.2-fold	↑ 9.7-fold	↗ 57%	↑ 11.6-fold	↑ 19.9-fold	↗ 72%
Me/(Me-MT)	↓ 22%	↓ 42%	↓ 23%	↗ 32%	↓ 39%	↓ 31%	↔
17 months							
Zn/(MT × 7)	↓ 28%	↓ 55%	↔	↗ 2.4-fold	↓ 62%	↓ 29%	↗ 88%
Cu/(MT × 12)	↓ 23%	↓ 48%	↔	↗ 2.1-fold	↓ 58%	↓ 24%	↗ 80%
Cd/(MT × 7)	↔	↑ 5.3-fold	↑ 12.5-fold	↗ 2.4-fold	↑ 16.5-fold	↑ 22.6-fold	↗ 37%
Me/(Me-MT)	↔	↓ 29%	↑ 23%	↗ 74%	↓ 21%	↓ 42%	↗ 81%
24 months							
Zn/(MT × 7)	↓ 22%	↓ 52%	↓ 49%	↔	↓ 58%	↓ 20%	↗ 90%
Cu/(MT × 12)	↓ 20%	↓ 48%	↓ 46%	↔	↓ 56%	↔	↗ 95%
Cd/(MT × 7)	↔	↔	↑ 3.9-fold	↔	↑ 7.5-fold	↑ 15.4-fold	↗ 2-fold
Me/(Me-MT)	↔	↓ 39%	↓ 26%	↔	↓ 50%	↓ 24%	↗ 54%

[1] The rats received the 0.1% aqueous AE or not and Cd in their diet at the concentration of 0, 1, and 5 mg/kg. [2] In this table, only statistically significant ($p < 0.05$) changes compared to the control group (↓, percentage decrease; ↑, percentage increase or a factor of increase) and the respective group that received Cd alone (↗, percentage increase or a factor of increase) are indicated. ↔, without change ($p > 0.05$) compared to the control group; ↔, without change ($p > 0.05$) compared to the respective group treated with Cd alone. The Zn/(MT × 7) in the duodenum of the control group reached 3.490 ± 0.160, 2.792 ± 0.207, 2.915 ± 0.424, and 2.375 ± 0.234, after 3, 10, 17, and 24 months, respectively, whereas the Cu/(MT x 12) was reached 0.140 ± 0.005, 0.095 ± 0.007, 0.128 ± 0.017, and 0.091 ± 0.009. The Cd/(MT × 7) reached 0.003 ± 0.0002, 0.0012 ± 0.0001, 0.001 ± 0.0001, and 0.0017 ± 0.0002, while the Me/(Me-MT) was 3.633 ± 0.164, 2.888 ± 0.214, 3.044 ± 0.442, and 2.468 ± 0.243 after 3, 10, 17, and 24 months, respectively. [3] An increase in the Zn/(MT × 7), Cu/(MT × 12), Cd/(MT × 7), or Me/Me-MT ratios compared to the control group or any other experimental group indicates a rise in the pool of MT-unbound metals (Zn, Cu, Cd, or all of the metals, respectively), while a decrease in these ratios reflects a drop in the pool of non-MT-bound metals. Zn/(MT × 7), the pool of MT-unbound Zn; Cu/(MT × 12), the pool of MT-unbound Cu; Cd/(MT × 7), the pool of MT-unbound Cd; Me/(Me-MT), the pool of MT-unbound metals (Zn, Cu, and Cd).

Feeding of the rats with the 1 and 5 mg Cd/kg diets changed the degree of Zn, Cu, and Cd binding to MT, as well as the total pool of MT-unbound metals in the liver, kidney, and duodenum in different ways, depending on the level and duration of the treatment (Tables 3–5). Generally, low and moderate exposure to Cd increased the liver, kidney, and duodenal MT saturation with this toxic metal, except for a lack of impact on the pool of non-MT bound Cd in the liver in the case of the low-level treatment (Tables 3–5). Moreover, as is evident from the detailed data presented in Tables 3–5, exposure to both levels of Cd alone decreased the pool of MT-unbound Zn and Cu, as well as the total pool of MT-unbound metals in the liver, kidney, and duodenum, with only a few exceptions (Tables 3–5).

The administration of AE to the animals fed with the diets containing 1 and 5 mg Cd/kg either had no impact or increased the pool of non-MT-bound Cd in the liver, kidney, and duodenum (Tables 3–5). Moreover, the intake of AE under exposure to Cd significantly (partially or completely), prevented the decrease in the pool of MT-unbound Zn and Cu, as well as in the total pool of metals unbound to this protein, induced by Cd (Tables 3–5). The ANOVA/MANOVA analysis (Tables S12–S14) revealed that the modifying impact of AE consumption while feeding with the diets containing 1 and 5 mg Cd/kg on the degree of Zn, Cu, and Cd binding to the liver, kidney, and duodenal MT, as well as on the total pool of MT-unbound metals, was the result of independent action of the extract ingredients ($F = 4.22–79.3$, $p < 0.05–0.001$) and/or their interaction with this toxic metal ($F = 4.49–80.1$, $p < 0.05–0.001$).

4. Discussion

The present study is the first to investigate and reveal the modifying effect of prolonged consumption of a polyphenol-rich AE on the body status of Zn and Cu under chronic low-level and moderate exposure to Cd via diet. Although the study was focused first of all on the influence of AE on the metabolism of both bioelements in the conditions of chronic intoxication with this toxic metal, it has also provided new important data on their body status in the case of chokeberry extract consumption under the standard diet, and on the influence of low-level and moderate lifetime exposure to Cd on the metabolism of these necessary elements. It has been reported that repeated excessive exposure to Cd results in the redistribution of Zn and Cu in the organism, mainly consisting in the retention of these bioelements in the liver and kidneys in the MT-bound form and a decrease in their concentrations in the serum and other organs and tissues, including bone tissue [15,22,36,38,52–55]. However, there is no complex evaluation of the influence of long-term low-level exposure on the body status of Zn and Cu, properly reflecting the environmental exposure to Cd occurring in industrialised countries nowadays [14–21,48,49].

The relevance of the used experimental model for investigating the impact of Cd or/and AE on the body status of Zn and Cu is confirmed by the same daily intake of particular bioelements in all groups throughout the experiment, as well as the same intake of Cd at particular levels of exposure regardless of whether this xenobiotic was administered alone or in conjunction with AE, and the same consumption of AE in the groups receiving the extract alone and together with Cd. The fact that the daily intake of Zn and Cu in all of the groups during the first 3 months of the study was higher than that during the other experimental periods resulted from higher Zn and Cu content in the breeding diet administered during the first 3 months, compared to the maintenance diet used thereafter, as well as from lower body mass of the young animals compared to the older ones, with similar food consumption [7].

The present study has revealed that treatment with Cd, reflecting moderate human exposure to this heavy metal (5 mg Cd/kg diet) and corresponding to general population's the low-level exposure in industrialised countries (1 mg Cd/kg diet), may cause disorders in the metabolism of Zn and Cu. Percentage changes in the values of estimated indices of the body status of Zn and Cu induced by the 1 mg Cd/kg diet were relatively low. However, the fact that they occurred at such low exposure levels makes them very important, as Cd is a common and unavoidable pollutant of food products [13]. The decreased total pool of Zn in internal organs and the sum of this bioelement content in the liver and kidneys as a result of 3 months of administration of the diet containing 1 mg Cd/kg, despite the

lack of statistically significant changes of its concentration and content in particular internal organs or in the serum and bone tissue concentrations, indicate a slight deficiency of this bioelement in the organism, associated with its deficiency in the liver and kidneys. The decreased total pool of Zn in internal organs after 3 months of feeding with the 1 mg Cd/kg diet, together with its unaffected total pool after 10 and 17 months, and the fact that dietary intake of this bioelement during the first 3 months of the study was higher than thereafter, suggest that a young organism may be particularly susceptible to Zn deficiency even in the case of low-level exposure to Cd. The slight decrease in the content of Zn in internal organs of the rats fed with the 1 mg Cd/kg diet for 3 months, including the sum of its content in the liver and kidneys (being the main organs of this bioelement storage in the body), may be explained by interactions at the stage of gastrointestinal absorption and transport of both metals in the organism [33,56]. This is confirmed by the decreased Zn concentration in the duodenal tissue and stomach of these animals, as well as being consistent with the available literature data on Cd–Zn interactions, including our own findings [15,22–27,33–39,45,47,52–54]. Interactions between Cd and Zn at the absorption stage from the digestive tract are related, first of all, to metals' ability to both synthesise MT in the duodenum and bind to it [33]. Moreover, these elements may compete for uptake by this protein and zinc-regulated transporter proteins [33,56]. The unchanged value of the Abs_{Zn} in the Cd_1 group after 3 months, reflecting Zn bioavailability from the digestive tract (estimated based on the difference between its daily oral intake and faecal excretion), does not rule out a slight decrease in its absorption. The decreased value of the Abs_{Zn} in this experimental group after 10 months confirms the capability of Cd to decrease Zn absorption from the gastrointestinal tract in the case of low-level exposure.

The next very important finding is that low-level lifetime (24 months in our experimental model) exposure to this xenobiotic, unlike exposure in youth only (3 months in our experimental model), may result in the enhanced retention of Zn in the liver and kidneys, and thus also enhance its total pool in internal organs (in spite of its decreased content in the spleen). This is in good agreement with the literature data on this bioelement accumulation in the liver and kidneys under environmental exposure to Cd [15,45,52] and intoxication with this heavy metal in animal models [22,37,45]. The detailed mechanism of Zn retention in the liver and kidneys, which is connected with Cd accumulation and MT biosynthesis in these organs, is widely reported [22,33,37,38]. An increase in the concentrations of Cd and MT in the liver and kidneys may be accompanied with a simultaneous increase in the content of Zn and Cu [22,37], as was observed in the present study in the rats fed for 24 months with the diet containing 1 mg Cd/kg.

It should be emphasised that the lifetime moderate treatment with Cd, unlike the low-level exposure, did not result in Zn retention in the liver and kidneys in spite of the slightly increased (by 5%) concentration of this bioelement in the kidney, as well as Cd accumulation in the liver and kidney [7] and markedly enhanced MT concentration in these organs. However, the higher total pool of Zn in internal organs, as well as its content in the liver and kidney after 10 months of exposure to the 5 mg Cd/kg diet together with the increased Zn content in the kidney after 17 months, show that under moderate exposure to Cd, a rise in the content of Zn in the liver and kidneys did occur as well, but due to higher Cd [7] and MT concentrations in these organs it took place after a shorter period of exposure than in the case of low-level treatment, and it was temporary. The fact that 24 months of moderate exposure to Cd resulted in an unchanged total pool of Zn in internal organs and its content in the liver and kidneys after their previous rise allow us to suppose that in the last phase of lifetime moderate exposure to Cd, interactions between Cd and Zn, including those in the gastrointestinal tract resulting in a decrease in Zn absorption, might occur. Because of the accumulation of Zn in internal organs during the earlier stage of exposure, the possibly decreased intake of this bioelement at the further stage might ensure that the body content of this bioelement is kept at the correct level, as was reflected in the proper concentration and content of Zn in tissues and organs after 24 months. Moreover, detailed analysis of the results on the body status of Zn in the females fed with the 5 mg Cd/kg diet allowed us to conclude that the increased value of the Abs_{Zn} noted after 24 months of the

experiment might result from the enhanced retention of this bioelement in the gastrointestinal tract or might be a compensatory mechanism preventing Zn deficiency in the organism.

The results of the present study show that in the case of low-level exposure to Cd, a young organism is also susceptible to Cu deficiency, like in the case of Zn, while lifetime intoxication with this toxic metal also results in an increase in the total pool of this bioelement in internal organs. Both the deficiency of Cu at a young age and its increased total pool in internal organs due to lifetime low-level exposure were associated with a change of its concentration and content in the kidneys. The decreased Abs_{Cu} and Ret_{Cu} in the young animals fed for 3 months with the diet containing 1 mg Cd/kg confirm that decreased gastrointestinal absorption of Cu and its lower retention in the body were the cause of the lower pool of this element in internal organs, mostly determined by its decreased content in the kidneys. The clear tendency to increase the Cu content in the kidney of the animals treated with the 1 mg Cd/kg diet for 24 months, together with the higher sum of its content in the liver and kidneys in spite of the unchanged concentration and content of this element in the liver, show that the increase in the total pool of Cu in internal organs of these animals is connected with its accumulation in the kidney. The possible mechanism explaining the changes of the body status of Cu seems to be analogous to the case of Zn; however, under lifetime low-level exposure, Cu was mainly retained in the kidneys, while Zn was mainly retained in the liver and kidneys.

The findings of the present study on the impact of 24 months of administration of a diet containing 1 mg Cd/kg on the liver and kidney status of Zn and Cu are in agreement with the results obtained by Noël and others [47] in female rats exposed for 13 weeks to a 10 mg Cd/kg diet and by Piasek and others [53] in pregnant rats intoxicated via diet with 3 and 5 mg Cd/kg b.w. Moreover, they are consistent with our previous results in male rats treated with 50 mg Cd/L in drinking water for 12 weeks [38] and 6 months [22].

Based on the results of the present study, it can be concluded that under low-level and moderate chronic exposure to Cd, changes may occur in the bone concentrations of bioelements necessary for the proper metabolism of the bone tissue, such as Zn and Cu [57], and the changes depend on the level and duration of exposure on the one hand and the kind of bone tissue on the other hand. Generally, the changes noticed in the bone concentrations of Zn and Cu consisted in their deficiency (with only a few exceptions). The decrease in the bone concentrations of Zn and Cu may be explained by Cd-induced disorders in the bone tissue metabolism (inhibition of bone formation and stimulation of bone resorption) noted by us in the females maintained on the 1 and 5 mg Cd/kg diets [5,9], and it may be related to the impact of this xenobiotic on the body status of these bioelements. However, based on our knowledge, we are unable to explain the proper reason for the increase in the bone concentration of Cu. Detailed analysis of the results on the impact of Cd on the bone tissue concentrations of Zn and Cu shows that the low-level lifetime exposure to this heavy metal may only cause a slight and transient decrease in Zn concentration in the regions of the skeleton where compact bone predominates and may have no impact on the concentration of Cu in both trabecular and compact bone regions of the skeleton, while moderate exposure may affect the concentrations of Zn and Cu in both kinds of bone tissue. However, the impact may also be transient and, after a very long period of exposure (24 months), only the concentration of Cu in the regions of the skeleton abundant in the trabecular bone tissue may decrease. The decrease in the bone tissue concentrations of Zn and Cu is in agreement with our previous findings [54,55]; however, this change at such low exposure has not been reported until now.

Our interest in the present study was focused first of all on the impact of prolonged (from 3 up to 24 months) consumption of AE on the body status of Zn and Cu under chronic low-level and moderate exposure to Cd. Moreover, the study allowed us to evaluate the effect of the extract intake on the metabolism of these necessary elements at a very low-level exposure to this toxic metal resulting from its unavoidable trace presence in the standard diet (0.06 mg/kg in our experimental model [7]).

A very important question related to the enhanced consumption of polyphenol-rich products is the risk of decreased bioavailability of divalent bieoelements due to polyphenolic compounds' ability

to bind these elements and form stable, unabsorbable complexes [28,31,32,43,46]. Detailed analysis of the effects on the body status of Zn and Cu in the rats fed with the diet without Cd addition (the AE group) has revealed that long-term and even lifetime (24 months) consumption of AE in the daily dose from 31.1 to 154.7 mg/kg b.w. (corresponding to polyphenols consumption in the dose of 41.5–101.7 mg/kg b.w.) did not disturb the body metabolism of both bioelements in spite of the high content of polyphenols (cyanidin derivatives) and other compounds (fibers and pectins) capable of binding Zn and Cu ions (Zn^{2+} and Cu^{2+}) in *Aronia* berries [1,6,28,31,32]. Although the extract intake modified (increased or decreased compared to the proper values of the control group) the concentrations and contents of Zn and Cu in some tissues and organs at some time points, the total pool of these elements in internal organs and their concentrations in the serum and bone tissue were unaffected throughout the 3- to 24-month consumption of the extract, with only a few exceptions. The decrease in the serum Cu concentration that occurred in the young animals was transient, but it may suggest that we have to be careful in the case of prolonged administration of *Aronia* products to young individuals. The declined values of the Abs_{Zn} and Abs_{Cu} due to AE administration show that active compounds present in AE may be able to interact with these bioelements in the lumen of the gastrointestinal tract. However, the lack of evidence of Zn and Cu deficiencies after 3 months of AE administration, except for the decreased Cu concentration in the serum, shows that the decrease in the absorption of these bioelements was not long-lasting. Moreover, the Zn and Cu contents in the diet administered to the animals during the first 3 months of the experiment, higher than thereafter, might counteract the possible negative effect of AE on the gastrointestinal absorption of these bioelements. The decreased values of the Abs_{Zn} and Abs_{Cu}, noted after 10 months of AE consumption, may be explained by Zn and Cu binding by some ingredients of the extract [1,6,28,31,32]; however, these had no influence on the body status of both bioelements. The finding that even long-term consumption of AE together with the diet containing trace amounts of Cd has no negative influence on the body status of Zn and Cu is a very important result of this study. It indicates that rational amounts of *A. melanocarpa* berries containing-products may be included in the daily diet without the risk of disturbances in the metabolism of these elements. This finding is of practical use because the consumption of *Aronia* products is widely recommended due to their multidirectional beneficial action on health [1].

The most important finding of the present study, of high practical use, is the revelation that the consumption of AE under low-level and moderate chronic exposure to Cd offers effective protection from the destruction of the body status of Zn and Cu induced by this heavy metal. However, it is important to underline that apart from the protective (partial or complete) impact of AE against changes of various indices of the body status of Zn and Cu, the extract administration also influenced (decreased or increased) some indices of the body status of both bioelements unchanged by Cd alone. This mitigates the enthusiasm generated by our research results. Detailed analysis of the results showed that administration of the extract while feeding with the 1 mg Cd/kg diet completely prevented Zn and Cu deficiencies in internal organs in the young organism, and Cu deficiency in internal organs in the adult organism (after 17 months of the experiment), as well as the retention of both elements in the body due to lifetime Cd exposure. AE administration was also capable of completely counteracting the disorders in the body status of Zn caused by exposure to the 5 mg Cd/kg diet, but it decreased the concentration of this bioelement in the serum unaffected by Cd alone. The main negative effect of AE consumption was destroying the body status of Cu as a result of lifetime use of the extract in the case of moderate Cd treatment, consisting in its deficiency in internal organs, and the increase in the serum concentration of this bioelement. However, it is important to stress that the unfavourable changes of various indices of the body status of Zn and Cu due to AE consumption under exposure to Cd were transient and not marked.

The results of the ANOVA/MANOVA analysis allow us to recognise that the beneficial impact of AE administration under exposure to the 1 and 5 mg Cd/kg diets on the body status of Zn and Cu was caused by independent action of the extract ingredients and their interaction with Cd.

The independent impact of AE might be related to the ability of ingredients present in chokeberry (cyanidin derivatives, fibers, and pectins) to chelate Zn and Cu [6,28,31,32], as well as to AE's ability to inhibit MT biosynthesis in the liver, kidney, and duodenal tissue in the conditions of exposure to Cd. Although the administration of AE alone had no impact on the concentration of MT, its administration to the animals exposed to Cd markedly decreased this protein concentration and even increased the pool of non-MT-bound Cd in the liver, kidney, and duodenal tissue. The results of the ANOVA/MANOVA analysis clearly show that this was caused not only by the interaction of the extract ingredients with Cd, but also by their independent action. It is important to underline that our recent findings [11,58] indicate that the AE-caused decrease in the concentration of MT, responsible for Cd detoxification [22,37], as well as the increase in the pool of non-MT-bound Cd in the liver, is not associated with an increase in its toxicity. On the contrary, we have observed a protective effect of the extract on the liver of the female rats exposed to the 1 and 5 mg Cd/kg diets [11,58]. It has been revealed that polyphenolic compounds, including cyanidin derivatives (the main polyphenolic fraction of the AE) are able to chelate Zn and Cu [6,28,31,32]. Our more recent in vitro study has revealed that 0.1% AE, as well as cyanidin 3-galactoside present in the extract, are capable of chelating Zn and Cu (data in preparation for publication). The interactive impact of AE may be explained by its indirect action resulting from the influence on the body burden of Cd [7] due to the ability of the extract ingredients to bind Cd ions [6,28] and a decrease in MT concentration. Recently, we have reported that administration of the extract under exposure to 1 or 5 mg Cd/kg diets importantly decreased the body burden of this heavy metal, especially including its accumulation in the liver and kidneys, due to its decreased gastrointestinal absorption and increased urinary excretion [7]. Since the Cd content in the body was lower, the unfavourable effects of this toxic metal's interactions with Zn and Cu were also less advanced. Thus, due to its ability to complex Zn and Cu ions and decrease Cd accumulation and MT biosynthesis, AE was able to counteract Cd-induced Zn and Cu retention in internal organs. Moreover, AE's ability to protect against Cd-induced increase in the total pool of Cu in internal organs due to lifetime exposure to the 1 mg Cd/kg diet might result from the increased urinary excretion of this element noted in the Cd_1 + AE group after 24 months of the experiment. The fact that the extract administration entirely prevented the deficiency in the total pool of Zn and Cu in internal organs of a young organism caused by low-level exposure to Cd shows that the content of Zn and Cu in the diet administered to young animals was sufficient to compensate the possible negative effect resulting from the ability of the extract ingredients to complex these bioelements in the gastrointestinal tract. It is important to emphasise that AE administration not only partially or entirely protected against changes in Zn and Cu concentrations and contents in tissues, but it also had a beneficial impact on the pool of MT-unbound bioelements in the liver, kidney, and duodenum. The Cd-induced decrease in the pool of MT-unbound Zn and Cu may indicate hampered gastrointestinal absorption of these bioelements and their insufficient availability to ensure the normal course of physiological processes dependent on them. Thus, revealing that the administration of AE maintains the pool of MT-unbound Zn and Cu at the proper level is another argument for the beneficial effect of the extract consumption under low-level and moderate exposure to Cd. The modifying impact of AE administration on bone tissue concentrations of Zn and Cu may be explained by the beneficial impact of the extract consumption on bone turnover that we have recently reported [5,9] and by the protection against Cd accumulation in the bone tissue (only at the moderate exposure) [7], and it might be related to the influence of the extract consumption on the body status of these bioelements. Wider discussion of the results of the present study on the impact of AE on the body status of Zn and Cu is impossible because of a lack of literature data on this subject.

We are not only aware of the achievements of the present study, but also its limitations. One of them is the inability, at this stage of our investigation, to exactly explain the mechanisms of the beneficial impact of AE on the body status of Zn and Cu. Moreover, while discussing the results of the present study on the possibility of the protective use of AE against disorders in the body status of Zn and Cu resulting from low-level and moderate exposure to Cd, we cannot ignore the fact that

AE administration when feeding with 1 and 5 mg Cd/kg diets did not provide complete protection against all Cd-induced changes in the body status of Zn and Cu. In some cases, it also influenced the Cd alone-unchanged values of the estimated indices of the body status of these bioelements, resulting in an increase or decrease compared to the proper values of the control group and/or the values determined in the respective Cd group. At this stage of investigation, we are unable to explain the cause of these changes; however, they were innumerous and only occurred at some time points. Still, they limit the importance of our findings and thus require further study. The changes in Zn and Cu concentrations or contents in some tissues due to AE administration to female rats exposed to Cd may be related to the impact of the extract on the total body status of particular bioelements. It should also be taken into account when interpreting the results that the parameters describing the body status of Zn and Cu at particular time points were not evaluated in the same animals, but in various subgroups within particular experimental groups. This may also explain, at least to some extent, why some favourable effects of AE consumption under Cd exposure were or were not observed at some time points. We are also aware that since our findings come from an experimental female rat model, they only refer to the body status of Zn and Cu in females, and an analogous study in a male rat model is necessary. However, in spite of the limitations described above, the present study has provided reliable evidence that shows that the consumption of polyphenol-rich chokeberry products seems to be a promising strategy in the protection against Cd toxicity, and that efforts should be focused on investigation aimed at confirming their effectiveness in humans. First of all, it seems necessary to investigate the dependence between the consumption of these products and the health status of inhabitants of industrialised countries, including the indices of the body status of Zn and Cu.

5. Conclusions

In summary, the current study provides the first evidence that the consumption of AE under chronic low-level and moderate exposure to Cd may offer significant protection against most of the changes in the body status of Zn and Cu caused by this xenobiotic. However, it may also influence (increase or decrease) some of the indices of the metabolism of these bioelements unchanged by Cd. Although the unfavourable effects of the administration of the extract in the case of exposure to Cd were innumerous, transient, and not marked, and only occurred at some time points, they limit the importance of our findings and show that efforts should be focused on ruling out the risk of destroying the metabolism of Zn and Cu in the organism exposed to Cd due to the intake of *Aronia* products. Our results indicate that the influence of AE consumption on the body status of Zn and Cu under low-level and moderate exposure to Cd may be mediated by MT and that the beneficial effect of the extract may be related to its ability to maintain the pool of MT-unbound Zn and Cu at the proper level. Moreover, revealing that even long-term consumption of AE in the standard diet containing only trace amounts of Cd had no negative influence on the body status of Zn and Cu shows that incorporation of chokeberry products into the diet of healthy individuals with the aim of protecting their health should not disturb the body status of necessary bioelements such as Zn and Cu. On the basis of the findings of the present study, it seems that rational amounts of chokeberry products may be included in the daily diet unpolluted by Cd without the risk of destroying Zn and Cu metabolisms; however, their potential prophylactic use under exposure to Cd requires further study to exclude any unfavourable impact on the metabolism of these essential elements. These results, together with our previous findings on the beneficial impact of the administration of AE against some effects of the toxic action of Cd [5,7,9–11,58], allow us to conclude that *Aronia* berries and their products may be very promising natural agents for effective use in the prevention against various effects of this heavy metal action, including disorders in the metabolism of Zn and Cu. However, the occurrence of some unfavourable changes of various indices of the body status of Zn and Cu due to the consumption of AE under exposure to Cd somewhat reduces the enthusiasm arising from our studies, allowing us to conclude that products made from chokeberries may be promising preventive agents against various

unfavourable actions of this xenobiotic. Thus, further studies on this subject are warranted to confirm their effectiveness and recognise health hazards related to their use.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/9/12/1374/s1, Supplementary Material—Zinc, Supplementary Material—Copper; Table S1: Analytical quality of zinc (Zn) and copper (Cu) measurements in certified reference materials, Table S2: Effect of the extract from the berries of *Aronia melanocarpa* (AE) on cadmium (Cd) concentration in the liver and kidney of rats exposed to this toxic metal, Table S3: The daily intake of zinc (Zn) and copper (Cu) with diet in particular experimental groups during the 5-day balance study, Table S4: Main and interactive effects of cadmium (Cd) and the extract from the berries of *Aronia melanocarpa* (AE) on apparent absorption (Abs_{Zn}), retention in the body (Ret_{Zn}), and faecal (FE_{Zn}) and urinary (UE_{Zn}) excretion of zinc (Zn), Table S5: Main and interactive effects of cadmium (Cd) and the extract from the berries of *Aronia melanocarpa* (AE) on zinc (Zn) concentration in the serum and tissues of rats exposed to the 1 mg Cd/kg diet, Table S6: Main and interactive effects of cadmium (Cd) and the extract from the berries of *Aronia melanocarpa* (AE) on zinc (Zn) concentration in the serum and tissues of rats exposed to the 5 mg Cd/kg diet, Table S7: Main and interactive effects of cadmium (Cd) and the extract from the berries of *Aronia melanocarpa* (AE) on zinc (Zn) content in internal organs, Table S8: Main and interactive effects of cadmium (Cd) and the extract from the berries of *Aronia melanocarpa* (AE) on apparent absorption (Abs_{Cu}), retention in the body (Ret_{Cu}), and faecal (FE_{Cu}) and urinary (UE_{Cu}) excretion of copper (Cu), Table S9: Main and interactive effects of cadmium (Cd) and the extract from the berries of *Aronia melanocarpa* (AE) on copper (Cu) concentration in the serum and tissues of rats exposed to the 1 mg Cd/kg diet, Table S10: Main and interactive effects of cadmium (Cd) and the extract from the berries of *Aronia melanocarpa* (AE) on copper (Cu) concentration in the serum and tissues of rats exposed to the 5 mg Cd/kg diet, Table S11: Main and interactive effects of cadmium (Cd) and the extract from the berries of *Aronia melanocarpa* (AE) on copper (Cu) content in internal organs, Table S12: Main and interactive effects of cadmium (Cd) and the extract from the berries of *Aronia melanocarpa* (AE) on metallothionein (MT) concentration in the liver and the degree of zinc (Zn), copper (Cu), and Cd binding to this protein, Table S13: Main and interactive effects of cadmium (Cd) and the extract from the berries of *Aronia melanocarpa* (AE) on metallothionein (MT) concentration in the kidney and the degree of zinc (Zn), copper (Cu), and Cd binding to this protein, Table S14: Main and interactive effects of cadmium (Cd) and the extract from the berries of *Aronia melanocarpa* (AE) on metallothionein (MT) concentration in the duodenum and the degree of zinc (Zn), copper (Cu), and Cd binding to this protein, Figure S1: The body retention of zinc (Ret_{Zn}) and copper (Ret_{Cu}) in particular experimental groups, Figure S2: Zinc (Zn) content in the kidney, liver, and heart in particular experimental groups, Figure S3: Zinc (Zn) content in the spleen and brain in particular experimental groups, Figure S4: Copper (Cu) content in the kidney, liver, and heart in particular experimental groups, Figure S5: Copper (Cu) content in the spleen and brain in particular experimental groups.

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Author Contributions: Sylwia Borowska took part in designing the research, conducted the study, performed statistical analysis and interpreted the results, and wrote the paper; Małgorzata M. Brzóska took part in designing the research, supervised the study, supervised analysis and interpretation of the results and the manuscript preparation, as well as corrected the final version of the article; Małgorzata Gałażyn-Sidorczuk participated in the study; Joanna Rogalska participated in the study. All authors approved the final version of the manuscript.

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Abbreviations

AAS	atomic absorption spectrometry
Abs_{Cu}	apparent absorption of copper
Abs_{Zn}	apparent absorption of zinc
AE	extract from the berries of *Aronia melanocarpa*
A. melanocarpa	*Aronia melanocarpa*
Cd	cadmium
$CdCl_2 \times 2\frac{1}{2}\ H_2O$	cadmium chloride
Cd/(MT × 7)	the pool of metallothionein-unbound cadmium
Cu	copper
Cu/(MT × 12)	the pool of metallothionein-unbound copper
CV	coefficient of variation
F AAS	flame atomic absorption spectrometry
GF AAS	flameless atomic absorption spectrometry with electrothermal atomisation in a graphite furnace
FE_{Cu}	faecal copper excretion
FE_{Zn}	faecal zinc excretion
HCl	hydrochloric acid
HNO_3	nitric acid
Me	metal (zinc, copper, or cadmium)
Me/Me-MT	the total pool of metallothionein-unbound metals
MT	metallothionein
NaCl	sodium chloride
Ret_{Cu}	copper retention in the body
Ret_{Zn}	zinc retention in the body
SD	standard deviation
SE	standard error
UE_{Cu}	urinary copper excretion
UE_{Zn}	urinary zinc excretion
Zn	zinc
Zn/(MT × 7)	the pool of metallothionein-unbound zinc

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Dietary Intake of Flavonoids and Ventilatory Function in European Adults: A GA^{2}LEN Study

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Abstract: Background: Flavonoids exert anti-inflammatory properties and modulate oxidative stress in vitro, suggesting a protective effect on lung function, but epidemiological studies examining this association are scarce. Methods: A stratified random sample was drawn from the GA^{2}LEN screening survey, in which 55,000 adults aged 15 to 75 answered a questionnaire on respiratory symptoms.

Post-bronchodilator spirometry was obtained from 2850 subjects. Forced vital capacity (FVC), the ratio between the forced exhaled volume in 1 second (FEV_1) and FVC (FEV_1/FVC), FVC below lower limit of normal (FVC < LLN), and FEV_1/FVC < LLN were calculated. Intake of the six main subclasses of flavonoids was estimated using the GA^{2}LEN Food Frequency Questionnaire. Adjusted associations between outcomes and each subclass of flavonoids were examined with multivariate regressions. Simes' procedure was used to test for multiple comparisons. Results: A total of 2599 subjects had valid lung function and dietary data. A lower prevalence of FVC < LLN (airway restriction) was observed in those with higher total flavonoid (adjusted odds ratio (aOR), higher vs. lowest quintile intake 0.58; 95% Confidence Interval (CI) 0.36, 0.94), and pro-anthocyanidin intakes (aOR 0.47; 95% CI 0.27, 0.81). A higher FEV_1/FVC was associated with higher intakes of total flavonoids and pro-anthocyanidins (adjusted correlation coefficient (a β-coeff 0.33; 0.10, 0.57 and a β-coeff 0.44; 95% CI 0.19, 0.69, respectively). After Simes' procedure, the statistical significance of each of these associations was attenuated but remained below 0.05, with the exception of total flavonoids and airway restriction. Conclusions: This population-based study in European adults provides cross-sectional evidence of a positive association of total flavonoid intake and pro-anthocyanidins and ventilatory function, and a negative association with spirometric restriction in European adults.

Keywords: flavonoids; pro-anthocyanidins; lung function; GA^{2}LEN

1. Introduction

Flavonoids are polyphenolic plant secondary metabolites ubiquitously present in vegetables, fruits, and beverages. Various biological properties of relevance for lung health have been identified for these compounds, including antioxidant, anti-allergic, and immune-modulating activities [1]. There is a considerable body of experimental evidence (in vivo and in vitro) on the anti-inflammatory properties of flavonoids, particularly pro-anthocyanidins, on lung health. Experimental evidence shows that pro-anthocyanidins can reduce lung mRNA expression of pro-inflammatory cytokines such as Interferon-γ, Interleukin (IL)-4, and IL-13 [2], and down-regulate nitric oxide production in asthma [3]. Trifolirhizin, another flavonoid, has been demonstrated to inhibit in a dose-response manner, the production of IL-6 and tumour necrosis factor-α (TNF-α) in lung cancer cells [4]. Pro-anthocyanin-enriched blackcurrant extract and epigallocatechins were shown to reduce eosinophilic-driven airway inflammation in human alveolar cells [5].

Epidemiological studies have shown a beneficial effect of a higher intake of fruits and vegetables on chronic respiratory health in adults, most of which refers to incidence of asthma and chronic obstructive pulmonary disease (COPD) [6,7] or to prevalence of asthma symptoms [8]. These benefits have been attributed to the high content of flavonoids present in hard fruits such as apples [9,10], though this effect was not confirmed in one population-based study [11]. Epidemiological evidence on the association between flavonoids and lung function is scant and limited to the assessment of few flavonoid subclasses (e.g., flavonols and flavan-3-ols). A recent population-based study in young adults found that a higher forced vital capacity (FVC) was associated with a higher intake of catechins and of total fruit [12]. There are also observational studies showing a slower decline of forced exhale volume in 1 second (FEV_1) [13,14] and FVC [14], indirectly attributed to flavonoids. In asthmatic subjects, a higher FEV_1 was associated with having a higher intake of soy genistein (a type of polyphenol) [15].

The GA^{2}LEN survey is the first multi-national, population-based study investigating the role of all main major subclasses of flavonoids on ventilatory function in a representative sample of European adults.

2. Materials and Methods

2.1. The GA²LEN Study: Screening and Clinical Surveys

The core protocol for the GA²LEN survey required 18 European participating centres to identify a random sample of at least 3000 adults aged 15–74 years from an available population-based sampling frame. In 2008–2009, potential participants were sent a short questionnaire by mail, and at least three attempts were made to elicit a response. The questionnaire collected information on age, gender, smoking, and the presence of symptoms of asthma (including age of onset), chronic rhino-sinusitis, and allergic rhinitis. Four sub-samples were selected to define cases and controls: (1) those with self-reported asthma and at least one respiratory symptom reported in the last 12 months ('asthma'); (2) those having chronic sinusitis (defined following the European Position Paper on Rhinosinusitis and Nasal Polyps (EP³OS) Group criteria, that is, the presence of at least two of the following symptoms for at least 12 weeks in the past year: (i) nasal blockage, (ii) nasal discharge, (iii) facial pain or pressure, or (iv) reduction in sense of smell with at least one of the symptoms being nasal blockage or nasal discharge); (3) those who had both 'asthma' and 'chronic sinusitis'; and (4) those who had none of these conditions [16].

Participants selected were invited in for further tests. Relevant to this analysis they had performed spirometry using a New Diagnostic Design (NDD) Easy-One spirometer before and after 200 μg of short-acting beta-agonist, salbutamol inspired through a Clement Clarke Able spacer. After at least three acceptable and two reproducible maneuvers were obtained by trained technicians, the lung function was defined as the highest of two values for FVC and FEV_1 taken from acceptable forced expiratory maneuvers that did not vary by more than 200 mL. Only spirometry that met the European Respiratory Society/American Thoracic Society (ERS/ATS) criteria were accepted [17]. All spirometries were checked centrally for quality control.

2.2. Estimates of Diet and Flavonoid Intake

Usual consumption of 250 foods was estimated using the GA²LEN food frequency questionnaire (FFQ), which was designed to assess dietary intake across countries in Europe using a single, standardised instrument, and validated in five countries participating in GA²LEN ClinicalTrials.gov Identifier: NCT03251157) [18]. The FFQ collected data on a wide range of foods, including 37 vegetables and 28 fruits, as well as foods rich in soy and grains. The FFQ also included a general question to ascertain use of nutritional supplements ('Do you regularly take any nutritional supplements?').

On the FFQs, respondents sometimes left individual items blank. This was assumed to denote zero intake of these foods; however, if 20% of the items were blank, the FFQ was considered incomplete, and the subject was excluded from further analyses. Participants were also excluded if they had extreme values of total energy intake, which might suggest an unrealistic response. We calculated the expected basal metabolic rate (BMR) with the given age, weight, and sex, and excluded subjects with a ratio of energy intake to expected BMR that was either below the 0.05th sample centile or above the 99.5th sample centile for their country.

Total energy intake (TEI) was calculated using the latest available food composition estimates from the British Food Composition Table [19]. A database for the assessment of intakes of different flavonoid subclasses was constructed. We used the updated and expanded US Department of Agriculture (USDA) flavonoid content of foods and the pro-anthocyanidin databases [20,21]. Intakes were derived for the six main flavonoid subclasses habitually consumed in general population [22]: (1) flavanones (eriodictyol, hesperetin, and naringenin); (2) anthocyanins (cyanidin, delphinidin, malvidin, pelargonidin, petunidin, and peonidin); (3) flavan-3-ols (catechins and epicatachins); (4) flavonols (quercetin, kaempferol, myricetin, and isohamnetin); (5) flavones (luteolin and apigenin); and (6) polymers (proanthocyanidins, theaflavins, and thearubigins). Total flavonoid intakes were derived by the addition of the six component subclasses. We also examined associations with the

pro-anthocyanidins subclass, which were derived separately, summing monomers, dimers, trimers, 4- to 6-mers, 7- to 10-mers, and >10-mers.

2.3. Statistical Analyses

Sampling probability weights were used to standardise prevalences by gender and age to a European Standard Population. For each centre, the sample mean of the FEV_1/FVC ratio and the FVC was estimated as well as the sample prevalence of a low FEV_1/FVC ratio or a low FVC according to the National Health and Nutrition Examination Survey (NHANES) III norms for Caucasians. For each flavonoid group, total intake was split into quintiles. For each centre, the odds of the FEV_1/FVC ratio being below the lower limit of normal comparing the lowest and highest quintile of flavonoid consumption was calculated. These odds ratios for all centres were then combined into a single estimate using random effects meta-analysis [23]. For continuous measures of lung function (FEV_1/FVC and FVC), their association with each flavonoid subclass was assessed against the per-quintile increase of each flavonoid subclass. Associations were examined using two models of adjustments. Model 1 included adjustment for height, age, and sex, and Model 2 added the variables whether a case or a control (as defined in the GA^2LEN survey), body mass index (BMI), smoking status (never, ex-smoker, current smoker), occupation, age at completion of full time education, use of nutritional supplements, total fruit and vegetable intake, and TEI. Simes' procedure was used to correct statistical estimates derived from multiple testing [24].

We examined the correlation of each of the subclasses of flavonoids and these ranged between 0.28 (flavan-3-ols) and 0.40 (pro-anthocyanidins), therefore we did not use energy-adjusted values of flavonoids and instead adjusted for TEI in the models.

Written informed consent was obtained from all participants. The GA^2LEN Follow-Up Survey was granted ethical approval by the UK's National Ethics Research Committee No. 07/H0604/121.

3. Results

A total of 2599 adults (mean age 47.2 $\pm$ 14.5 years) had valid lung function estimates (Table 1). With the exception of Belgians, the BMI for all participant countries indicated that the participants were, on average, overweight. Around half of the participants reported never having smoked, whilst just over 15% declared that they were current smokers. Nearly two-thirds of the sample studied was defined as a 'case'. Use of nutritional supplements varied across countries, with Finnish adults reporting the highest proportion of intakes of nutritional supplements and Portuguese the lowest. Intake of fruits and vegetables averaged three portions of each per person per day in the whole sample.

Table 1. General characteristics of the study population (n = 2599) [#].

Variables	Countries Participating in the GA²LEN Nutrition Survey									
	Denmark Odense (268)		Finland Helsinki (122)		Sweden Total (1085)		UK Total (139)		Portugal Coimbra (233)	
Age, years; mean (SD)	47.9	(14.2)	45.8	(14.5)	45.5	(15)	51.3	(13.2)	47.2	(14.5)
Males, n (%)	115	(42.9)	47	(38.5)	471	(43.4)	54	(38.8)	80	(34.3)
Height, m (SD)	1.7	(0.1)	1.7	(0.1)	1.7	(0.1)	1.7	(0.1)	1.6	(0.1)
BMI (m^2/kg)	26.9	(4.7)	26.5	(4.3)	26	(4.7)	27.8	(5.8)	26.6	(5.3)
Never smokers, n (%)	119	(44.4)	60	(49.2)	578	(53.3)	63	(45.3)	148	(63.5)
Ex-smokers, n (%)	71	(26.5)	30	(24.6)	366	(33.7)	55	(39.6)	51	(21.9)
Current smokers, n (%)	78	(29.1)	32	(26.2)	141	(13)	21	(15.1)	34	(14.6)
Cases/controls	180/88		62/60		774/311		95/44		169/64	
* FVC, L	4.1	(1.0)	4.0	(1.0)	4.1	(1.0)	3.7	(1.0)	3.8	(1.0)
* FEV_1/FVC	78.4	(8.6)	80	(7.9)	78.2	(8.6)	75.4	(10.8)	80.5	(8.3)
FVC < LLN, n (%)	36	(13.4)	10	(8.2)	141	(13)	20	(14.4)	16	(6.9)
FEV_1/FVC < LLN, n (%)	32	(11.9)	6	(4.9)	130	(12)	25	(18)	25	(10.7)
Nutritional supplements, n (%)	107	(39.9)	52	(42.6)	285	(26.3)	49	(35.3)	15	(6.4)
Total fruit intake, g/day	328	(263.8)	278.2	(219.4)	320.7	(242.5)	328.8	(238.3)	549.2	(403.7)
Total vegetable intake, g/day	342.6	(223.8)	415.5	(297.5)	355.8	(206.8)	343.2	(207.8)	454.5	(321.1)

Variables	Countries Participating in the GA²LEN Nutrition Survey									
	Belgium Ghent (107)		Germany Total (305)		The Netherlands Amsterdam (174)		Poland Total (166)		Total 2599	
Age, years, mean (SD)	44.5	(14.4)	48.7	(15.2)	52.7	(13.6)	51.6	(15)	47.4	(14.8)
Males, n (%)	47	(43.9)	116	(38)	88	(50.6)	65	(39.2)	1083	(41.7)
Height, m (SD)	1.7	(0.1)	1.7	(0.1)	1.7	(0.1)	1.7	(0.1)	1.7	(0.1)
BMI (m^2/kg)	24.5	(4)	26.9	(5)	27.1	(4)	27.7	(5.2)	26.5	(4.8)
Never smokers, n (%)	60	(56.1)	148	(48.5)	70	(40.2)	79	(47.6)	1,325	(51)
Ex-smokers, n (%)	32	(29.9)	108	(35.4)	67	(38.5)	48	(28.9)	828	(31.9)
Current smokers, n (%)	15	(14)	49	(16.1)	37	(21.3)	39	(23.5)	446	(17.2)
Cases/Controls	60/47		142/163		111/63		108/58		1701/898	
* FVC, L	4.3	(1.0)	3.9	(1.0)	4.1	(1.0)	3.8	(1.0)	4.0	(1.0)
* FEV_1/FVC	79.4	(9.5)	78.8	(7.7)	77.1	(8.4)	77	(10.4)	78.3	(8.8)
FVC < LLN, n (%)	9	(8.4)	55	(18)	11	(6.3)	13	(7.8)	311	(12)
FEV_1/FVC < LLN, n (%)	10	(9.3)	25	(8.2)	20	(11.5)	20	(12)	293	(11.3)
Nutritional supplements, n (%)	33	(30.8)	81	(26.6)	72	(41.4)	39	(23.5)	733	(28.2)
Total fruit intake, g/day	333.1	(235.1)	314.7	(273.2)	289.5	(211.8)	465.2	(466.9)	347.3	(291.2)
Total vegetable intake, g/day	244.8	(126.1)	261.9	(187.2)	224.5	(130.5)	482.8	(429.6)	349.1	(246.9)

FVC, forced vital capacity; FEV_1, forced exhaled volume in 1 second; LLN, lower limit of normal. * Post-bronchodilator; SD standard deviation; 'n' number of participants; '%' percentage; [#] Based on individuals with complete dietary data and valid (quality controlled) lung function measurements.

The distribution of total flavonoid intake is shown in Figure 1, and that of other subclasses in Table 2. Overall, the median intake of total flavonoids was 291 mg/person/day. Germany and Belgium had the lowest intake. Poland, the Netherlands, and Portugal had the highest intakes of total flavonoids. The Scandinavian countries and the UK had an intermediate intake. The main contributors to total flavonoid intake are summarised in Table 3. Fruits were the main source, followed by tea, wine, chocolate, and vegetables. As expected, flavonoid polymers were the main source of flavonoids in most food groups. Citrus fruits were the predominant source of flavonones. Fruits (mostly berries and citrus) and wine were the main contributors of anthocyanins, whilst flavonols and flavones were mainly derived from vegetables (Table 3).

The association between FVC and spirometric restriction (FVC < LLN) with flavonoids is presented in Table 4. In the basic model (adjusted by height, age, and sex only), total flavonoid intake and several most subclasses were positively associated with a 30 mL higher FVC. After controlling for further confounders (BMI, whether a case or a control, intake of nutrient supplements, education, smoking status, total fruit and vegetable intake, and TEI), these associations were attenuated and were no longer statistically significant. With regards to spirometric restriction, a 42% lower risk of FVC < LLN was observed in those with the highest quintile of total flavonoid intake (odds ratio (OR) 0.58; 95% CI 0.36, 0.94). Similarly, after adjusting for the full set of potential confounders, a 53% lower risk of FVC < LLN was observed in those adults with the highest intake of anthocyanins and pro-anthocyanidins. After applying the Simes' procedure, the statistical significance of the adjusted associations remained (Table 4).

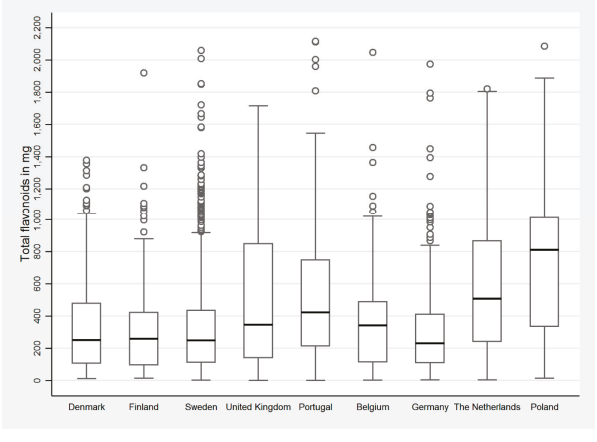


Figure 1. Total flavonoid intake in the adults participating in the GA^{2}LEN Follow-Up Survey who had valid lung function data (n = 2599).

Table 5 shows the association between FEV_1/FVC and FEV_1/FVC < LLN with flavonoid intake. A higher FEV_1/FVC was associated with a higher intake of flavonoids, polymers, and pro-anthocyanidins. After Simes' procedure, each of these associations remained statistically significant. Airflow obstruction (FEV_1/FVC < LLN) was not associated with any of the flavonoid exposures studied.

The meta-analyses in Figure 2 illustrate the adjusted associations between FEV_1/FVC and total flavonols and pro-anthocyanidins per centre and the overall effect. These associations showed evidence of moderate but not statistically significant evidence of heterogeneity between countries in the analyses with pro-anthocyanidins (I^2 = 38.2%, p-value = 0.10), whilst the meta-analysis for pro-anthocyanidins showed no evidence of variations between countries, as reflected by the zero heterogeneity in these associations.

Table 2. Dietary intake of main flavonoid subclasses in adults from countries participating in GA^{2}LEN.

Flavonoid Subclass	Dietary Intake of Flavonoids across Countries (Median Intake in mg, IQR)									
	Denmark Odense (268)		Finland Helsinki (122)		Sweden Total (1085)		UK Total (139)		Portugal Coimbra (233)	
Total flavonoids	251.1	(106.3 to 479.7)	260.5	(95.2 to 427.2)	249.4	(113 to 439.1)	345.5	(140.3 to 848.8)	426.5	(215.8 to 749.1)
Flavanones	4.5	(1.3 to 20.9)	8.3	(1.8 to 23.6)	5.3	(1.4 to 21)	5	(1.8 to 18.3)	17.1	(2.9 to 60.3)
Anthocyanins	7.5	(2.4 to 16.3)	5.9	(2.1 to 13)	6.5	(2.1 to 15.1)	9.8	(3.7 to 30.3)	22.1	(6.9 to 47.5)
Flavan-3-ols	30.6	(14 to 69)	24.4	(10 to 56.3)	29.4	(13 to 65.3)	43.7	(14.8 to 183.5)	44	(20.9 to 78.2)
Flavanols	16.3	(9.3 to 29.3)	19.5	(10.8 to 33.1)	16.9	(9.2 to 28.2)	16.7	(9.1 to 32.7)	26.6	(12.9 to 44.6)
Flavones	2.1	(0.9 to 4.4)	2.4	(1.3 to 4.5)	2	(0.9 to 4.3)	1.7	(0.6 to 3.2)	2.6	(1.1 to 5.7)
Polymers	175.7	(66.1 to 316.3)	162.9	(62.5 to 260.2)	159.5	(66.4 to 283.3)	251.5	(75.7 to 601.5)	266.8	(106.5 to 459.8)
Pro-anthocyanidins	135.3	(68.6 to 261.1)	152.8	(58.7 to 270.7)	126.6	(62.9 to 245.7)	195.2	(76.7 to 376.6)	286.7	(113.1 to 465.1)
Flavonoid Subclass	**Dietary Intake of Flavonoids across Countries (Median Intake in mg, IQR)**									
	Belgium Ghent (107)		Germany Total (305)		The Netherlands Amsterdam (174)		Poland Total (166)		Total 2599	
Total flavonoids	341.4	(113.9 to 489.4)	231.7	(110.2 to 413.8)	507	(242.1 to 867.4)	817.3	(341.6 to 1029.3)	291.2	(126.8 to 569.4)
Flavanones	10.3	(2.3 to 27.9)	4.9	(1.1 to 21.6)	7	(2.3 to 29)	6.7	(1.8 to 28)	5.7	(1.5 to 22.5)
Anthocyanins	10.5	(2.7 to 21)	5.5	(1.7 to 13.3)	8.1	(2.8 to 22.1)	9.2	(2.3 to 26.3)	7.5	(2.4 to 19.6)
Flavan-3-ols	32.9	(13.6 to 83.8)	38.1	(13.2 to 77.3)	84.1	(35.5 to 186.2)	176.3	(54.9 to 200.1)	37.9	(15.4 to 90.4)
Flavanols	13.1	(7 to 23.7)	11	(5.2 to 20.2)	17.8	(9.2 to 30.6)	34	(21.3 to 51.8)	17.2	(9.2 to 30.8)
Flavones	1.4	(0.5 to 2.5)	1.1	(0.4 to 2.3)	1.5	(0.6 to 2.5)	2.1	(0.7 to 5.8)	1.9	(0.7 to 3.9)
Polymers	216.5	(71.1 to 325.5)	132.1	(63.7 to 268.1)	341.7	(129.5 to 622.3)	585.9	(243 to 687.5)	198.4	(72.9 to 375.2)
Pro-anthocyanidins	214.9	(80.5 to 343.8)	136.1	(69 to 261.4)	190.8	(101.5 to 327.8)	192.7	(105.5 to 311.8)	154.6	(72.5 to 284.3)

IQR: interquartile range.

Table 3. Provenance and contribution of flavonoid subclasses in the diet of participants in the GA^{2}LEN Follow-Up Study.

Flavonoid Subclasses (mg)	Food Groups (mg/% Total Dietary Sources)										
	All Fruit	Citrus Fruit	Hard Fruit	Berries	Other Fruit	Vegetables	Chocolate	Nuts	Legumes	Tea & Coffee	Wine & Beer
Total flavonoids	289.93	36.58	124.08	2.91	64.09	14.9	30.87	1.43	0.23	152.9	21.07
Flavanones	7.51%	59.21%	0.00%	0.10%	0.19%	2.08%	0.00%	0.06%	0.00%	0.00%	2.47%
Anthocyanins	5.66%	9.00%	4.38%	28.94%	13.18%	1.96%	0.00%	2.59%	0.42%	0.00%	9.43%
Flavan-3-ols	6.86%	1.58%	7.07%	4.61%	8.93%	0.00%	16.33%	3.26%	21.38%	31.41%	26.85%
Flavonols	3.45%	1.49%	4.41%	1.17%	1.87%	79.15%	0.00%	1.38%	21.62%	2.91%	9.00%
Flavones	0.34%	1.62%	0.14%	0.45%	0.29%	16.81%	0.00%	0.00%	0.00%	0.00%	1.23%
Polymers	76.18%	27.11%	84.02%	64.74%	75.54%	0.00%	83.67%	92.71%	56.59%	65.68%	51.02%
Pro-anthocyanidins	234.07	10.11	110.84	1.98	50.75	0.00	34.64	1.40	0.18	15.1	14.92

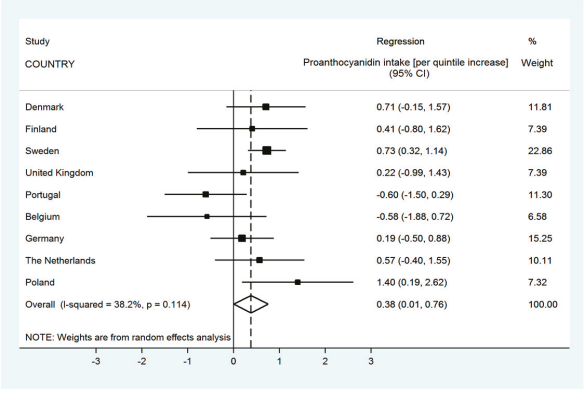
Table 4. Association of post-bronchodilator spirometric values of FVC and FVC < LLN with dietary intake of flavonoids.

Flavonoid Subclass (Quintiles; mg)	FVC (Lt) (Continuous) Effect Size (Regression Coefficient and 95% Confidence Interval) Per-Quintile Increase in Flavonoid Intake						FVC < LLN (Binary) OR (95% Confidence Interval) Highest Vs. Lowest Quintile of Flavonoid Intake						
	Model 1 (n = 2599)		p-Value	Model 2 (n = 2599)		p-Value	Model 1 (n = 2599)		p-Value	Model 2 (n = 2599)		p-Value	p-Value after Simes' Procedure
Total flavanoids	0.03	(0.02 to 0.05)	<0.0001	0.02	(−0.002 to 0.03)	0.09	0.47	(0.31 to 0.71)	<0.0001	0.58	(0.36 to 0.94)	0.03	0.07
Flavanones	0.03	(0.02 to 0.05)	<0.0001	0.02	(−0.002 to 0.03)	0.08	0.47	(0.31 to 0.71)	0.0003	0.60	(0.37 to 0.97)	0.04	0.07
Anthocyanins	0.04	(0.02 to 0.05)	<0.0001	0.02	(−0.01 to 0.04)	0.14	0.37	(0.24 to 0.57)	<0.0001	0.47	(0.26 to 0.83)	0.01	0.04
Flavan-3-ols	0.02	(0.002 to 0.03)	0.03	<0.001	(−0.02 to 0.02)	0.97	0.58	(0.39 to 0.86)	0.01	0.75	(0.49 to 1.14)	0.17	
Flavonols	0.03	(0.02 to 0.05)	0.0001	0.01	(−0.004 to 0.03)	0.12	0.52	(0.35 to 0.76)	0.0008	0.66	(0.41 to 1.04)	0.07	
Flavones	0.02	(0.01 to 0.04)	0.003	0.01	(−0.01 to 0.02)	0.54	0.58	(0.40 to 0.85)	0.01	0.79	(0.51 to 1.22)	0.28	
Polymers	0.03	(0.02 to 0.05)	0.0001	0.01	(−0.003 to 0.03)	0.10	0.52	(0.34 to 0.79)	0.002	0.65	(0.41 to 1.04)	0.07	
Pro-anthocyanidins	0.04	(0.02 to 0.05)	<0.0001	0.02	(−0.002 to 0.04)	0.07	0.41	(0.26 to 0.63)	0.0001	0.47	(0.27 to 0.81)	0.01	0.04

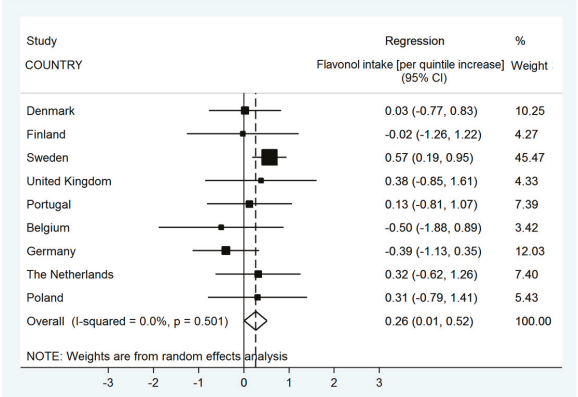
Model 1 Adjusted by height, age, and sex; Model 2 Adjusted by height, age, sex, whether a case or a control, BMI, smoking status (never, ex-smoker, current smoker), occupation, age at completion of full time education, use of nutritional supplements, total fruit intake, and total energy intake (TEI), Lt: liters.

Table 5. Association of post-bronchodilator ratio FEV_1/FVC and FEV_1/FVC < LLN with dietary intake of flavonoids.

Flavonoid Subclass (Quintiles; mg)	FEV_1/FVC (Continuous) Effect Size (Regression Coefficient and 95% Confidence Interval) Per-Quintile Increase in Flavonoid Intake							FEV_1/FVC < LLN (Binary) OR (95% Confidence Interval) Highest vs. Lowest Quintile of Flavonoid Intake						
	Model 1 (n = 2599)		p-Value	Model 2 (n = 2599)		p-Value	p-Value after Simes' Procedure	Model 1 (n = 2599)		p-Value	Model 2 (n = 2599)		p-Value	p-Value after Simes' Procedure
Total flavanoids	0.50	(0.29 to 0.72)	<0.0001	0.33	(0.10 to 0.57)	0.01	0.02	0.58	(0.39 to 0.86)	0.01	0.61	(0.39 to 0.97)	0.04	0.12
Flavanones	0.16	(−0.05 to 0.37)	0.15	0.07	(−0.30 to 0.17)	0.57		0.71	(0.47 to 1.08)	0.11	0.84	(0.51 to 1.37)	0.49	
Anthocyanins	0.34	(0.13 to 0.56)	0.002	0.12	(−0.15 to 0.39)	0.39		0.58	(0.39 to 0.86)	0.01	0.73	(0.43 to 1.24)	0.25	
Flavan-3-ols	0.42	(0.21 to 0.63)	0.0001	0.20	(−0.02 to 0.42)	0.07		0.65	(0.44 to 0.97)	0.03	0.73	(0.47 to 1.11)	0.14	
Flavonols	0.34	(0.13 to 0.56)	0.002	0.18	(−0.06 to 0.42)	0.14		0.77	(0.52 to 1.14)	0.19	0.92	(0.58 to 1.47)	0.74	
Flavones	0.31	(0.1 to 0.52)	0.004	0.17	(−0.07 to 0.4)	0.16		0.80	(0.54 to 1.18)	0.26	1.01	(0.63 to 1.6)	0.97	
Polymers	0.48	(0.27 to 0.69)	<0.0001	0.32	(0.09 to 0.55)	0.01	0.02	0.55	(0.37 to 0.81)	0.002	0.56	(0.36 to 0.88)	0.01	0.09
Pro-anthocyanidins	0.58	(0.37 to 0.79)	<0.0001	0.44	(0.19 to 0.69)	0.001	0.004	0.58	(0.39 to 0.85)	0.01	0.61	(0.37 to 0.99)	0.04	0.12



(**a**)



(**b**)

Figure 2. The association of FEV1/FVC and intakes (mg) of pro-anthocyanidins (**a**) and flavonols (**b**) (Meta-analysis of adjusted effect estimates (β-coefficient); n = 2599).

4. Discussion

This multi-centric population-based study in European adults showed that those with a higher intake of pro-anthocyanidins, had a higher FEV_1/FVC, a lower risk of airway obstruction (FEV_1/FVC < LLN) and a lower risk of spirometric restriction (FVC < LLN). Total flavonoid intake was also associated with a higher FEV_1/FVC and with a lower risk of spirometric restriction. The statistical significance of each of these associations remained after applying Simes' procedure, with the exception of the associations observed with FEV_1/FVC < LLN. The direction of these associations was fairly consistent across countries, with moderate or no evidence of heterogeneity between them.

To our knowledge, this is the first multi-national population-based study to examine the association between objective measures of airway restriction and obstruction with the intake of a wide range of flavonoids. Our results suggest that a higher intake of flavonoids might contribute to a better ventilatory function and reduce the risk of spirometric restriction. Two other observational studies have suggested this, although they examined three subclasses of flavonoids, and the effect of pro-anthocyanidins was not investigated. The study of Garcia-Larsen recently showed that catechin intake was associated with a 70 mL higher FVC in a population-based study of young adults, when comparing those in the highest versus the lowest quintile of intake [14]. Similarly, Tabak et al. showed

that total intake of catechins was associated with a 43 mL higher FEV_1 and negatively associated with self-reported symptoms in Dutch adults [25].

After controlling for multiple comparisons, our results showed no evidence of an association with airflow obstruction, but with spirometric restriction. FVC has been linked more strongly to survival and to comorbidities than airflow obstruction [26]. This remains largely unexplained, other than a strong relation to early life factors such as birth weight. The fact that a beneficial association was observed with FVC < LLN and not with measures of airway obstruction, suggest that pro-anthocyanidins might contribute to preserving ventilatory function and might, in turn, prevent an accelerated decline of FVC, thus reducing the risk of general mortality and co-morbidities [27]. Although such an effect would need to be confirmed in longitudinal studies, the findings in the GA^{2}LEN population justify the need for such studies.

Mechanistic effects from experimental studies support our findings. An experimental study using a mouse model to investigate the effect of pro-anthocyanidins on lung inflammation showed that the animals fed with this flavonoid had a significantly reduced production of pro-inflammatory cytokines produced by Th2 cells [2]. The antioxidant activity of pro-anthocyanidins has also been demonstrated in murine model of induced allergic asthma [3,26], where mice treated with the flavonoid extract had significantly lower nitric oxide levels [3], airway hyper-responsiveness [26], and their lung tissue levels of various cytokines, chemokines, and growth factors were lower than those in the mice that did not receive the pro-anthocyanidin extract [27]. In human alveolar cells, pro-anthocyanidin extract has been shown to reduce the IL-4-dependent eosinophil expression [5].

Earlier evidence in humans and rodents showing that flavonoids are poorly absorbed and often excreted un-metabolised [28], has been reappraised in the light of more recent studies. The study of Czek and colleagues showed that specific flavonoids such as anthocyanidins are metabolised to a structurally diverse range of metabolites [29], and that these circulate in plasma in adults for up to 48 hours after ingestion [30]. In animals fed with procyanidin-enriched supplement, metabolites of pro-anthocyanidins (mainly glucoronide forms of epicatechins) were detected in plasma and also found in lung tissue, up to 18 h after ingestion [31]. Metabolites of anthocyanins have also been detected after experimental ingestion in animals [32,33]. These findings suggest that the bioavailability of flavonoids expands beyond the gastro-intestinal tract and that they might play a key role in a number of anti-inflammatory processes in the airways [34].

In the GA^{2}LEN study, we observed that with the exception of Germany, there was a consistent trend across countries for a reduced risk of spirometric restriction with higher in those with the highest intakes of pro-anthocyanidins, with no statistically significant evidence of heterogeneity. The risk reduction of 57% has clinical and public health relevance. The intake of pro-anthocyanidins in the GA^{2}LEN participants was on average 192 mg and came largely from fruit intake. This amount can be achieved by eating 3–4 units of hard fruits a day, which is an attainable target in the general adult population. The statistical significance of this association was retained after applying Simes' procedure. Although the statistical significance of the positive association between FVC and intake of pro-anthocyanidins was attenuated after Simes' procedure, the size of the effect, and the consistent trend across countries found in this study, would confirm the laboratory evidence showing a protective effect of these specific flavonoids.

The associations between flavonoid subclasses and respiratory outcomes were controlled for use of nutritional supplements. Nearly a third of the participants in this study reported taking nutritional supplements, a strong trend widespread in Europe [35]. Bioactive forms of various polyphenols, including flavonoids, are often present in nutritional supplements, and our results are unlikely to be explained by this use.

The strengths of the current study include that we used a representative sample of the European adult population and used several measures of airway restriction and airway obstruction that underwent rigorous quality control before being considered acceptable. We also assessed a wide range of flavonoid subclasses. Dietary flavonoid intakes were calculated from a database developed

using recent USDA databases for flavonoids and pro-anthocyanidins [21,22]. These datasets allowed us to quantify a broad range of flavonoid subclass intakes providing more robust evidence than previous studies on flavonoid intake and lung diseases. The FFQ used in the current study included a comprehensive list of fruits and vegetables, as well as other dietary sources known to have flavonoids. The FFQ was validated in a sample of this study population [17] and reflects the usual dietary intake of these individuals as well as facilitating international comparisons. We also validated the FFQ for intake of flavonoids and found that the FFQ is an accurate instrument to capture dietary sources of flavonoids [36].

This study has some limitations. High flavonoid intake may be representative of a healthy lifestyle in general, and although we adjusted for a range of lifestyle variables, as well as fruit and vegetable intake, residual or unmeasured confounding cannot be ruled out. The cross-sectional nature of our study prevents us from establishing a causal inference in the association between flavonoids and lung function. However, recent longitudinal findings from the elderly adults from the Normative Aging Study showed that a higher intake of anthocyanins was associated with slower FVC decline over 15 years [37], which lends further support to our hypothesis that a higher intake of flavonoids might preserve lung function and possibly reduce the risk of COPD. Epidemiological studies on lung health and flavonoids are still scant, with the exception of lung cancer, for which there is increasing evidence of a protective role of dietary flavonoids [38]. Replications of our findings in longitudinal studies are warranted to confirm the role in reducing the risk of lung disease.

5. Conclusions

In conclusion, this study provides for the first time, observational evidence for a positive association of pro-anthocyanidins and possibly total flavonoid intake and lung function in a multi-centric sample of European adults. The magnitude of the effect is clinically significant and of public health relevance. Increasing the consumption of dietary sources of flavonoids is an achievable target and can contribute to maintaining lung function, and possibly to preventing its accelerated decline.

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Author Contributions: V.G.L. and P.G.J.B. conceived and designed the analyses. N.T. and D.C. are postgraduate research students at Imperial College London and contributed to the analyses. J.F.P. and V.G.L. led the analyses. A.C. advised on the analytical interpretation flavonoid classification and interpretation of results, and provided the composition tables with content estimates for flavonoids. V.G.L. wrote the final version of the manuscript, with contributions from all co-authors. T.V.Z., T.T., M.A., T.H., T.K., P.M.M., G.B., M.L.K., J.M., E.N.M., B.R., C.L., A.T.B., C.B., B.F., C.J. and K.T. contributed data from their centres and interpretation and discussion of the findings. All co-authors approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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Coffee Intake Decreases Risk of Postmenopausal Breast Cancer: A Dose-Response Meta-Analysis on Prospective Cohort Studies

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Abstract: Aim: A dose-response meta-analysis was conducted in order to summarize the evidence from prospective cohort studies regarding the association between coffee intake and breast cancer risk. **Methods:** A systematic search was performed in electronic databases up to March 2017 to identify relevant studies; risk estimates were retrieved from the studies and linear and non-linear dose-response analysis modelled by restricted cubic splines was conducted. A stratified and subgroup analysis by menopausal and estrogen/progesterone receptor (ER/PR) status, smoking status and body mass index (BMI) were performed in order to detect potential confounders. **Results:** A total of 21 prospective studies were selected either for dose-response, the highest versus lowest category of consumption or subgroup analysis. The dose-response analysis of 13 prospective studies showed no significant association between coffee consumption and breast cancer risk in the non-linear model. However, an inverse relationship has been found when the analysis was restricted to post-menopausal women. Consumption of four cups of coffee per day was associated with a 10% reduction in postmenopausal cancer risk (relative risk, RR 0.90; 95% confidence interval, CI 0.82 to 0.99). Subgroup analyses showed consistent results for all potential confounding factors examined. **Conclusions:** Findings from this meta-analysis may support the hypothesis that coffee consumption is associated with decreased risk of postmenopausal breast cancer.

Keywords: coffee; caffeine; breast cancer; receptor; postmenopausal; dose-response; meta-analysis

1. Introduction

Breast cancer is the most frequently diagnosed cancer and among the leading causes of cancer death among females [1–3]. Worldwide and European estimates of women with a diagnosis of breast cancer occurring in the last 5 years were over 6.2 and 1.8 million, respectively, in 2012 [4]. Significant improvements in early diagnosis and treatment have led to decreased mortality in the last

two decades [5,6]. However, evaluating potential risk factors and improving preventive actions is needed in order to decrease the global burden of such disease.

A recent summary of scientific literature provided insightful the evidence of the potential benefits of coffee on human health [7]. Most of evidence relied on observational prospective cohort studies, suggesting that moderate-to-high coffee can be overall associated with lower risk of all-cause, cardiovascular and cancer mortality compared to lower consumption [8]. These protective effects are likely to be mediated by coffee active compounds, including but not limited to, coffee polyphenols, which have been shown to have anti-diabetic, anti-carcinogenic, anti-inflammatory and anti-obesity properties [9–11]. Among others, coffee consumption has been hypothesized to affect the risk of female cancers (breast, endometrial and ovarian cancers) and particularity the risk of breast cancer in post-menopausal women. A recent analysis of dietary patterns of women participating in the Nurses' Health Study II pointed out that a low intake of green leafy vegetables, cruciferous vegetables and coffee during adolescence and early adulthood may increase the incidence of premenopausal breast cancer, thus posing the basis for further research on long-term associations and cumulative effects [12].

Previous quantitative evidence syntheses on breast cancer risk often reported contrasting results, either when assessing the risk estimates for extreme categories of consumption and in a dose-response manner [13–17]. For instance, the meta-analysis carried out by Li and colleagues—on 16 cohort and 10 case-control studies—found a borderline significant association (RR: 0.96; 95% CI: 0.93–1.00) when comparing highest versus lowest coffee consumption; results limited to cohort studies were not significant. Interestingly, a significant inverse association between coffee drinking and breast cancer risk was documented in women without oestrogen receptor (ER-negative subgroup) [18]. Similarly, Jiang and colleagues analysed 20 case-control and 17 cohort studies and reported a borderline association with breast cancer risk of the highest coffee consumption category compared to the lowest (RR: 0.97; 95% CI: 0.93–1.00); yet, a significant inverse association was present in postmenopausal women and in women with breast related cancer antigen 1 (BRCA1) mutations (the latter being highlighted as strong association) [19]. Therefore, coffee consumption might have a protective role for specific subgroups of individuals (i.e., based on receptor and menopausal status) [18,19]; Since the latest meta-analyses from Jing et al. and Li et al., several new prospective studies have been published on the association between coffee intake and breast cancer. Thus, the aim of this study was to update current evidence on the association between coffee consumption and risk of breast cancers, analysing results obtained from prospective studies only, in order to summarize the evidence and provide new insights on potential effect modification of putative confounding factors.

2. Methods

We followed Meta-Analysis of Observational Studies in Epidemiology (MOOSE) protocols throughout preparing background, search strategy, methods and reporting the results, discussion and conclusion of meta-analysis (Table S1).

2.1. Search Strategy

Articles were retrieved by searching two different electronic databases (PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and EMBASE (http://www.embase.com/) and were limited to publications in English language between the earliest available online indexing year and March 2017. The following search strategy based on the conjunction of the three terms: (i) coffee OR caffeine OR beverages AND (ii) breast AND (iii) cancer OR carcinoma OR neoplasm (Table S2) was adopted. Titles and abstracts of all identified studies were independently reviewed by two authors. Based on recent guidelines proposed to draft the highest level of evidence in nutritional science [20], eligibility criteria for study inclusion in the meta-analysis were based on the following criteria: (1) a prospective design; (2) coffee consumption as the exposure of interest; (3) incidence of breast cancer as the outcome; (4) the measure of association (relative risk or hazard ratio) with 95% confidence interval provided for 3 or more quantitative categories of coffee consumption. Hand searching the reference lists of obtained

manuscripts was also performed to find additional studies not previously detected. In the case of duplicated published cohorts, the one with the largest number of cases/entire cohort or with the longest follow-up for endpoint of interest was included.

2.2. Data Extraction

Using a standardized extraction form, data were abstracted from all identified studies. The following information was obtained from each article: (1) first author name; (2) year of publication; (3) study cohort name; (4) country; (5) sex of participants; (6) age range of the study population at baseline; (7) categories of coffee consumption; (8) type of coffee; (9) follow-up period; (10) distribution of cases and person-years/number of participants, across categories of exposure; (11) relative risks or hazard ratios, with 95% CIs for all categories of exposure; (12) covariates used in adjustments. Extraction of data was conducted independently by two authors. Discrepancies were resolved through a consensus discussion, The Newcastle-Ottawa Quality Assessment Scale was used to assess the quality of included studies [21].

2.3. Statistical Analysis

For the purpose of this meta-analysis, relative risks (RRs) or hazard ratios (HRs) with 95% confidence intervals (CIs) for all categories of coffee consumption were extracted based on the most fully adjusted models. Pooled effects were assessed by random-effect meta-analyses in which RRs and HRs were treated as equivalent measure of risk [22] and the term of the relative risk refer to both of them. Heterogeneity was assessed using I^2 statistic and the Cochran's Q test. The I^2 statistic represented the amount of total variation that could be attributed to heterogeneity and its values $\leq$25%, 25–50%, 50–75% and >75% indicated no, small, moderate and significant heterogeneity, respectively. The P values of Q test of less than 0.1 were accepted as statistically significant. The relationship between coffee consumption and risk of breast cancer was firstly determined by highest versus lowest analysis. The stability of the results was assessed through a sensitivity analysis in which one study at a time was excluded. Potential confounders or effect modifiers and sources of heterogeneity were verified in subgroup analysis and publication bias was tested visually by detecting asymmetry of funnel plots.

Based on retrieved data (amount of exposure, distributions of cases and person-years or number of participants and RRs/HRs with 95% CIs) for each category (at least three) of coffee consumption, a dose-response meta-analysis was performed. Within the studies, the mean or median intake, alternatively the midpoint of the range of intake of coffee consumption was assigned to the corresponding RR/HR with the 95% CI. Right-unbounded highest categories of exposure were assumed to have the same width as the adjacent one. Both linear and non-linear dose-response relationship between coffee intake and risk of total and postmenopausal breast cancer was assessed by random-effect meta-analysis performed in two stages. In the first stage, the generalized least-squares (GLS) method reported by Greenland and Orsini was implemented and study-specific coefficients were calculated based on retrieved data across categories of coffee consumption and taking into account the intraclass (within study) correlation of RRs/HRs [23,24]. Non-linear dose-response analysis was modelled by restricted cubic splines with 3 knots at fixed percentiles (25%, 50% and 75%) of the distribution [25]. In the second step of the random-effect meta-analysis summary statistics from each study were combined. The between-study variance in linear dose-response meta-analysis and the between-study covariance matrices in non-linear dose-response meta-analysis was assessed by DerSimonian and Laird estimator or multivariate extension of the method of moments, respectively. P-value for non-linearity was calculated by testing the value of the coefficient of the second spline of zero. All analyses were performed with R software version 3.0.3, to conduct dose-response meta-analysis the package dosresmeta was used (Development Core Team, Lucent Technologies, Vienna, Austria).

3. Results

3.1. Study Characteristics

The systematic search identified 1724 studies, of which 1533 were excluded after reviewing the title and 162 after reviewing the abstract (Figure 1). Of the 29 publications selected for evaluation of full-text article, 8 were excluded for the following reasons: (1) article did not provide risk with confidence intervals; (2) article did not have prospective design; (3) article provided data only on genetic polymorphism; (4) article did not provide data for general population.

For the meta-analysis on the association between coffee consumption and breast cancer risk 21 studies were eligible [26–46]. Several cohorts, namely VIP (Västerbotten Intervention Programme), NOWAC (Norwegian Women and Cancer), E3N (Etude Epidémiologique auprès de femmes de la Mutuelle Générale de l'Education Nationale) and EPIC-NL (European Prospective Investigation into Cancer and Nutrition—The Netherlands) [26,29,40,42] were excluded from the main analysis, as part of theirs cases are included in the multicentre study EPIC [27]. However, an alternative analysis was performed by including these cohorts and excluding EPIC study. Two articles were used only for subgroup analysis [33,41]. Studies eligible for the main analysis comprised 1,068,098 participants and 36,597 breast cancer cases. Selected characteristics of the studies included in the meta-analysis are described in Table 1. Eight studies provided relative risk for postmenopausal [27,28,30,31,33,37,43,45] and for premenopausal status [27,28,32,37,41,43,45,46]. Seven studies were conducted in North America [28,30–32,34,37,46], 6 in Europe [27,35,36,39,43,45] and 2 in Asia [38,44]. The follow-up in prospective cohort studies ranged from about 5 to 26 years.

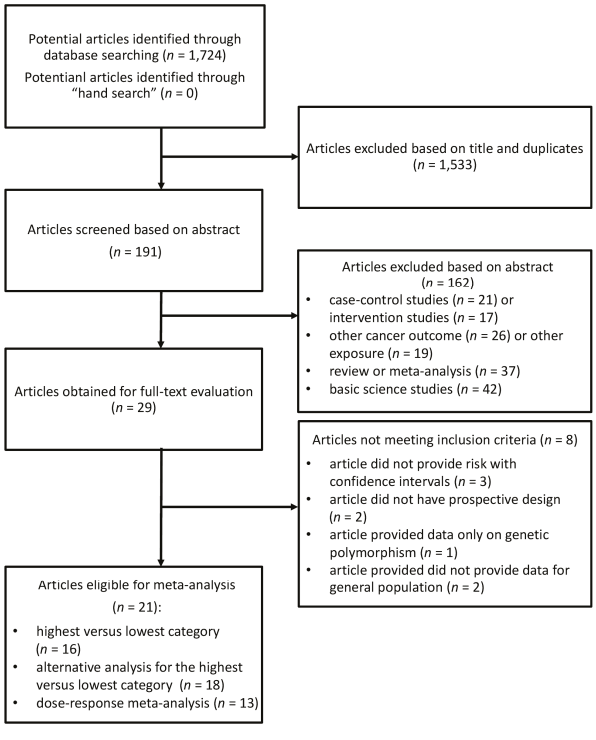

Figure 1. Flow chart of selection of studies reporting on the association between coffee consumption and breast cancer risk.

Table 1. Selected characteristics of the studies included in the meta-analysis. RR = relative risk. HR = hazard ratio.

Author, Year	Cohort Name, Country	Years of Study, Follow-Up	Cases; Total Population	RR (95%CI) for Highest vs. Lowest Category of Coffee Consumption	Adjustments
Vatten, 1990 [45]	National Health Screening Service, Norway	1974-NR, 12 years (mean)	155; 14,593	≥7 cups/day vs. ≤2 cups/day; RR = 0.80 (0.50, 1.40)	Age.
Hoyer, 1992 [36]	The Glostrup Population Studies, Denmark	1964–1990, 26 years (maximum)	51; 5207	≥7 cups/day vs. ≤2 cups/day; RR = 1.70 (0.70, 4.30)	Social class, age at menarche, menopause status, number of full-term pregnancies, height, weight, BMI, alcohol, smoking.
Folsom, 1993 [30]	Iowa Women's Health Study, USA	1986–1990, 5 years	580; 34,388	≥4 cups/day vs. 0 cups/day; RR = 1.02 (0.79, 1.30)	Age, waist/hip ratio, number of live births, age at first live birth, age at menarche, FHBC (family history of breast cancer), family history × waist/hip ratio and family history × number of live births.
Key, 1999 [38]	The Radiation Effects Research Foundation's Life Span Study, Japan	1969–1993, 24 years (maximum)	427; 34,759	≥5 cups/week vs. ≤1 cups/week; RR = 1.19 (0.93, 1.52)	Age, calendar period, city, age at time of bombings, radiation dose.
Michels, 2002 [41]	Swedish Mammography Cohort, Sweden	1987–1997, 9.5 years	1271; 59,036	≥4 cups/day vs. ≤1 cup/day; HR = 0.94 (0.75, 1.28)	Age, family history of breast cancer, height, BMI, education, parity, age at first birth, alcohol consumption, total caloric intake.
Suzuki, 2004 [44]	Cohort I-Cohort II, Japan	1984-NR Cohort I, 9 years;1990-NR Cohort II, 7 years	103; 8799 Cohort I; 119; 11,288 Cohort II	≥1 cup/day vs. never; RR = 0.81 (0.55, 1.18)	Age, types of health insurance, age at menarche, menopausal status, age at first birth, parity, mother's history of breast cancer, smoking, alcohol drinking, BMI, consumption frequencies of black tea.
Hirvonen, 2006 [35]	Supplementation en Vitamines et Mineraux Antioxydants (SU.VI.MAX), France	1994–2002, 6.6 years (median)	95; 4396	≥3 cups/day vs. ≤1 cup/day; RR = 1.10 (0.66, 1.84)	Age, smoking, number of children, use of oral contraception, family history of breast cancer, menopausal status.
Ganmaa, 2008 [31]	National Health Service I, USA	1976–2002, 22 years	5272; 85,987	≥4 cups/day vs. <1 cup/month; RR = 0.92 (0.82, 1.03)	Age months, smoking status, BMI, physical activity, height, alcohol intake, family history of breast cancer in mother or a sister, history of benign breast disease, menopausal status, age at menopause, use of hormone therapy, age at menarche, parity and age at first birth, weight change after 18, duration of postmenopausal hormone use, tea intake.
Ishitani, 2008 [37]	Women's Health Study, USA	1992–2004, 10 years (average)	1188; 38,432	≥4 cups/day vs. almost never; RR = 1.08 (0.89, 1.30)	Age, randomized treatment assignment, BMI, physical activity, total energy intake, alcohol intake, multivitamin use, age at menopause, age at menarche, age at first pregnancy lasting ≥6months, number of pregnancies lasting ≥6months, menopausal status, postmenopausal hormone use, prior hysterectomy, prior bilateral oophorectomy, smoking status, family history of breast cancer in mother or a sister, history of benign breast disease.
Larsson, 2009 [39]	Swedish Mammography Cohort (SMC), Sweden	1987–2007, 17.4 years (mean)	2952; 61,433	≥4 cups/day vs. <1 cup/day; RR = 1.02 (0.87, 1.20)	Age, education, BMI, height, parity, age at first birth, age at menarche, age at menopause, use of oral contraceptives, use of postmenopausal hormones, family history of breast cancer and intakes of total energy, alcohol and tea.
Wilson, 2009 [46]	National Health Service II, USA	1991–2005, 14 years	1179; 90,628	≥3 cups/day vs. <1 cup/day; RR = 0.92 (0.77, 1.11)	BMI, height, OC use, parity and age at first birth, age at menarche, family history of breast cancer, history of benign breast disease, smoking, physical activity, animal fat, glycaemic load, alcohol and energy.

Table 1. *Cont.*

Author, Year	Cohort Name, Country	Years of Study, Follow-Up	Cases; Total Population	RR (95%CI) for Highest vs. Lowest Category of Coffee Consumption	Adjustments
Bhoo-Pathy, 2010 [26]	European Prospective Investigation into Cancer and Nutrition (EPIC-NL), Netherlands	1993–2007, 9.6 years (average)	681; 27,323	>5 cups/day vs. <1 cup/day; HR = 0.94 (0.72, 1.24)	Age, smoking status, educational status, BMI, alcohol intake, energy intake, energy-adjusted saturated fat intake, energy-adjusted fibre intake, tea intake, physical activity level, ever prior use of oral contraceptives, presence of hypercholesterolemia, family history of breast cancer, age at menarche, parity and cohort.
Boggs, 2010 [28]	Black Women's Health Study, USA	1995–2007, 12 years	1268; 52,062	≥4 cups/day vs. never; RR = 1.03 (0.77, 1.39)	Age, energy intake, age at menarche, BMI at age 18, family history of breast cancer, education, geographic region, parity, age at first birth, oral contraceptive use, menopausal status, age at menopause, female hormone use, vigorous activity, smoking status, alcohol intake.
Nilsson, 2010 [42]	Västerbotten Intervention Programme (VIP), Sweden	1992–2007, 15 years (maximum)	587; 32,178	≥4 cups/day vs. <1 cup/day; HR = 0.92 (0.68, 1.25)	Age, sex, BMI, smoking, education, recreational physical activity.
Fagherazzi, 2011 [29]	Etude Epidémiologique auprès de femmes de l'Education Nationale (E3N), France	1993–2005, 11 years (median)	2868; 67,703	>3 cups/day vs. never; HR = 1.02 (0.90, 1.16)	Age, total energy intake, ever use of oral contraceptives, age at menarche, age at menopause, number of children, age at first pregnancy, history of breast cancer in the family and years of schooling, current use of postmenopausal hormone therapy (for postmenopausal women only), personal history of benign breast disease, menopausal status, BMI.
Gierach, 2012 [32]	NIH-AARP Diet and Health Cohort Study, USA	1995–2006, 9.8 years (average)	9915; 198,404	≥4 cups/day vs. never; RR = 0.98 (0.91, 1.07)	Age, race/ethnicity, education, BMI, smoking status and dose, alcohol, proportion of total energy from fat, age at first live birth, menopausal hormone therapy use, history of breast biopsy, family history of breast cancer.
Bhoo-Pathy, 2015 [27]	European Prospective Investigation into Cancer and Nutrition (EPIC), Multicentre	1992–2010, 11 years (average)	10,198; 335,060	Highest quartile vs lowest quartile; HR = 1.00 (0.98, 1.03) for premenopausal women; HR = 0.99 (0.98, 0.99) for postmenopausal women	Age at menarche, ever use of oral contraceptives, age at first delivery, ever breastfeeding, smoking status, educational level, physical activity level, alcohol intake, height, weight, energy intake from fat source, energy intake from non-fat source, total saturated fat intake, total fibre intake, ever-use of postmenopausal hormones.
Harris, 2015 [33]	Swedish Mammography Cohort (SMC), Sweden	1987–2012, 15 years	1603; 37,004	Highest quartile vs lowest quartile; HR = 0.86 (0.72, 1.04)	Age, energy intake, height, BMI, education, oral contraceptive use, hormone replacement therapy use, age at menarche, age at menopause, family history of breast cancer, history of benign breast disease, smoking status, physical activity, alcohol intake.
Hashibe, 2015 [34]	Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial, USA	1992–2011, 13 years (maximum)	1703; 50,563	≥2 cups/day vs. <1 cup/day; RR = 0.97 (0.87, 1.08)	Age, sex, race and education, drinking frequency.
Oh, 2015 [43]	Swedish Women's Lifestyle and Health study, Sweden	1991–2012, 11 years (average)	1395; 42,099	≥5 cups/day vs. ≤2 cups/day; RR = 0.81 (0.70, 0.94)	Age, BMI, duration of breastfeeding, alcohol consumption.
Lukic, 2016 [40]	Norwegian Women and Cancer study (NOWAC), Norway	1991–2013, 13.1 years (average)	3277; 91,767	≥7 cups/day vs. ≤1 cup/day; HR = 0.87 (0.71, 1.06)	Menopausal status, smoking status, age at smoking initiation, number of pack-years, exposure to cigarette smoke during childhood, duration of education, BMI, physical activity level, alcohol consumption, number of children, age at first birth, ever use of oral contraceptives, duration of oral contraceptive use, use of hormone replacement therapy, maternal history of breast cancer, total energy intake, intake of fibres, intake of processed meat, intake of red meat, height, participation in mammography screening.

3.2. Summary Relative Risk for the High vs. Lowest Category of Coffee Consumption

The summary RR of breast cancer for highest vs. lowest category of coffee consumption was RR = 0.96, 95% CI: 0.93, 1.00 with small heterogeneity I^2 = 7%, p = 0.37 (Figure 2, Table 2); no publication bias was found after visual inspection of funnel plot (Figure S1). Two studies [36,38] were at higher risk of bias and provided very small heterogeneity to the overall analysis, despite no specific reasons have been identified; we may hypothesize that quality of the data was not optimal since the studies were not conducted specifically on coffee as variable of interest but this is only speculative and no data can support this explanation. Since several cohorts (VIP, NOWAC, E3N, EPIC-NL) share the cases with EPIC multicentre study an alternative analysis by excluding EPIC study and including the others was performed. The summary RR of breast cancer for highest vs. lowest category of coffee consumption in alternative analysis was RR = 0.96, 95% CI: 0.93, 1.00 with no evidence of heterogeneity I^2 = 0%, P = 0.48; indicating the stability of the results. In the stratified analysis, we found a significant inverse association between coffee consumption and breast cancer risk among postmenopausal women (RR = 0.92, 95% CI: 0.88, 0.98 with no evidence of heterogeneity I^2 = 0%, p = 0.57). Stratified analyses were carried out for receptor status and BMI: none of them showed a significant association between coffee intake and cancer risk; yet, similar point estimates and confidence intervals were found for the receptor-negative group and for the overweight and obese group. Interestingly, results were not influenced by coffee type (caffeinated versus decaffeinated), nor for time of follow-up. Adjusted analyses were carried out for smoking, alcohol intake, physical activity and education, without evidence of effect modification.

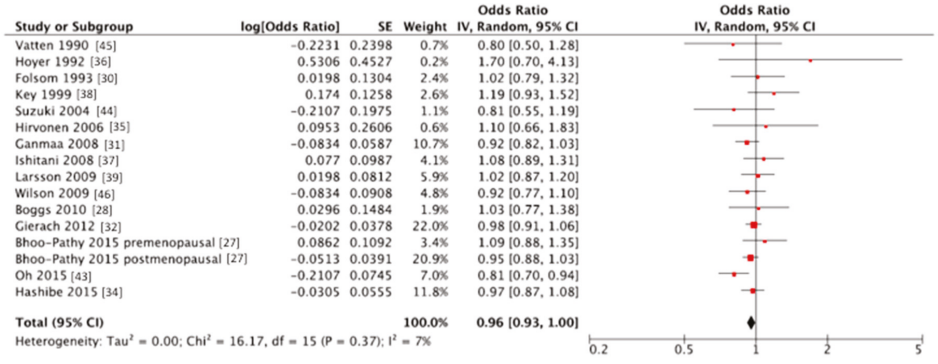

Figure 2. Forest plot of summary relative risks (RRs) of breast cancer for the highest versus lowest (reference) category of coffee consumption. SE = standard error; CI = confidence interval; IV = instrumental variable.

Table 2. Subgroup and additional analyses of studies reporting risk of breast cancer for the highest versus lowest (reference) category coffee consumption.

Subgroup	No. of Datasets	RR (95% CI)	I^2	$P_{heterogeneity}$
Total	16	0.96 (0.93, 1.00)	7%	0.37
(Alternative analysis)	18	0.96 (0.93, 1.00)	0%	0.48
Geographical location				
North America	7	0.97 (0.93, 1.02)	0%	0.84
Europe	7	0.95 (0.87, 1.05)	36%	0.15
Asia	2	1.01 (0.70, 1.47)	63%	0.10
Menopausal status				
Premenopausal	8	0.98 (0.89, 1.07)	0%	0.46
Postmenopausal	8	0.92 (0.88, 0.98)	0%	0.57

Table 2. *Cont.*

Subgroup	No. of Datasets	RR (95% CI)	I^2	$P_{heterogeneity}$
Receptor status				
ER+/PR+	5	0.97 (0.89, 1.07)	0%	0.40
ER+/PR- or ER-/PR+	5	0.98 (0.82, 1.17)	0%	0.60
ER-/PR-	5	0.92 (0.79, 1.07)	0%	0.82
Coffee type				
Caffeinated	6	0.96 (0.91, 1.01)	0%	0.45
Decaffeinated	6	0.97 (0.90, 1.04)	0%	0.67
BMI				
<25 kg/m^2	5	0.98 (0.87, 1.10)	0%	0.42
≥25 kg/m^2	5	0.91 (0.79, 1.04)	0%	0.69
Duration of follow-up				
<10 years	3	0.97 (0.80, 1.18)	0%	0.55
≥10 years	13	0.97 (0.92, 1.01)	20%	0.24
Adjustment for smoking				
No	7	0.95 (0.86, 1.05)	41%	0.12
Yes	9	0.97 (0.93, 1.01)	0%	0.67
Adjustment for alcohol intake				
No	5	1.02 (0.88, 1.19)	0%	0.41
Yes	11	0.96 (0.92, 1.00)	13%	0.32
Adjustment for physical activity				
No	10	0.97 (0.90, 1.04)	27%	0.20
Yes	6	0.96 (0.91, 1.01)	0%	0.58
Adjustment for education				
No	10	0.95 (0.87, 1.04)	32%	0.16
Yes	6	0.98 (0.93, 1.02)	0%	0.86

3.3. Dose-Response Meta-Analysis

Thirteen studies [26,28,29,31,32,34,37–40,42,43,45] were eligible for dose-response meta-analysis of prospective cohort studies on coffee consumption and breast cancer risk. Six studies [32,34,41–43,45] provided risk estimates for postmenopausal woman only. In linear dose-response meta-analysis a significant association between coffee consumption and breast cancer risk was found (Figure 3, Table 3). Compared with no coffee consumption, the pooled relative risks for breast cancer were 0.99, 95% CI: 0.98, 1.00 for one cup/day, 0.98, 95% CI: 0.96, 0.99 for two cups/day, 0.97, 95% CI: 0.94, 0.99 for three cups/day, 0.96, 95% CI: 0.93, 0.99 for four cups/day, 0.95, 95% CI: 0.91, 0.98 for five cups/day, 0.93, 95% CI: 0.89, 0.98 for six cups/day and 0.92, 95% CI: 0.88, 0.98 for seven cups/day. When taking into account postmenopausal woman only, the association between coffee consumption and risk of breast cancer was stronger.

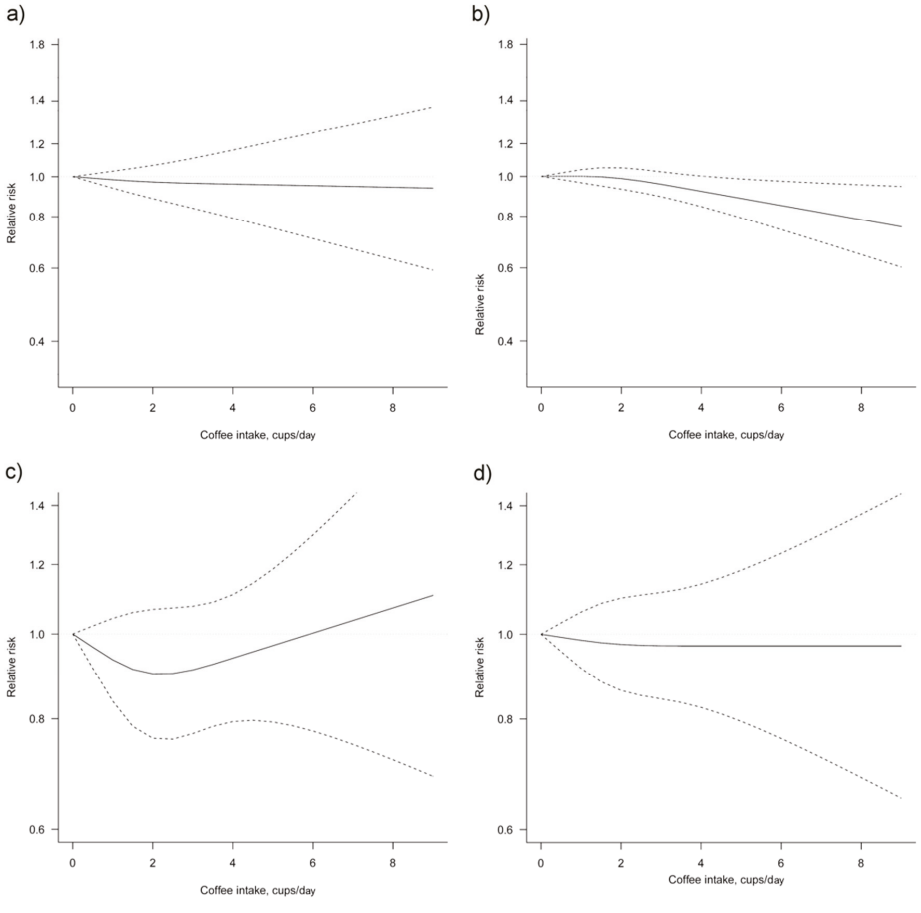

Figure 3. Dose-response association between coffee consumption and breast cancer risk (**a**) non-linear, total analysis; (**b**) non-linear, postmenopausal; (**c**) non-linear, receptor status ER-/PR-; (**d**) non-linear, BMI $\geq$ 25 kg/m^2. Solid lines represent relative risk, dashed lines represent 95% confidence intervals.

Table 3. Dose-response meta-analysis of prospective cohort studies on coffee consumption and breast cancer risk.

	No. of Datasets (No. of Studies)	Coffee Intake (Cups/Day)								I^2 (%)	$P_{heterogeneity}$	$P_{non\text{-}linearity}$
		0	1	2	3	4	5	6	7			
Total												
Non-linear	13 (13)	Ref.	0.98 (0.94, 1.03)	0.97 (0.88, 1.06)	0.96 (0.84, 1.11)	0.96 (0.79, 1.16)	0.95 (0.75, 1.22)	0.95 (0.71, 1.28)	0.95 (0.67, 1.34)	-	0.69	0.52
Linear	13 (13)	Ref.	0.99 (0.98, 1.00)	0.98 (0.96, 0.99)	0.97 (0.94, 0.99)	0.96 (0.93, 0.99)	0.95 (0.91, 0.98)	0.93 (0.89, 0.98)	0.92 (0.88, 0.98)	-	0.58	NA
Postmenopausal												
Non-linear	6 (6)	Ref.	1.00 (0.96, 1.04)	0.99 (0.93, 1.05)	0.96 (0.89, 1.03)	0.92 (0.84, 1.00)	0.88 (0.79, 0.99)	0.85 (0.74, 0.97)	0.82 (0.69, 0.96)	0	0.71	0.14
Linear	6 (6)	Ref.	0.97 (0.95, 1.00)	0.95 (0.90, 1.00)	0.92 (0.86, 1.00)	0.90 (0.82, 0.99)	0.88 (0.78, 0.99)	0.85 (0.74, 0.99)	0.83 (0.70, 0.99)	39.6	0.14	NA
BMI > 25 kg/m^2												
Non-linear	5 (5)	Ref.	0.98 (0.91, 1.06)	0.97 (0.86, 1.10)	0.97 (0.84, 1.12)	0.97 (0.82, 1.14)	0.97 (0.80, 1.18)	0.97 (0.76, 1.24)	0.97 (0.72, 1.30)	10.1	0.35	0,77
Linear	5 (5)	Ref.	0.99 (0.96, 1.02)	0.98 (0.92, 1.05)	0.97 (0.88, 1.08)	0.96 (0.84, 1.10)	0.95 (0.81, 1.13)	0.95 (0.77, 1.16)	0.94 (0.74, 1.19)	39	0.16	
ER-/PR-												
Non-linear	5 (4)	Ref.	0.93 (0.84, 1.04)	0.9 (0.76, 1.07)	0.91 (0.77, 1.08)	0.94 (0.79, 1.11)	0.97 (0.79, 1.19)	1.00 (0.77, 1.30)	1.04 (0.75, 1.43)	0	0.88	0.25
Linear	5 (4)	Ref.	0.99 (0.95, 1.03)	0.98 (0.91, 1.06)	0.97 (0.86, 1.10)	0.97 (0.82, 1.13)	0.96 (0.78, 1.17)	0.95 (0.75, 1.20)	0.94 (0.71, 1.24)	66.3	0.43	NA

4. Discussion

The present dose-response meta-analysis, which included 13 prospective studies and followed over 1 million people, did not show a significant association between coffee consumption and overall breast cancer risk in the non-linear model. However, an inverse relationship has been found when the analysis was restricted to post-menopausal women (RR 0.92, 95% CI: 0.88–0.98).

The meta-analysis performed by Li and colleagues [18], on 16 cohort and 10 case-control studies, showed a borderline significant inverse association between coffee intake and the risk of breast cancer (RR: 0.96, CI 95%: 0.93–1.00 for highest versus lowest analysis; RR: 0.98, CI 95%: 0.97–1.00 for an increment of 2 cups per day). Statistical significance was reached only for those women without oestrogen receptor (ER-negative, RR: 0.81, 95% CI: 0.67–0.97). In our study, such finding was not confirmed. The work carried out by Jiang and colleagues [19], which included 17 prospective and 20 case-control studies, found no significant association between coffee consumption and breast cancer risk (highest versus lowest analysis: RR: 0.98, CI 95% 0.95–1.02; dose-response analysis: RR: 0.98, 95% CI: 0.92–1.05 for an increment of 2 cups per day). Such relationship became significant for post-menopausal women, with a stronger inverse relationship for those with BRCA1 mutation. Overall results are mostly confirmed in our meta-analysis, even though we included a higher number of individuals and performed alternative analyses to avoid overlap of cohorts. However, while our analysis confirmed a statistically significant relationship between coffee intake and breast cancer in post-menopausal women, we were not able to draw insights on the role of BRCA status (evidence derived from case-control studies, not eligible for our meta-analysis). Moreover, similarly to our study, the meta-analysis of Jiang and colleagues differentiated the analysis according to follow-up duration and found similar results between studies with long (>10) and short (<10 years) follow-up.

Several studies associated coffee consumption with health benefits, including decreased risk of cancers (e.g., colorectal, endometrial and prostate cancers) [7,13,14]. Several compounds have been considered responsible for such potential protective effects, including polyphenols (such as chlorogenic acids), diterpens (such as cafestol and kahweol) but also melanoidins (generated during the roasting process) and trigonelline [47–50]. Data from in vitro and in vivo studies suggest that coffee could interfere with different stages of the cancerous process, including induction of DNA damage caused by pro-carcinogens and reactive oxygen species (ROS), activation of proto-oncogenes and inactivation of onco-suppressor genes, loss of apoptosis and growth control, induction of angiogenesis and consequent metastatic process [11].

Our study revealed significant results for post-menopausal women. Several mechanisms have been proposed to act specifically in the etiopathogenesis of female cancers: for instance, caffeine and coffee intake have been inversely associated with free estradiol levels in premenopausal women, either directly or indirectly (relationship mediated by the sex hormone-binding globulin-SHBG) [51–53]. SHBG, which is the major carriers of sex-steroids, lowers the circulating free levels of oestrogens and the positive association between coffee intake and SHBG has been documented in post-menopausal women as well [54]. Similarly, coffee has been associated with the inhibition of CYP19, or aromatase, the enzyme converting androgens into oestrogens [51]. Circulating oestrogens are well-established risk factors for breast cancer [55].

Despite the complexity of the described mechanisms, it is crucial to understand the pathways through which coffee may decrease risk of cancer. Our finding, showing a similar association for caffeinated and decaffeinated coffee, may suggest a limited role of caffeine on breast cancer risk (the literature highlights the role of caffeine on neurotransmitters, therefore on neurological, cardiorespiratory and gastrointestinal diseases) [7]. It is therefore more plausible that the action on carcinogenesis is mediated by other coffee compounds [11]. The beneficial effects may be related to the antioxidant ability, as coffee is one of the major contributor to dietary antioxidant intake worldwide [56,57]. However, based on the observational nature of the studies involved in this research, we cannot rule out the possibility of existence of confounding factors or effect modifiers that can indirectly explain the potential benefits of coffee toward breast cancer risk. For

instance, one of the stronger evidence regarding coffee consumption regards liver and, more generally, metabolic health [58]. Several studies showed a better metabolic status occurring in individuals characterized by high consumption of coffee [10,59–68]. We found no difference in the relationship between coffee intake and breast cancer risk according to BMI; nevertheless, obesity is a well-recognized risk factor for breast cancer and hormonal impairment consequent to excess body weight may be inversely associated with consumption of coffee [69,70]. The metabolic syndrome, also known as insulin resistance syndrome, is a multifactorial disease well related to breast cancer risk of incidence and recurrence [71,72] with a crucial role in the activation of many endocrine and immune factors linked to breast cancer cell proliferation, survival and chemo-resistance. Among different nutritional approach aimed to revert metabolic syndrome in breast cancer patients, it was recently shown that consumption of coffee in adults up to three cups a day reduces the risk of type-2 diabetes, the metabolic syndrome as well as non-alcoholic fatty liver disease (NAFLD) [58,73]. Several studies showed that the association is rather stronger with the overall metabolic status than with individual components of the syndrome [67,74]. A crucial endocrine role in breast cancer risk and progress may be related to low levels of adiponectin (having anti-cancer effect) and high levels of leptin (having insulin and IGF1-like effects in cancer cells) in the blood [75,76]; interestingly, in a cross-sectional study, it was demonstrated that coffee consumption has a significant positive associations with adiponectin and inverse associations with leptin as well as with the inflammatory marker high sensitivity C-reactive protein (hs-CRP) indicating interesting endocrine and metabolic effects of coffee consumption [77].

In our study, we investigated the possible role of confounding factors in determining the association between coffee consumption and breast cancer risk. It has been reported that smoking status, education, physical activity and BMI, may cluster together with dietary choices that may affect cancer risk (i.e., consumption of meat, fruit and vegetable) [78]. Besides BMI, for which some studies provided specific RRs and we were able to perform the aforementioned stratified risk analysis, in our study the results of studies grouped according to adjustment for specific variables, such as smoking status, education and physical activity, did not weakened the results, rather they shortened CIs despite yet not significant, suggesting that better designed cohort studies properly adjusting for potential confounding factors may enhance the overall quality of comprehensive quantitative synthesis and leading to significant results toward lower risk of breast cancer associated with higher consumption of coffee.

Another indirect confounding effect may be related to alcohol consumption: alcohol is another known risk factor for breast cancer through the alteration of biological pathways associated to hormone levels, the production of carcinogens through metabolism of ethanol and the inhibition of the one-carbon metabolism and nucleotide biosynthesis [79,80]. Some descriptive studies on beverage drinking patterns suggest that increased coffee/tea consumption might be associated with alcohol intake [81–86]. However, our results showed that studies adjusting for alcohol intake provided lower risk estimates for the association between coffee and breast cancer risk, suggesting that adjustment for such lifestyle factors is crucial when exploring the potential effects of coffee on health. Coffee drinking has been associated with smoking habits, which in turn may exert detrimental effects on health [87]; in contrast, coffee might be a part of an overall healthier dietary pattern, which in turn might be associated with lower risk of cancer due to a synergistic effect of several components rather than an individual food or beverage [88]. More in-depth studies with further evaluation of genetic parameters are ongoing but additional research is needed to conclude a causal relation between coffee consumption and risk of cancer.

Besides the aforementioned limitations related to the original design of the studies included in this meta-analysis, the presented results should be considered in light of some other limitations. No data on methods of preparation have been provided in the studies, leading to possible heterogeneity of results due to differences in quantity and quality of bioactive compounds [89]. Finally, due to the observational nature of the studies included in the meta-analysis we cannot exclude the possibility of recall bias and reverse causation.

5. Conclusions

In conclusion, overall we observed no significant association between coffee intake and breast cancer risk but coffee consumption may represent a protective factor for post-menopausal breast cancer risk. Further evidences taking into account population subsets and specific strata are extremely needed to corroborate the retrieved associations.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/10/2/112/s1, Figure S1: Funnel plot for breast cancer risk of the highest versus lowest (reference) category of coffee consumption, Table S1: Meta-Analysis of Observational Studies in Epidemiology (MOOSE) checklist, Table S2: Search strategy.

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Author Contributions: A.L., A.M. and M.B. conceived the study; A.L. wrote introduction and discussion in consultation with P.D.P., S.B., P.R., V.Q. and M.B.; A.M. carried out the statistical analysis and wrote methods and results.

Conflicts of Interest: The authors declare no conflict of interest.

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nutrients

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Review

Phytochemical and Pharmacological Properties of *Capparis spinosa* as a Medicinal Plant

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Abstract: Over the past decades, there has been increasing attention on polyphenol-rich foods including fruits and vegetables on human health. Polyphenols have been shown to possess some potential beneficial effects on human health and they are widely found in foods consumed by populations worldwide. *Capparis spinosa* (*C. spinosa*) is an important source of different secondary metabolites of interest to humankind. The traditional therapeutic applications of *C. spinosa* have been reported in Ancient Romans. Numerous bioactive phytochemical constituents have been isolated and identified from different parts (aerial parts, roots and seeds) of *C. spinosa* which are responsible alone or in combination for its various pharmacological activities. Therefore, this paper is a review of publications on the phytochemical and pharmacological properties of *C. spinosa*. There is insufficient evidence to suggest that *C. spinosa* or its extracts are able to improve the biomarkers of cardiovascular disease and diabetes. However, these studies used different parts of *C. spinosa* plant, methods of preparation and types of solvents, which cause the evaluation of activity of *C. spinosa* difficult and involve quite heterogeneous data. There is also evidence, although limited, to suggest benefits of *C. spinosa* in improving human health. Therefore, the relationship between *C. spinosa* and improved human health outcomes requires further study.

Keywords: *Capparis spinosa*; caper; cardiovascular disease; traditional medicine; flavonoids; rutin

1. Introduction

Medicinal plants have been used since ancient times as therapeutic agents for the management of health and treatment of diseases because they possess health-promoting effects and contain bioactive components [1]. According to the World Health Organization (WHO) [2], 80% of the world's populations rely mainly on traditional medicine. In China, 30–50% of the overall medicinal consumption is estimated from the preparations of traditional medicine [3]. Approximately 90% of the population in Germany reported that they have used natural remedies for certain health purposes [2]. Therefore, there is increasing use and popularity of traditional medicine in both the developing and industrialized countries [4], demonstrating that the global market for traditional medicine continues to be strong. The international market for herbal medicines has hit over $60 billion yearly and it continues to increase gradually [4]. Therefore, medicinal plants such as *Capparis spinosa* (*C. spinosa*) continue to play a major role in healthcare systems [4].

C. spinosa is one of the most important economical species in the *Capparidaceae* family which has a wide range of diversity (i.e., about 40–50 genera and 700–900 species) [5]. *Capparidaceae* has been known to be closely related to the family of the *Brassicaceae* (*Cruciferae*) that is rich in glucosinolates and flavonoids [5]. *C. spinosa* is also known as Caper, wild watermelon (in China) [6], Cappero (in Italy), Alaf-e-Mar (in Persian) [7] and Alcapparo (in Spain) [8]. *C. spinosa* is a dicotyledonous perennial shrub

which can grow up to 1 m high and has extensive root systems [9]. It is native to the Mediterranean basin and widely distributed from Morroco to Crimea, Armernia, Iran [10]. Several countries such as Greece, Italy, Spain and Turkey have widely produced *C. spinosa* [11]. For example, Spain and Turkey produce about 1000 and 4500 tonnes of *C. spinosa* a year, respectively [8].

Few articles [12,13] have reviewed some of the chemical compounds and health benefits of *C. spinosa* in different aspects including its potential for sustainability. Therefore, the aim of our work was to summarise and review the phytochemical of *C. spinosa* including aerial parts, roots and seeds with reference to their pharmacological activity shown in animal and human studies. In addition, our work has also added significantly to the current understanding of the bioactive compounds isolated from the roots and seeds of *C. spinosa*, which has not been adequately covered in the previous literature reviews.

1.1. Search Strategy

An electronic literature search was conducted using Cochrane Library, PubMed, Medline (OvidSP) and Google Scholar until October 2017. Additional references were hand-searched. Search terms included combinations of the following using Boolean markers: *Capparis spinosa*, *C. spinosa*, *Capparis*, caper, phytochemical, pharmacological and antioxidant. The search was restricted to English language publications that addressed the phytochemical constituents and/or pharmacological properties of *C. spinosa*.

1.2. Taxonomic Revision of Genus Capparis

There are 813 plant name records that matched *Capparis* in the plant database 'The Plant List' (at http://www.theplantlist.org). When *C. spinosa* was searched in the same database, 22 plant name records were found. There have been 250 different species in the genus of *Capparis* which are recognized morphologically. However, the use of morphological markers of the *Capparis* species has its limitations in defining the subspecies and varieties because of the free hybridization of different *Capparis* species and the presence of their intermediate forms [14]. Therefore, the classification of *Capparis* remains to be very ambiguous and controversial [15]. Aichi-Yousfi et al. [16] demonstrated that the use of amplified fragment length polymorphism (AFLP) analysis was efficient to make definitive discrimination among the genetic diversity and relationship between various species of *Capparis*. The authors used three primer combinations of AFLP markers to genotype 213 *Capparis* accessions belonging to six Tunisian *Capparis* species [16].

1.3. Cultivation of C. spinosa

C. spinosa which is an aromatic plant is usually cultivated in tropical and subtropical regions [10]. The most common propagation of *C. spinosa* is vegetative cuttings [10]. It can flourish under dry hot conditions in either well-drained or poor soils. In addition, *C. spinosa* is salt tolerant and resistant to drought [17]. Although *C. spinosa* can be grown in a wide range of environmental conditions, it is generally grown on sandy loam soils with low alkalinity [18]. It grows and flowers from May to October covering the summer drought [19]. Since it has deep, extensive root systems and can be grown in harsh environments, it has been recommended for the prevention of land degradation and soil erosion control [17].

1.4. Traditional Uses of C. spinosa

Different parts of *C. spinosa* including fruits and roots have been used as a traditional herbal remedy since ancient times for its beneficial effects on human diseases [20]. Ancient Egypt and Arab consumed the roots of *C. spinosa* to treat liver and kidney diseases; Ancient Romans used *C. spinosa* for the treatment of paralysis; Moroccans used *C. spinosa* to treat diabetes [8]. In the Northern areas of Pakistan, the root barks of *C. spinosa* have been used to treat splenomegaly, mental disorders and tubercular glands [21]. In China, *C. spinosa* has been used in traditional Uighur Medicine for

the treatment of rheumatoid arthritis and gout [6]. In Iran, *C. spinosa* is used to treat hemorrhoids and gout [22]. Table 1 shows the main traditional uses of *C. spinosa* used to ease symptoms and treat diseases.

Table 1. Main traditional uses of *Capparis spinosa* (*C. spinosa*) to ease symptoms and treat diseases.

Treated Symptoms and Diseases
Toothache
Fever
Headache
Menstruation
Rheumatism
Convulsions
Gout
Skin disease
Kidney disease
Liver disease
Diabetes
Hemorhoids
Ulcers
Sciatica

2. Phytochemical Properties of *C. spinosa*

Since *C. spinosa* has several beneficial health effects on human diseases, the chemical and bioactive components of *C. spinosa* have been extensively investigated and reported by numerous analytical studies [11,23–36]. Table 2 demonstrates the important constituents isolated from *C. spinosa*. These studies have focused on the bioactive compounds of different parts of *C. spinosa* such as leaves [27,37], buds [11], fruits [23–26], roots [31–33] and seeds [34–36]. In general, *C. spinosa* has a wide range of bioactive compounds such as alkaloids, flavonoids, streroids, terpenoids and tocopherols [38].

Since *C. spinosa* is rich in flavonoids, many studies have identified and quantified flavonoids found in *C. spinosa*. Flavonoid compounds such as rutin and quercertin are detected in *C. spinosa* [39]. Flavonoids have received considerable attention for their positive effects on health because of their anti-oxidative property [40]. Flavonoids are hydroxylated phenolic compounds and are found in plants [39]. Flavonoids have a 15-carbon skeleton, which consists of two benzene rings and a heterocyclic pyrane ring [41]. Flavonoids can be divided into several classes based on their structures such as flavones, flavonols, flavanones, isoflavonoids and others [41]. The consumption of flavonoids is suggested to reduce the risk of cardiovascular disease (CVD) and promote human health [41]. Rutin strengthens capillaries and inhibit platelet clump formation in the blood vessels due to its high radical scavenging and antioxidant properties [42]. In addition, rutin reduces low density lipoprotein (LDL) cholesterol level, which is associated with improve CVD risk biomarkers [43]. Quercetin has been linked with a reduced risk of CVD because of its anti-hypertensive and anti-platelet aggregating properties [44].

2.1. Aerial Parts

Most of the isolation and identification of alkaloids and related compounds have been focused on the fruits of *C. spinosa*. A study by Fu et al. [23] reported that the fruits of *C. spinosa* were shown to contain Cappariloside A, stachydrin, hypoxanthine and uracil. Another study by Çaliş et al. [25] reported that two glucose-containing 1*H*-indole-3-acetonitrile compo*unds*, 1*H*-indole-3-acetonitrile 4-*O*-β-(6′-*O*-β-glucopyranosyl)-glucopyranoside and 1*H*-indole-3-acetonitrile 4-*O*-β-glucopyranoside were identified from mature fruits of *C. spinosa* using spectroscopic methods. In addition, capparine A, capparine B, flazin, guanosine, 1*H*-indole-3-carboxaldehyde, 4-hydroxy-1*H*-indole-3-carboxaldehyde apigenin, kaempferol and thevetiaflavone were identified in the fruits of *C. spinosa* [26]. There are also

other new identified alkaloids including capparisine A, capparisine B, capparisine C that were also isolated from the fruits of *C. spinosa* [45]. Tetrahydroquinoline acid was isolated from the fruits and stems of *C. spinosa* using column chromatorgraphy [46].

Table 2. Summary of some important constituents isolated from *C. spinosa*.

Parts of *C. spinosa* Plant	Compounds Identified	Extraction Method	References
Aerial parts	Capparilosides A Stachydrin Hypoxanthine Uracil	Chromatographic method	Fu et al. (2007) [23]
	1*H*-indole-3-acetonitrile 4-*O*-β-(6′-*O*-β-glucopyranosyl)-glucopyranoside 1*H*-indole-3-acetonitrile 4-*O*-β-glucopyranoside Indole-3 acetonitrile glycosides	Spectroscopic method	Çaliş et al. (1999) [25]
	Capparine A Capparine B Flazin Guanosine 1*H*-indole-3-carboxaldehyde 4-hydroxy-1*H*-indole-3-carboxaldehyde Apigenin Kaempferol Thevetiaflavone	Spectroscopic method	Zhou et al. (2010) [26]
	Capparisine A Capparisine B Capparisine C	Chromatographic method	Yang et al. (2010) [45]
	Tetrahydroquinoline Benzofuranone enantiomers 2-(4-hydroxy-2-oxo-2,3-dihydrobenzofuran-3-yl)acetonitrile	Chromatographic method	Zhang et al. (2014) [46]
	Rutin	Chromatographic method	Mollica et al. (2017) [37]
	Kaempferol-3-glucoside Kaempferol-3 rutinoside Kaempferol-3-rhamnorutinoside	Chromatographic method	Rodrigo et al. (1992) [28]
	Kaempferol 3-*O*-rutinoside Isorhamnetin 3-*O*-rutinoside	Chromatographic method	Siracusa et al. (2011) [29]
	Quercetin 3-*O*-glucoside Quercetin 3-*O*-glucoside-7-*O*-rhamnoside Quercetin 3-*O*-[6‴-α-L-rhamnosyl-6″-β-D-glucosyl]-β-D-glucoside	Chromatographic method	Sharaf et al. (2000) [30]
	Kaempferol 3-rhamnosyl-rutinoside Kaempferol 3-rutinoside Quercetin 3-rutinoside	Chromatographic method	Inocencio et al. (2000) [11]
	Ginkgetin Isoginkgetin Sakuranetin Quercetin-3-*O*-rutinoside	Spectroscopic method	Zhou et al. (2011) [47]
	Quercetin-7-rutinoside	Chromatographic method	Sharaf et al. (1997) [48]
	Glucocapparin	Chromatographic method	Matthaus & Ozcan (2002) [49]
Roots	Capparispine Cadabicine 26-*O*-β-D-glucoside Capparispine 26-*O*-β-D-glucoside	Spectroscopic method	Fu et al. (2008) [27]
	Stachydrine	Chromatographic method	Khatib et al. (2016) [32]
	3-hydroxy-7-methoxy-2-methyl-4*H*-1,4-benzoxazine-4-carbaldehyde	Chromatographic method	Boga et al. (2011) [33]
Seeds	Glucocapparin	Chromatographic method	Matthaus & Ozcan (2002) [49]

Rodrigo et al. [28] reported that fresh *C. spinosa* were shown to contain rutin, kaempferol-3-glucoside, kaempferol-3 rutinoside and kaempferol-3-rhamnorutinoside. Of these

flavonoids, rutin was the most abundant [28]. Ramezani et al. [27] reported that the content of rutin in fruits, flowers and leaves of *C. spinosa* was 6.03, 43.72 and 61.09 mg/100 g of dried powder, respectively. It is suggested that rutin may be beneficial to health including cardio-protective versatile, cholesterol-lowering, anti-cancer and anti-inflammatory.

A study by Siracusa et al. [29] reported that rutin, kaempferol 3-*O*-rutinoside and isorhamnetin 3-*O*-rutinoside were isolated from wild-grown *C. spinosa* using High-Performance Liquid Chromatography/Ultraviolet–Visible-Diode Array Spectroscopy/Electrospray Ionization-Mass Spectrometry (HPLC/UV-Vis-DAD/ESI-MS). The authors reported that about half of the total amount of phenolic compounds was rutin (quercetin-3-*O*-rutinoside) followed by kaempferol 3-*O*-rutinoside [29].

In addition, other flavonoids such as quercetin 3-*O*-glucoside, quercetin 3-*O*-glucoside-7-*O*-rhamnoside, quercetin 3-*O*-[6‴-α-L-rhamnosyl-6″-β-D-glucosyl]-β-D-glucoside were also identified in the aerial parts of *C. spinosa* [30]. Another study by Zhou et al. [47] reported that ginkgetin, isoginkgetin, sakuranetin, kaempferol-3-*O*-rutinoside and quercetin-3-*O*-rutinoside were isolated in fruits of *C. spinosa*. The authors also reported that ginkgetin, isoginkgetin and sakuranetin were the first time to be identified in fruits of *C. spinosa* [47]. It is suggested that ginkgetin and isoginkgetin may have anti-inflammatory and neuroprotective effects. A study by Sharaf et al. [48] demonstrated that kaempferol-3-rutinoside, quercetin-3-rutinoside, quercetin-7-rutinoside, quercetin 3-glucoside-7-rhamnoside were isolated in stems and leaves of *C. spinosa*.

Afsharypuor et al. [31] reported that leaves and ripe fruits of *C. spinosa* contained the glucosinolates degradation products which were methyl, isopropyl and set-butyl isothiocyanates. Glucosinolates are secondary plant metabolites that are of pharmacological interest because they may have a role in the prevention of diseases and reducing the risk of carcinogenesis. Similarly, Çaliş et al. [25] also reported that indole-3 acetonitrile glycosides was detected in fruits of *C. spinosa.*

Some studies also investigated the flavonoids in flower buds of *C. spinosa* [11,49]. Inocencio et al. [11] used HPLC coupled with a diode-array detector to isolate kaempferol 3-rhamnosyl-rutinoside, kaempferol 3-rutinoside and quercetin 3-rutinoside from the flower buds of *C. spinosa*. The authors reported that rutin (quercetin-3-*O*-rutinoside) was the most abundant compound followed by kaempferol 3-rutinoside [11]. Also, they found that 10 g of *C. spinosa* would provide approximately 65 mg of flavonoid glycosides [11]. The main glucosinolates in shoots and buds of *C. spinosa* was glucocapparin, which contributed 90% of the total glucosinolates [49]. It is suggested that the composition of glucosinolates may vary according to the bud size of *C. spinosa.*

Other classes of compounds such as benzofuranone enantiomers 2-(4-hydroxy-2-oxo-2,3-dihydrobenzofuran-3-yl)acetonitrile were also isolated from the stems and fruits of *C. spinosa* using column chromatography [46]. Two new (6S)-hydroxy-3-oxo-α-ionol glucosides and a prenyl glucoside were isolated from the mature fruits of *C. spinosa* using chromatographic methods [24]. In addition, *p*-hydroxybenzoic acid, vanillic acid, protocatechuric acid, butanedioic acid, uracil, uridine and daucosterol were isolated from fruit of of *C. spinosa* using chromatographic methods [50]. The *p*-methoxy benzoic acid was isolated and identified from the aerial parts of *C. spinosa* using Nuclear Magnetic Resonance (NMR) spectroscopy [51].

In a study investigating the spontaneous volatile emission of different aerial parts of *C. spinosa*, Ascrizzi et al. [52] reported that 178 volatile organic compounds (VOCs) were determined by headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography (GC)/MS. The major VOCs emitted by leaves of *C. spinosa* were germacrene D (20%) and decanal (15%) [52]. The flower bud of *C. spinosa* mainly emitted isopropyl tetradecanoate (26%), (*E*)-nerolidol (17%) and hexahydrofarnesyl acetone (11%) [52]. For the fruits of *C. spinosa*, the majority of the VOCs were β-caryophyllene (40%), α-guaiene (12%), bicyclogermacrene (11%) and macrene B (8%) [52].

2.2. Roots

Limited studies have been focused on the isolation and identification of bioactive compounds from the roots of *C. spinosa*. Similar to leaves and fruits of *C. spinosa*, the roots of *C. spinosa* were shown to contain glucosinolates degradation products including methyl, isopropyl and set-butyl isothiocyanates [31]. Fu et al. [53] isolated several new spermidine alkaloids (i.e., capparispine, cadabicine 26-*O*-β-D-glucoside and capparispine 26-*O*-β-D-glucoside) from the roots of *C. spinosa* and used NMR spectroscopy to establish their structures. Stachydrine [32] and 3-hydroxy-7-methoxy-2-methyl-4*H*-1,4-benzoxazine-4-carbaldehyde [33] were also determined from the roots of *C. spinosa* using proton (^{1}H) NMR.

2.3. Seeds

Ascrizzi et al. [52] reported that the seeds of *C. spinosa* emitted 99% of the total emission profile were sesquiterpene hydrocarbons with the most abundant compounds being β-caryophyllene (45%) followed by α-guaiene (15%), bicyclogermacrene (12%) and germacrene B (8%).

Similar to roots of *C. spinosa*, less attention has been devoted to the seeds of *C. spinosa*. The total content of glucosinolates in seeds of *C. spinosa* (dry weight basis) ranged from 42.6 to 88.9 μmol/g and more than 95% of the total amount of glucosinolates was glucocapperin [36], which is similar to main glucosinolates in shoots and buds of *C. spinosa*. The seeds of *C. spinosa* are also a potential important source of oils for industrial, nutritional and pharmaceutical purposes. The oil content of *C. spinosa* seeds ranged from 27 to 38 g/100 g [36]. Three studies [34–36] have shown that the seeds of *C. spinosa* contained two major unsaturated fatty acids, linoleic acid (25–51%) and oleic acid (15–37%), which can improve cardiovascular health. In addition, the seed oils of *C. spinosa* also contain other fatty acids such as myristic acid (1%), stearic acid (3%), palmitic acid (12%), palmitoleic acid (4%), *cis*-vaccenic acid (19%), linolenic acid (1%), behenic acid (1%), eicosenoic acid (<1%), eicosanoic acid (1%) and lignoceric acid (<1%) [36].

Sterols such as stigmasterol, sitosterol, campesterol and avenasterol were also determined from the seed oils of *C. spinosa* [36]. The total amount of sterols in the seed oils of *C. spinosa* ranged from 4962 to 10,008 mg/kg. In addition, the seed oils of *C. spinosa* are also rich in tocopherols with γ- and δ- tocopherols as the major vitamin E active compounds [36]. The total amount of tocopherols in the seed oils of *C. spinosa* ranged from 249 to 1985 mg/100 g [36]. Citrostadienol, cycloartanol, gramisterol, hexadecanol, octadecanol, β-amyrin and tetracosanol were also determined from the seeds of *C. spinosa* [36].

3. Pharmacological Effects of *C. spinosa*

Although many studies using various parts of *C. spinosa* have reported diverse pharmacological activities including anti-diabetic and anti-hypertensive, there is still no conclusive information regarding the association between *C. spinosa* and its health benefits. This is because only a very few studies that involved human subjects examined the effect of *C. spinosa* consumption on human health. Similar to other plants [54,55], although there exists literature related to the health benefits of *C. spinosa*, studies often infer a causal correlation between a bioactive substance of *C. spinosa*, and the observe health outcomes. This approach is likely to be an oversimplification of the complex mechanisms in the body that will eventually lead to the observed health outcomes. Therefore, the conclusions of such studies on the effects of changes in the dietary intake should be interpreted with caution because the health outcomes may not be attributed to the action of a single bioactive substance [56]. Table 3 illustrates major pharmacological effect of *C. spinosa*.

Table 3. Overview on major pharmacological effects of *C. Spinosa*.

Pharmalogical Effects	Models	Parts of *C. spinosa* Plant Used	References
Anti-diabetic	Streptozotocin-induced diabetic rats	Fruits	Eddouks et al. (2005) [57]
	Highly glucose tolerant and high fat diet-fed mice	Fruits	Lemhadri et al. (2007) [58]
	Type 2 diabetic patients	Fruits	Huseini et al. (2013) [59]
	Streptozotocin-induced diabetic rats	Leaves	Mollica et al. (2017) [37]
Anti-obesity	Streptozotocin-induced diabetic rats	Fruits	Eddouks et al. (2005) [57]
	Highly glucose tolerant and high fat diet-fed mice	Fruits	Lemhadri et al. (2007) [58]
Cholesterol-lowering	Streptozotocin-induced diabetic rats	Fruits	Eddouks et al. (2005) [57]
	Streptozotocin-induced diabetic rats	Fruits	Jalali et al. (2016) [60]
	Type 2 diabetic patients	Fruits	Huseini et al. (2013) [59]
Anti-hypertensive	Spontaneously hypertensive rats	Fruits	Ali et al. (2007) [61]
Antimicrobial	Cell culture	Roots	Boga et al. (2011) [33]
	Cell culture	Roots and fruits	Mahboubi & Mahboubi (2014) [22]
	Cell culture	Stem barks and shoots	Gull et al. (2015) [62]
	Cell culture	Aerial parts	Masadeh et al. (2014) [63]
Anti-inflammatory	Swiss albino mice	Leaves	El Azhary et al. (2017) [64]
	Human peripheral blood mononuclear cells	Leaves	Moutia et al. (2016) [65]
	Male Sprague-Dawley rats	Roots	Maresca et al. (2016) [66]
	Mouse-bone marrow derived dendritic cells	Fruits	Hamuti et al. (2017) [67]
Antihepatotoxic	Albino rats of Wistar strain	Aerial parts	Gadgoli & Mishra (1999) [51]
	Diabetic rats		Kazemian et al. (2015) [68]

3.1. Anti-Diabetic

In a randomised, double-blind, placebo-controlled clinical trial of 54 type 2 diabetic patients, Huseini et al. [59] reported that patients who took 1200 mg *C. spinosa* fruit extracts daily for 2 months had a significantly lower level of glycosylated hemoglobin and fasting blood glucose than the control group ($p = 0.043$ and 0.037, respectively). The findings of their study demonstrated an improvement in hypertriglyceridemia and hyperglycemia in diabetic patients [59]. In addition, no renal and hepatic adverse events were reported in the patients [59]. The possible mechanism is that *C. spinosa* decreases the rate of carbohydrate absorption and exert its postprandial hypoglycemic effect in the gastrointestinal tract [58]. Therefore, the consumption of *C. spinosa* may be beneficial and safe for controlling and treating blood glucose levels in diabetic patients.

3.2. Anti-Obesity

Eddouks et al. [57] demonstrated that there was a significant weight loss in diabetic rats fed with the aqueous extract of powdered fruits of *C. spinosa* (20 mg/kg) after 2 weeks ($p < 0.01$). Another study by Lemhadri et al. [58] reported that repeated oral administration of aqueous extracts of *C. spinosa* was associated with a significant loss of body weight in high fat diet-fed mice after 2 weeks ($p < 0.01$). Therefore, it is suggested that *C. spinosa* may be used for weight loss management. However, further studies are needed to confirm such findings.

3.3. Cholesterol-Lowering

C. spinosa has been reported to be associated with improved plasma lipid parameters. Huseini et al. [59] reported a significant decrease in the triglyceride level of type 2 diabetic patients who were supplemented with 1200 mg *C. spinosa* fruit extracts daily for 2 months ($p = 0.029$). The possible mechanism for the cholesterol-lowering effect of *C. spinosa* is that *C. spinosa* decreases the activity of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMG-CoA reductase), which plays an important role in the biosynthesis of cholesterol [69]. Therefore, *C. spinosa* may be useful for the treatment of fatty liver disease and metabolic syndrome because it plays an important role in the inhibition of gluconeogenesis in liver.

3.4. Anti-Hypertensive

C. spinosa has the potential to be used for the treatment of hypertension. In a study of spontaneously hypertensive rats, Ali et al. [61] reported that when the aqueous extract of powdered fruits of *C. spinosa* (150 mg/kg) was administered for 20 days, the systolic blood pressure was significantly decreased after 8 days ($p < 0.05$), 12 days ($p < 0.01$) and 16 days ($p < 0.001$). In addition, there was also a significant increase in the concentration of urinary sodium ($p < 0.001$), potassium ($p < 0.001$) and chloride ($p < 0.001$) after 20 days [61]. No change in heart rate was observed during the period. In addition, there was also no difference in the activity of plasma angiotensin-converting enzyme (ACE) and renin after 20 days [61]. It is suggested that *C. spinosa* decreases blood pressure by increasing the excretion of renal electrolytes and inhibiting the ACE activity. The inhibition of ACE activity is associated with a decrease in blood pressure [70]. Therefore, *C. spinosa* may play an important in the reduction of blood pressure.

3.5. Anti-Microbial

In a study investigating the antibacterial activity of *C. spinosa*, Boga et al. [33] reported that the growth rate of *Deinococcus radiophilus* (*D. radiophilus*) was significantly inhibited by the addition of aqueous extracts from roots of *C. spinosa* as compared to the control. However, no inhibition was shown on the growth rate of *Escherichia coli* (*E. coli*) by the addition of aqueous extracts from roots of *C. spinosa* [33].

Another study by Mahboubi and Mahboubi [22] evaluated the antimicrobial activity of aqueous, ethanolic, ethyl acetate and methanolic extracts of *C. spinosa* roots and fruits. The authors reported that the aqueous extracts of *C. spinosa* roots exhibited a higher antimicrobial activity against a wide range of microorganisms than that of fruit aqueous extracts of *C. spinosa* fruits [22]. The authors demonstrated that the aqueous extracts from roots of *C. spinosa* possessed inhibitory effect against bacteria and fungi including *Staphylococcus aureus* (*S. aureus*), *Staphylococcus saprophyticus* (*S. saprophyticus*), *Staphylococcus epidermidis* (*S. epidermidis*), *Streptococcus pyogenes* (*S. pyogenes*), *Streptococcus mutans* (*S. mutans*), *E. coli*, *Salmonella typhimurium* (*S. typhimurium*), *Shigella dysenteriae* (*S. dysenteriae*), *Shigella flexneri* (*S. flexneri*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Bacillus subtilis* (*B. subtilis*), *Bacillus cereus* (*B. cereus*), *Candida albicans* (*C. albicans*), *Candida glabrata* (*C. glabrata*), *Aspergillus flavus* (*A. flavus*), *Aspergillus parasiticus* (*A. parasiticus*) and *Aspergillus niger* (*A. niger*) [22].

Gull et al. [62] reported that the methanolic extracts of *C. spinosa* stem barks and shoots had the greatest antibacterial activity against *B. subtilis* with growth inhibition zones of 26.8 mm and 24.6 mm, respectively. While the methanolic extracts of *C. spinosa* fruits had the highest growth inhibition zones (24.9 mm) against *Pasteurella multocida* (*P. multocida*) followed by *B. subtilis* (23.9 mm), *E. coli* (20.9 mm) and *S. aureus* (17.7 mm) [62]. On the other hand, the highest antibacterial activity of the methanolic extracts of *C. spinosa* flowers and roots was observed against *E. coli* with growth inhibition zones of 26.5 mm and 23.9 mm, respectively [62]. In addition, polysaccharides of *C. spinosa* leaves have been suggested to exhibit antimicrobial activity. Mazarei et al. [71] reported that polysaccharides of *C. spinosa* leaves had a higher antimicrobial activity against *E. coli*, *S. dysenteriae* and *Salmonella typhi (S. typhi).*

Mahasneh [72] reported that butanol extract of *C. spinosa* showed a moderate to good antifungal activity against *C. albicans* and *A. flavus*. In addition, ethanol extracts of *C. spinosa* exhibited weak cytotoxicity against *Helicobactor pylori* (*H. pylori*) isolates [63]. The infection of *H. pylori* is associated with several gastroduodenal diseases such as gastric cancer [73]. It is suggested that the method of preparing extracts and the type of solvent used may affect the microbial activity of *C. spinosa*.

3.6. Anti-Inflammatory

In an in vivo mouse model, El Azhary et al. [64] reported that Swiss albino mice treated with *C. spinosa* leaf extracts had a significantly reduced edema than control, demonstrating the

anti-inflammatory activity of *C. spinosa*. In addition, the authors also found that *C. spinosa* had significantly decreased the dermis thickness and immune cell infiltration in the inflammatory site. Similarly, Moutia et al. [65] reported that *C. spinosa* leaf extract was shown to exhibit anti-inflammatory activity in vitro on human peripheral blood mononuclear cells (PBMC) obtained from healthy subjects. The authors also found that PBMC treated with the aqueous fraction of *C. spinosa* leaf extract had a significant increase in interleukin (IL)-4 gene expression (an anti-inflammatory cytokine) and a significant decrease in IL-17 gene expression (pro-inflammatory cytokine) [65]. Therefore, these studies suggested that *C. spinosa* leaf extracts exhibit anti-inflammatory activity by inhibiting the pro-inflammatory cytokines expression and immune cell infiltration [64,65].

C. spinosa root extracts were reported to relieve pain in complete Freund's adjuvant (CFA)-induced rheumatoid arthritis and mono-iodoacetate (MIA)-induced osteoarthritis male Sprague-Dawley rats [66]. It is suggested that the pain reliever effect of *C. spinosa* roots could be due to the presence of spermidine alkaloids which have anti-inflammatory effects [66]. Similarly, the lyophilised methanolic extracts from flowering buds of *C. spinosa* was also reported to exhibit anti-inflammatory effect by reducing the production of reactive oxygen species (ROS), nitric oxide (NO) and prostaglandins (PGE_2) induced by IL-1β on human chondrocytes [74]. These findings were consistent with another study by Feng et al. [75] who demonstrated that the fraction eluted by ethanol-water (50:50, *v*/*v*) from *C. spinosa* fruits was shown to exhibit the most significant anti-arthritic response in male Wistar rats, providing additional evidence that *C. spinosa* possesses anti-inflammatory effects.

Similarly, *C. spinosa* exhibited anti-inflammatory activity to suppress cytokine production of dendritic cells (DC), induced by lipopolysaccharide (LPS). In a study investigating the effect of *C. spinosa* fruit ethanol extracts on the maturation of mouse bone marrow-derived DC Hamuti et al. [67] reported that different types of *C. spinosa* extracts exhibited different effects on the maturation of DC. DC is the key regulator in many aspects in homeostasis of immune system [76] and inflammatory skin disorders [77]. These different types of *C. spinosa* extracts were labelled as *C. spinosa* extract (CSE) 2 water (W), CSE middle-layered isolated (M) W, CSE3W, CSE2 dimethyl sulfoxide (D), CSEMD and CSE3D according to their methods of preparation which were either dissolving in water or dimethyl sulfoxide (DMSO) at a concentration of 200 mg/mL. The authors reported that CSEMD and CSEMW dose-dependently increased the expression of cluster of differentiation (CD) 40, CD80 and CD86 [67]. CSE2W was shown to significantly increase the expression of CD40 but not CD80 [67]. Although CSE3D and CSE3W had significantly inhibited the secretions of pro-inflammatory cytokines including IL-1β, IL-12p40, IL-6 and tumor necrosis factor (TNF)-γ induced by LPS, CSE3D had a higher inhibitory activity than CSE3W [67].

3.7. Antihepatotoxic

A study by Gadgoli and Mishra [51] evaluated the effects of *C. spinosa* on the antihepatotoxic on rats against paracetamol and carbontetrachloride induced toxicity in vivo. In addition, the authors also investigated the effects of *C. spinosa* on galactosamine and thioacetamide induced toxicities in vitro [51]. In their study, methanol soluble fraction of the aqueous extract of aerial parts of *C. spinosa* was reported to exhibit significant reductions in serum glutamyl pyruvate transaminase (SGPT), serum glutamyl oxalacetate transaminase (SGOT), alkaline phosphatase and total bilirubin in paracetamol and carbontetrachloride induced hepatotoxicity in albino rats of Wistar strain [51]. In addition, the authors also reported that aerial parts of *C. spinosa* showed significant anti-hepatotoxic activity in galactosamine and thioacetamide induced hepatotoxicity in isolated rat hepatocytes [51].

Kazemian et al. [68] reported that both groups of diabetic rats receiving 0.2 g/kg and 0.4 g/kg of hydroalcoholic extract of *C. spinosa* had a significant reduction of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) ($p < 0.05$ for both groups) after 4 weeks of treatment. Therefore, it is suggested that *C. spinosa* extract does not cause any toxic effect on the liver [68].

3.8. Other Pharmacological Properties

C. spinosa also exhibits potential protective effects against cognitive impairment. In a study investigating the effect of *C. spinosa* on the learning and memory damage induced by chronic administration of LPS (175 μg/kg) in young male Sprague-Dawley rats, Goel et al. [78] reported that the aqueous extract of *C. spinosa* buds had significantly reduced the neurodegeneration in the first region in the hippocampal circuit (CA1) region of hippocampus, suggesting that *C. spinosa* could be used to treat cognitive disorders.

In addition, another study by Mohebali et al. [79] reported that the expression of β-secretase enzyme (BACE)-1, presenilin protein (PSEN)-1 and PSEN-2 and amyloid precursor protein (APP) genes were significantly down regulated in amyloidogenic related genes in amyloid-beta (Aβ) peptide-injected Wister albino rats treated with hydroalcoholic extracts of *C. spinosa* leaves and fruits as compared to the control group. Another study by Turgut et al. [80] reported that the extracts of *C. spinosa* seeds significantly protected against the damage of DNA bands and attenuated cognitive impairment induced by D-galactose in male, Bagg albino, laboratory-bred strain of the House Mouse (BALB/c) mice. Chronic neurodegenerative diseases such as Alzheimer's disease are associated with the aggregation and misfolding of proteins. The accumulation of Aβ peptide is the key protein involved in the development of Alzheimer's disease and the accumulation of Aβ peptide can be caused by malfunction in β- and γ- secretase enzymes. BACE is encoded by BACE-1; γ-secretase enzyme is encoded by PSEN-1 and PSEN-2 [81].

C. spinosa also exhibits inhibitory effects on human immunodeficiency virus (HIV)-1 reverse transcriptase [82]. Lam and Ng [82] isolated a protein with an N-terminal amino acid sequence from the seeds of *C. spinosa* that inhibited HIV-1 reverse transcriptase. The protein exhibited some similarly to imidazoleglycerol phosphate synthase [82]. Lam and Ng [82] reported that the protein possessed HIV-1 reverse transcriptase inhibitory activity with the half maximal inhibitory concentration (IC_{50}) of 0.23 μM. It is suggested that the inhibition is due to the protein-protein interaction which causes homologous retroviral reverse transcriptase to be inhibited by HIV-protease [82].

3.9. Adverse Effects

Currently, the consumption of *C. spinosa* is not associated with any adverse effects according to the published literature, providing evidence that *C. spinosa* is safe to consume [83].

4. Conclusions

A literature review has highlighted that *C. spinosa* exhibits important pharmacological effects because *C. spinosa* is rich in many bioactive compounds including flavonoids. Therefore, given the few clinical studies cited, it is very risky to highlight the potential role of *C. spinosa* on treatment such as diabetes, hypertension and obesity. This suggests that there are many opportunities for the food and healthcare industry to explore the health benefits of *C. spinosa* because there is a potential growth market for *C. spinosa*. However, the majority of the studies that reported beneficial effects of *C. spinosa* on health are animal-based studies. Moreover, these studies used different parts of *C. spinosa* plant, types of solvents and methods of preparation, which cause the evaluation of activity of *C. spinosa* difficult. In addition, these studies involve quite heterogeneous data.

Therefore, future prospective research should screen for individual polyphenol constituents that possess health-promoting properties in *C. spinosa*. This is because a cause-effect relationship between *C. spinosa* and its health effects can only be established when the composition of *C. spinosa* is properly characterised and standardised. In addition, there is limited evidence of randomized controlled trials on humans to support such health beneficial effects of *C. spinosa* when compared to other plants such as walnuts that have high economic interests to the food industry. The underlying mechanism influencing human health by the consumption of *C. spinosa* still remains unclear. The effect of short- and long-term consumption of *C. spinosa* on human health therefore needs to be further evaluated.

Furthermore, the role of the gut microbiota in the degradation of polyphenolic compounds present in the plant has not been considered. This aspect should be put forward in the perspectives [84].

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Aged Oolong Tea Reduces High-Fat Diet-Induced Fat Accumulation and Dyslipidemia by Regulating the AMPK/ACC Signaling Pathway

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Abstract: While oolong tea (OT) has been shown to induce weight loss and reduce fat accumulation, the mechanisms remain poorly defined, especially for aged OT. In this study, five groups of mice (n = 9/group) were used including a normal diet with vehicle treatment, and a high-fat diet (HFD) with vehicle or the water extracts from aged OTs (EAOTs, three different storage years) by oral gavage at 1000 mg/kg·BW for 6 weeks. Body weight, fat accumulation, and serum biochemical parameters were used to evaluate obesity. The morphology of hepatocytes and adipocytes was analyzed by being stained with hematoxylin and eosin. The levels of p-AMPK, p-ACC (and non-phosphorylated versions), CPT-1 and FAS were determined by Western blotting and immunohistochemistry. EAOTs decreased HFD-induced body weight, fat accumulation, serum levels of triglyceride, total cholesterol, and low-density lipoprotein cholesterol, while enhancing the serum high-density lipoprotein cholesterol level. At the same time, EAOTs clearly alleviated fatty liver and reduced the size of adipocytes in the epididymal fat, especially in the 2006 group. Most importantly, EAOTs increased the phosphorylation of AMPK and ACC, and up-regulated the expression of CPT-1 but down-regulated the expression of fatty acid synthase, TNF-α and iNOS. Thus, EAOTs may inhibit obesity by up-regulating energy expenditure and fatty acid oxidation while inhibiting fatty acid synthesis and inflammation.

Keywords: anti-obesity; anti-inflammation; water extract; aged oolong tea

1. Introduction

High energy intake, malnourished diets containing lots of fat, and refined carbohydrates coupled with a sedentary lifestyle are believed to contribute to the global obesity epidemic [1,2]. Obesity tends to result in other pathological disorders, such as diabetes, atherosclerosis, hypertension, and cancer [3–5]. It is estimated that the prevalence of obesity will increase by 7% and 10% among men and women, respectively, by 2020 [6]. As one of the most popular beverages consumed worldwide, tea has been shown in numerous clinical trials to affect body weight and fat metabolism in humans [7] and in rodents [8] fed high-fat diets (HFDs). Oolong tea (OT) is a kind of partially fermented Chinese tea, and it is considered to have a stronger weight loss effect than any other kinds of tea. Previous research has shown that OT leaves can remarkably decrease rats' body weight [9]. However, the effects of the water

extract from aged OTs (EAOTs, three different storage years) on fat deposition and inflammation, as well as potential mechanisms of action, were not adequately studied. During storage, the contents of tea polyphenols, soluble sugar, and other ingredients changed [10]. As these ingredients are the material basis of OT's anti-obesity and anti-inflammation effects, it is reasonable to speculate that the physiological effects of OT would change with storage as well.

The aim of this study was to investigate the effects of EAOTs on anti-obesity and anti-inflammation. Additionally, we analyzed the protein expression following EAOT treatments to evaluate HFD-induced pathways of alleviating obesity and inflammation. Therefore, the present study could demonstrate new insights into the anti-obesity and anti-inflammation mechanism induced by EAOTs.

2. Materials and Methods

2.1. Materials

Dancong Oolong tea (a kind of OT, stored in 2016, 2006, 1996 separately) was purchased from Dapuxiyan Tea Group Co. Ltd. in Meizhou, Guangdong, China.

2.2. Preparation of EAOTs

Powder prepared from OT was extracted three times by placing in boiling distilled water for 30 min each time (tea/water, 1:20 *w*:*v*). These extracted solutions were combined and centrifuged, then concentrated at 60 °C. Finally, the solution was dried by lyophilization.

2.3. Determination of Ingredients in EAOTs

The water content was measured by comparing the weight difference between before and after heating in a 130 °C oven for 3 h, and the content of free amino acids was determined by the ninhydrin method. In addition, anthrone–sulfuric acid colorimetric assay was used to determine the total soluble sugar content, and the Folin-phenol method for tea polyphenols.

2.4. Animals

Male, 7-week-old C57BL/6J mice were purchased from Beijing Huafukang Bioscience Co. Ltd. (Beijing, China). All experimental procedures were conducted in accordance with institutional guidelines for the care and use of laboratory animals, and all efforts were made to minimize animal suffering. The protocols were approved by the Ethical Committee of Tea Research Institute (2015-005) at Guangdong Academy of Agricultural Sciences.

Mice were individually housed in standardized conditions for animal facilities: a 12-h light/dark cycle (off at 7 pm), 23 ± 2 °C room temperature, and 55 ± 5% relative humidity. Mice had ad libitum access to water and food (Beijing Huafukang Bioscience Co., Ltd.).

2.5. Diet-Induced Obesity

After a week of adaptation, 45 C57BL/6J were randomly divided into five groups as follows: (i) the control group (n = 9); (ii) the group fed an HFD (Model) (n = 9); (iii) the group fed an HFD and 1000 mg/kg·BW EAOTs stored in 2016 (2016) (n = 9); (iv) mice fed an HFD and 1000 mg/kg·BW EAOTs stored in 2006 (2006) (n = 9); and (v) the group fed an HFD and 1000 mg/kg·BW EAOTs stored in 1996 (1996) (n = 9). Control mice were fed a normal diet (containing protein 18%, fat 4%, carbohydrate 62%, fiber 5%, minerals 8%, and vitamins 3%, *w*:*w*; Beijing Huafukang Bioscience Co., Ltd., 1022), and mice in the other four groups were fed an HFD (45% calories from fat, 20% calories from protein, 35% calories from carbohydrate; Beijing Huafukang Bioscience Co., Ltd., H10045) for 18 weeks, during which time all mice were not administered EAOTs.

2.6. EAOT Treatment

After 18 weeks, the mice in all groups remained on the same diet as before. Mice from three EAOT treatment groups were given, by gavage, 1000 mg/kg·BW EAOTs daily for approximately 6 weeks, while the control group and the model group mice were given, by gavage, distilled water at the same time. Body weight, food, and water intake were recorded once a week. At the end of the experimental period, the mice fasted for 12 h, and then blood samples were collected by cardiac puncture technique under carbon dioxide anesthesia. Thereafter, the mice were euthanized with an anesthetic overdose, and were perfused transcardially through the ascending aorta with normal saline so as to remove any blood clots from tissues and organs. The liver was rapidly removed, weighed, and washed thoroughly with phosphate buffer saline (PBS, pH 7.4). Half hepatic lobar was preserved in 10% buffered formalin solution for histopathological examinations. The rest of the liver was homogenized in ice-cold phosphate buffer saline, then stored in liquid nitrogen for various biochemical and molecular assays. The entire procedure was carried out under cold conditions.

2.7. Lee's Index

Lee's index is similar to the human body mass index, and mainly reflects the proportion of body fat in the total weight. It can characterize the degree of obesity more accurately than body weight [11]. The smaller the Lee's index, the more slender the body. After the animals were sacrificed, their body weight and length were measured to calculate the Lee's index.

$$\text{Lee's} = [\text{weight (g)}/\text{length (cm)}]^{1/3}$$

2.8. Serum Chemistry Analysis

After being kept at room temperature for 30 min, blood samples were centrifuged at 13,200 r/min for 20 min at 4 °C, and subsequently stored at −80 °C. The serum was used to measure triglycerides (TGs), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels (iMagic-V7 Automatic Analyzer, ICUBIO, Beijing, China).

2.9. Histological Examination

The liver and epididymal fat sections fixed in 10% neutral buffered formalin were dehydrated in graded concentrations of alcohols, and embedded in paraffin. Then the tissue was sectioned at 4 μm and stained with hematoxylin and eosin (H&E). The pathological changes were assessed and photographed on an Olympus BX-53 microscope (Olympus, Tokyo, Japan).

2.10. Western Blotting Analysis

Protein from each liver sample was extracted with lysis buffer at 4 °C. Then, the extracts were centrifuged at 13,200 r/min and 4 °C for 30 min, and the supernatants of these tissues were used for Western blotting analyses. The protein samples were separated on 10% SDS-PAGE gels and electrophoretically transferred onto PVDF membranes. The membranes were blocked at room temperature with 5% non-fat dry milk in TBST for 1 h, and then incubated overnight at 4 °C with the indicated primary antibodies as follows: AMP-activated protein kinase α (AMPKα), p-AMPKα (Thr172), acetyl-CoA carboxylase (ACC), p-ACC (Ser79), camitinepalmitoyl transferase 1 (CPT-1), fatty acid synthase (FAS), iNOS (Cell Signaling Technology, Danvers, MA, USA), TNF-α (Abcam, Cambridge, MA, USA), and β-actin (Sigma-Aldrich, St. Louis, MO, USA). After washing three times with TBST, the blots were hybridized with secondary antibodies conjugated to horseradish peroxidase. The proteins were visualized by enhanced chemiluminescence.

2.11. Immunohistochemistry (IHC)

The liver paraffin samples were sliced 4 μm thick and were dewaxed in xylene and rehydrated through a graded series of ethanol concentrations. Then, the slides were pretreated with a microwave antigen retrieval technique and blocked with 5% BSA. The slices were incubated overnight at 4 °C with diluted primary antibodies (Cell Signaling Technology, Danvers, MA, USA). After three washes with PBS for 5 min, the sections were incubated with secondary antibodies (Boster, Wuhan, China) for 30 min at 37 °C. Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG (Beyotime, Shanghai, China) were used for enhanced chromogenic reaction. After washing, the DAB chromogen was added for 3 min before counterstaining with hematoxylin. Images of immunostained liver tissues were taken with an Olympus BX-53 microscope (Olympus, Tokyo, Japan).

2.12. Statistical Analysis

Repeated measures analysis of variance (ANOVA) was conducted, and Tukey's post-hoc test was used to adjust for multiple comparisons. A p-value < 0.05 was considered statistically significant, and Prism 6.0 software for Windows (GraphPad Software, La Jolla, CA, USA) was used to conduct statistical analyses. Data are presented as mean ± standard error of the mean (SEM) for effects of EAOT treatment on body weight, food intake, water consumption, and Lee's index, and mean ± standard deviation (SD) for all other results.

3. Results

3.1. The Ingredients of EAOTs

As shown in Table 1, the free amino acids content was significantly lower in aged oolong tea, because of the degradation and polymerization during the storage time. Meanwhile, microorganisms may also have consumed protein and decreased this content.

Table 1. The ingredients of water extract from aged oolong teas (EAOTs) (g/g).

Sample	Water (%)	Free Amino Acids (%)	Total Soluble Sugar (%)	Tea Polyphenols (%)
2016	0.07 ± 0.01 ^{a}	5.13 ± 0.03 ^{a}	7.08 ± 0.25 ^{a}	39.7 ± 0.3 ^{a}
2006	0.08 ± 0.01 ^{a}	3.99 ± 0.19 ^{b}	8.18 ± 0.08 ^{b}	46.5 ± 0.6 ^{b}
1996	0.06 ± 0.01 ^{a}	3.81 ± 0.04 ^{b}	8.39 ± 0.08 ^{b}	34.0 ± 0.5 ^{c}

The value is the mean ± SD (n = 3). Values marked with different lower-case letters in superscript format indicate significant differences; values marked with the same lower-case letters in superscript format indicate no significant differences.

The content of total soluble sugar markedly increased with the storage time, as macromolecular carbohydrates gradually decomposed when stored.

However, with the storage time, the change in tea polyphenols content was nonlinear. The 2006 group had a higher tea polyphenol content compared to the other two groups. Similar results were also seen in other studies, although for a shorter period [12].

3.2. Body Weight, Food and Water Intake, and Lee's Index

The effect of EAOTs on obesity was investigated using male C57BL/6J mice with HFD-induced obesity. Although the average body weight did not significantly differ between the model group and three treatment groups at week 0, the latter had significantly lower body weight than the model group, and had even reached the level similar to the control group from the second week to the end of the trial (Figure 1A). The 2006 group showed slightly lower body weight than the 2016 and 1996 groups, but the difference was not statistically significant. However, no marked differences were observed in daily food and water intake among all groups, showing that EAOTs did not suppress food and

water intake (Figure 1B,C). At the end of the animal test, Lee's index was also measured to evaluate the degree of mice obesity. In the groups fed the HFD and EAOTs, Lee's indexes were significantly prevented compared to that of the mice fed only the HFD (Figure 1D).

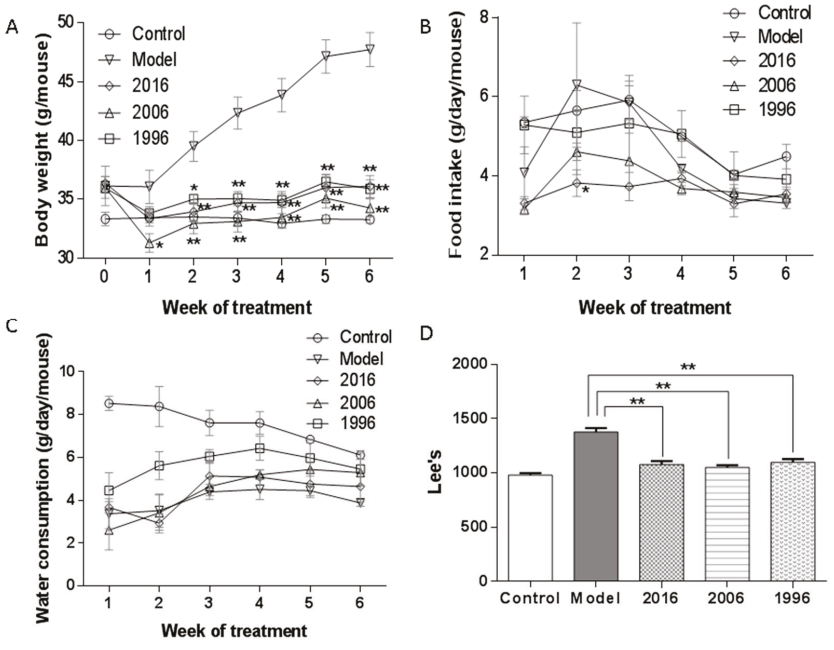

Figure 1. Effects of EAOT treatment on (**A**) body weight; (**B**) food intake; (**C**) water consumption; and (**D**) Lee's index. During 6 weeks of different storage years of EAOT treatment, body weights, food intake, and water consumption were recorded once a week. After 6 weeks of administration, Lee's index was measured. Data were mean ± SEM (n = 9). * $p < 0.05$ versus model; ** $p < 0.01$ versus model.

3.3. EAOTs Attenuate Fatty Liver and Adiposity in HFD-Induced Obese Mice

The effects of EAOTs on fat accumulation were studied by anatomic observation and by analyzing the organ index of white adipose tissues. Gross observation revealed that the mice in the model group showed a large accumulation of white fat, while it decreased markedly after being treated with EAOTs, especially in the 2006 group (Figure 2A). In Figure 2B, the model group mice had developed fatty livers. Their liver sizes were larger than those of the control group. They also became yellow-brown and the liver surfaces were uneven, whereas the liver changes of mice treated with EAOTs were equal to those of the control mice. Moreover, EAOTs greatly decreased the size of epididymal and pararenal adipose tissue masses (Figure 2C,D).

At the end of the animal experiment, the body weight, organ weight, and adipose tissue weight of the mice were measured. As shown in Table 2, the hepatic indexes were evidently diminished in EAOT-treated obese animals, as well as the fat indexes. Moreover, the intestinal fat indexes of the mice in the EAOT treatment groups were sharply decreased to a level similar to those in the control group, and the pararenal fat indexes in the 2006 group mice showed no significant differences with those of the mice in the control group. As a whole, the administration of EAOTs inhibited the increase of the white fat index, particularly in the 2006 group. In contrast, the model group mice had a markedly reduced kidney index, comparable to that seen in the control group. After EAOT treatment, this index

increased to a level similar to that of the control mice (Table 2). However, there were no significant differences between each group in the sizes of kidney (Figure 2D), implying that this drop in model group was mainly because of the increase in body weight.

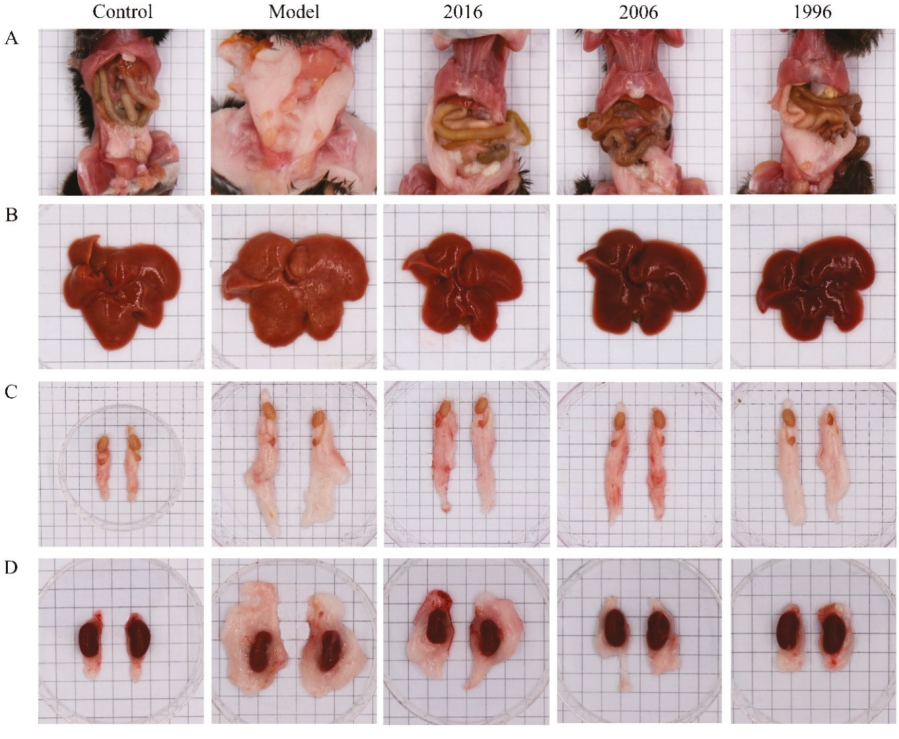

Figure 2. EAOTs attenuate fatty liver and adiposity in high-fat diet (HFD)-induced obese mice. Representative images of (**A**) whole body; (**B**) liver; (**C**) epididymal fat; (**D**) and pararenal fat in all groups.

Table 2. Organ index of mice fed a HFD and EAOTs for 6 weeks.

Group	Liver/Weight (%)	Kidney/Weight (%)	Epididymal Fat/Weight (%)	Intestinal Fat/Weight (%)	Pararenal Fat/Wright (%)	White Fat/Weight (%)
Control	44.5 ± 0.1 ^{a}	13.0 ± 1.0 ^{a}	1.30 ± 0.46 ^{a}	5.14 ± 2.43 ^{a}	4.15 ± 1.98 ^{a}	9.31 ± 4.32 ^{a}
Model	45.2 ± 0.5 ^{a}	9.39 ± 0.88 ^{b}	5.35 ± 0.29 ^{b}	23.9 ± 5.3 ^{b}	30.6 ± 3.3 ^{b}	56.3 ± 6.1 ^{b}
2016	36.4 ± 0.1 ^{b}	11.9 ± 0.9 ^{a}	3.02 ± 1.16 ^{c}	10.4 ± 5.4 ^{a}	16.4 ± 8.0 ^{c}	24.7 ± 16.3 ^{c}
2006	37.0 ± 0.1 ^{b}	12.4 ± 1.5 ^{a}	2.77 ± 0.75 ^{c}	7.73 ± 3.76 ^{a}	11.8 ± 6.1 ^{a,c}	16.4 ± 5.6 ^{a,c}
1996	37.0 ± 0.1 ^{b}	12.4 ± 1.6 ^{a}	2.91 ± 1.07 ^{c}	9.20 ± 4.65 ^{a}	12.8 ± 6.5 ^{c}	19.7 ± 9.3 ^{a,c}

The value is the mean ± SD (n = 3). Values marked with different lower-case letters in superscript format indicate significant differences; values marked with the same lower-case letters in superscript format indicate no significant differences.

3.4. Effects of EAOTs on Serum Levels in HFD-Induced Obesity Mice

Obese people usually suffer from hyperlipidemia, so the serum biochemistry was evaluated to assess the anti-obesity effects of EAOTs in HFD mice. The model group had the highest TG, TC, and LDL-C, and the HDL-C level was lowest in the model group (Table 3). EAOT treatments resulted in

a dramatic decrease in TG, TC, and LDL-C, and a notable increase of HDL-C, which were similar to those of the control mice. Moreover, a moderately lower concentration of TG, TC, and LDL-C was found in the 2006 group than in the 2016 and 1996 groups (Table 3). Together, these results clearly revealed that EAOTs could inhibit hyperlipidemia in obese animals.

Table 3. Serum levels of triglycerides (TGs), total cholesterol (TC), high-density lipoprotein (HDL), and low-density lipoprotein (LDL).

Group	TG (mmol/L)	TC (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)
Control	1.41 ± 0.30^{a}	4.18 ± 0.58^{a}	2.78 ± 0.37^{a}	0.55 ± 0.12^{a}
Model	1.95 ± 0.34^{b}	9.25 ± 1.30^{b}	2.26 ± 0.24^{a}	1.46 ± 0.33^{b}
2016	1.45 ± 0.22^{a}	6.20 ± 0.59^{c}	4.03 ± 0.44^{b}	1.00 ± 0.11^{c}
2006	1.13 ± 0.26^{a}	5.74 ± 0.46^{c}	3.80 ± 0.36^{b}	0.86 ± 0.10^{c}
1996	1.19 ± 0.24^{a}	6.42 ± 0.84^{c}	4.21 ± 0.58^{b}	0.97 ± 0.24^{c}

The value is the mean ± SD (n = 3). Values marked with different lower-case letters in superscript format indicate significant differences; values marked with the same lower-case letters in superscript format indicate no significant differences.

3.5. Effect of EAOTs on Accumulation of Lipid Droplets in Liver and Epididymal Fat

To determine whether the observed decrease in liver weight was due to a reduced accumulation of fat, we stained representative liver with H&E. As shown in Figure 3, the model group mice showed an increase in lipid accumulation and pronounced steatosis in the liver, which was characterized by prominent ballooning injury, vacuolation, and hepatocellular hypertrophy. EAOTs clearly alleviated fatty liver, and lipid deposition was markedly decreased, especially in the 2006 group (Figure 3A).

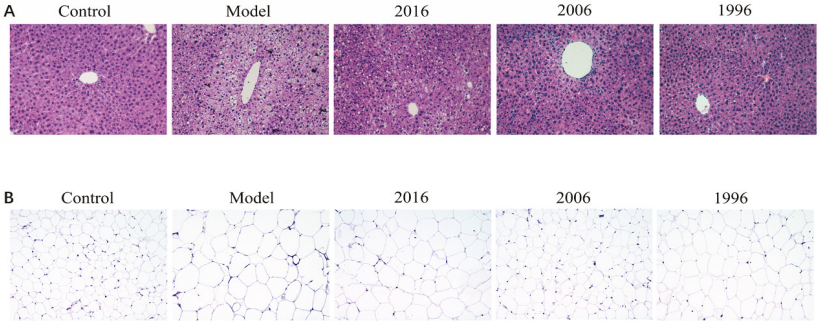

Figure 3. Effect of EAOTs on accumulation of lipid droplets in liver and epididymal fat. Representative hematoxylin and eosin (H&E) staining images of (**A**) liver and (**B**) epididymal adipose tissue (scale bar 20 μm).

For adult mice, the effect of obesity on adipocyte is mainly the increase of cell volume. We also examined the epididymal adipose tissue histology of obese mice in the absence and presence of EAOTs. Enlargement of adipocytes in the epididymal fat of mice in the model group was clear, and it was revealed that the size of adipocytes in the epididymal fat was definitely suppressed in the EAOT treatment groups compared with the model group. Moreover, the diameter of lipid droplets in the 2006 group was similar to that in the control group (Figure 3B).

3.6. EAOTs Activate AMPK and ACC Phosphorylation

AMPK is able to dissipate excessively stored energy by stimulating fatty acid oxidation. To investigate the effect of EAOTs on AMPK activation in mice liver, immunoblot analysis was

performed. AMPK activation was determined by measuring the phosphorylation level of the AMPKα subunit at Thr172, which reflects the activation of AMPK [13]. In the liver tissue of obese mice, the HFD led to a radical decrease in the phosphorylation degree of AMPKα compared to the control group, while EAOT treatments resulted in exponentially increased AMPKα (Thr172) phosphorylation (Figure 4A). Moreover, the p-AMPKα/AMPK ratio decreased by 57.1% in the model group mice livers, whereas it increased in the mice fed the HFD and EAOTs (Figure 4B). In IHC, brown signals were mainly confined to p-AMPKα. The IHC signals were more intense and a significantly higher number of positive protein was observed in the livers of the mice in the treatment groups than that of the model mice, which showed mild signals and a lower number of positive protein (Figure 4C).

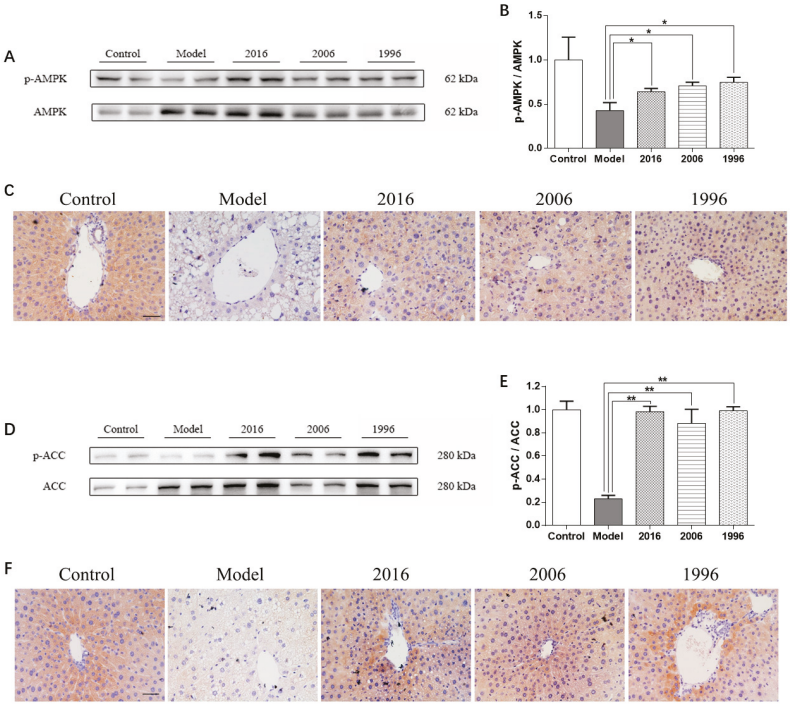

Figure 4. EAOTs activate AMPK and ACC phosphorylation. (**A**) Western blotting analysis of AMPK protein levels in mice liver and (**B**) densitometric quantification of them (n = 3 independent experiments); (**C**) Immunohistochemical staining of liver for p-AMPK; (**D**) Western blotting analysis of ACC protein levels in mice liver and (**E**) densitometric quantification of them (n = 3 independent experiments); (**F**) Immunohistochemical staining of liver for p-ACC. The scale bar represents 20 μm. Each value represents the mean ± SD (n = 9); * $p < 0.05$ versus model; ** $p < 0.01$ versus model.

ACC activation was determined by measuring the phosphorylation level of ACC at Ser79 where AMPK phosphorylates [14]. Similarly, the phosphorylation degree of ACC (Ser79) in the livers of the mice fed with the HFD was down-regulated compared with those in the control group. Furthermore, the HFD significantly reduced the p-ACC/ACC ratio (by 77.0%) in the livers of the mice. These HFD-induced down-regulations in the phosphorylation degree of ACC (Ser79) and the ratio of the p-ACC/ACC were markedly reversed in the livers of the mice in the treatment groups (Figure 4D,E). The phosphorylation of ACC (Ser79) was also assessed by IHC. The density of p-ACC (Ser79)-positive cells was radically increased in EAOT-treated mice compared to the model group (Figure 4F).

3.7. EAOTs Activate CPT-1 Expression and Inhibit FAS Expression

To better understand the molecular mechanism by which EAOTs mediate the beneficial effects on obese animals described above, two proteins were measured. As shown in the immunoblot analysis, CPT-1, which promotes fatty acid oxidation, was significantly higher in the treatment groups than in the model group (Figure 5C). This was confirmed by immunohistochemistry. The number of CPT-1-positive cells significantly increased with EAOT treatment (Figure 5A).

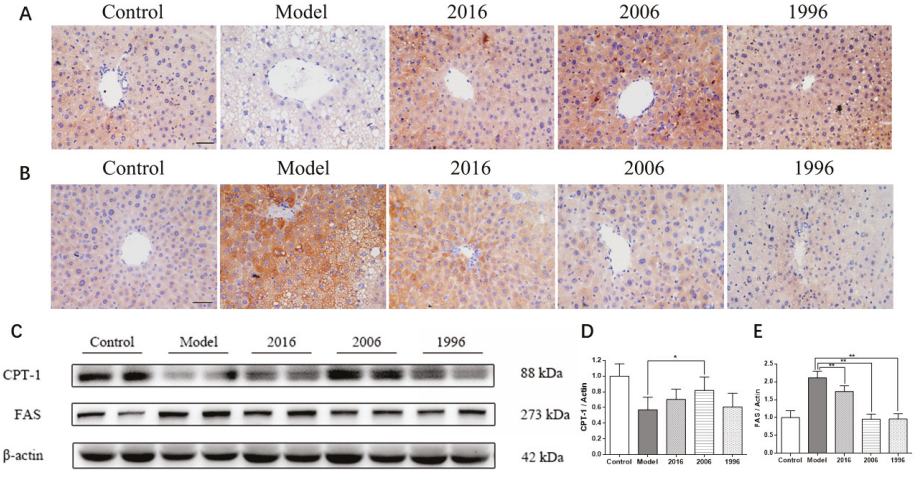

Figure 5. EAOTs activate CPT-1 expression and inhibit FAS expression. Immunohistochemical staining of liver for (**A**) CPT-1 and (**B**) FAS; (**C**) Western blotting analysis of CPT-1 and FAS protein levels in mice liver and densitometric quantification of (**D**) CPT-1 and (**E**) FAS (n = 3 independent experiments). The scale bar represents 20 μm. Each value represents the mean ± SD (n = 9); * $p < 0.05$ versus model; ** $p < 0.01$ versus model.

Moreover, FAS, which enhances fat synthesis, was down-regulated in the EAOT groups, particularly in the 2006 and 1996 groups (Figure 5C). Consistently, the model group revealed intense staining around the lipid inclusions in comparison to the control groups, and EAOTs were able to significantly reduce FAS expression (Figure 5B). All these data indicate that EAOTs might alleviate HFD-induced metabolic abnormalities, at least in part, through regulating the expression of a certain set of metabolic proteins.

3.8. EAOTs Inhibit iNOS and TNF-α Expression

Obesity is usually associated with a large number of inflammatory cells and factors. Severe inflammation can aggravate the liver injury, cause the decline of liver function, and weaken the glucolipid metabolism ability and lipid metabolism [15]. As shown in Figure 6A, the protein expression of iNOS and TNF-α in livers of the model group mice showed a marked increase compared to those of the control group, while the treatment groups had a lower iNOS and TNF-α protein expression level in the liver than those in the model group. Especially in the 2006 group, the expression levels decreased markedly compared to the model group. These results showed that EAOTs can relieve liver inflammation to a certain degree by inhibiting inflammation factors, iNOS and TNF-α.

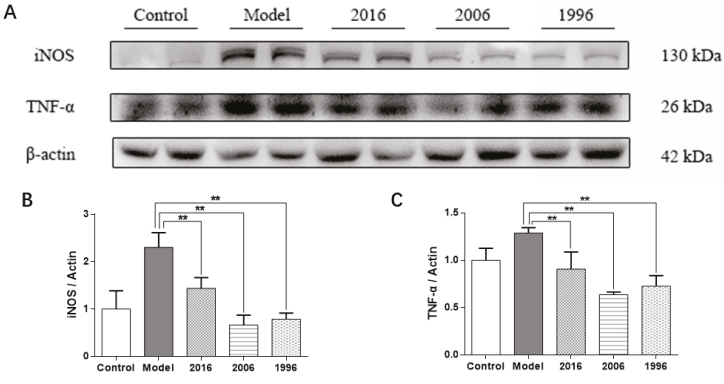

Figure 6. EAOTs inhibit iNOS and TNF-α. (**A**) Western blotting analysis of iNOS and TNF-α protein levels in mice liver and densitometric quantification of (**B**) iNOS and (**C**) TNF-α (n = 3 independent experiments). Each value represents the mean ± SD (n = 9); ** $p < 0.01$ versus model.

4. Discussion

In the present study, we provided evidence that EAOTs significantly improved obesity phenomena, such as body weight, Lee's index, fatty liver, adiposity, and hyperlipidemia in mice with HFD-induced obesity. The reduction of body fat accumulation and organ index in the study also indicated that EAOTs modulated obesity in mice. Furthermore, proteins up-regulating fat consumption were higher in the treatment groups, particularly in the 2006 group, and proteins related to fat synthesis and inflammation were down-regulated in the EAOT groups. These results revealed that obesity seemed to be alleviated within six weeks when C57BL/6J mice were given EAOTs daily.

White adipose tissues (WATs) are optimized to store energy in large lipid droplets for later use [16]. The accumulation of body fat in WATs leads to both hypertrophy and hyperplasia of white adipocytes [17]. These changes are associated with obesity-related diseases such as type-2 diabetes and an inflammatory response [18,19]. Previous studies have demonstrated that AMPK is a master energy sensor that integrates nutrients, hormones, and stress signals to maintain whole-body energy homeostasis [20]. Activated AMPKα, namely p-AMPKα, is able to stimulate catabolism and inhibit anabolism, leading to an increase of fat consumption. Rasmus's study has proved that AMPK in muscle is activated in response to exercise [21]. Moreover, p-AMPK can inactivate ACC1, leading to inhibition of de novo fatty acid and cholesterol synthesis. Phosphorylation of ACC2 by p-AMPK, on the other hand, causes increases of fatty acid oxidation [22]. In addition, the AMPK-ACC pathway is able to suppress the TG production because ACC is the rate-limiting enzyme for the synthesis of malonyl-CoA, which is a critical substrate for fatty acid biosynthesis and a potent inhibitor of fatty acid oxidation [23]. Various phytochemicals used to treat metabolic diseases are often able to stimulate AMPK activation [24–26]. In the present study, EAOTs markedly induced AMPKα and ACC phosphorylation in the liver, and increased the p-AMPKα/AMPK and p-ACC/ACC ratio, suggesting that AMPK may be the key mechanism for the beneficial effects of EAOTs on fatty liver.

This speculation was further supported by the observation that EAOTs activated CPT-1, whose expression is inhibited by ACC in the mouse liver. The phosphorylation of ACC promoted by p-AMPKα decreased the activation of Malonyl CoA [27], which is a fatty acid precursor, as well as an inhibitor of CPT-1. Therefore, p-AMPKα promotes the expression of CPT-1 by improving the level of p-ACC and decreasing the expression of Malonyl CoA. CPT-1 is the rate-limiting enzyme of mitochondrial fatty acid oxidation [28], which can increase the capacity of fatty acid flux into the mitochondria. In other studies [29], drugs affect the lipid regulatory system by activating the

AMPK-ACC-CPT-1 pathway. Therefore, it is reasonable to speculate that EAOTs relieve disorders of lipid metabolism by activating the AMPK-ACC-CPT-1 pathway.

FAS is a key lipogenic enzyme in mammals, and it catalyzes all the reactions in the conversion of acetyl-CoA and malonyl CoA to palmitate [30]. Its concentration is sensitive to nutritional and hormonal status in lipogenic tissues such as liver and adipose tissues. The nutritional regulation of FAS occurs mainly via changes in FAS gene transcription [31]. Our study has shown that FAS expression is significantly inhibited by EAOT administration, also suggesting that EAOTs' anti-obesity effects are partly due to the inhibition of expression of fatty acid and TG synthesis-related proteins. However, more experiments are needed to understand the effect of EAOTs on anti-obesity in other body parts.

In addition, it has been reported that AMPK activation led to decreased production of inflammatory mediators in macrophages [32]. In our study, EAOTs stimulated AMPKα phosphorylation and decreased expression of inflammatory factor TNF-α, a potent inflammatory inducer, as well as iNOS. It appears that EAOT-induced AMPK might also contribute to the suppression of inflammatory responses, which would lead to improvements in metabolic disorders.

While the 2006 group showed better anti-obesity and anti-inflammatory effects than the other EAOT treatment groups, it is difficult to speculate which ingredients contributed to this difference, if only relying on the data in this study. As shown in Table 1, we determined the content of free amino acids, total soluble sugar, and tea polyphenols in EAOTs. The free amino acids content was significantly lower in aged oolong tea, and the content of total soluble sugar markedly increased with the storage time. The tea polyphenols content was the highest in 2006 EAOTs, the second highest in 2016 EAOTs, and the lowest in 1996 EAOTs. After correlation analysis, these three ingredients were not related to EAOTs' anti-obesity and anti-inflammatory effects. It may be the effect of undetermined ingredients, or the combined effect of one known ingredient and other unclear substances, that contributes to these effects. Actually, this question is exactly what we plan to research later. HPLC-MS will be used to determine and analyze more comprehensive ingredients.

In conclusion, EAOT administration has a beneficial effect, reducing body weight gain, adipose tissue weight, and adipocyte size, as well as lipid levels in serum and liver in HFD-induced obese mice. Moreover, EAOTs affect the lipid metabolic regulatory system in the liver by activating the expression of FAS and changing the activation of the AMPK-ACC-CPT-1 pathway, which is known to play a central role in lipid metabolism regulation that facilitates catabolism of fuel storage. In addition, EAOTs could have an impact on inflammation, which protects the liver function and energy metabolism indirectly. Taken together, these results suggest that, as a novel AMPK activator, EAOTs would be a potential candidate for the treatment of obesity and metabolic disorders.

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Conflicts of Interest: The authors declare no conflict of interest.

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Review

Dietary Inflammatory Index and Cardiovascular Risk and Mortality—A Meta-Analysis

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Abstract: Diet and chronic inflammation have been suggested to be risk factors in the development of cardiovascular disease (CVD) and related mortality. The possible link between the inflammatory potential of diet measured through the Dietary Inflammatory Index (DII®) and CVD has been investigated in several populations across the world. The aim of this study was to conduct a meta-analysis on studies exploring this association. Data from 14 studies were eligible, of which two were case-control, eleven were cohort, and one was cross-sectional. Results from the random-effects meta-analysis showed a positive association between increasing DII, indicating a pro-inflammatory diet, and CVD. Individuals in the highest versus the lowest (reference) DII category showed a 36% increased risk of CVD incidence and mortality, with moderate evidence of heterogeneity (relative risk (RR) = 1.36, 95% confidence interval (CI): 1.19, 1.57; heterogeneity index I^2 = 69%, $p < 0.001$). When analyzed as a continuous variable, results showed an increased risk of CVD risk and mortality of 8% for each one-point increase in the DII score. Results remained unchanged when analyses were restricted to the prospective studies. Results of our meta-analysis support the importance of adopting a healthier anti-inflammatory diet for preventing CVD incidence and related mortality. In conclusion, a pro-inflammatory diet is associated with increased risk of CVD and CVD mortality. These results further substantiate the utility of DII as tool to characterize the inflammatory potential of diet and to predict CVD incidence and mortality.

Keywords: diet; cytokines; nutrition; inflammation; epidemiology; dietary inflammatory index; cardiovascular diseases; meta-analysis

1. Introduction

Chronic inflammation is characterized by the continuous presence of pro-inflammatory cytokines through increased blood flow during tissue injury, as a consequence of histamine released by damaged mast cells [1], and is known to play a major role in the development of cardiovascular diseases (CVD) and related mortality [2]. Previous research has indicated that whole diet and various dietary

components have a direct association with inflammation [3–6]. Consumption of fruit and vegetables has been shown to reduce levels of inflammation, while consumption of red meat has been shown to increase inflammation [7,8]. Various dietary components also have been implicated in playing a major role in the development of various CVDs [8–10]. In a recent meta-analysis, increased intake of processed meat has been shown to be associated with increased risk of CVD mortality [11]. Increased adherence to healthier dietary patterns characterized by increased intake of plant-based foods such as fruit and vegetables, nuts, and whole grains and adherence to a healthier dietary pattern has been shown to help prevent and to manage CVD [12–14]. Finally, an increased intake of polyphenols, anti-oxidants with ability to decreases oxidative stress and inflammation through scavenging free radicals [15], was shown to be associated with decreased risk of overall and CVD-related mortality [16].

Until 2009, there was no tool that could take into account the entire diet and determine its inflammatory potential. Researchers from University of South Carolina have developed a dietary tool called the Dietary Inflammatory Index (DII®) that places individuals on a continuum from maximally pro-inflammatory to maximally anti-inflammatory diet. The first version of DII was developed based on literature published on various dietary components and inflammation through 2007 [17]. In the first version, 927 peer reviewed articles were scored based on the direction and significance of the association between various dietary components and each of the six inflammatory markers (C-reactive protein (CRP); interleukin (IL)-6, -1β, -4, and -10; and tumor necrosis factor (TNF)-α). In 2014, the DII® was refined and improved with literature review extending through 2010 [18], wherein the number of articles that were reviewed and scored increased to 1943. The DII categorizes individuals' diets according to their inflammatory potential on a continuum from maximal pro-inflammatory to maximal anti-inflammatory. A higher DII score indicates a more pro-inflammatory diet, whereas a lower DII score represents a more anti-inflammatory diet. The DII can be calculated from various dietary assessment tools, with food frequency questionnaires (FFQs) being the most commonly used tool [19–21]. The DII has been construct validated in several studies, in the first validations study DII has been shown to be associated with changes in high-sensitivity-CRP in the Seasonal Variation in Blood Cholesterol Study [17,20]. Subsequently, the DII has been validated in several studies from around the world with different measures of inflammation such as CRP, IL-6, and TNF-α-R2 [19,22–25]. Apart from the validation studies, DII has also been shown to be associated with various chronic inflammation-related health outcomes such as cancer incidence [26–28], all-cause and cancer-specific mortality [29–31], respiratory conditions such as asthma [32,33] and cognitive disorders [34,35]. In a recent meta-analysis from nine studies that looked at the association between DII and colorectal cancer (CRC), individuals in the highest versus the lowest (reference) DII category showed an overall 40% increased risk of colorectal cancer (CRC), with moderate evidence of heterogeneity (relative risk (RR) = 1.40, 95% confidence interval (CI): 1.26, 1.55; I^2 = 69%, $p < 0.001$) [36]. Several studies have found positive associations between DII score and CVD incidence and CVD mortality [9,10,14,37–44]. Using results from qualifying studies, the purpose of the current meta-analysis was to investigate the cumulative association between the inflammatory potential of diet, as measured by the DII score, and CVD risk and CVD mortality.

2. Methods

We followed the Meta-Analysis of Observational Studies in Epidemiology (MOOSE) protocol throughout the design, execution, analysis and reporting of the present meta-analysis [45].

2.1. Search Strategy and Study Selection

A comprehensive literature search was conducted using PubMed (National Library of Medicine) and Excerpta Medica database (EMBASE); databases were screened from the earliest available online indexing year up to September 2017, with Title/Abstract and MESH terms restriction. The following search terms were included: (dietary inflammatory index or inflammatory diet or anti-inflammatory diet or dietary score) and (CVD or cardiovascular disease or coronary heart disease (CHD) or ischemic heart disease (IHD) or myocardial infarction or stroke or heart attack or hypertension). Two authors

performed the search independently. We included observational studies with any type of design (prospective, case-control and cross-sectional) that evaluated the association between the DII and cardiovascular disease risk and mortality. Studies were eligible if they provided corresponding risk estimates, such as RRs (risk ratios), HRs (hazard ratios), or ORs (odds ratios). We excluded studies that reported insufficient statistics or evaluated other dietary scores as exposure. Reference lists of included manuscripts also were "hand searched" for additional studies not previously identified. When a duplicate report from the same study/cohort was identified, we included the report that provided the largest number of cases/entire cohort or had the longest follow-up for each endpoint of interest. Two authors assessed full texts of potentially relevant articles independently.

2.2. Data Extraction

Data were extracted from each eligible study using a standardized extraction form. The following information was collected: (i) name of the first author; (ii) year of publication; (iii) study cohort or name; (iv) country; (v) number of participants; (vi) sex of participants; (vii) age range or mean age of the study population at baseline; (viii) follow-up period; (ix) endpoints and cases; (x) measures of risk or association with 95% confidence intervals (CIs) for the highest versus the lowest category of exposure and for one-point increase of the DII score (when available); and (xi) covariates used for adjustment.

The quality of eligible studies was assessed in accordance to the Newcastle–Ottawa Quality Assessment Scale [46], which consist of three parameters of quality: selection (four points), comparability (two points), and outcome (three points), with a score of seven or more points reflecting high quality.

2.3. Statistical Analysis

In this meta-analysis, ORs and HRs were deemed equivalent to RRs [47]. Random-effects models were used to calculate pooled RRs (with 95% CIs) of CVD incidence and CVD mortality for the highest compared to the lowest category of exposure and for a one-point increase in the DII score. Risk estimates of CVD occurrence and mortality for one-point increase of the DII score (continuous), were estimated for the studies not reporting the measure but providing sufficient data to estimate it. Heterogeneity was assessed using the Q test and I^2 statistic. The significance for the Q test was defined as $p < 0.10$. The I^2 statistic represents the amount of total variation that could be attributed to heterogeneity. I^2 values $\leq$ 25%, 25–50%, 50–75% and >75% indicated no, small, moderate, and high heterogeneity, respectively. A sensitivity analysis was conducted by excluding one study at the time in order to assess the stability of results. Subgroup analyses were conducted by type of cardiovascular event, sex, geographical, and adjustment for body mass index (BMI), smoking, education and physical activity. Publication bias was assessed by visual observation of funnel plot. All analyses were performed with Review Manager (RevMan) version 5.2 (The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark).

3. Results

3.1. Study Characteristics

The database search identified 63 studies, of which 19 were excluded after reviewing the title, and 27 based on abstract (Figure 1). Of the remaining 17 publications, three were excluded for the following reasons: (i) article reported on cardiovascular risk factors, not CVD events; (ii) article did not report on general population, it used a clinic population with possible prior CVD; and (iii) article provided data on total/all-cause mortality, from which CVD deaths could not be distinguished.

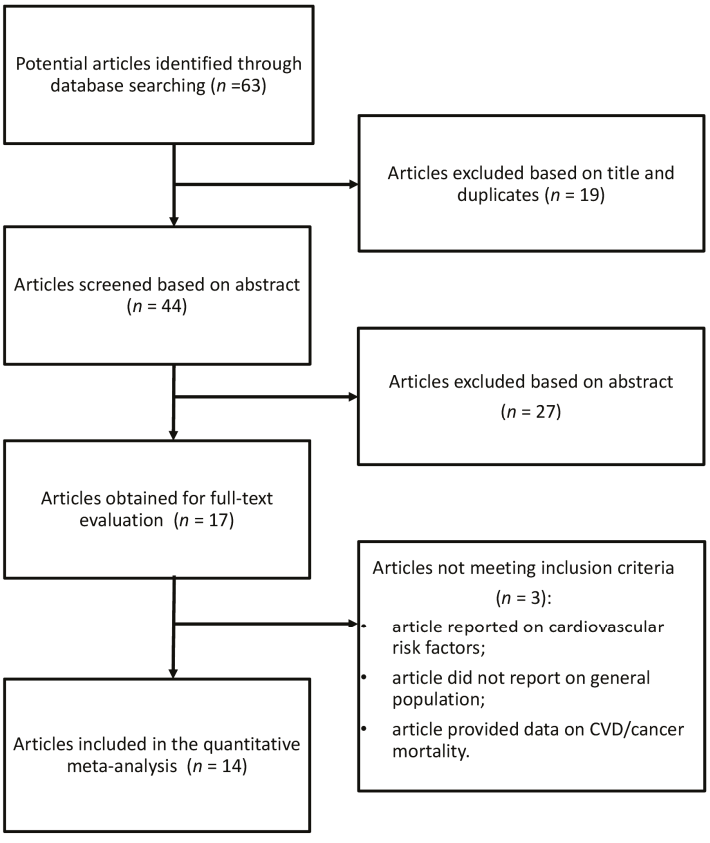

Figure 1. Selection of relevant studies reporting on the association between Dietary Inflammatory Index (DII®) and cardiovascular disease (CVD) occurrence and mortality.

For the analysis on the association between DII and CVD risk and mortality, 14 studies were eligible [9,10,29,37–44,48–50]. Eleven reports were prospective studies [10,29,37–43,48,49], one study had cross-sectional design [50] and two studies were case-control [9,44]. Case-control studies were used only for the subgroup analysis of risk of myocardial infarction. Prospective studies included the following cohorts: the Iowa Women's Health Study (IWHS), National Health and Nutrition Examination Survey (NHANES)-III study, Swedish Mammography Study, Whitehall II study, MONICA/KORA Augsburg study, Supplémentation en Vitamines et Minéraux AntioXydants (SU.VI.MAX), The Australian Longitudinal Study on Women's Health (ALSWH), Geelong Osteoporosis Study (GOS), Prevención con Dieta Mediterránea (PREDIMED), Seguimiento Universidad de Navarra (SUN) and Calcium Intake Fracture Outcome Study (CAIFOS). Studies eligible for the meta-analysis comprised 161,337 participants and 15,738 cases. Main characteristics of the studies included in the meta-analysis are described in Table 1. Six studies provided relative risk for CVD incidence [10,38,39,48–50], six for CVD mortality [29,37,40–43], three for CHD mortality [29,37,42], three for IHD/CHD [42,49,50], three for stroke [48–50], four for myocardial infarction [9,44,48,49], and two for angina pectoris [48,50]. Three studies were conducted in North America [29,43,50], eight in Europe [9,10,39–42,44,48] and three in Australia [37,38,49]. The follow-up time in prospective cohort studies ranged from about 4.3 to 26 years.

Table 1. Main characteristics of the studies included in the meta-analysis.

Author, Year	Study Design	Study Name, Country	Follow-Up (Years)	Cases; Total Population	Cases/Controls or Person Years of Observations or Total Number of Subjects for Lowest Quantile of DII	Cases/Controls or Person Years Observations or Total Number of Subjects for Highest Quantile of DII	Gender	Adjustments
Garcia-Arellano, 2015 [10]	Cohort	PREDIMED, Spain	4.3	277 incident cases; 7216	49/7641 [a]	79/7960	MF	Age and sex, overweight/obesity, waist-to-height ratio, total energy intake, smoking status, diabetes, hypertension, dyslipidemia, family history of premature cardiovascular disease, physical activity and educational level.
O'Neil, 2015 [38]	Cohort	GOS, Australia	5	76 incident cases; 1363	NA	NA	M	Age, diabetes, systolic and diastolic blood pressure (BP), smoking history, activity level, waist circumference, and total daily energy consumption.
Ramallal, 2015 [39]	Cohort	SUN, Spain	8.9	117 incident cases; 18,794	24/41,240 [a]	37/42,345	MF	Age, cardiovascular risk factors (hypertension, dyslipidemia, diabetes, smoking status, family history of cardiovascular disease), total energy intake, physical activity, body mass index (BMI), educational level, and other cardiovascular diseases (tachycardia, atrial fibrillation, aortic aneurysm, pulmonary embolism, deep vein thrombosis, peripheral artery disease, heart valve disease, or pacemaker placement), special diet at baseline, snacking, average time sitting, average time spent watching television.
Shivappa, 2015 [29]	Cohort	IWHS, USA	14	6528 CVD deaths; 37,525	1615/195,996 [a]	1665/192,198	F	Age, BMI, smoking status, pack-years of smoking, HRT use, education, prevalent diabetes, prevalent hypertension, prevalent heart disease, prevalent cancer, total energy intake.
Shivappa, 2015 [43]	Cohort	NHANES III, USA	13.5	1235 CVD deaths; 12,366	368/4183 [b]	437/4119	MF	Age, sex, race, diabetes status, hypertension, physical activity, BMI, poverty index, and smoking.
Shivappa, 2015 [40]	Cohort	SMC, Sweden	15	2399 CVD deaths; 33,747	445/*n*	560/*n*	F	Age, energy intake, BMI, education, smoking status, physical activity, and alcohol intake.
Neufcourt, 2016 [48]	Cohort	SUVIMAX, France	11.4	292 CVD incidence; 7743	63/22,432 [a]	89/21,471	MF	Sex, energy intake without alcohol, supplementation group, number of 24-h records, education level, marital status, smoking status, physical activity, BMI.
Vissers, 2016 [49]	Cohort	ALSWH, Australia	11	526 CVD incidence; 6972	71/1626 [b]	264/5346	F	Age, energy, diabetes, hypertension, smoking status, education, menopausal status and HRT use, physical activity and alcohol consumption.
Wirth, 2016 [50]	Cross-sectional	NHANES, USA	NA	1734 prevalent cases; 15,693	505/3393	373/3531	MF	Family member smoking status, personal smoking status, age, and BMI.

Table 1. *Cont.*

Author, Year	Study Design	Study Name, Country	Follow-Up (Years)	Cases; Total Population	Cases/Controls or Person Years of Observations or Total Number of Subjects for Lowest Quantile of DII	Cases/Controls or Person Years Observations or Total Number of Subjects for Highest Quantile of DII	Gender	Adjustments
Boden, 2017 [9]	Nested Case-control	NSHDS, Sweden	6.4	1389 acute myocardial infarction cases; 5555 matched controls	210/1056 [c]	344/1056	MF	Total energy intake, BMI, physical activity, systolic blood pressure, total serum cholesterol, diabetes, smoking, and postsecondary academic education.
Bondonno, 2017 [37]	Cohort	CAIFOS, Australia	15	269 deaths; 1304	55/4368 [b]	83/4072	F	Age, BMI, energy intake, energy expended in physical activity, socioeconomic status, use of low-dose aspirin, use of antihypertensive medication, use of statins, current or previous smoking, prevalent ASVD (atherosclerotic vascular disease) and treatment.
Shivappa, 2017 [44]	Case-control	NA, Italy	NA	682 cases; 682 controls	154/171 [c]	225/171	MF	Age, sex, and total energy intake, education, tobacco smoking, BMI, occupational physical activity at age 30–39, coffee consumption, history of hypertension, history of hyperlipidemia, history of diabetes and family history of acute myocardial infarction in first-degree relatives.
Shivappa, 2017 [42]	Cohort	MONICA/KORA, Germany	For CVD mortality: 25.8 and 16.7 years for Survey 1 and Survey 3 For CVD incidence: 21.4 and 13.9 years for S1 and S3	399 CVD related deaths; 1297 men 213 incidence cases; 1297 men	50/324 [b] 40/324	74/324 66/324	M	Age, survey, BMI, place of residence, actual hypertension, education level, diabetes, physical activity, energy intake, ratio of total cholesterol and HDL cholesterol, smoking status.
Shivappa, 2017 [41]	Cohort	Whitehall II, UK	22	264 CVD deaths; 7627	84/2456 [b]	107/2434	MF	Age, sex and ethnicity, occupational grade, living alone, smoking habits, alcohol consumption, physical activity, BMI, antecedent of CVD, use of lipid-lowering drugs, HDL-cholesterol, hypertension, type 2 diabetes and longstanding illness.

[a] Denominator is person years of observations; [b] Denominator is the total number of subject in the DII category; [c] Denominator is the total number of controls in the DII category. DII®: Dietary Inflammatory Index; PREDIMED: Prevención con Dieta Mediterránea; GOS: Geelong Osteoporosis Study; SUN: Seguimiento Universidad de Navarra; IWHS: Iowa Women's Health Study; NHANES: National Health and Nutrition Examination Survey; SMC: Swedish Mammography Study; SUVIMAX: Supplémentation en Vitamines et Minéraux AntioXydants; ALSWH: The Australian Longitudinal Study on Women's Health; NSHDS: Northern Sweden Health and Disease Study; CAIFOS: Calcium Intake Fracture Outcome Study; NA: not applicable; CVD: cardiovascular disease. HRT: hormone replacement Therapy; HDL: high density lipoproteins; M: male, MF: female.

3.2. DII and CVD Risk and Mortality

The overall analysis of the association between the DII and CVD (either risk or mortality) is shown in Figure 2. Individuals in the highest versus the lowest (reference) DII group of exposure had a 36% increased risk of CVD (RR = 1.36, 95% CI: 1.19, 1.57). There was evidence of heterogeneity (I^2 = 65%, $p < 0.001$) but no evidence of publication bias after examining the funnel plot (Figure S1). A sensitivity analysis conducted by excluding one study at a time revealed that the results from the IWHS were the largest contributor to the heterogeneity and its exclusion reduced heterogeneity to 38% ($p = 0.10$) with substantially unchanged effect size (RR = 1.41, 95% CI: 1.24, 1.61). The analysis considering the DII score as a continuous variable showed an increased risk of CVD of 8% for each one-point increase of the score (RR = 1.08, 95% CI: 1.04, 1.12) (Figure 3).

Despite that results were affected by heterogeneity, no publication bias was evident after examination of the funnel plot (Figure S2).

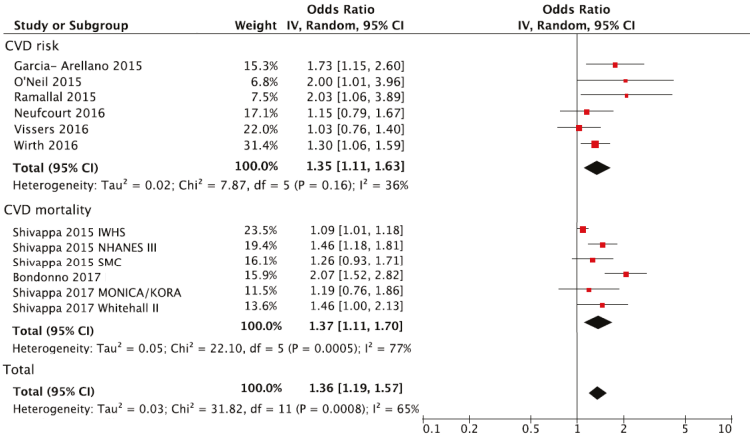

Figure 2. Forest plot of summary relative risks (RRs) of CVD occurrence and CVD mortality for the highest vs. lowest (reference) category of DII. IWHS: the Iowa Women's Health Study; NHANES: National Health and Nutrition Examination Survey; SMC: Swedish Mammography Study.

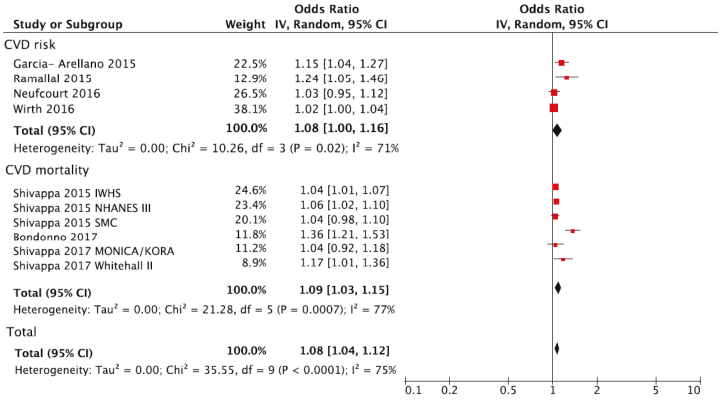

Figure 3. Forest plot of summary relative risks (RRs) of CVD occurrence and CVD mortality for one-point increase of DII.

The analysis for CVD risk and CVD mortality showed similar effect sizes (Figure 2), with a substantial increased risk of events in individuals with the highest DII compared to the lowest group (Table 2). However, results for CVD mortality were affected by high heterogeneity (I^2 = 77%, $p < 0.001$). Assessment of risk estimates for individual outcomes among CVD showed no significant results besides the association between DII and myocardial infarction; however, statistical power of these analyses was limited due to the reduced number of available datasets (Table 2).

Table 2. Analyses of studies exploring the association between DII and CVD, total and individual outcomes. Risk estimates refer to the highest vs. lowest (reference) category of DII.

Subgroup/Additional Analysis	No. of Datasets (Studies)	RR (95% CI)	I^2	*p*
CVD risk and mortality				
Total	12 (12)	1.36 (1.19, 1.57)	65%	<0.001
CVD risk				
Total	6 (6)	1.35 (1.11, 1.63)	36%	0.16
IHD/CHD	3 (3)	1.18 (0.89, 1.58)	37%	0.20
Stroke	3 (3)	1.10 (0.60, 2.00)	65%	0.06
Myocardial infarction	5 (4)	1.43 (1.09, 1.89)	38%	0.17
Angina pectoris	2 (2)	0.79 (0.56, 1.12)	0%	0.73
CVD mortality				
Total	6 (6)	1.37 (1.11, 1.70)	77%	<0.001
CHD mortality	3 (3)	1.37 (0.88, 2.12)	68%	0.05

IHD: Ischemic Heart Disease; CHD: Coronary Heart Disease.

3.3. Subgroup Analyses

Several subgroups were conducted to assess whether some variables of interest might be responsible for the heterogeneity observed between results of studies exploring the relation between DII and CVD (either risk or mortality; Table 3). The analysis revealed that increased risk of CVD in individuals with high versus low DII was significant only in women and in studies conducted in Europe and North America, but not in men and studies conducted in Australia (Table 3). Moreover, among the variables investigated, adjustment for BMI and physical activity was found to be crucial for obtaining significant results compared to studies that did not adjust for such variables (Table 3).

Table 3. Subgroup analyses of studies exploring the association between DII and CVD (either risk or mortality). Risk estimates refer to the highest vs. lowest (reference) category of DII.

Subgroup/Additional Analysis	No. of Datasets (Studies)	RR (Relative Risk) (95% CI)	I^2	*p*
Study design				
Cross-sectional	1 (1)	1.30 (1.06, 1.58)	NA	NA
Prospective cohort	11 (11)	1.38 (1.18, 1.62)	68%	<0.001
Sex				
Male	2 (2)	0.95 (0.70, 1.30)	39%	0.20
Female	5 (5)	1.39 (1.05, 1.82)	86%	<0.001
Geographical area				
North America	3 (3)	1.25 (1.03, 1.51)	75%	0.02
Europe	6 (6)	1.37 (1.16, 1.61)	0%	0.51
Australia	3 (3)	1.58 (0.93, 2.67)	81%	0.005
Follow-up duration				
<10 years	3 (3)	1.85 (1.36, 2.51)	0%	0.89
≥10 years	9 (9)	1.30 (1.12, 1.49)	66%	0.003
Adjusted for smoking				
No	0	NA	NA	NA
Yes	12 (12)	1.36 (1.19, 1.57)	65%	<0.001

Table 3. *Cont.*

Subgroup/Additional Analysis	No. of Datasets (Studies)	RR (Relative Risk) (95% CI)	I^2	*p*
Adjusted for BMI				
No	2 (2)	1.33 (0.71, 2.51)	67%	0.08
Yes	10 (10)	1.39 (1.19, 1.61)	69%	<0.001
Adjusted for education				
No	5 (5)	1.54 (1.29, 1.83)	41%	0.15
Yes	7 (7)	1.20 (1.04, 1.37)	33%	0.17
Adjusted for physical activity				
No	2 (2)	1.16 (0.98, 1.37)	60%	0.11
Yes	10 (10)	1.44 (1.23, 1.67)	42%	0.08

4. Discussion

This meta-analysis, which used results from 14 studies that have examined the association between the DII and CVD, showed strong evidence of association between increasing inflammatory potential of diet and CVD risk and related mortality. Therefore, increasing intake of healthy and anti-inflammatory dietary components such as fruits and green leafy vegetables and decreasing intake of pro-inflammatory components such as processed meat and sugar-sweetened drinks may play a vital role in reducing the risk of CVD and related mortality.

Up until the development of the DII, all dietary indices fell into one of three categories: (1) those based on dietary recommendations such as the Healthy Eating Index-2010 (HEI-2010) [51], or the Alternative Healthy Eating Index (AHEI) [52], both based on the US Dietary Guidelines or the Dietary Approaches to Stop Hypertension (DASH) [53], which was promoted by the National Heart, Lung, and Blood Institute; (2) those related to adherence to a particular foodway or cuisine such as the Mediterranean Dietary Index (MDI) [54]; or (3) those derived from a particular study using some kind of regression technique such as principal components analysis or reduced rank regression [55]. These indices have been examined with CVD incidence and mortality as outcome in the past [56]. In a meta-analysis conducted in 2015 that examined results from 15 prospective studies, high-quality diets, which are indicated by having higher HEI, AHEI, and DASH score, showed a significant risk reduction (RR) for CVD incidence or mortality (RR = 0.78, 95% CI 0.75 to 0.81; $p < 0.00001$; $I^2 = 45\%$, 95% CI 13% to 66%) [56]. Higher values for each of these indices represent a healthier diet. Each of them suffers from idiosyncrasies of the approach that include, as a common shortcoming, a narrow range of exposure variability. None was developed specifically to assess the inflammatory potential of the diet. By contrast, the DII was designed to reflect all evidence from a wide variety of human populations as well as from laboratory animal and cell culture experiments. The second advantage of the DII is that it is grounded in research which involved reviewing and scoring 1943 peer reviewed publications that looked at the association between various dietary components and inflammatory markers and is not dependent on a single study or a few studies within the same or similar populations. Results from a cross-sectional study in the USA indicated that the DII score was negatively correlated with the previously mentioned dietary indices (HEI-2010 ($r = -0.65$, $p < 0.01$), AHEI ($r = -0.55$, $p < 0.01$), and the DASH ($r = -0.52$, $p < 0.01$)) [57] and, in large cohort study from Australia, DII was inversely correlated with the Mediterranean Diet Score (MDS) ($r = -0.45$, $p < 0.01$) [58]. Apart from showing strong and consistent association between increasing DII and CVD risk, the DII has also been successfully validated with different markers of inflammation in studies across different populations in different countries [22,25,33,59]. This shows that the DII captures unique properties of diet that are usually not incorporated in a general healthy diet. Presumably, inflammation is the additional aspect of diet that is not captured by other dietary indices and patterns. The 45 dietary components used to derive DII are called food parameters because within this list there are several macro- and micronutrients; food items such as garlic, ginger and onions; and important bioactive polyphenols such as flavonoids. All six groups of flavonoids (isoflavones, flavanol, flavan-3-ol, anthocyanidins, flavones and flavanones) are

included in the DII calculation and all of these have a negative literature-derived inflammatory effect score [18].

Multiple theories exist that explain the observed consistent association between DII and CVD risk and related mortality. One of them is the effect of pro-inflammatory diet on increasing levels of cytokines like IL-1 and TNF-α which, in turn, causes attraction and migration of inflammatory cells into vascular tissue [60,61]. These inflammatory markers also increase the expression of cellular adhesion molecules such as selectins and cadherins, which mediate adhesion of white blood cells to the vascular endothelium [62].

A report published from NHANES-III showed that pro-inflammatory diet was associated with an increased risk of CVD mortality in addition to all cause and all-cancer mortality among prediabetic patients [63]. Modified versions of DII have been tested before in relation to CVD risk and mortality. Georgousopoulou et al., in a study from the ATTICA cohort evaluated the association between anti-inflammatory diet and 10-year CVD incidence [64]. In this study, authors calculated a modified version of the DII and have called it Dietary Anti-Inflammation Index (D-AII). The difference between DII and D-AII is that in the D-AII calculation, the *z*-scores are not converted to centered percentiles and instead are directly multiplied by inflammatory effect scores. The scores ranged from 10 to 77. After adjusting for multiple confounding variables, an anti-inflammatory diet, was borderline significantly inversely associated with 10-year CVD incidence (OR 3rd tertile vs. 1st tertile = 0.98, 95% CI: 0.96–1.01) [64]. Finally, in a report from the Spanish cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC) study, the authors again have modified the DII and have called the index Inflammatory Score of Diet (ISD) [65]. The difference between DII and ISD is that instead of standardizing the intake values to means and standard deviations from the global database, authors have standardized the intake values to means and standard deviations of the study population. Subjects classified in the fifth quintile of the ISD (more pro-inflammatory diets) were at higher risk of cardiovascular diseases mortality (RR = 1.89, 95% CI: 1.48–2.40) as compared with those in the first quintile [65].

In addition to its strengths, this meta-analysis also has some limitations. First, in the majority of the studies used for this meta-analysis, DII was derived from dietary assessment tools that were based on self-report using food frequency questionnaires and 24-h recalls, which carries an inherent degree of recall bias and can lead to a potential misclassification of the exposure. Second, the DII score was calculated only once at baseline and human diet might change during study follow-up. However, previous research has shown that adult dietary patterns seem to be relatively stable over time [66]. Third, substantial heterogeneity was observed across studies pooling the CVD risk and mortality. One possible explanation for this could be the differences in the number of food parameters used to calculate the DII score in different studies; another reason could be the different demographic characteristics, and follow-up duration across these studies. Finally, only four studies were eligible for the analysis on CVD risk and six studies for the analysis on CVD mortality.

In conclusion, results from this meta-analysis of a large pool of DII and CVD risk and mortality studies suggests that increasing inflammatory potential of diet as evidenced by higher DII score was independently associated with the increased risk of CVD and related mortality. Hence, educating and encouraging people to consume diets rich in anti-inflammatory food components and low in pro-inflammatory components should play a crucial role in in reducing the risk of developing CVD. Future steps would include exploring how a DII compliant dietary intervention (i.e., aiming to lower overall DII score) would help in preventing newly incident CVD in high-risk populations and in preventing recurrence among those already living with a CVD.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/10/2/200/s1, Figure S1: Funnel plot for (a) CVD (cardiovascular disease) occurrence and mortality, (b) CVD occurrence and c) CVD mortality for the highest versus lowest (reference) category of dietary inflammatory index (DII®); Figure S2: Funnel plot for (a) CVD occurrence and mortality, (b) CVD occurrence and (c) CVD mortality for 1-point increase of DII.

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Conflicts of Interest: The authors declare no conflict of interest. However, we have the following disclosure: James R. Hébert owns controlling interest in Connecting Health Innovations LLC (CHI), a company planning to license the right to his invention of the dietary inflammatory index (DII®) from the University of South Carolina in order to develop computer and smart phone applications for patient counseling and dietary intervention in clinical settings. Michael D. Wirth and Nitin Shivappa are employees of CHI.

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Resveratrol in Patients with Minimal Hepatic Encephalopathy

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Abstract: Background: Minimal Hepatic Encephalopathy (MHE) is characterized by an impairment of social interaction, emotional behavior, sleep disorders, physical and mental symptoms, and diminished Quality of Life (QoL). The aim of our study is evaluating the potential liver health promoting a perspective of Resveratrol (RV) activities and evaluate whether RV treatment may improve health related quality of life (HRQL) and reduce depression and anxiety in patients with MHE. Methods: *W*e evaluated depression using the Beck Depression Inventory test, anxiety with State-trait anxiety inventory test, quality of life through SF-36 test, and ammonia serum levels in 70 MHE patients that were randomized into two groups. Results: In the comparison between RV group and placebo group we observed a decrease in Back Depression Inventory (BDI) ($p < 0.001$), in State-trait anxiety inventory (STAI) ($p < 0.001$), and improve in physical function ($p < 0.001$), in role physical ($p < 0.05$), in body pain ($p < 0.05$), in general health ($p < 0.001$), in vitality ($p < 0.05$), and in social function ($p < 0.001$). Conclusions: Resveratrol showed efficacy in the treatment of depression, anxiety, and ammonia serum levels, and improved the quality of life Of MHE patients.

Keywords: depression; anxiety; resveratrol; ammonia; quality of life; minimal hepatic encephalopathy

1. Introduction

Minimal Hepatic Encephalopathy (MHE) is a frequent and common complication of cirrhosis, affecting nearly 80% of cirrhotic patients. Physical examination in MHE is often normal; patients may present subtle abnormalieties, interfering with executive function, including their working memory and orientation, impairment of social interaction, emotional behavior, sleep disorders, physical and mental symptoms, and diminished Quality of Life (QoL) [1].

This underrecognized and underdiagnosized syndrome often presents minimal changes in personality and in memory, intellectual function, concentration, and coordination.

These disorders impinge on the health related quality of life and have a negative prognostic value in relation to the occurrence of both bouts of overt hepatic encephalopathy and death [2].

Resveratrol (RV) is a natural phytoalexin, which is isolated from the roots of with hellebore (Veratrum grandiflorum Hoes). RV is present in two isoforms, cis/trans, and trans isomer is the

biologically active one. It has been report that RV plays role as antinflammatory, antioxidants, antiviral, and exerts anticancer activities through many different mechanisms [3–11].

RV showed protective effect against neuro-inflammation inhibiting reactive oxygen species (ROS) production and mitogen-activated protein kinases (MAPK) signal transduction pathways, and also inactivating signalling pathways of both nuclear factor-kappa b (NF-κB) and silent mating type information regulation 2 homolog 1 (SIRT1) [12,13]. SIRT belongs to a protein family consisting of seven individual members, which are involved in cellular metabolism, mitochondrial function, and stress responses modulation [14].

The RV attracted the interest of researchers for the combined antidepressant and anti-inflammatory effects [11].

The aim of the current study was the evaluation of RV in liver health, in health related quality of life (HRQL), and in reduction of depression and anxiety in patients with MHE.

2. Patients and Methods

2.1. Study Design

Seventy-five patients with cirrhosis and minimal cognitive impairment regarding selective attention and executive function were recruited between May 2013 and November 2016 at Cannizzaro Hospital in Catania (Italy).

The diagnosis of cirrhosis was based on case history, clinical examination, biochemical, endoscopic, and ultrasounds finding; or, when needed, on liver biopsy.

This observational study was approved by the research ethics committee of Cannizzaro Hospital.

All of the eligible patients were approached and asked to participate in the study; those who accepted signed the written the informed consent after reading carefully the patients information leaflet.

75 patients with diagnosis of MHE were enrolled in the study: three withdrew consent, two overt hepatic encephalopathy (Figure 1). The 70 patients were randomized into two groups, using permuted-block randomization with an allocation ratio 1:1 and block size of four. Random numbers were assigned according to the sequency of their inclusion and patients received respective study products. Group A were treated with RV in pills 19.8 mg, *N*-acetyl cysteine 600 mg, lactoferrin 23.6 g (Resvis Alfa-sigma, Bologna, Italy). Group B were treated with placebo: *N*-acetyl cysteine 600 mg, lactoferrin 23.6 g. The diagnosis of HE was made on the evaluation of consciousness, intellectual behavior, and neuromuscular functions. Mental status was assessed and graded on the basis of West-Haven Criteria [15]. The schedule was to receive for 90 days a supply of either RV or placebo. The measurement was made every month, both for efficacy and for tolerability tests.

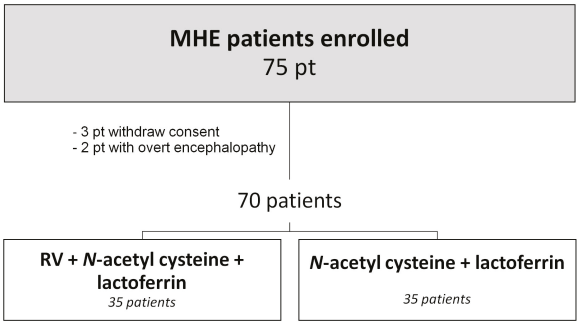

Figure 1. Study design. MHE = Minimal Hepatic Encephalopathy; RV=Resveratrol.

2.2. Protocol

The inclusion criteria were age 25–65 years.

Cirrhotic patients who performed abnormally on psychometric tests but performed normally on clinical neuropsychiatric examination were diagnosed with MHE. MHE was diagnosed according to guidelines from the final report of the Working Party at the 11th World Congress of Gastroenterology in Vienna in 1998.

The exclusion criteria were: pregnancy and breast feeding; past history of overt hepatic encephalopathy (OHE); use of interferon or psychoactive drugs, such as benzodiazepines, psychotropic drugs, antiepileptic; alcohol abuse, history of diabetes or renal impairment; significant head injury; arterial hypertension. Patients' characteristic included educational status, Child-Turcotte Pugh class, and etiology of cirrhosis [16].

Male patients were considered to have alcohol related cirrhosis if their alcohol intake was more than 50 g and female patients if their intake was more than 30 g for more than five years and if testing showed no viral metabolic or immunologic cause. Chronic hepatitis B and C were diagnosed when testing was positive for the viral markers Hepatitis B surface Antigen (HBsAg) and Hepatitis C virus antibody (anti-HCV) respectively.

All of the patients underwent to Psychometric Hepatic Encephalopathy Score (PHES) [17,18], Short Form 36 Health Survey (SF-36) [19–22], Beck Depression Inventory (BDI) [23], and State-Trait Anxiety Inventory (STAI) [22].

2.2.1. Diagnosis of MHE

The use of neuropsychometric battery tests have been limited to research of minimal HE. We performed the Psycometric Hepatic Encephalopathy Score (PHES), which consists of five psychometric tests that measure psychomotor speed and precision, visual perception, visus-spatial orientation, visual construction, concentration, attention, and memory [17,18].

2.2.2. Neuropsychological Testing

PHES Test Battery

PHES Scores a neuropsychological battery composed of five different tests: Number connection test A (NET-A) and B (NET-B), line tracing test, serial dotting test, and digit symbol test [15,16]. The test detects attention and psychomotor speed, areas that were most affected by HE. PHES score is obtained based on sum of the result of each test [17,18].

2.2.3. SF-36 (Short Form—36 Health Survey)

SF-36 questionnaire measures with eight domain scales and 36 items, health status. [17]

SF-36 provides two summary scores: physical component and mental component and eight health status scales: Physical functioning, role limitation due to physical problems, bodily pain, General health perceptions, vitality, social functioning, role limitation due to emotional problems, and mental health.

Patients completed the questionnaire and the resulting scores were transformed onto a scale from 0 (the worst possible score) to 100 (the best possible score), as recommended by questionnaires originators [19–22].

2.2.4. Beck Depression Inventory (BDI)

BDI is the most widely used instrument for detecting depression. It is a 21-item self report rating inventory that measures characteristic attitudes and symptoms of depression, including mood, sense of failure, pessimism, guilt, punishment, self-dissatisfaction, self dislike, self accusation, crying, suicidal ideas, irritability, social withdrawal, indecisiveness, body image change, and work difficulty. Each answer is scored from 0 to 3. Higher total score indicate more severe depressive symptoms. Index

score of ≤9 is considered to be within normal range, a score of 10–15 shows minimal depressive symptomatology, a score of 16–31 points toward mild depression, a score of 32–47 is in favor of moderate depression, and a score of >47 indicates severe depression [23]. The maximum total score is 63, whereas the minimum is 0.

2.2.5. State-Trait Anxiety Inventory (STAI)

Patients' anxiety was measured using the 20-items state-trait anxiety inventory (STAI). Each of 20 items related to anxiety was scored as 1 (not at all), 2 (somewhat), 3 (moderately), 4 (very much) with higher score indicating more anxiety.

The scale is scored by summing the 20 responses (range 20–80) [24].

2.2.6. Clinical and Laboratory Assessment

Medical history and physical examination had been performed for all patients. Laboratory tests included renal and liver function test, albumin, α fetoprotein, hemogram, prothrombin time index, fasting blood sugar, and venous ammonia concentration. The ammonia was determined according to the enxymatic determination of ammonia with glutamate dehydrogenase in a rapid and interference-free photometric determination (340 nanometer) of NH_4^+ in native blood plasma as according to De Fonseca-Wollheim method [25]. Blood was immediately taken by refrigerated transport sent to the laboratory for determination within 15 min from blood sampling. To stage cirrhosis Child-Pugh score was carried out.

Cirrhosis and Viral etiology were evaluated, considering alcohol consumption for cirrhosis and measuring through ELISA HBsAg levels and anti HCV levels for viral tests. When indicated, medical examination for autoimmune liver disease, Wilson disease, hemochromatosis, and Budd-Chiari Syndrome were performed.

2.2.7. Neurological Assessment

Detailed neurological, psychiatric and mental state assessment had been performed to avoid undiagnosed psychiatric disorders.

2.2.8. Efficacy and Tolerability Assessment

The primary efficacy measures were changed at the beginning and at the end of the study period in aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl-transpeptidase (γ-GT), albumin, alkaline phosphatase (ALP), prothrombin time, and ammonia.

Laboratory assessments included hemochrome, glycemia, creatininemia, and blood urea were monitored at baseline and monthly until the end of the trial. Each subject of both the groups underwent ultrasonography (US) examination of the liver, electrocardiography, and blood pressure with the use of standard techniques [26].

2.3. Statistical Analysis

Descriptive statistics, chi-square tests (for categorical variables), and *t*-tests (for continuous variables) were used to compare demographic and clinical characteristics. p values ≤ 0.05 were considered to be statistically significant. Means, actual numbers, and percentages were used to describe data. Statistical Analyses were performed by two-way analysis of variance (ANOVA), as well as by controlling for Bonferroni's multiple correction.

3. Results

Demographics characteristics were similar between the two groups at baseline. At enrolment, no significant differences were observed in biohumoral tests, at etiologic factors in cirrhosis (Table 1).

3.1. Effects of RV on Biohumoral Findings

In the group that were treated with RV, we observed a significant decrease ($p < 0.001$) in urea, in NH4, in AST, in ALT, and γ-GT.

In the placebo group we observed a decrease ($p < 0.05$) in AST and ALT.

The comparison between RV group and placebo group shows a significant decrease in NH4 ($p < 0.001$) in AST ($p < 0.001$) and in ALT ($p < 0.001$) (Table 2).

Table 1. Clinical characteristics.

Characteristics	Resveratrol Group 35 pt	Placebo Group 35 pt	*p*
Male/Female	25/10	23/12	N.S
Age (range)	39–60	35–60	N.S
SBP (mmHg)	144.00 ± 18.20	145.00 ± 19.10	N.S
DBP (mmHg)	82.10 ± 10.40	81.80 ± 10.50	N.S
Heart Rate (bpm)	77 ± 8	75 ± 9	N.S
BMI (kg/m^2)	25.80 ± 2.40	26.10 ± 2.70	N.S
Smokers/No Smokers	15/20	14/21	N.S
Cirrhosis etiology			
Post Hepatitis B	10	11	N.S
Post Hepatitis C	16	14	N.S
Alcoholism	3	2	N.S
Unknown	6	8	N.S
Child-Pugh Class			
Grade A	20	19	N.S
Grade B	15	16	N.S

SBP = Systolic Blood Pressure; DBP = Diastolic Blood Pressure; BMI: body mass index; N.S = not significant.

Table 2. Comparison of clinical parameters.

Characteristics	Resveratrol Group 35			Placebo Group 35			After RV→Placebo
	Before	After	*p*	Before	After	*p*	*p*
Urea	51.80 ± 6.10	48.20 ± 6.40	0.019	51.40 ± 5.90	50.20 ± 6.10	N.S	N.S
Ammonia	66.20 ± 7.80	41.40 ± 6.80	<0.001	62.80 ± 7.40	59.70 ± 6.70	N.S	<0.001
AST	87.80 ± 10.70	40.40 ± 9.80	<0.001	80.40 ± 9.80	75.20 ± 8.70	0.022	<0.001
ALT	96.40 ± 11.80	78.40 ± 8.70	<0.001	91.70 ± 10.20	84.20 ± 10.20	0.003	0.013
γ-GT	46.10 ± 6.70	40.40 ± 6.40	<0.001	40.80 ± 6.40	40.50 ± 6.10	N.S	N.S
Prothrombine time	15.40 ± 1.80	15.00 ± 1.20	N.S	15.50 ± 1.70	15.10 ± 1.60	N.S	N.S
Bilirubin	2.00 ± 0.40	2.10 ± 0.50	N.S	2.10 ± 0.40	2.00 ± 0.50	N.S	N.S
Albumin	3.50 ± 0.50	3.70 ± 0.80	N.S	3.60 ± 0.50	3.60 ± 0.70	N.S	N.S

AST = Aspartate aminotranferase; ALT = alanine aminotransferase; γ-GT = gamma-glutamyl transpeptidase; RV = Resveratrol; N.S = not significant.

3.2. Effects of RV in Depression, in Anxiety and in Quality of Life

Anxiety and quality of life. The comparison between before and after RV treatment showed a decreased in BDI $p < 0.001$ in STAI ($p < 0.001$) and increased in physical function ($p < 0.001$), in role physical ($p < 0.05$), in Body pain ($p < 0.001$), in general health ($p < 0.001$), in vitality ($p = 0.002$), and in social function ($p < 0.001$).

In the comparison between the RV group and the placebo group, we observed a decrease in BDI ($p < 0.001$), in STAI ($p < 0.001$), an improvement in physical function ($p < 0.001$), in role physical ($p < 0.05$), in body pain ($p < 0.05$), in general health ($p < 0.001$), in vitality ($p < 0.05$), and in social function ($p < 0.001$) (Figure 2; Table 3).

3.3. Adverse Events

No serious adverse events have been observed in both groups.

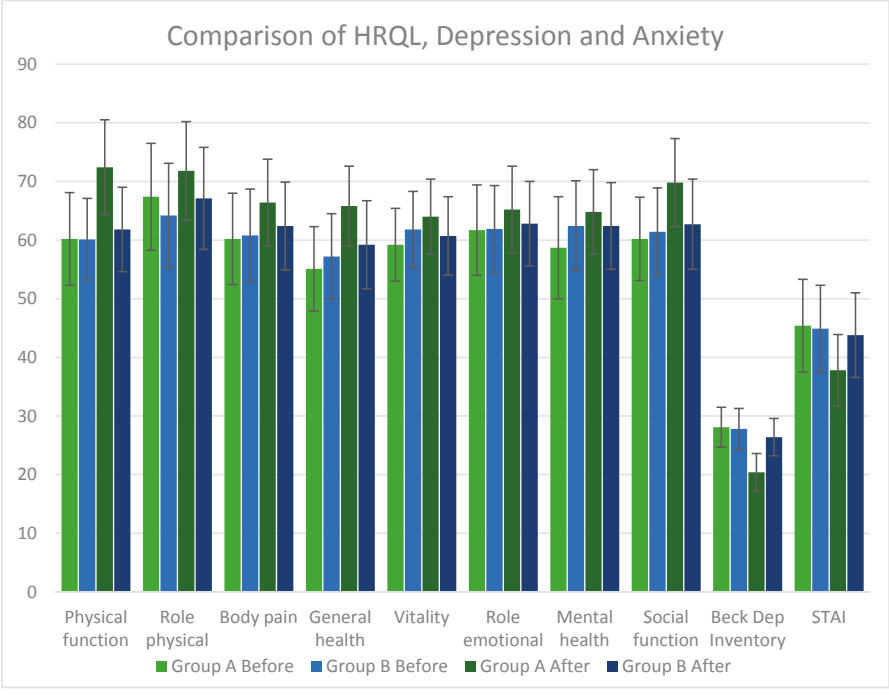

Figure 2. Comparison of health related quality of life (HRQL), Depression, and Anxiety. Group A = Resveratrol Group; Group B = Placebo Group; STAI = State-Trait Anxiety Inventory.

Table 3. Comparison of HRQL, Depression, and Anxiety.

Characteristics	Resveratrol Group		*p*	Placebo Group		*p*	After treatment RV vs. Placebo
	Before	After		Before	After		*p*
Physical function	60.20 ± 7.90	72.40 ± 8.10	<0.001	60.10 ± 7.00	61.80 ± 7.20	N.S	<0.001
Role physical	67.40 ± 9.10	71.80 ± 8.40	N.S	64.20 ± 8.90	67.10 ± 8.70	N.S	0.025
Body pain	60.20 ± 7.80	66.40 ± 7.40	0.001	60.80 ± 7.90	62.40 ± 7.50	N.S	0.028
General health	55.10 ± 7.20	65.80 ± 6.80	<0.001	57.20 ± 7.30	59.20 ± 7.50	N.S	<0.001
Vitality	59.20 ± 6.20	64.00 ± 6.40	0.002	61.80 ± 6.50	60.70 ± 6.70	N.S	0.039
Role emotional	61.70 ± 7.70	65.20 ± 7.40	N.S	61.90 ± 7.40	62.80 ± 7.20	N.S	N.S
Mental health	58.70 ± 8.70	64.80 ± 7.20	0.002	62.40 ± 7.70	62.40 ± 7.40	N.S	N.S
Social function	60.20 ± 7.10	69.80 ± 7.50	<0.001	61.40 ± 7.50	62.70 ± 7.70	N.S	<0.001
Beck Depression Inventory	28.10 ± 3.40	20.40 ± 3.20	<0.001	27.80 ± 3.50	26.40 ± 3.20	N.S	<0.001
STAI	45.40 ± 7.90	37.80 ± 6.10	<0.001	44.90 ± 7.40	43.80 ± 7.20	N.S	<0.001

4. Discussion

In the present study, we tested a cohort of patients with MHE. Our result reveals the presence of moderate depression, anxiety, and a decrease of QoL in subjects with MHE.

The pathogenesis of MHE has been associated with impairment in cerebral energy metabolism with alteration in glucose utilization, glycolysis, and mitochondrial dysfunction. It is thought that impaired mitochondrial function and the down-regulation of the expression of key anti-oxidation enxzymes contribute to an increase in oxidative damage to membrane lipids [27–33].

It has been demonstrated that RV has remarkable health benefits. One of the major etiopathogenetic factors that are involved in the development of HE is represented by abnormal accumulation in the blood of ammonia. Nonetheless although ammonia seems to have a central role in HE, there is evidence that neurotransmission abnormalities, injury to astrocytes, and microglia activation contribute to the development of HE [34,35]. Hyperammonemia represents a major contributing factor for the

development of HE. Recent studies have demonstrated that ammonia exerts effect on many signal transduction pathways, gene expression, and post-translational modifications and influences gene expressions via alteration in micro-RNA expression [36]. Micro-RNAs are small non-coding RNA sequences that play a role in gene silencing at the level of gene transcription as well as translation, and can be regulated by oxidative stress. In RV group we observed a decreased in ammonia serum levels, in depression, in anxiety, and an increase in quality of life (QoL).

RV improves astroglial function and it may play a role in the modulation of glutamatergic metabolism having a cytoprotective action [37,38].

Neurotransmission impairment, caused by an imbalance between the inhibitory and excitatory neurotransmission systems toward a net increase of inhibitory system, is suggested to participate in the pathogenesis of the disease. Several reported findings are consistent with an increase in γ-aminobutiric acid ergic (GABAergic) tone in HE, such as a greater resistance to drug that decreases GABEergic agonists [39,40]. There is also evidence indicating disturbed glutamatergic tone, such as an increase in cortical release of glutamate (Glu) and a decrease in Glu uptake by astrocytes and neurons, leading to high Glu levels in the brain extracellular fluid, lower expression of the astroglia-specific Glu transporter, and a decrease of Glu receptors. An increase in intra-astrocytic glutamine due to the high ammonia levels seems to be a key factor for the development of Alzheimer type II astrocytes.

Microglial activation, which progresses with the development of HE and brain edema, and increases in expression of genes coding for proinflammatory cytokines have been documented in HE.

RV seems to have protective effects in geriatric disorders including Alzheimer's disease. RV can activate SIRT1, which belong to the sirtuine family of proteins [41]. SIRT1 is involved in epigenetic processes via histones and nonhistone proteins deacetylation and its-regulated pathway is associated with inflammation, cells viability, senescence, and also metabolism.

Murine models demonstrated antipsychotic and anxiolytic properties [42]. Neurological disorders are thought to occur via oxidative and and inflammatory to the CNS [43–45].

Studies have reported NF-κB, Akt and STAT3 inhibition by RV. Activation of SIRT-1, a protein recently associated with depressive-like states, inhibits NF-κB via histone deacetylation operated by RV [46,47]. Another SIRT-1 downstream target is represented by AKT, a serine/threonine kinase, which is linked to axonal and neuronal regulation, regeneration and survival [48,49].

RV oxidative and glial inflammation modulation had been demonstrated to prevent ammonia toxicity in astroglial cells.

High levels of ammonia can reduce serotonin and noradrenaline levels in the CNS, resulting in low alertness and attention-associated sleep complaints [10]. Neurobehavioral abnormalities are the major clinical component of HE and have shown to be associated with increased levels of inflammatory cytokines [50]. RV may serve as an antidepressant agent, also exerting anti-inflammatory effects [51,52] and alleviating the consequence of stress with alterations involvement in the immune functions in brain that cause deleterious modifications in the neuronal signaling [53].

The decrease in depression justifies the efficacy of RV in depressed patients with RV.

The pathophysiological mechanisms involved in HE were inflammation, oxidative stress, impaired blood-brain barrier, permeability, neurotoxins, and impaired energy metabolism of the brain [54–58].

Another pathogenic mechanism in HE associated with energy disturbances is the alteration in neurotransmission system, such as the glutamatergic and GABA-ergic systems, resulting in neuronal disinhibition. In vivo preclinical studies had also proposed the involvement of CNS neurotransmitters, including catecholamines, glutamate, GABA, histamine, serotonin, and melatonin [59–65].

In animal experimental models, RV shows a potent inhibitory monoamine oxidase A and oxidase B and activate serotonine and noradrenaline system [66–70].

There are limited data showing the consequences of RV intake regarding the effects in humans.

This study has several limitations. In fact, we excluded patients with poor HRQoL due to their use of forbidden medications, which could also explain the lack of major changes. We also did not

examine confounding factors or potential factors of depressed patients with MHE because the numbers of patients were not statistically powerful.

Such studies need to be increased in number in order to evaluate the role of RV as a modulator of behaviour in MHE patients. However, treatment of minimal HE remains a huge unmet need and a big concerted effort is needed to better defines the multiple pathophysiological mechanisms.

5. Conclusions

This clinical trial suggests that Resveratrol, as carnitine derivates and sylibin [71–73] is helpful in reducing ammonia serum levels and exhibit sufficient bioavailability at the dosages used without any adverse events. Thus, this study provide a rationale for further testing of resveratrol ub future clinical trials and for studying its effectiveness in treating other neurological disorders.

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Author Contributions: All authors contributed in the same way to the manuscript and read and approved the final version.

Conflicts of Interest: The authors declare no conflict of interest

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