Special Issue Reprint

Food Components in Health Promotion and Disease Prevention

Edited by
Luigi Brunetti and Annalisa Chiavaroli

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Food Components in Health Promotion and Disease Prevention

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About the Editors

Luigi Brunetti

Luigi Brunetti is a Full Professor of Pharmacology at G. d'Annunzio University, Chieti, Italy, and he is currently the Chairman of the Department of Pharmacy. He completed a Medical Degree cum laude at Catholic University School of Medicine, Rome, in 1984; a specialization (residency) in Endocrinology in 1987; and a Ph.D. in Endocrinology and Metabolism in 1993. He was formerly an Assistant Professor in Pharmacology at Catholic University and G. d'Annunzio University, an Associate Professor at G.d'Annunzio University, and the Dean of School of Pharmacy at G. d'Annunzio University. His main research activities include exploring chemical compounds of medicinal plants and their possible therapeutic effects and preclinical studies of neuroendocrine pharmacology, in particular regarding energy metabolism, feeding and body weight regulation, and emotional disorders.

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Editorial

Food Components in Health Promotion and Disease Prevention

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In recent years, more plant-based sources of functional foods have been shown to be effective in preventing, reducing, and treating chronic inflammatory and metabolic diseases, and promoting health [1]. There is a great deal of interest in food with reference to bioactive compounds present in natural products, such as vegetables and fruits extracted from wastes and by-products, since they can exert various beneficial effects by virtue of their anti-inflammatory and antioxidant properties [2].

This Special Issue contains thirteen papers, which explore the possible protective role of foods, their components, and natural antioxidant compounds, together with an accurate evaluation of qualitative and quantitative chemical composition of foods and their ingredients. The contributions covered a variety of chronic inflammatory and metabolic disorders such as cardiovascular disease, metabolic syndrome and ulcerative colitis, exploiting different experimental approaches, as well as attracting reviews.

Interestingly, Recinella's article (contribution 1) studied the protective effects of aged black garlic water extract alone or in association with multivitamins on mouse heart specimens exposed to *E. coli* lipopolysaccharide. The authors showed that the extract exerted beneficial actions on isolated samples, as corroborated by the inhibitory activities on multiple pro-inflammatory and oxidative stress-related biomarkers. The protective effects could be related to the high content in the extract of two polyphenolic compounds known for their preventive action at the cardiovascular level: catechin and gallic acid [3–5].

In this context, the effect of *Aronia melanocarpa* fruit juice supplementation was evaluated on age-related coronary arteries by Daskalova and collaborators (contribution 2). The results highlighted that the treatment had a positive impact in the experimental model used, supporting the potential use of antioxidant-rich foods in age-related diseases. In agreement, black chokeberry (*Aronia melanocarpa* L.) intake showed effectiveness in recent studies in attenuating obesity-induced colonic inflammation, in preventing gut microbiota dysbiosis, and in improving intestinal lesions in inflammatory bowel diseases (IBDs) [6–8]. Chokeberry fruits, due to their high polyphenol content, could soon be recommended to prevent and treat many metabolic disorders [9].

In addition, Alonso-Bastida and collaborators (contribution 3) investigated glycemic variation in physically active persons consuming different macronutrients; their findings revealed the beneficial effects in glucose levels of eating healthy foods before carbohydrates. Further studies are needed to develop alternative solutions using a correct sequence of food intake for metabolic and neurodegenerative diseases [10].

Another interesting study (contribution 4) focused on sodium levels in Malaysian street food and found that processed foods present in the main street food dishes contain high quantities of sodium, higher than the recommended daily dose. It is certainly necessary to reduce the salt content in these foods and suggest replacements with products low in sodium and simultaneously rich in potassium, as demonstrated by Marklund and collaborators [11].

Based on these results, a subsequent study (contribution 5) was conducted to better understand the correlation between correct eating behaviors and consumption of dietary fiber (DF), associating them with frequency of food label reading with reference to DF. The

findings showed that greater awareness and education of the population on the accurate reading of food labels, evaluating the presence of dietary fiber, encourages consumers to purchase increasingly healthier foods [12].

Furthermore, there is an ever-increasing interest in plant extracts, including tea leaf extracts, which act as inhibitors of pathogens. Recently, Liu and collaborators (contribution 6) examined the antibacterial activity of four varieties of tea extracts, highlighting green tea extracts as the most effective against a variety of Gram-positive and Gram-negative bacteria, attributable to the interaction of catechin with the bacterial cell membrane. In this context, various in vitro studies of antibacterial effect exerted by green tea catechin were reported [13,14].

Moreover, garlic extracts have also proven effective in IBD as attested by the study by Recinella and collaborators (contribution 7): both tested extracts exerted protective effects in the colon probably due to the high presence of catechin as also confirmed by other studies improving the potential role of this natural compound as a therapeutic target [15].

Additionally, a further study investigated the composition, possible anti-tumor effects, and molecular mechanisms of extracts from a mushroom (contribution 8). The findings demonstrated that *L. hatsudake* extracts were effective at both inducing apoptosis and arresting the proliferation of cancer cells and further validate the effectiveness of mushrooms and the possible use of their bioactive ingredients against cancer [16,17].

Among the articles, there is a study (contribution 9) on gamma aminobutyric acid-enriched fermented oyster (Crassostrea gigas) extract (FO) efficacy in activating osteoblast differentiation and bone formation; the obtained results confirmed previous data and proposed a future FO use to improve bone health [18].

Interestingly, Matisek and collaborators (contribution 10) studied the potential neuroprotective effects of bioactive compounds present in Beetroot/Carrot Juice in neurodegenerative diseases showing that the drink induced beneficial actions attributable to the activity of natural ingredients with an increase in intracellular Ca^{2+} in neurons, preventing the toxic effects of heavy metals and laying the foundation for future studies on the neuroprotective effects of bioactive compounds [19].

In a review (contribution 11), the authors focused their attention on ellagitannins (ETs) present in many plants and responsible for beneficial health effects on various diseases. They concentrated both on highlighting the most important and innovative characteristics exerted by hydrolysable tannins and gut microbiota-derived metabolites as well as examining their protective activities on the gut–brain axis. In addition, numerous studies confirmed protective actions exerted by ETs on several pathologies including various forms of cancer and toxic compounds. However, further studies are certainly necessary to better understand the potential applications and the toxicological and pharmacological profile of these substances [20–22].

Metabolic syndrome is the combination of conditions that together increase the risk of coronary heart disease, diabetes, stroke, and other serious diseases. Eating habits and lifestyle demonstrate a huge impact on the development of metabolic syndrome. Among natural products, there are several bioactive compounds that regulate lipid and carbohydrate metabolism and improve digestion in the human body. In their review, Kania-Dobrowolska and collaborators (contribution 12) focused on dandelion, an edible plant rich in active compounds effective in the treatment and prevention of metabolic syndrome. Several studies showed that dandelion's extracts can exert multidirectional effects revealing both hypolipidemic and hypoglycemic actions and antiobesity, antioxidant, and antiplatelet activity, corroborating its possible use in the treatment of diabetes and cardiovascular diseases. However, further research is needed to investigate the mechanism of action, safety, and biological activity [23].

Finally, in another review (contribution 13), an objective overview was provided on variations in tumor necrosis factor-alpha levels in subjects with different body mass index and on the effectiveness of a diet composed of different foods and products containing mixed bioactive compounds in overweight/obese subjects. There was no clear correlation

between the responses of this cytokine and changes in body weight. According to the authors, it is necessary to use new methods that allow us to consider TNF-α as a biomarker of response to diet [24].

In summary, all research articles and reviews in this Special Issue provide new insights to further investigate the role of foods and natural antioxidant compounds in the management and prevention of chronic inflammatory and metabolic diseases.

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

List of Contributions

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Article

Anti-Inflammatory and Vasorelaxant Effects Induced by an Aqueous Aged Black Garlic Extract Supplemented with Vitamins D, C, and B12 on Cardiovascular System

Lucia Recinella [1,†], Maria Loreta Libero [1,†], Valentina Citi [2], Annalisa Chiavaroli [1,*], Alma Martelli [2,3,4,*], Roberta Foligni [5], Cinzia Mannozzi [5], Alessandra Acquaviva [1,6], Simonetta Di Simone [1], Vincenzo Calderone [2,3,4], Giustino Orlando [1], Claudio Ferrante [1], Serena Veschi [1], Anna Piro [1], Luigi Menghini [1], Luigi Brunetti [1] and Sheila Leone [1]

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Abstract: Multiple studies demonstrated biological activities of aged black garlic, including anti-inflammatory, antioxidant, and cardioprotective effects. We aimed to investigate the protective effects of an aged black garlic water extract (ABGE) alone or in association with multivitamins consisting of combined Vitamins D, C, and B12, on mouse heart specimens exposed to E. coli lipopolysaccharide (LPS). Moreover, we studied the hydrogen sulphide (H_2S) releasing properties and the membrane hyperpolarization effect of the Formulation composed by ABGE and multivitamins, using Human Aortic Smooth Muscle Cells (HASMCs). ABGE, vitamins D and C, and the Formulation suppressed LPS-induced gene expression of cyclooxygenase (COX)-2, tumor necrosis factor (TNF)-α, interleukin (IL)-6, nuclear factor-kB (NF-kB), and inducible nitric oxide synthase (iNOS) on mouse heart specimens. The beneficial effects induced by the extract could be related to the pattern of polyphenolic composition, with particular regard to gallic acid and catechin. The Formulation also increased fluorescence values compared to the vehicle, and it caused a significant membrane hyperpolarization of HASMCs compared to ABGE. To conclude, our present findings showed that ABGE, alone and in association with multivitamins, exhibited protective effects on mouse heart. Moreover, the Formulation increased intracellular H_2S formation, further suggesting its potential use on cardiovascular disease.

Keywords: aged black garlic; hydrogen sulfide; oxidative stress; inflammation

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1. Introduction

Cardiovascular disease (CVD) represents the main cause of mortality all over the world. In this context, it has been demonstrated that inflammation and oxidative stress play a pivotal role in the development of CVD such as heart failure, acute coronary syndromes, atherosclerosis, and hypertension [1–4]. In agreement, an association between alterations in levels of pro-inflammatory and pro-oxidant markers and CVD has been found [5]. Various herbal extracts, particularly in pharmacological associations, were shown to be effective in decreasing the burden of inflammation and oxidative stress [6–9]. Moreover, hydrogen sulfide (H_2S), a gaseous molecule, is critically involved in CVD. To this end, several

preclinical and clinical studies suggested its protective role in heart failure, myocardial infarction, and hypertension [10].

Aged black garlic (ABG), obtained from fresh garlic (*Allium sativum* L.) and fermented under high temperatures (60–90 °C) and high humidity (80–90%) for a specific time period, exerts beneficial effects in various experimental paradigms. In particular, ABG induced multiple biological activities, including antioxidant, antiallergic, antidiabetic, anti-inflammatory, cardiovascular, hepatoprotective, neuroprotective, and anticarcinogenic effects [11–19]. The protective effects induced by aged garlic on different types of CVD have been demonstrated in several studies [20]. In agreement, aged garlic was found able to decrease high blood pressure in humans [21]. Furthermore, black garlic extract was found able to modify serum levels of triglycerides and cholesterol [12]. Various phytoconstituents, including phenolics, S-allyl cysteine (SAC), and hydroxycinnamic acid derivatives were found in BG, with respect to raw garlic [12,13].

The beneficial properties of garlic in CVD have been hypothesized to be related to polyphenolic compounds and SAC [22]. Multiple studies showed significant differences in the total phenolic content of black and fresh garlic. In particular, the content in phenolic compounds is 5–8-times higher in black garlic than that in fresh garlic [23].

The maintenance of cardiovascular health is also attributed to vitamins, such as vitamin B 12, vitamin C, and vitamin D [24].

In particular, vitamin D supplementation improves the cardiac function [25], while vitamin C supplementation, thanks to its antioxidant properties, is effective in the prevention or treatment of several cardiovascular diseases [26]. Furthermore, vitamin B12 deficiency promotes the onset of different CVD, including myocardial infarction, stroke, and other circulatory health problems [27].

The present study aims to investigate the potential antioxidant and anti-inflammatory effects of an ABG water extract (ABGE), alone or in association with multivitamins consisting of the combined Vitamin D, C, and B12 Formulation on mouse heart specimens exposed to *Escherichia coli* lipopolysaccharide (LPS), a known proinflammatory agent. In this context, we evaluated gene expression of various biomarkers involved in inflammation and oxidative stress, including cyclooxygenase (COX)-2, tumor necrosis factor (TNF)-α, interleukin (IL)-6, nuclear factor-kB (NF-kB), and inducible nitric oxide synthase (iNOS). In addition, we studied the H_2S releasing properties and the membrane hyperpolarization effect of ABGE, as well as the Formulation composed by ABGE and multivitamins using Human Aortic Smooth Muscle Cells (HASMCs).

The ABGE was also investigated in order to identify and quantify the polyphenolic content using high-performance liquid chromatography coupled with a photo diode array detector (HPLC-DAD) analytical method.

2. Materials and Methods

2.1. Preparation of ABGE

ABG cloves were supplied as dried material by il Grappolo S.r.l. (Soliera, Modena, Italy). Preparation of ABGE was performed as previously reported [28,29]. The detailed protocol is enclosed as supplementary materials.

2.2. Total Polyphenol Content of ABGE

Total polyphenol content was determined according to the Folin-Ciocalteu method, as described in Savini et al. (2017) [30] with some modifications. The detailed protocol related to total polyphenol content of ABGE is described in the Supplementary Materials Section.

2.3. HPLC-DAD-MS Analysis of Phenolic Compounds

The extract was analyzed for phenol quantitative determination using a reversed-phase HPLC-DAD-MS in gradient elution mode [31]. The details of the analysis are reported in Supplementary Materials (Tables S1 and S2).

2.4. Toxicological and Pharmacological Studies

2.4.1. Cell Line

H9c2 cells (rat cardiomyoblasts, ATTC, Rockville, MD, USA) were maintained in DMEM (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS), 1% of 100 unit/mL penicillin, and 100 mg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in T75 red cap tissue culture flasks, at 37 °C in a humidified atmosphere of 5% CO_2.

2.4.2. Cell Viability Assay

Cell viability was evaluated by MTT assay [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (Sigma, St. Louis, MO, USA), as previously described [32]. Briefly, H9c2 cell line was seeded in 96-well plates (5×10^3 cells/well) and it was pretreated with 10 μg/mL LPS for 24 h. Subsequently, both LPS-pretreated and not LPS-pretreated H9c2 cells were exposed to ABGE at various concentrations (1–100 μg/mL), or with vehicle (control) for a further 48 h. On the basis of results, we then performed a second set of experiments to evaluate the effects induced by the Formulation [ABGE (100 μg/mL) + Vitamin B12 (1 μg/mL) + Vitamin C (10 μg/mL) + Vitamin D (1 μg/mL)] and the vitamins alone [Vitamin B12 (1 μg/mL), Vitamin C (10 μg/mL) and vitamin D (1 μg/mL)] on H9c2 cell viability in both LPS- and not LPS-pretreatment. The detailed protocol is described in the Supplementary Materials section.

2.4.3. Ex Vivo Studies

Adult C57/BL6 male mice (3-month-old, weight 20–25 g) were housed in Plexiglas cages (2–4 animals per cage; 55 × 33 × 19 cm) and maintained under standard laboratory conditions (21 ± 2 °C; 55 ± 5% humidity) on a 14/10 h light/dark cycle, with ad libitum access to water and normal laboratory chow (RMH-B diet, Arie Blok animal feed, Woerden, the Netherlands). Housing conditions and experimentation procedures were strictly in agreement with the European Community ethical regulations (EU Directive no. 63/2010) on the care of animals for scientific research. According to the recognized principles of "Replacement, Refinement and Reduction in Animals in Research", heart specimens were obtained as residual material from vehicle-treated animals randomized in our previous experiments, approved by the local ethical committee ('G. d'Annunzio' University, Chieti, Italy) and Italian Health Ministry (Project no. 885/2018-PR).

After collection, isolated heart specimens were maintained in a humidified incubator with 5% CO_2 at 37 °C for 4 h (incubation period) in a RPMI buffer with added bacterial LPS (10 μg/mL), as previously described [33,34]. During the incubation period, the tissues were treated with ABGE (1 μg/mL, 10 μg/mL, 100 μg/mL), the Formulation [ABGE (10 μg/mL) + Vitamin B12 (1 μg/mL) + Vitamin C (10 μg/mL) + Vitamin D (1 μg/mL)], and the vitamins alone [Vitamin B12 (1 μg/mL), Vitamin C (10 μg/mL) and vitamin D (1 μg/mL)].

Extraction of total RNA was performed from the heart specimens using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), in agreement with the manufacturer's protocol. Contaminating DNA was removed using 2 units of RNase-free DNase 1 (DNA-free kit, Ambion, Austin, TX, USA). Determination of gene expression of COX-2, IL-6, NF-kB, TNF-α, and iNOS was performed by quantitative real-time PCR using TaqMan probe-based chemistry, as previously reported [7,35]. The detailed protocol is described in the Supplementary Materials Section.

2.4.4. Cell Line

HASMCs were cultured in Medium 231 (Life Technologies, Carlsbad, CA, USA) supplemented with a Smooth Muscle Growth Supplement (SMGS, Life Technologies, Carlsbad, CA, USA) and 1% of 100 units/mL penicillin and 100 mg/mL streptomycin (Sigma Aldrich, St. Louis, MO, USA) in tissue culture flasks at 37 °C in a humidified atmosphere and 5% CO_2, as previously described [36,37]. Cells were split 1:2 twice a week and used until passage 18.

2.4.5. Evaluation of H_2S Release on HASMCs

After 24 h, to allow cell attachment, the medium was replaced and cells were incubated for 30 min in the buffer standard (HEPES 20 mM, NaCl 120 mM, KCl 2 mM, $CaCl_2 \cdot 2H_2O$ 2 mM, $MgCl_2 \cdot 6H_2O$ 1 mM, Glucose 5 mM, pH 7.4, at room temperature), as previously described [36,37]. The detailed experimental procedure is reported in the Supplementary Materials Section.

2.4.6. Evaluation of the Membrane Hyperpolarizing Effects on HASMCs

After 24 h to allow cell attachment, the medium was replaced and cells were incubated for 1 h in the buffer standard containing the bisoxonol dye bis-(1,3-dibutylbarbituric acid) DiBac4(3) (Sigma Aldrich, St. Louis, MO, USA) 2.5 μM [38]. NS1619 (Sigma-Aldrich, St. Louis, MO, USA) 10 μM, a BK_{Ca} channel opener, was used as a reference drug. The ABGE (1–100 μg/mL), or the Formulation and the vitamins alone (Vitamin B12 1 μg/mL, Vitamin C 10 μg/mL and Vitamin D 1 μg/mL), were added to the cells, and the trends of fluorescence were followed for 35 min. The relative fluorescence decrease, linked to hyperpolarizing effects, was recorded every 2.5 min and was calculated as previously reported [38]. Six different experiments (n = 6) were performed.

2.5. Statistical Analysis

The data were analyzed by the licensed software GraphPad Prism version 6.0 (Graphpad Software Inc., San Diego, CA, USA). Analysis of means ± SEM for each experimental group was performed by one-way analysis of variance (ANOVA), followed by either the Newman-Keuls multiple comparison post hoc test or by the Bonferroni post hoc test [39]. The level of significance was set to 0.05. The Tukey-Kramer's Honest Significant Difference (HSD) test was used to compare the mean polyphenol contents of the extracts.

3. Results and Discussion

3.1. Total Polyphenol Content of ABGE

The ABGE provided a yield equal to 21.91 mg GAE/g DM in phenolic components [extraction yields of polyphenolic compounds obtained in ABGE (mg GAE/g DM): means ± SEM, 21.91 ± 1.07]. In our experiments, the ABGE showed a yield comparable to those reported by Najman et al. [35] (2021). Water extracts from conventional and organic black garlic have shown a content in polyphenolic components between 13.64 and 17.24 mg GAE/g DM [40]. In particular, a higher content in polyphenols was shown in black compared to fresh garlic, which was suggested to be dependent on various factors, including the garlic aging process (time, temperature, and relative humidity) [40].

3.2. HPLC-DAD-MS Analysis

The retention times, m/z ratio, as well as quantity (μg/mL) of the investigated phenolic compounds in ABGE are reported in Table 1. In this context, a total of 12 compounds were identified at a wavelength of 254 nm. Gallic acid (#1) and catechin (#4) were the prominent phytochemicals, as shown in Figure 1.

Results are only in part comparable to those reported in the literature [41]. A study performed by Moreno-Ortega and collaborators [42] (2020) has found an increase in phenolic compounds, such as gallic acid and epigallocatechin gallate, in black compared to fresh garlic. In addition, it is well known that each cultivar expresses a different analytes content dependent on cultivation methods. Different studies have confirmed that the bioactive compounds of ABG possess a wide range of pharmacological activities, such as hypolipidemic, anticancer, and cardiovascular effects [43], which have been suggested to be mainly due to its anti-inflammatory and antioxidant properties.

Table 1. Retention times, *m/z* ratio, as well as quantity (g/mL) of the investigated phenolic compounds in ABGE.

Peak Name	tR	m/z (Positive Ion)	Quantity (μg/mL)
Gallic Acid	8.80	171.12	24.495
3-Hydroxytirosol	11.71	155.16	1.713
Caftaric acid	12.93	313.23	1.773
Catechin	14.80	291.26	30.877
Gentisic acid	15.82	155.12	1.322
4-Hydroxybenzoic acid	16.20	193.12	n/a
Loganic acid	16.60	377.36	n/a
Chlorogenic acid	16.81	355.31	0.561
Vanillic acid	18.60	169.14	n/a
Caffeic acid	19.00	181.16	1.153
Epicatechin	19.41	291.26	1.338
Syringic acid	21.80	183.17	1.724
p-Coumaric acid	23.06	165.16	n/a
t-Ferulic acid	24.00	195.18	n/a
Benzoic acid	26.38	123.12	3.891
Rutin	27.16	611.52	n/a
Resveratrol	27.70	229.25	1.058

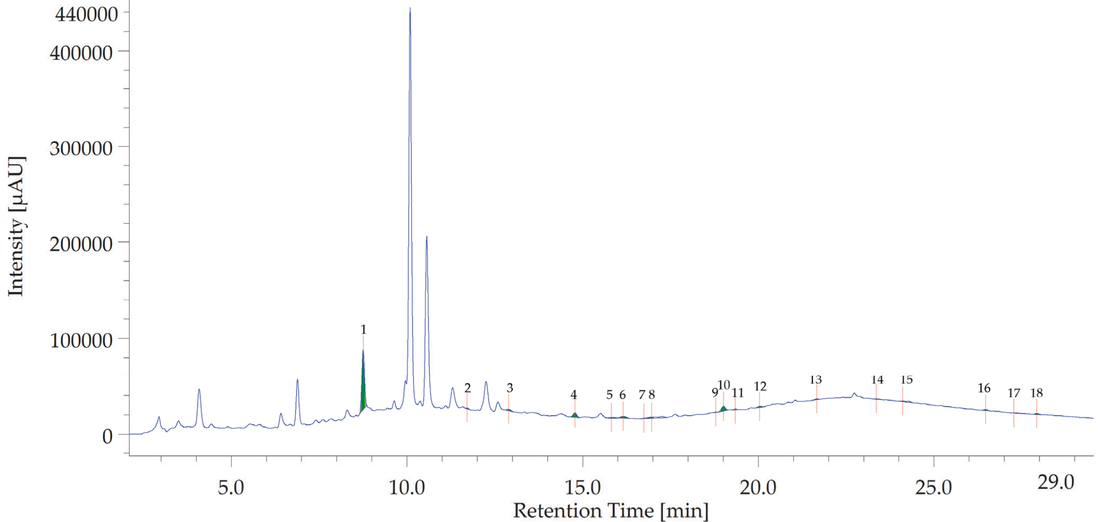

Figure 1. HPLC-DAD chromatogram of aged black garlic water extract (ABGE). The chromatographic analysis showed the presence of 12 phytochemicals: gallic acid (peak #1), 3-hydroxytyrosol (peak #2), caftaric acid (peak #3), catechin (peak #4), gentisic acid (peak #5), loganic acid (peak #7), chlorogenic acid (peak #8), caffeic acid (peak #10), epicatechin (peak #11), syringaldehyde (peak #13), benzoic acid (peak #16), and resveratrol (peak #18).

3.3. Toxicological and Pharmacological Studies

In the first series of experiments, we tested the effects of the ABGE (1–100 μg/mL) on the viability of cardiomyoblast (H9c2) cells. The experiments have been conducted both in basal conditions and after LPS-treatment for inducing an inflammatory status, in vitro. ABGE (1–100 μg/mL) did not alter H9c2 cell viability in basal conditions (Figure 2a). On the other hand, when H9c2 cells were treated with LPS, their viability was reduced, but ABGE (1–100 μg/mL) was able to revert the cytotoxicity (Figure 2b).

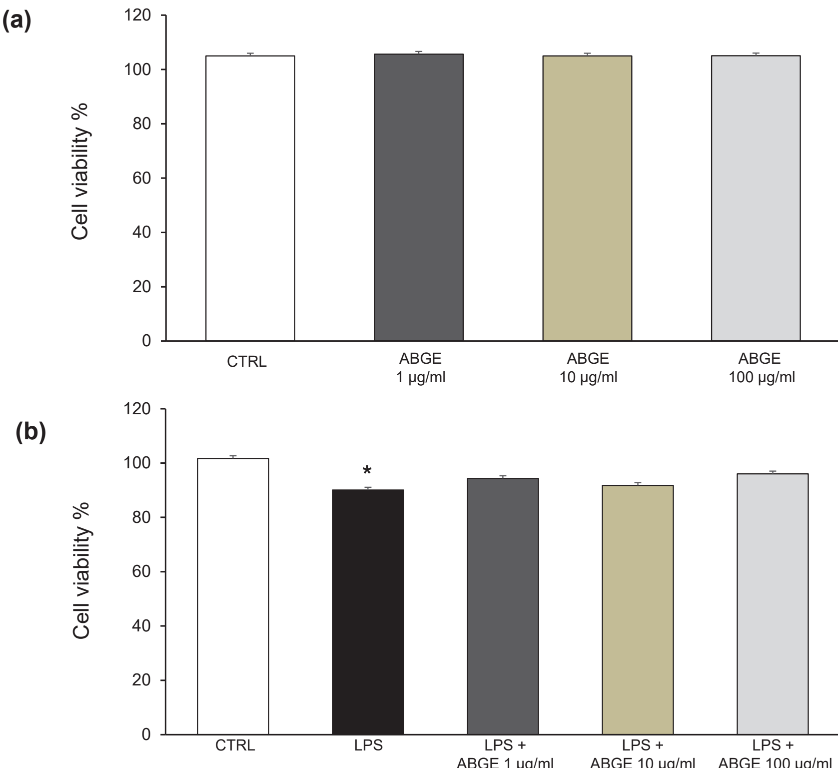

Figure 2. MTT assay of H9c2 cells exposed to aged black garlic water extract (ABGE) (1, 10, and 100 µg/mL) for 48 h, in basal (**a**) and after LPS pre-treatment (**b**) conditions. Data are reported as means ± SEM. ANOVA, Newman-Keuls multiple comparison post hoc test, * $p < 0.05$ vs. CTRL.

In particular, preclinical and clinical evidence has demonstrated that inflammation and oxidative stress play a crucial role in various CVD, including hypertension, fibrosis, diastolic dysfunction, left ventricular hypertrophy, heart failure, and ischemia/reperfusion damage [44].

Therefore, we investigated the protective effects induced by ABGE (1–100 µg/mL) in mouse heart specimens stimulated with LPS, which represents a validated model to study the modulatory activities of herbal extracts and drugs on inflammatory pathways and oxidative stress [33,34]. In particular, we evaluated the effects of ABGE (1–100 µg/mL) on pro-inflammatory and pro-oxidant mediators, such as COX-2, TNF-α, IL-6, NF-kB, and iNOS mRNA levels on isolated LPS-stimulated heart specimens, by RT-PCR analysis. This demonstrates the involvement of NF-κB in the transcription of various proinflammatory cytokines, such as TNF-α, and IL-6 [45], whose involvement in mediating cardiac dysfunction is well known [46].

In our ex vivo model, we observed that ABGE (10 and 100 µg/mL) significantly inhibited all markers investigated without showing a dose-dependent relationship (Figure 3a–e). In this context, polyphenol compounds have been suggested to induce cardioprotective effects by inhibiting oxidative stress and inflammation, as confirmed by a recently published study [7,47–51]. In particular, the beneficial activities induced by ABGE could be related to the pattern of polyphenolic composition, with particular regard to gallic acid and catechin. Accordingly, BenSaad and collaborators [52] (2017) reported that gallic acid inhibited LPS-induced prostaglandin E_2 and IL-6 production in RAW264.7 cells. Gallic acid was hypothesized to be able to exert a protective effect on rat liver mitochondria

by reducing oxidative stress induced by bisphenol A in ex vivo studies [53]. In addition, gallic acid pretreatment decreased levels of cardiac marker enzymes, including troponin T, which has been hypothesized to be involved in the myocardial damage reduction in rats [54]. Cardioprotective activities of catechins are also well known [55]. In particular, catechin administration attenuated coronary heart disease in a rat model by suppressing inflammation [56].

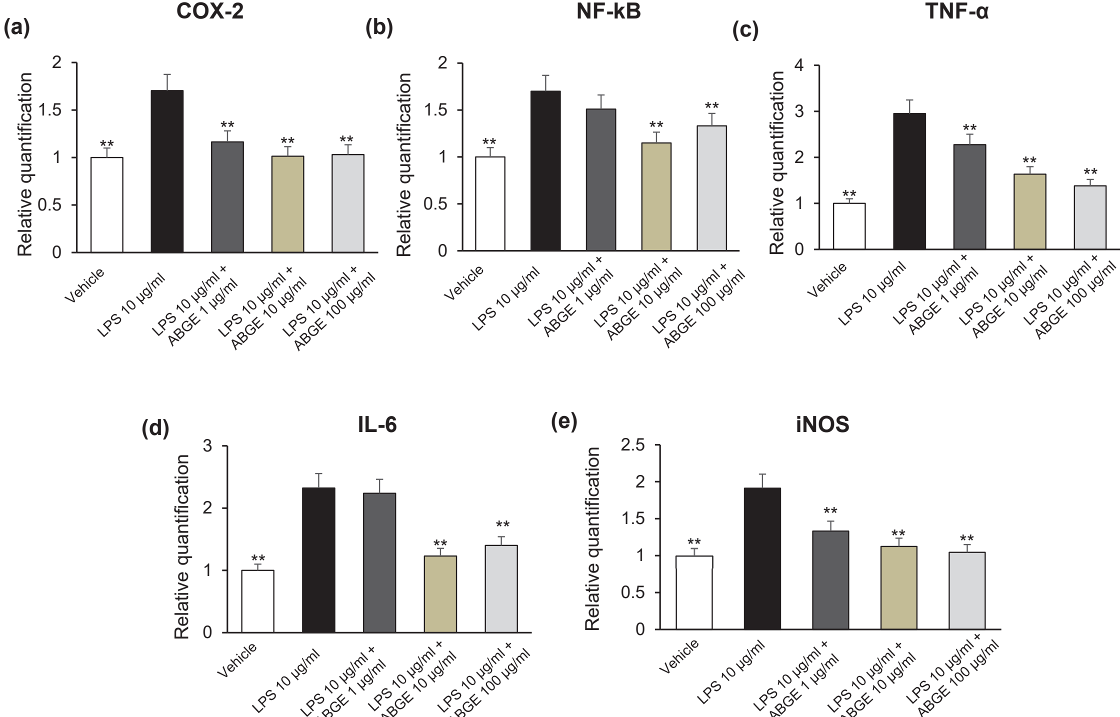

Figure 3. Effects of aged black garlic water extract (ABGE) (1, 10, and 100 μg/mL) on LPS-induced cyclooxygenase-2 (COX-2) (**a**), nuclear factor kB (NF-kB) (**b**), tumor necrosis factor α (TNF-α) (**c**), interleukin (IL)-6 (**d**), and inducible nitric oxide synthase (iNOS) (**e**) gene expression (RQ, relative quantification) in mouse heart specimens. Data are reported as means ± SEM. ANOVA, Newman-Keuls multiple comparison post hoc test. ** $p < 0.005$ vs. LPS.

Moreover, catechin, as well as being known for its antioxidant activities, has been described as an anti-inflammatory agent, being able to inhibit COX-2 expression [57,58].

Furthermore, black garlic was found to exert stronger antioxidant activity than fresh garlic, as confirmed by in vivo and in vitro experiments [41].

On the basis of these results, we performed a second series of experiments, aimed at evaluating the effects of the Formulation [ABGE (100 μg/mL) + Vitamin B12 (1 μg/mL) + Vitamin C (10 μg/mL) + Vitamin D (1 μg/mL)] on the viability of LPS-pretreated and not LPS-pretreated H9c2 cells. The results were compared with vitamins alone [Vitamin B12 (1 μg/mL), Vitamin C (10 μg/mL), and vitamin D (1 μg/mL)]. Our findings showed that the Formulation and the vitamins alone did not modify H9c2 cell viability in basal conditions (Figure 4a). In addition, the Formulation and the vitamins alone were able to contrast the cytotoxicity induced by LPS in H9c2 cells (Figure 4b).

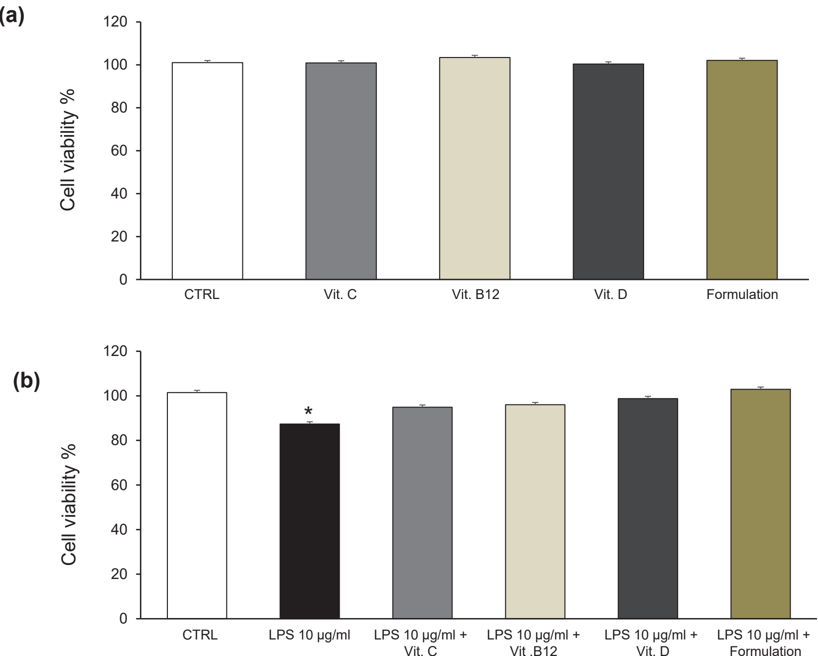

Figure 4. MTT assay of H9c2 cells exposed to Vitamin B12 (1 µg/mL), Vitamin C (10 µg/mL), vitamin D (1 µg/mL), and the Formulation [ABGE (100 µg/mL) + Vitamin B12 (1 µg/mL) + Vitamin C (10 µg/mL) + Vitamin D (1 µg/mL)] for 48 h in basal (**a**) and after LPS pre-treatment (**b**) conditions. Data are reported as means ± SEM. ANOVA, Newman-Keuls multiple comparison post hoc test * $p < 0.05$ vs. CTRL group.

Thereafter, we investigated the effects induced by the Formulation and the vitamins alone on COX-2, TNF-α, NF-kB, IL-6, and iNOS mRNA levels in mouse heart specimens treated with LPS.

As shown in Figure 5a–e, vitamins C and D as well the Formulation reduced gene expression of almost all markers tested in our ex vivo study. In particular, the Formulation was more effective than vitamins alone in blunting LPS-induced gene expression of IL-6, TNF-α, and NF-kB. Recent studies reported that vitamin D represents one of the mediators playing a pivotal role in the pathogenesis of CVD [59]. In agreement, vitamin D supplementation was able to decrease inflammation and oxidative stress [59,60], confirming its pivotal role in heart tissue. In particular, TNF-α and IL-6 secretion was decreased by vitamin D in monocytes and macrophages [61]. Vitamin D also exerts various potent antioxidant effects by downregulating intracellular oxidative stress-related protein oxidation, lipid peroxidation, and DNA damage [62].

Similarly, an inverse correlation between vitamin C supplementation and the risk of CVD has been suggested in various observational studies [63,64]. In this regard, the antioxidant effects of vitamin C have been shown to be involved in both prevention and treatment of CVD [65]. In particular, Ellulu [62] (2017) showed that vitamin C protected against oxidative stress via its effect on nitric oxide release as well as alleviating inflammation by down-regulating IL-6, TNF-α, and NF-kB mRNA levels [66]. As for vitamin B12, its deficiency can cause hyperhomocysteinemia, an independent risk factor for CVD [27]. Moreover, an association between vitamin B12 deficiency and increased incidence of inflammation and associated metabolic complications has been demonstrated by a number of studies [67,68]. Our present findings showed that vitamin B12 decreased LPS-induced gene expression of NF-kB, IL-6, and iNOS. In agreement, Birch and collaborators [69]

(2009) showed that vitamin B12 decreased NF-kB levels, which could represent a signaling molecule of vitamin B12 deficiency. Moreover, vitamin B12 was able to suppress IL-6 production, in vitro. Weinberg et al. [70] (2009) also reported that vitamin B12 is involved in the modulation of NOS function and NO synthesis in vivo.

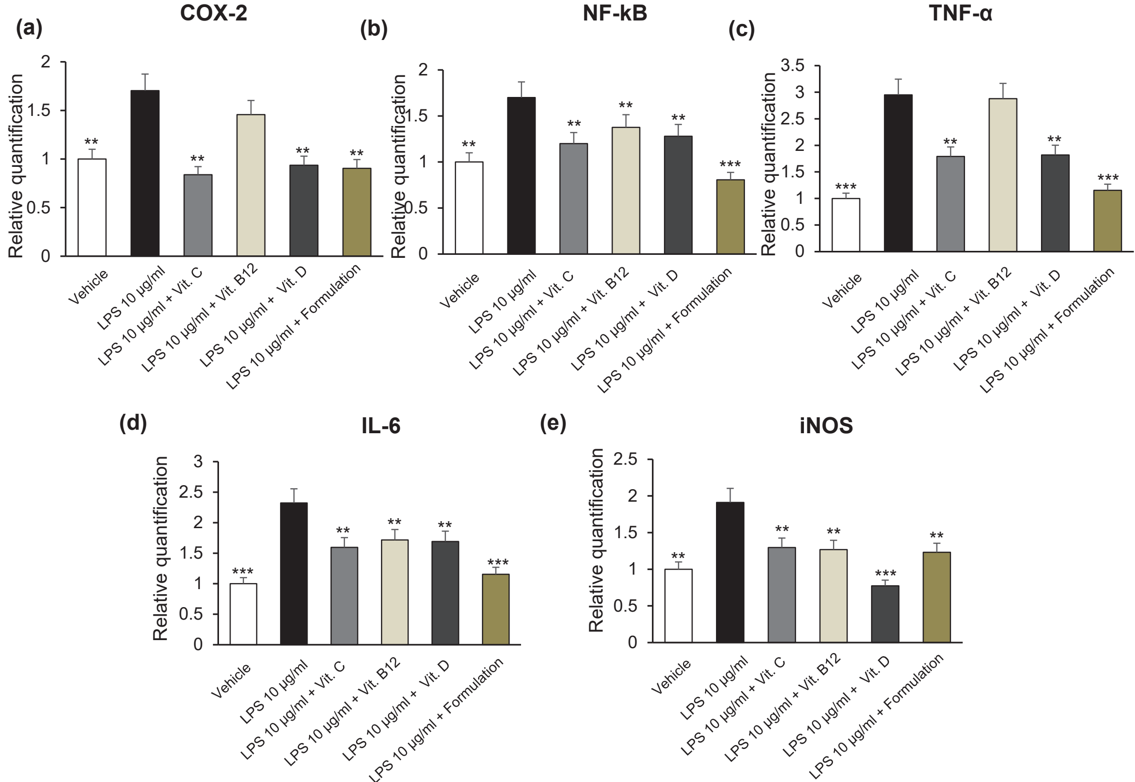

Figure 5. Effects of the Formulation [ABGE (100 µg/mL) + Vitamin B12 (1 µg/mL) + Vitamin C (10 µg/mL) + Vitamin D (1 µg/mL)] and the vitamins alone [Vitamin B12 (1 µg/mL), Vitamin C (10 µg/mL), Vitamin D (1 µg/mL)] on LPS-induced cyclooxygenase-2 (COX-2) (**a**), nuclear factor kB (NF-kB) (**b**), tumor necrosis factor-α (TNF-α) (**c**), interleukin (IL)-6 (**d**) and inducible nitric oxide synthase (iNOS) (**e**) gene expression (RQ, relative quantification) in mouse colon specimens. Data are reported as means ± SEM. ANOVA, Newman-Keuls multiple comparison post hoc test ** $p < 0.005$, *** $p < 0.001$ vs. LPS.

3.4. Evaluation of H_2S Release in HASMCs

H_2S has been suggested to be able to modulate many pathways related to cardiovascular pathophysiology [71]. In addition, it is one of the most important biological mediators involved in different pathological processes, where inflammation plays a predominant role, including CVD [72]. H_2S is known to be critically involved in garlic-induced cardioprotective effects [73–75]. In this context, H_2S was shown to play a key role in preventing the progression of cardiac hypertrophy to heart failure [76]. Considering the inhibitory effects induced by both ABGE (1–100 µg/mL) and the Formulation [ABGE (100 µg/mL) supplemented with Vitamin B12 (1 µg/mL) + Vitamin C (10 µg/mL) + Vitamin D (1 µg/mL)] on the investigated markers of inflammation and oxidative stress in our study, we also evaluated their potential effects on H_2S releasing properties using cultured HASMCs. DADS (300 µM) was used as a known H_2S releasing molecule and significantly increased the fluorescence index, thus indicating the intracellular H_2S formation (Figure 6a,b). We

showed that ABGE did not determine significant H_2S formation into the cells with respect to the vehicle (Figure 6a,b). Our findings are in agreement with those of Leitao et al. (2022) [77], showing that improvement of microvascular reactivity induced by aged garlic extract was not mediated by H_2S in older adults at CVD risk. Interestingly, the Formulation significantly increased fluorescence values compared to the vehicle, reflecting the H_2S formation inside the cells. In this context, we speculate that the presence of the vitamins into the Formulation allows the garlic extract to more easily cross the cell membrane and release H_2S. In this regard, B vitamins could act as cofactors of enzymes playing a key role in the sulfur network and modulate H_2S production [78]. Accordingly, Wilinski et al. [79] (2012) showed that vitamin D increased H_2S levels in a number of mouse organs, including the heart [79].

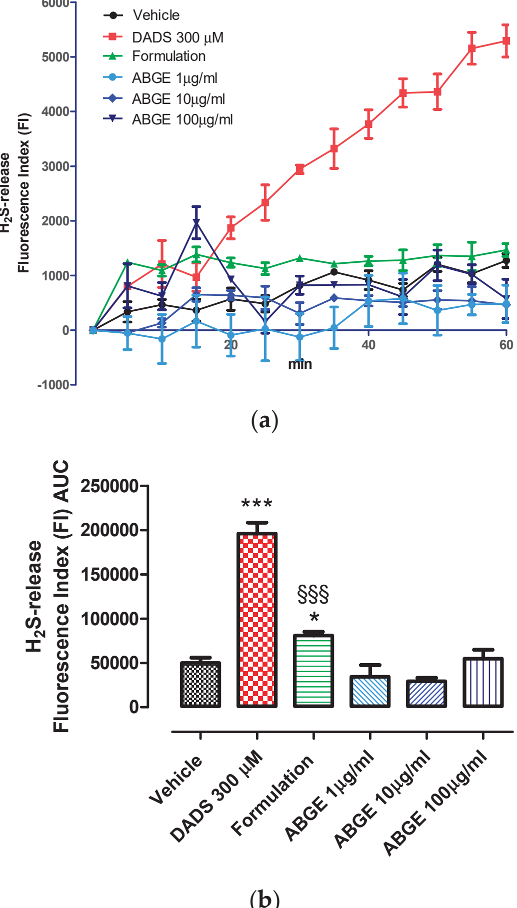

Figure 6. Fluorometric recording of H_2S-release inside HASMCs. (**a**) Time course of the fluorometric recordings of H_2S released by vehicle, ABGE (1–100 µg/mL), Formulation [ABGE (100 µg/mL) + Vitamin B12 (1 µg/mL) + Vitamin C (10 µg/mL) + Vitamin D (1 µg/mL)] and DADS 300 µM, during 60 min of observation: the increase in H_2S is expressed as FI. (**b**) The histograms show the total amount of H_2S released by vehicle, ABGE (1–100 µg/mL), Formulation [ABGE (100 µg/mL) + Vitamin B12 (1 µg/mL) + Vitamin C (10 µg/mL) + Vitamin D (1 µg/mL)] and DADS 300 µM in the 60 min of observation time, expressed as AUC. The vertical bars represent SEM, six different experiments were performed, each with six replicates (n = 6). ANOVA, Bonferroni post hoc test * $p < 0.05$, *** $p < 0.001$ vs. vehicle; §§§ $p < 0.001$ vs. ABGE.

An important finding of our study is that the Formulation tested has increased the release of H_2S, suggesting its potential role on CVD, including hypertension, thanks to its vasodilatation action [80]. In addition to its vasoprotective effects, H_2S could be critically involved in the pathogenesis of hypertension-related vascular dysfunction through its effects on blood pressure regulation, too, as well as inflammation [81–84].

3.5. Evaluation of Membrane Hyperpolarization of HASMCs

In addition, the effects of ABGE and the Formulation were evaluated on the membrane potential of cultured HASMCs. We showed that ABGE (1–100 µg/mL) did not modify membrane hyperpolarization. On the other hand, we showed that the Formulation caused a significant membrane hyperpolarization of HASMCs compared to ABGE (1–100 µg/mL) (Figure 7).

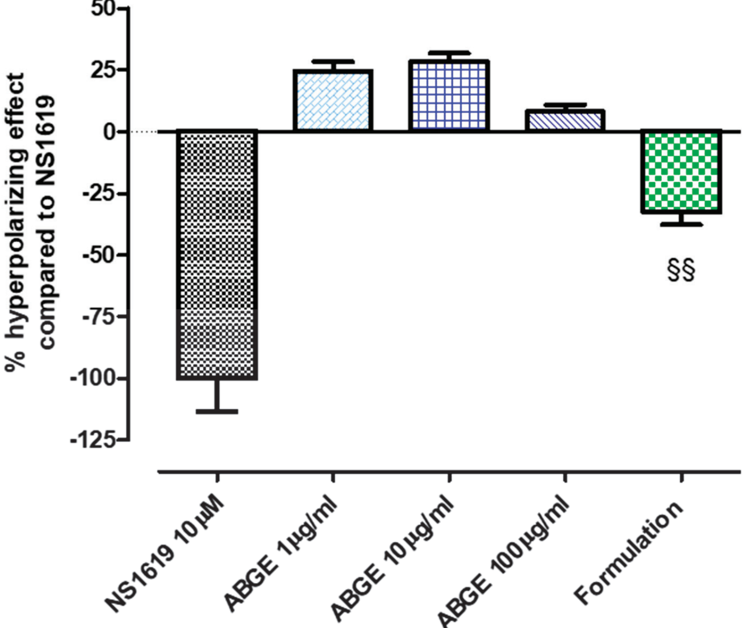

Figure 7. Hyperpolarizing effects in HASMCs. % hyperpolarization calculated as mean of changes in HASMCs membrane potential, followed for 35 min, induced by ABGE (1–100 µg/mL) or the Formulation [ABGE (100 µg/mL) + Vitamin B12 (1 µg/mL) + Vitamin C (10 µg/mL) + Vitamin D (1 µg/mL)]. Data are expressed as mean ± SEM, reported as % of the hyperpolarizing effect evoked by (1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazole-2-one (NS1619). Six different experiments were performed, each with six replicates (n = 6). The §§ indicates a significant difference from the effect evoked by ABGE (ANOVA, Bonferroni post hoc test §§ $p < 0.01$).

These results seem to suggest that the hyperpolarization is a consequence of the ability of the Formulation to release H_2S. Indeed, it is well known that compounds able to release H_2S, also called as H_2S-donors, exhibited the property to induce vascular smooth muscle hyperpolarization through the activation of different subtypes of potassium channels [36,85].

Experimental and clinical studies showed that ABG was able to exert beneficial effects on cardiometabolic alterations, which are usually related to metabolic syndrome [86,87]. Accordingly, Amor and collaborators [22] (2019) showed an improvement of metabolic syndrome following ABG treatment in rats [22]. In this context, the aging process was suggested to enhance the activity of bioactive compounds, including S-allylcysteine and

S-allylmercaptocysteine, whose cardioprotective effects are well known [88,89]. It is also well known that black garlic shows a reduced content of allicin when subjected to high temperatures during the production phase [87]. Moreover, Bradley and collaborators [73] (2016) suggested that allicin and alliin could not be the main bioactive compounds involved in the cardioprotective effects induced by aged garlic.

The content of phytochemicals in garlic has also been reported to be dependent on environmental, genetic, and agronomic factors [90].

Interestingly, gallic acid was found able to induce hyperpolarization of the cell membranes and excitation of muscles by binding to glutamate-gated chloride channels [91]. Furthermore, catechins have also been suggested to display inhibitory effects on voltage-dependent Ca^{2+} channels involving, albeit partially, membrane hyperpolarization deriving from the opening of K^+ channels [92]. Finally, we hypothesized that the beneficial effects of the Formulation are due to the presence of gallic acid, catechin, and vitamins.

In conclusion, our results showed that ABGE, alone and in association with multivitamins consisting of combined Vitamins D, C, and B12, exhibited protective effects, as confirmed by the inhibitory activities on multiple inflammatory and oxidative stress-related pathways on mouse heart specimens exposed to LPS. These effects could be related, at least in part, to the ABGE content in polyphenolic compounds, with particular regards to gallic acid and catechin. Moreover, the Formulation increased intracellular H_2S formation, and caused a significant membrane hyperpolarization of HASMCs, further suggesting its potential use on CVD. In this context, we speculate that the presence of the vitamins in the Formulation allows the garlic extract to more easily cross the cell membrane and release H_2S. However, further studies using independent experimental paradigms are necessary to accurately evaluate the in vivo activity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods12071558/s1, Table S1. Gradient Elution Conditions; Table S2. MS analysis conditions.

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Article

Impact on Glycemic Variation Caused by a Change in the Dietary Intake Sequence

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Abstract: This work presents an analysis of the effect on glycemic variation caused by modifying the macronutrient intake sequence in a person without a diagnosis of diabetes. In this work, three types of nutritional studies were developed: (1) glucose variation under conditions of daily intake (food mixture); (2) glucose variation under conditions of daily intake modifying the macronutrient intake sequence; (3) glucose variation after a modification in the diet and macronutrient intake sequence. The focus of this research is to obtain preliminary results on the effectiveness of a nutritional intervention based on the modification of the sequence of macronutrient intake in a healthy person during 14-day periods. The results obtained corroborate the positive effect on the glucose of consuming vegetables, fiber, or proteins before carbohydrates, decreasing the peaks in the postprandial glucose curves (vegetables: 113–117 mg/dL; proteins: 107–112 mg/dL; carbohydrates: 115–125 mg/dL) and reducing the average levels of blood glucose concentrations (vegetables: 87–95 mg/dL; proteins: 82–99 mg/dL; carbohydrates: 90–98 mg/dL). The present work demonstrates the preliminary potential of the sequence in the macronutrient intake for the generation of alternatives of prevention and solution of chronic degenerative diseases, improving the management of glucose in the organism and permeating in the reduction of weight and the state of health of the individuals.

Keywords: diet assistance; glucose curve; glucose homeostasis; glycemic variability; healthy person; macronutrient intake sequence

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1. Introduction

Consumption of food in human beings is an activity that provides the nutrients necessary for the adequate performance of the organism and prevents various diseases such as diabetes and cardiovascular diseases [1]. Consumption of any food generates elevations in glucose levels, defined as "glucose curves," because of a gradual elevation in blood glucose levels that is subsequently attenuated due to the homeostatic processes of glucose in the organism throughout time [2]. This period is called the postprandial glucose stage, which lasts 4–5 h for each meal taken [3]. Information on the magnitude, fluctuations, and different characteristics of glucose curves (peaks, plateaus, rise and decay times) is defined as "glycemic variability" [4], which has taken on great relevance for the generation of actions toward the development of nutritional interventions.

Prolonged postprandial glucose episodes and their high frequency generate one of the main risk factors for developing Type 2 Diabetes Mellitus (T2DM) [5,6] since the average blood sugar level throughout the day is above the basal glucose levels. T2DM is a multifactorial disease occurring in the adult population [7], where the main characteristic

is the presence of elevated blood glucose levels throughout the day (episodes of hyperglycemia) [8]. High glucose levels are mainly related to insulin resistance, a condition in which the different cells of the organism cannot assimilate the insulin hormone adequately. This condition leads to an increase in insulin secretion by the pancreas, which in turn facilitates the presence of hyperglycemia in the organism due to the low absorption of insulin by the cells [9]. This set of diseases is usually the product of carrying out many habits detrimental to health throughout the person's life before being diagnosed with T2DM [10].

T2DM is a chronic degenerative disease that leads to a degradation in the quality of life and facilitates the presence of cardiovascular complications [11] and renal, ocular, and liver diseases [12], these being a small part of the set of conditions related to T2DM. It should be noted that because of the disease control actions by the health sector, there is an added problem [13,14], ranging from the viewpoint of proper care for the community [15] to the economic aspect where the investment required to satisfy this need is growing every year [16,17]. Therefore, several alternatives have been developed to prevent the condition, some of them focused on informing society about how a healthy diet and regular physical activity reduce the chances of developing T2DM [18].

Diet is the main factor in the increase in glucose levels, so it is essential to control how we consume food. Several studies have addressed this problem, thus generating alternative solutions to reduce the impact of postprandial glucose, such as Dahl et al. [19], who demonstrated how semaglutide has beneficial effects on the reduction of postprandial glucose, triglycerides, glucagon, and gastric emptying in people with T2DM. Rayner et al. [20] similarly demonstrate the effects of lixisenatide in reducing gastric emptying by promoting postprandial glucose dynamics. Vlachos et al. [21] present an in-depth review of the subject, concluding that reducing carbohydrates in conjunction with a higher fiber intake positively affects postprandial glucose reduction.

How different macronutrients are ingested affects the glycemic variability of organisms, modifying the time to glucose elevation, the glucose curve magnitude, and the glucose decay time [22,23]. Sun et al. [24] developed a study of 16 healthy people in which the effect of the proper order for macronutrient intake on glycemic variation was evaluated. The study showed that consuming vegetables followed by proteins and concluding with carbohydrates is an effective strategy to reduce postprandial glucose and prevent the generation of T2DM. Kubota et al. [25] corroborate that the correct order of the food sequence reduces the episodes of postprandial hyperglycemia, improving weight loss and metabolic function.

Considering the alternatives presented in the various sources of information and considering that those related works generate an analysis on the modification of the sequence of macronutrient intake concerning tests of about 4 to 5 h of glucose monitoring, the main objective of this work is to obtain preliminary results on the effectiveness of a nutritional intervention based on the modification of the order of macronutrient intake in a healthy person. The particularity of this work is to generate continuous glucose measurements during three periods of 14 days in an individual for whom three types of nutritional interventions were developed. In this way, difficult-to-access information is obtained on the behavior of glucose curves derived from the proposed nutritional intervention. Considering the scope of the present work, this research is a first approximation for the development on a larger scale of nutritional interventions focused on the modification of the macronutrient sequence that allows the reduction of postprandial glucose levels in healthy people. This way, the necessary conditions are obtained to carry out this experimentation on a larger scale. The results of this research will allow the generation of information for the development of alternative solutions in the generation of metabolic and chronic degenerative diseases.

2. Materials and Methods

2.1. Quasiexperimental Study Design

The study methodology consists of the steps described below:

1. Generation of data: A series of body measurements, an indirect calorimetry test, and the development of a food reminder were developed in the participant to have an approximation of the nutritional status of the participant and to be able to propose the type of interventions in the sequence of macronutrient intake to follow so that there is no decompensation in the current type of food intake.
2. Implantation of the continuous glucose monitoring sensor: In each test, a new interstitial glucose sensor was implanted to generate data on glucose dynamics.
3. Daily diet (Test 1): Subsequently, Test 1 was developed, where glucose measurements were generated and focused on describing the variation of glucose levels in the face of the study subject's daily diet (mixture of macronutrients without having any order in food consumption).
4. Regular diet with ordered consumption of macronutrients (Test 2): Test 2 has the objective of obtaining the glucose dynamics when the sequence in the order of macronutrient consumption is modified without generating any change in the participant's regular diet.
5. Assisted diet with ordered consumption of macronutrients (Test 3): This test consists of generating measurements of glucose variation in the face of a modification in the participant's daily diet considering the change in the sequence of macronutrient intake.
6. Statistical analysis: Once the three different tests were generated, a statistical analysis of the results obtained was generated, which was the study's core. In this analysis, the impact of the sequence in macronutrient consumption was quantified and contrasted concerning the postprandial glucose curves generated in each dietary intake. For this purpose, the proportions of macronutrients consumed per intake were used and related to glucose concentrations, magnitudes of the postprandial glucose peaks, and times in which postprandial glucose stabilizes.

2.2. Ethics of Research

In the research developed, the health and integrity of the participant were not put at risk in any way, being an observational experiment. Each of the procedures developed was evaluated and authorized by the Ethics Committee of the Faculty of Medicine of the Autonomous University of the State of Morelos (CONBIOETICA-17-CEI-003-201-81112). It should be emphasized that the participant was informed of the procedures to be developed, and once informed and in agreement with the guidelines to be developed, the participant signed the letter of informed consent.

2.3. Instrumentation

The instrumentation used in this study consists of an interstitial continuous glucose monitoring (CGCM) system (Freestyle libre, Abbott®, Chicago, IL, USA) for the acquisition of glucose measurements, an indirect calorimetry system (KORR Medical®, West Valley City, UT, USA) to obtain the participant's daily energy consumption, a bioimpedance scale (BC-545 Segmental, Tanita® brand, Arlington Heights, IL , USA) and a stadiometer for the participant's body detection, and a food intake and physical activity diary for macronutrient counting and physical activity intensity.

There are no conflicts of interest in this research. There is no relationship between the suppliers of the instrumentation used in the experimentation. The instruments were purchased with funding from CONACYT (project number 320155) and TecNM (project numbers 14002.22P and 14003.22P).

2.4. Subject of Study

The proposed study has great difficulty in its development in population studies due to the strict discipline required to carry out the dietary sequence in the required order, the filling of the food intake and physical activity diaries, and the glucose monitoring. Therefore, this study was developed on a pilot basis in a physically active healthy person (without a diagnosis of diabetes or any chronic degenerative disease) considered by the

standards of a healthy person proposed by the World Health Organization (WHO) [26]. The participant is a 26-year-old male with a height of 1.78 m and a daily energy intake of 2810 calories (246 calories from physical activity, 591 calories derived from the participant's daily activities and lifestyle, and 1973 calories from energy consumed at rest). Body measurements were taken at the beginning of each test, described in Table 1, where weight, body mass index, abdominal circumference, and percentages of muscle, fat, and visceral fat are considered.

Table 1. Subject's body measurements at the start of each study.

Physiological Characteristics	Test 1	Test 2	Test 3
Body weight (kg)	65.4	67.1	64.6
Body mass index (kg/m^2)	20.6	21.2	20.4
Abdominal circumference (m)	0.79	0.79	0.79
Muscle percentage (%)	42.0	41.8	43.7
Fat percentage (%)	14.8	15.6	12.4
Visceral fat percentage (%)	3	3	3

To have certainty in the information, the participant received instructions to correctly fill out the food intake and physical activity recording instruments (both devices are standardized forms). In addition, the participant was instructed to record foods that were not consumed or added to the tools. This was combined with a 24-h reminder of the food consumed, carried out by trained personnel. Regarding continuous glucose monitoring, due to the conditions of the measuring instrument, the patient was instructed to take periodic manual measurements throughout the day, avoiding more than four hours between sizes (except for the participant's sleeping hours). The correct storage of glucose readings was corroborated with the report generated by the Abbott® platform. For more information on the monitoring system, we recommend consulting [27].

2.5. Food Sequence

Considering the three tests developed, in the case of Tests 2 and 3, the participant was assigned a sequence in the intake of macronutrients, sectioned according to the type of intake developed (breakfast, snack 1, lunch, dinner, and snack 2) and repeating the type of food and its quantity for 4 consecutive days exchanging the order in the consumption of macronutrients. (This is because the monotony of the food makes it difficult for participants to adhere to the needs of the experiment.) The sequence is presented regarding the symbology of the proposed macronutrients starting with the symbol on the left side and ending with the symbol on the right side (VF-CH-P-FT = 1. Vegetables and Fiber, 2. Carbohydrates, 3. Proteins of animal origin, 4. Fats). Table 2 presents the sequence used for each day of intake, denoting macronutrients as follows: P: Proteins of animal origin; CH: Carbohydrates; VF: Vegetables and Fiber; D: D: Dairy; FT: Fats; FR: Fruits.

2.6. Proportions of Macronutrients Ingested

Each food ingested by the participant was analyzed concerning its composition in carbohydrates, lipids, and proteins, obtaining for each macronutrient the weight (grams) and energetic quantity (calories) contained in the food. In addition, the percentage of energy provided by each macronutrient in each of the intakes analyzed was calculated. Table 3 presents the mean and standard deviations of the composition of each macronutrient analyzed in each intake developed for each of the three types of diets analyzed.

2.7. Diet for Each Test Developed

Throughout the experimentation, several menus were used to ensure proper adherence to the study by the participant. In the case of Tests 2 and 3, each of the menus was appropriately developed in such a way that the proportions of macronutrients ingested

were analogous. For the reader to have a clear idea of the menu composition, the following is an example for each of the menus developed in each test.

Table 2. Dietary sequence by day.

Test 2. Regular Diet with Ordered Food Consumption					
Day	**Breakfast**	**Snack 1**	**Lunch**	**Dinner**	**Snack 2**
1	D-P-CH	D-CH-FR	VF-CH-P-FT	P-CH-VF-FR	FT
2	P-CH-D	D-CH-FR	CH-P-CH-FT-VF	CH-VF-FR-P	FT
3	D-P-CH	D-CH-FR	P-CH-FT-VF-CH	VF-FR-P-CH	FT
4	P-CH-D	D-CH-FR	CH-FT-VF-CH-P	FR-P-CH-VF	FT
5	D-P-CH	D-CH-FR	FT-VF-CH-P-CH	P-CH-VF-FR	FT
6	P-CH-D	D-CH-FR	VF-CH-P-CH-FT	CH-VF-FR-P	FT
7	D	D-CH-FR	CH-P-CH-FT-VF	VF-FR-P-CH	FT
8	P-CH-D	D-CH-FR	P-CH-FT-VF-CH	FR-P-CH-VF	FT
9	D-P-CH	D-CH-FR	CH-FT-VF-CH-P	FT-CH-VF-FR	FT
10	P-CH-D	D-CH-FR	FT-VF-CH-P-CH	CH-VF-FR-FT	FT
11	D-P-CH	D-CH-FR	VF-CH-P-CH-FT	VF-FR-FT-CH	FT
12	P-CH-D	D-CH-FR	CH-P-CH-FT-VF	FR-FT-CH-VF	FT
13	D-P-CH	D-CH-FR	P-CH-FT-VF-CH	CH-P-VF-FT	FT
14	D	D-CH-FR	CH-FT-VF-CH-P	CH-P-FT	FT
Test 3. Assisted diet with ordered food consumption					
Day	**Breakfast**	**Snack 1**	**Lunch**	**Dinner**	**Snack 2**
1	CH-P-VF	D-FR	P-VF-CH	CH-P-VF-FT	FR-VF-FT
2	P-CH-VF	D-FR	CH-P-CH-VF	FT-P-CH-VF	FT-FR-VF
3	VF-P-CH	D-FR	CH-VF-P-CH	P-FT-VF-CH	VF-FT-FR
4	VF-P-CH	D-FR	VF-CH-P	VF-CH-FT-P	FR-VF-FT
5	CH-P-VF	D-FR	P-VF-CH	CH-P-D-VF	FR-CH-FT
6	P-CH-VF	D-FR-D	CH-P-VF-CH	VF-D-CH-P	FT-FR-CH
7	VF-P-CH	D-FR-D	VF-CH-P-CH	CH-VF-D-P	CH-FT-FR
8	VF-CH-P	D-FR-D	CH-VF-P	VF-D-P-CH	FR-CH-FT
9	P-D-VF-CH	D-FR	P-CH-VF-CH	CH-D-VF-FT	FR-CH-FT
10	VF-CH-P-D	D-FR	CH-P-CH-VF	VF-FT-CH-D	CH-FR-FT
11	CH-P-D-VF	D-FR	VF-CH-P-CH	FT-D-VF-CH	FT-CH-FR
12	P-D-VF-CH	D-FR-D	P-VF-CH	D-VF-FT-CH	FR-CH-FT
13	VF-P-D-CH	D-FR-D	CH-P-CH-VF	CH-P-VF-FT	CH-FT-FR
14	CH-P-D-VF	D-FR-D	VF-CH-P	CH-D-VF-FT	FT-FR-CH

- Test 1: Breakfast, smoothie with the following items, 1 banana (100 g), 33 g amaranth, 84 g oatmeal, and milk (500 mL), 2 eggs, and 2 corn tortillas (developed at 7:50 h). Lunch, 15 g of chickpeas, a piece of bread, and 500 mL of water (developed at 13:10 h). Snack 1, smoothie with the following items, 25 g whey protein, and 350 mL of milk (developed at 19:00 h). Dinner, 1 sandwich consisting of 40 g turkey ham and 25 g of cheese, and 350 mL of milk (developed at 19:15 h). Snack 2, 30 peanuts (developed at 21:00 h).
- Test 2: Breakfast, 240 mL of whole milk, 2 scrambled eggs, 2 corn tortillas, and 500 mL of water (developed at 7:40 h). Snack 1, a smoothie with the following items, 25 g whey protein, 84 g oatmeal, 1 banana (100 g), and 500 mL of water (developed 13:20 h). Snack 2, 3 nuts (developed at 21:30 h). Lunch, a salad (lettuce and tomato), 60 g rice, 100 g beef steak, 3 corn tortillas, 15 peanuts, and 500 mL of water (developed at

15:30 h). Dinner, 60 g chicken breast, 120 g salad (potato, carrot, and pea), 50 g jicama, 18 pieces of grapes, and 500 mL water (developed at 20:30 h).

- Test 3: Breakfast, 2 slices of white bread, 40 g of turkey ham, 60 g of salad (alfalfa sprouts, lettuce, and tomato), 23 g avocado, and 500 mL of water (developed at 8:15 h). Snack 1, 25 g whey protein, 100 g Greek yogurt, and 110 g apple (developed at 11:30 h). Lunch, 60 g chicken, 60 g bell peppers and onion, 2 tortillas, 100 g, and 1000 mL water (developed at 16:15 h). Dinner, 2 baked corn tostadas, 90 g tuna, 50 g onion and tomato, 23 g avocado, and 750 mL of water (developed at 19:00 h). Snack 2, 150 g of pineapple, 100 g of jicama, 15 peanuts, and 500 mL of water (developed at 21:40 h.).

Table 3. Proportions of macronutrients ingested.

Aspect Analyzed	Test 1	Test 2	Test 3
Total calories (Cal)	Breakfast: 1079.60 ± 95 Lunch: 919.29 ± 332 Dinner: 707.47 ± 275 Snack 1: 435.66 ± 12 Snack 2: 241.13 ± 158	Breakfast: 332.27 ± 102 Lunch: 1197.30.0 ± 399 Dinner: 572.44 ± 218 Snack 1: 316.50 ± 136 Snack 2: 86.23 ± 30	Breakfast: 335.00 ± 63 Lunch: 490.01 ± 92 Dinner: 387.36 ± 57 Snack 1: 596.21 ± 27 Snack 2: 260.38 ± 44
Carbohydrates (g)	Breakfast: 141.50 ± 14 Lunch: 136.46 ± 85 Dinner: 79.38 ± 36 Snack 1: 36.51 ± 7 Snack 2: 28.03 ± 18	Breakfast: 40.82 ± 7 Lunch: 182.10 ± 70 Dinner: 52.98 ± 13 Snack 1: 40.51 ± 33 Snack 2: 2.69 ± 1	Breakfast: 30.18 ± 8 Lunch: 65.43 ± 7 Dinner: 33.85 ± 5 Snack 1: 73.10 ± 6 Snack 2: 41.50 ± 6
Carbohydrate (Cal) (%) Energy proportion	Breakfast: 566.00 ± 58 → (52 ± 3)% Lunch: 545.86 ± 343 → (55 ± 17)% Dinner: 317.53 ± 146 → (44 ± 7)% Snack 1: 146.04 ± 31 → (33 ± 6)% Snack 2: 112.13 ± 75 → (47 ± 18)%	Breakfast: 162.87 ± 30 → (47 ± 15)% Lunch: 728.40 ± 282 → (58 ± 12)% Dinner: 211.94 ± 54 → (39 ± 11)% Snack 1: 149.74 ± 137 → (40 ± 17)% Snack 2: 10.76 ± 4 (12 ± 3)%	Breakfast: 120.74 ± 33 → (35 ± 5)% Lunch: 261.72 ± 31 → (54 ± 8)% Dinner: 135.40 ± 20 → (34 ± 1)% Snack 1: 292.42 ± 26 → (48 ± 2)% Snack 2: 166.03 ± 26 → (63 ± 1)%
Lipids (g)	Breakfast: 37.30 ± 7 Lunch: 26.96 ± 11 Dinner: 25.22 ± 12 Snack 1: 16.02 ± 0.2 Snack 2: 8.28 ± 5	Breakfast: 7.61 ± 4 Lunch: 33.15 ± 12 Dinner: 26.36 ± 5 Snack 1: 4.25 ± 4 Snack 2: 7.18 ± 2	Breakfast: 14.07 ± 2 Lunch: 8.40 ± 2 Dinner: 15.73 ± 5 Snack 1: 17.32 ± 0.1 Snack 2: 8.59 ± 2
Lipid Calories (Cal) (%) Energy proportion	Breakfast: 335.42 ± 69 → (30 ± 4)% Lunch: 242.70 ± 103 → (30 ± 15)% Dinner: 227.02 ± 109 → (30 ± 6)% Snack 1: 144.26 ± 2 → (33 ± 1)% Snack 2: 74.56 ± 49 → (35 ± 22)%	Breakfast: 68.54 ± 42 → (18 ± 8)% Lunch: 301.14 ± 113 → (27 ± 10)% Dinner: 105.44 ± 23 → (39 ± 17)% Snack 1: 38.26 ± 18 → (12 ± 5)% Snack 2: 64.65 ± 23 → (75 ± 6)%	Breakfast: 126.68 ± 24 → (38 ± 5)% Lunch: 75.64 ± 24 → (15 ± 2)% Dinner: 141.58 ± 45 → (36 ± 7)% Snack 1: 155.95 ± 1 → (26 ± 1)% Snack 2: 77.36 ± 18 → (29 ± 2)%
Protein (g)	Breakfast: 44.53 ± 4 Lunch: 32.68 ± 9 Dinner: 40.72 ± 18 Snack 1: 36.3 ± 3 Snack 2: 13.60 ± 16	Breakfast: 25.26 ± 11 Lunch: 42.11 ± 11 Dinner: 26.36 ± 5 Snack 1: 32.12 ± 6 Snack 2: 2.70 ± 1	Breakfast: 21.89 ± 3 Lunch: 38.16 ± 15 Dinner: 27.59 ± 6 Snack 1: 36.95 ± 0.5 Snack 2: 4.24 ± 1
Protein Calories (Cal) (%) Energy proportion	Breakfast: 178.15 ± 17 → (16 ± 3)% Lunch: 130.73 ± 37 → (14 ± 3)% Dinner: 162.91 ± 75 → (24 ± 9)% Snack 1: 145.3 ± 15.67 → (33 ± 4)% Snack 2: 54.43 ± 67 → (16 ± 15)%	Breakfast: 101.07 ± 46 → (26 ± 10)% Lunch: 167.15 ± 46 → (14 ± 3)% Dinner: 105.44 ± 23 → (21 ± 10)% Snack 1: 128.48 ± 25 → (46 ± 16)% Snack 2: 10.80 ± 6 → (12 ± 4)%	Breakfast: 87.57 ± 14 → (26 ± 3)% Lunch: 152.64 ± 63 → (30 ± 7)% Dinner: 110.3 ± 27 → (28 ± 7)% Snack 1: 147.83 ± 2 → (24 ± 1)% Snack 2: 16.98 ± 4 → (6 ± 1)%

2.8. Glucose Curve

The analysis of glycemic variability considers information on the dynamics of the glucose curves produced at each dietary intake. Figure 1 shows a contrast between the glucose measurement (left graph) and the respective magnitude of the curve generated after a meal (right graph). The beginning of the curve is the moment when food intake is generated, followed by the absorption of macronutrients by the organism, followed by a pronounced elevation in glucose levels until reaching the maximum peak, from where a decrease in glucose begins because of the homeostatic regulation process generated by the organism, thus generating abrupt changes in glucose derived from the effect of insulin secretion. Once the decline is complete, glucose stabilizes, thus attenuating the postprandial glucose curve generated. The magnitude of the glucose elevation (right graph) is calculated by subtracting the initial measurement from the glucose curve analyzed in each of the measurements over the time of the curve, thus having a magnitude of 0 mg/dL at the beginning, which over time can have positive or negative glucose concentration values due to the different types of absorption of the macronutrients ingested.

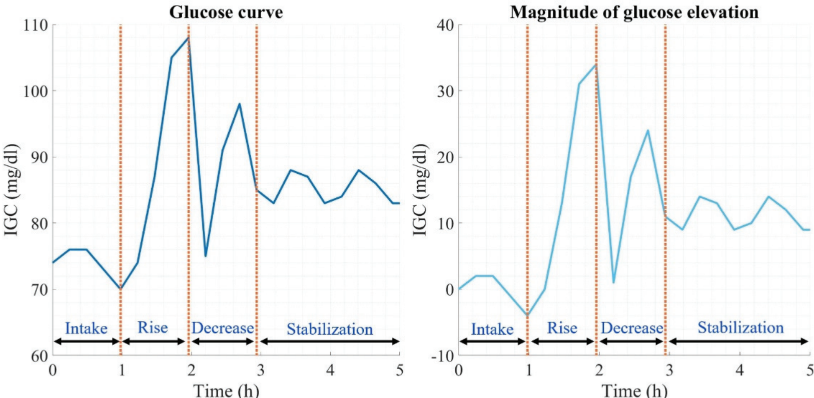

Figure 1. Glucose curve description.

3. Results

3.1. Glucose Measurement

In each test, 14 days of glucose measurements were generated. The result of the glucose variation in each test is presented in Figure 2, positioning in the upper part the glucose dynamics according to a daily diet (Test 1), in the central part the dynamics according to a daily diet modifying the sequence in the macronutrient intake (Test 2), and finally, in the lower part the glucose variation according to a modification in the diet and sequence of macronutrient intake (Test 3). The difference between tests is clear according to each of the glucose curves, being greater in Test 1 since there is no fixed schedule for food intake, contrary to what happened in Test 3, where the time in the glucose curves is constant. Consequently, the behavior of glucose is more homogeneous.

Quantitatively, the average variation between each of the tests is described in Table 4, where the glucose average data, the glucose management indicator (based on that proposed by Leelarathna et al. [28]), and the glucose coefficient of variation (considering that submitted by Rodbard [29]) are presented. These data were calculated for total glucose measurements in each test performed over the 14 days of size. The results demonstrate how a higher glucose variation coefficient correlates with lower glucose concentrations and a glucose management indicator. This phenomenon is visible when comparing Test 1 results with those in Test 3.

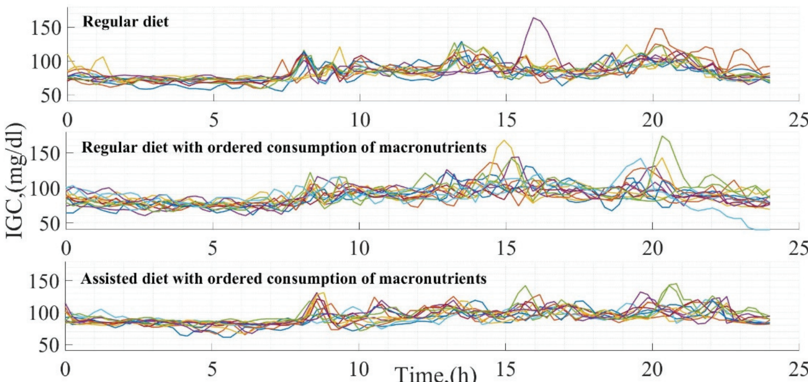

Figure 2. Glucose measurements in each test developed.

Table 4. Glucose statistics at the end of each test.

Test Number	Glucose Average (mg/dL)	Glucose Management Indicator (%)	Coefficient of Glucose Variation (%)
Test 1	84.06 ± 13.77	5.32	16.39
Test 2	88.88 ± 14.49	5.43	16.31
Test 3	93.13 ± 11.93	5.53	12.81

Five types of intakes were generated for each day throughout the tests. The postprandial glucose average of each intake developed throughout every test is presented in Table 5, where the postprandial glucose concentrations are lower at breakfast (ranging between 83–89 mg/dL) due to starting from a condition close to the basal level, contrary to dinner where the glucose ranges between 95–100 mg/dL because the glucose curve starts from a higher level since the time gaps between each intake avoid the homeostasis to reach a basal level after a postprandial period.

Table 5. Postprandial glucose average at each meal.

Intake	Test 1 (mg/dL)	Test 2 (mg/dL)	Test 3 (mg/dL)
Breakfast	83.02 ± 1.87	87.49 ± 2.28	89.78 ± 2.27
Lunch	90.39 ± 4.02	98.31 ± 5.60	100.37 ± 2.83
Dinner	96.53 ± 4.62	95.10 ± 7.02	100.77 ± 3.58
Snack 1	91.32 ±3.66	89.34 ± 4.59	90.48 ± 2.26
Snack 2	96.96 ± 3.43	87.66 ± 1.81	92.94 ± 3.84

3.2. Food Sequence Modification Effect on Glucose

There are marked differences between the intakes analyzed in each test. Therefore, this work explored the magnitudes, elevation, and stabilization times in the different glucose curves developed in each intake. For evaluating the impact of the sequence of macronutrients in the dietary intake, each of the glucose curves was grouped among three different types of patterns:

1. Carbohydrate intake at the beginning;
2. Vegetable and fiber intake at the beginning;
3. Animal protein intake at the beginning.

The results of this analysis are presented in Table 6, highlighting the following aspects: (a) Higher maximum glucose peaks occur when carbohydrates are consumed first. (b) The consumption of vegetables and fiber or proteins generates lower glucose average levels in

contrast to an early consumption of carbohydrates. (c) Early consumption of carbohydrates generates shorter periods of elevation and stabilization in the glucose curves in contrast to an early consumption of proteins or vegetables, resulting in higher glucose average levels.

Table 6. Glycemic variation in relation to the first macronutrient ingested.

First Macronutrient Ingested	Glucose Average (mg/dL)	Maximum Peak (mg/dL)	Peak Time (h)	Stabilization Time (h)
Carbohydrates	Breakfast : 90.72 ± 4 Lunch : 98.48 ± 3 Dinner : 95.40 ± 7	Breakfast : 115.50 ± 11 Lunch : 123.62 ± 12 Dinner : 125.66 ± 20	Breakfast : 3.02 ± 1.2 Lunch : 2.63 ± 1.8 Dinner : 2.36 ± 1.3	Breakfast : 3.54 ± 1.0 Lunch : 3.02 ± 1.2 Dinner : 3.42 ± 0.8
Vegetables and Fiber	Breakfast : 87.81 ± 4 Lunch : 93.67 ± 14 Dinner : 95.74 ± 3	Breakfast : 113.33 ± 5 Lunch : 119.55 ± 16 Dinner : 117.44 ± 6	Breakfast : 3.10 ± 1.0 Lunch : 2.34 ± 1.9 Dinner : 2.80 ± 1.3	Breakfast : 3.99 ± 1.3 Lunch : 2.96 ± 1.1 Dinner : 3.58 ± 0.9
Proteins of animal origin	Breakfast : 87.72 ± 5 Lunch : 99.91 ± 3 Dinner : 82.02 ± 11	Breakfast : 112.24 ± 11 Lunch : 123.14 ± 14 Dinner : 107.00 ± 24	Breakfast : 2.82 ± 0.9 Lunch : 2.51 ± 1.5 Dinner : 2.38 ± 1.9	Breakfast : 3.44 ± 1.0 Lunch : 3.70 ± 0.9 Dinner : 3.48 ± 1.0

As a complement to Table 6, Figure 3 illustrates the dynamics of the glucose curves when the first macronutrient ingested is carbohydrates. It shows three graphs corresponding to the three main intakes (breakfast, lunch, and dinner). Each chart has two types of colors, red (referring to the results obtained in Test 2) and blue (Test 3), representative of the dynamics of the intake developed throughout the experiment, thus illustrating the postprandial glucose during a period of 5 h of measurement. Considering the numerical results presented, the postprandial glucose dynamics show lower elevations at breakfast (maximum glucose peaks below 130 mg/dL) than at lunch, where glucose peaks reach values close to 150 mg/dL, and dinner with top mounts above 150 mg/dL.

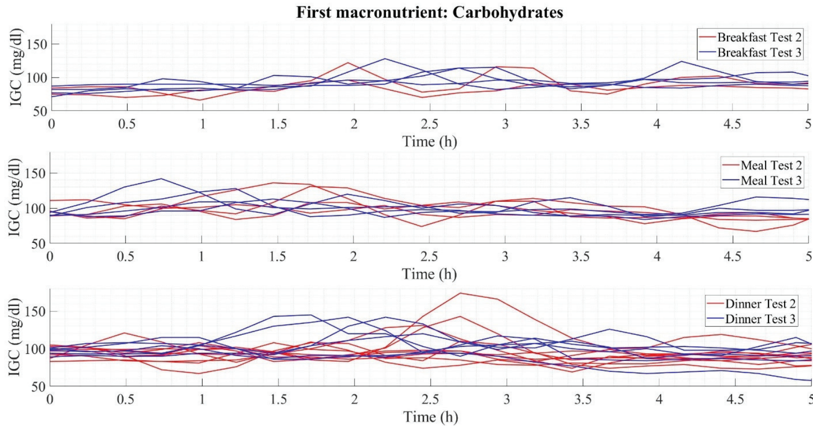

Figure 3. Glycemic variability in early carbohydrate consumption.

4. Discussion

Recently, the concept regarding the sequence and frequency of macronutrient intake has gained strength due to the benefits it generates for the organism [30]. Paoli et al. [31] proposed an example of this, where the modification of the frequency of intakes generates benefits in the reduction of intestinal inflammation, improving autophagy, and stress resistance. Henry et al. [32] allude to how consuming vegetables before carbohydrates is a strategy capable of optimizing glycemic control and positively influencing postprandial glucose. On the other hand, King et al. [33] describe how consuming a small dose of

whey protein before a macronutrient meal mix stimulates insulin generation and improves postprandial glucose in people with T2DM.

Considering the current need for the generation of information and alternative solutions for the prevention of T2DM, this work developed the necessary experimentation to determine the effects of the sequence of macronutrient intake on glucose reduction after the adoption of dietary regimens that promote the decrease in glucose levels evaluated during periods of 14 days. The results presented in a preliminary way the effectiveness of the adoption of nutritional regimens focused on the anticipated consumption of vegetables, fiber, or protein for the reduction and good condition of glucose levels. The best results came from an early intake of vegetables and fiber with an average glucose of 87–95 mg/dL and peak and stabilization times starting at 2.34 and 2.96 h, respectively. In this way, pronounced postprandial glucose episodes are avoided, and the shape of the glucose curves is flattened, in contrast to early carbohydrate intake, which has peak values of 130–150 mg/dL.

The development of the experimentation presents significant difficulty for the participant and the researcher who carries it out, because a dietary plan must be designed for each participant, thus promoting adherence to the menu and guaranteeing the correct performance of the experiment. The gradual variety of the menus is of utmost importance since it favors the participant's comfort and decreases the probability of desertion during the experimentation. The participant's correct development of the experiment must be corroborated with the filling of food diaries and continuous glucose measurements. In the case of sample scaling, it is advisable to consider those mentioned above, thus favoring the conditions for correct development in the experimentation. For population studies, two experimental periods with a duration of 14 days per period should be developed. In the first one, the glycemic variation is evaluated under an assisted diet, and in the second one, under a similar diet, but in this case varying the sequence in which the different types of macronutrients are ingested. It is recommended that there be a 14-day rest period between each of the tests. Otherwise, adherence to experimentation is challenging in the second experiment stage.

The complexity of this type of research illustrates the difficulties that exist for participants in adhering to a dietary regimen [34,35]. Consequently, the primary concentration of this type of research is limited to evaluating a single glucose curve in a population of healthy individuals, as is the case of Sun et al. [24] with a population of 16 healthy individuals. Alternatively, research is limited to evaluating people with gestational diabetes mellitus (GDM), as proposed in Yong et al. [36], where, like what is presented in this work, glycemic variation is analyzed in 10 women with GDM, exchanging the sequence of macronutrients and measuring glucose with a GCM. The results of this work agree with Sun et al. [24] and Yong et al. [36], where an early consumption of vegetables, fiber, or protein reduces postprandial glucose. Taking these works as a reference point, both use only one feeding plan due to a shorter duration of the experimentation, contrary to the case presented in this work, where the time of investigation makes it necessary to change the feeding plan periodically. Classifying the macronutrients consumed in these studies is similar to the method used in this research, where food is classified according to the predominant macronutrient in its composition.

The particularity of this work is focused on four specific points:

1. Development of an analytical study on the effect of food sequence on postprandial glucose curves and the impact on glucose levels throughout the research period, with statistical analysis being a fundamental part of generating the results obtained;
2. Experimentation time of 42 days divided into three different tests;
3. Periodic change in meal plans to achieve patient adherence to experimentation;
4. Contrast between three different conditions of glycemic variation derived from the tests proposed (the basis for experimentation on a more significant number of population).

The experimentation developed is a pilot test that serves as a reference to evaluate the feasibility of carrying it out in a larger population under specific conditions of degradation in glucose homeostasis. Although the measures that must be taken to develop the experi-

mentation are extensive, the benefits gained from it are significant. These can be included in the wide range of nutritional alternatives that can be proposed for the management and prevention of diabetes. One of the main benefits is the possibility of attending to the problem without generating an extra economic cost derived from its treatment. The present work opens a window of opportunities for developing several topics focused on managing and preventing metabolic diseases from a nutritional point of view.

5. Conclusions

In this work, three types of nutritional studies were developed to analyze the effect of managing the order of macronutrient intake. The results are consistent with the literature, indicating that early consumption of vegetables, fiber, or protein reduces the size of the postprandial glucose curves, thus decreasing blood glucose levels and improving glucose homeostasis in the organism. Considering that the work is a pilot test, based on the results obtained and the recommendations proposed to carry out the experimentation, it is feasible to develop it on a larger sample scale. This research is a potential milestone for the generation of knowledge focused on improving glucose homeostasis in different treatments for diabetes. This work generates the possibility of creating alternatives for the prevention and control of type 2 diabetes based on changes in the dietary sequence and in conjunction with pharmacological treatment (in the case of diabetes) that does not generate an extra economic expense for the health sectors and the people treated in them.

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Informed Consent Statement: The participants involved in the research signed the letter of informed consent and expressed their approval for the development of the experimental tests.

Data Availability Statement: The data are available from the corresponding author.

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Article

Street Food in Malaysia: What Are the Sodium Levels?

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Abstract: Street food is a major source of food in middle- and low-income countries as it is highly accessible and inexpensive. However, it is usually perceived as unhealthy due to the high levels of sodium, sugar, and fat content. However, there is little analytical data on the sodium levels in the street foods of Malaysia. This study started with a survey to determine the most frequently available street foods in every state in Malaysia, followed by food sampling and the analysis of sodium (reported mg/100 g sample). Street food in the snack category contained the highest amount of sodium (433 mg), followed by main meals (336.5 mg) and desserts (168 mg). Approximately 30% of the local street food in this study was deep-fried. Snacks from processed food (8%) contained high sodium content (500–815 mg). Fried noodles and noodle soup contained the highest amount of sodium (>2000 mg sodium) based on per serving. Most main dishes that use a variety of sauces contained high amounts of sodium. These findings were recorded in the Malaysian Food Composition Database. Moreover, this study could raise awareness and serve as baseline data for future interventions on the sodium content in the street foods of Malaysia.

Keywords: street food; sodium content; main meal; snack; dessert; Malaysia

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1. Introduction

According to the World Health Organization Report [1], non-communicable diseases (NCD) account for more than 70% of all deaths globally. Additionally, the number of NCD deaths is rising, and is expected to reach 52 million by 2030 [2]. In Malaysia, approximately 85,000 deaths were due to NCDs [1]. The Malaysian Community Salt Study [3], a population-based survey, reported that Malaysian adults consume a high amount of salt daily (7.9 g/day). This amount exceeds the recommended maximum salt intake of 5 g/day by the World Health Organization [4], resulting in hypertension, which affects one in three Malaysian adults [5]. Hypertension increases the risk of stroke, heart disease, and chronic kidney disease [6–8].

Due to the high burden of disease associated with excess salt intake, the Ministry of Health Malaysia (MOH) developed a national salt reduction strategy in 2010, to reduce daily salt intake by 30% by 2025 [9]. A proposed approach is the monitoring of sodium content in foods, which is then recorded in the available Malaysian Food Composition Database (MyFCD). This information is critical for food and health education, food research, and change monitoring. Since the implementation of the salt reduction strategy, sodium intake is monitored from the nutrition labels of packaged foods [9]. Nevertheless, little is

known about the sodium content in local street foods [10]. This information is important because street foods are an increasingly common food source due to their accessibility, convenience, low cost, availability, and wide range of options [11]. Thus, this study aimed to determine the most frequently available street foods in every state in Malaysia and their sodium content. This data will be useful in developing effective strategies to reduce the salt content in local street foods.

2. Materials and Methods

Malaysia comprises 13 states and 3 federal territories (Kuala Lumpur, Labuan, and Putrajaya). The total population of Malaysia in 2022 is estimated at 32.7 million as compared to 32.6 million in 2021, with an annual population growth rate of 0.2%. The highest percentage distribution by ethnic group is Bumiputra (69.9%), followed by Chinese (22.8%), Indians (6.6%), and others (0.7%) [12]. In 2021, 77.7% of Malaysia's total population lived in urban areas and cities [13]. There is currently no specific legislation for street foods and their sodium content. Street food stalls are randomly distributed throughout the cities and villages.

Data collection was carried out in two phases. Phase I was a survey to determine the most frequently available street food in all 13 states and the federal territory of Kuala Lumpur. This survey started in early 2020 but was stopped due to a series of nationwide lockdowns. The survey resumed in December 2020 when the government allowed interstate travel once more. This was followed by Phase II, where street foods in all states of Malaysia were sampled from May to December 2021. The analysis of the sodium content in all samples started in early 2022. The flowchart of the whole study is illustrated in Figure S1. The procedures in this study were approved by the Research Ethics Committee of the National University of Malaysia with reference number UKM PPI/111/8/JEP-2020-433.

2.1. Phase I: Survey of Street Foods in All States of Malaysia

Phase I of this study was a field survey of locally available street foods conducted in 13 states and the federal territory of Kuala Lumpur in Malaysia. Street food is described as ready-to-eat food and beverages made and sold by vendors and hawkers, especially in the street and other similar public places. Based on the definition by the Food & Agriculture Organization (FAO) [14], street food can be distinguished from formal food service operations (e.g., cafes and restaurants) and includes foods from pushcarts, bicycles, baskets, balancing poles, or stalls without four permanent walls. The criteria for the street food stall to be selected in the study was one that did not have a fixed building or was confined within four walls (based on the definition by the FAO). This included individual stalls and stalls in the day and night markets. For each state, the operational night and morning markets were identified from the city council websites. A few night or morning markets in every state were surveyed.

A survey form (Table S1) was used to record information about all street foods available from each location, such as the state, district, name of the street food, category of the street food (e.g., main meal, snack, or dessert), and the preparation method. The total frequency of each street food surveyed in each state was determined. The top 15 most frequently available street foods among each of the categories for every state were then identified and listed. The street foods selected were also based on the expected sodium content (e.g., desserts composed mostly of sugar were not included).

2.2. Phase II: Food Sampling

2.2.1. Sampling the Three Categories of Street Food Samples for Each State

A total of 15 frequently available street foods (7 main meals, 5 snacks, and 3 desserts), determined from the survey in Phase I, were sampled from every state. A total of 210 street food samples from all 14 states in Malaysia were analyzed for their sodium contents. The sampling of the street food was carried out according to a local method [15]. Each type of

street food selected for sodium analysis was purchased from two different stalls within the same state. Since food sampling was carried out during the COVID-19 pandemic, the operating hours of street food stalls were affected and some of the stalls ceased operation. Thus, this study was limited by the availability of food. After purchasing the street food samples, they were stored in an ice box and transported (5 °C for 1.5 h) to the laboratories to protect the food from spoilage.

2.2.2. Preparation of Street Food Samples for Sodium Analysis

Preparation and analysis of street food samples were conducted in the food analysis laboratory based on the method in [15]. Each sample was weighed together with the packaging still intact using the top pan balance. Inedible portions (e.g., bones) were identified and removed. Following this, the same type of street food that was purchased from two different stalls was mixed and homogenized in a food processor. The homogenized samples were then kept in an airtight container and stored in a −20 °C freezer, before preparation for sodium analysis. Approximately 1–3 g samples were weighed into a 50 mL polypropylene tube, and 30 mL of 30% nitric acid was added into the tube for wet digestion. The closed tube was placed on a hot block at 95 °C for 1.5 h. The tube was then cooled to room temperature and topped up to 50 mL with deionized water. The stock standard used for sodium analysis had a concentration of 10,000 ppm. A series of intermediate standards (i.e., 0.1, 0.5, 1.0, 2.0, 5.0, 10, 20, 30, 50, and 100 ppm) were prepared from the stock standard. Each intermediate standard was prepared in a 1 L volumetric flask with diluted nitric acid. The sodium content was then analyzed in duplicates using inductively coupled plasma–optical emission spectrometry (Agilent Technologies, Santa Clara, CA, USA, 5100 ICP-OES) based on the protocol in [16]. Each sample was analyzed in duplicate and reported as mean ± standard deviation. The LOD and LOQ of this instrument were 0.0454 and 0.1610 mg/kg, respectively.

2.2.3. Sodium Content Classification

There is no classification standard for high sodium ready-to-eat foods (i.e., street foods) in Malaysia. The sodium classification in Malaysia is meant for packaged foods with nutritional labeling. These packaged foods can be classified as low sodium (<120 mg/100 g sample), very low sodium (<40 mg/100 g sample), or sodium-free (5 mg/100 g sample). Hence, this study utilized the UK's traffic light labeling scheme for classifying low sodium (<120 mg/100 g sample), medium sodium (between 120 and 600 mg/100 g sample), and high sodium (>600 mg/100 g sample) foods [17]. This classification is in line with the classifications used by the Food and Drugs (Composition and Labeling) Regulations in Hong Kong [18] and China (GB/T 23789-2009) [19].

2.3. Statistical Analyses

Here, IBM (Armonk, NY, USA) Statistical Package for Social Sciences (SPSS) version 25.0 was used to analyze the data. For Phase I, a descriptive test was performed to analyze the 15 most common street foods in Malaysia and sort them by food categories. The test included the preparation method, type, and category of the selected street foods. For Phase II, a descriptive test was used to analyze the average sodium content found in the selected street foods. Inferential statistical analysis, such as the one-way ANOVA test, was used to compare the average sodium content between the selected foods according to the food category and group. If the food groups and categories had unequal sample sizes and both one-way ANOVA and homogeneity of variance were significant, a Games–Howell post hoc test was performed to identify the specific differences between the three groups and categories.

3. Results

A total of 68 districts with 380 street food locations were surveyed. The highest number of locations surveyed (184 locations) was in Johor, whereas the lowest (4 locations) was in Kelantan. Among the total 10,520 types of street food surveyed in all states of Malaysia,

the most common category of street food was snacks (40%), followed by main meals (37%), and then desserts (23%) (Table 1). Based on the survey, the main cooking method for street foods was deep-frying (28%), followed by steaming (13.3%), pan frying (11.1%), boiling (10.5%), grilling (9.4%), stir-frying (7.3%), stewing (6.1%), fermenting (5.3%), baking (4.9%), simmering (2.3%), roasting (9.4%), braising (9.4%), smoking (0.1%), and blanching (0.1%)

Table 1. The total number of street foods surveyed in this study by food category, districts, and locations of every state.

Regions	Coasts	States	Number of Districts Surveyed	Number of Locations Surveyed	Street Foods Category			Total Street Food by States
					Main Meals	Snacks	Desserts	
West Malaysia	West Coast	Selangor	7	17	792	1000	553	2345
		Federal Territory of Kuala Lumpur	7	14	883	935	328	2146
		Negeri Sembilan	1	6	333	305	164	802
		Melaka	1	5	203	480	156	839
		Johor	8	194	207	220	143	570
		Kedah	3	11	118	64	35	217
		Perlis	2	8	42	47	20	109
		Perak	11	40	90	65	37	192
		Penang	4	34	113	44	43	200
	East Coast	Terengganu	7	10	495	504	396	1395
		Pahang	8	14	362	356	241	959
		Kelantan	2	4	92	102	172	366
East Malaysia	Not applicable	Sabah	4	8	54	55	28	137
		Sarawak	3	15	103	57	83	243
		Total	68	380	3887	4234	2399	10520

A total of 210 samples were analyzed. There were 41 types of street foods from more than 1 state and 53 types of street foods from 1 particular state. The sodium content of similar street foods was averaged. The study reported the average sodium content in all 94 types of street foods as being in the range of 3.9–815.0 mg/100 g sample. Explanation on each street food can be found in Table S2.

The average sodium content of street foods between the East Coast and West Coast of Malaysia was compared based on the food categories (21 and 63 main meals, 15 and 42 snacks, and 9 and 27 desserts, respectively). The sodium content in all three food categories between the two coasts was not significantly different ($p > 0.05$). On both coasts, snacks had the highest sodium content (411–506 mg/100 g sample), followed by main meals (327–392 mg/100 g sample), and then desserts (186–191 mg/100 g sample).

The average sodium content of street foods between West and East Malaysia was compared based on the food categories (84 and 14 main meals, 57 and 12 snacks, and 36 and 7 desserts, respectively). The average sodium content in the snack category for both West and East Malaysia had the highest sodium content (450–481 mg/100 g sample), followed by main meals (376–404 mg/100 g sample), and then desserts (92–190 mg/100 g sample). The sodium content in the snacks and main meal categories between the two regions was not significantly different ($p > 0.05$). However, only desserts in West Malaysia reported significantly higher sodium content (190 mg/100 g sample) as compared to East Malaysia (92 mg/100 g sample) ($p < 0.05$).

Table 2 displays the average sodium content for each food category and food group. For food categories, snacks contained the highest average amount of sodium, followed by main meals and desserts. Both main meals and snacks contained significantly higher sodium contents when compared to desserts ($p < 0.05$). For food groups, processed foods contained the highest average amount of sodium compared to cooked dishes and local cakes. Most of the street foods were cooked dishes (63%).

Table 2. The average sodium content in different street food categories and groups.

Food Category	Percentage (%)	Average Sodium Content (mg/100 g)
Snacks	40	433.0 ± 198.1 [a]
Main meals	37	336.5 ± 148.2 [a]
Desserts	23	168.0 ± 134.7 [b]
Food Group	**Percentage (%)**	**Average sodium content (mg/100 g)**
Processed foods	8	509.1 ± 144.0 [a]
Cooked dishes	63	421.8 ± 207.5 [a]
Local cakes	29	214.0 ± 138.7 [b]

[a,b] Different letters indicate significant differences across the column ($p < 0.05$), based on the Games–Howell post hoc test.

Table 3 shows the sodium content in processed foods prepared as street foods. Only 8% of the street foods were prepared using processed foods, and most of the processed foods had medium to high sodium contents (451–815 mg sodium/100 g sample). Fried fish balls, fried chicken with cheese, fried crab meatballs, fried sausages, and fried chicken balls were categorized as processed foods prepared as street foods that contain high amounts of sodium (605–815 mg/ 100 g sample). The other street food samples in the snack category with high sodium content were fish-based snacks or keropok lekor (780.4 ± 109.5 mg/100 g sample) and seaweed pickles (761.0 ± 42.4 mg/100 g sample).

Table 3. Sodium content in processed food prepared as street food.

No	Name of Street Food (*n* = Number of States in Which the Street Food Was Sampled)	Street Food Category	mg Sodium/100 g (Mean ± std dev)	mg Sodium/ Serving (Household Measurement)
1	Fried fish ball (*n* = 3)	Snack	815.0 ± 47.7	1530.9 (6 pieces)
2	Fried chicken with cheese (*n* = 1)	Snack	706.0 ± 24.0	1087.2 (1 piece)
3	Fried crab meatball (*n* = 2)	Snack	690.5 ± 18.4	553.6 (6 pieces)
4	Fried sausage (*n* = 3)	Snack	690.3 ± 74.4	683.4 (3 pieces)
5	Fried chicken ball (*n* = 1)	Snack	605.5 ± 12.0	601.3 (6 pieces)
6	Beef burger (*n* = 1)	Main meal	584.0 ± 2.8	584.0 (1 piece)
7	Fried sausage with cheese (*n* = 1)	Snack	563.0 ± 9.9	802.3 (3 pieces)
8	Chicken nuggets (*n* = 5)	Snack	519.1 ± 73.8	327.0 (3 pieces)
9	Pizza (*n* = 1)	Snack	485.0 ± 12.7	5432.0 (1 whole regular)
10	Chicken burger (*n* = 3)	Main meal	451.8 ± 102.0	682.3 (1 piece)

Table 4 shows that 46% of the main meal category consisted of noodles and rice, such as fried noodles, noodles with gravy, fried rice, and other cooked rice, which contained medium to high amounts of sodium. Noodle-based dishes, such as *soto*, fried noodles, *bakso*, and noodle soup contained more than 2000 mg sodium per serving. Fried kuey teow, *char kuey teow*, and noodles with curry or soy sauce gravy contained almost 2000 mg sodium per serving. *Laksa* (rice-based noodles with gravy made from fish) from Penang and Perak also contained almost 2000 mg sodium per serving.

Table 5 displays the rest of the street foods that contained medium sodium content (121.5–586.5 mg/100 g sample). This included 19 snacks, 15 desserts, and 7 main meals. There were 17 low sodium street foods (<120 mg/100 g sample) in this study. Most of the low sodium street foods (82.4%) were in the dessert category (Table 5).

Table 4. Sodium content in noodle and rice-based street food with high (>600 mg/ 100 g sample) and medium (120–599 mg/100 g sample) sodium contents.

High Sodium Content (>600 mg/100 g)				
No.	**Name of Street Food (n = Number of States in Which the Street Food Was Sampled)**	**Street Food Category**	**mg Sodium/100 g (Mean ± Std Dev)**	**mg Sodium/ Serving (Household Measurement)**
1	Fried noodles (n = 10)	Main meal	704.2 ± 225.4	2185.5 (1 plate)
2	*Kolo mee* (n = 1)	Main meal	625.0 ± 1.4	1152.5 (1 bowl)
Medium Sodium Content (120–599 mg/100 g)				
No.	**Name of Street Food (n = Number of States in Which the Street Food Was Sampled)**	**Street Food Category**	**mg Sodium/100 g (Mean ± Std Dev)**	**mg Sodium/ Serving (Household Measurement)**
1	*Bakso* (n = 1)	Main meal	468.5 ± 3.5	2576.8 (1 bowl)
2	Fried *kuey teow* (n = 7)	Main meal	437.1 ± 78.6	1477.8 (1 plate)
3	Noodle soup (n = 1)	Main meal	431.0 ± 29.7	2249.8 (1 bowl)
4	*Char kuey teow* (n = 2)	Main meal	418.3 ± 127.6	1565.5 (1 plate)
5	Fried vermicelli @ fried *mihun* (n = 11)	Main meal	398.6 ± 133.6	797.2 (1 plate)
6	*Soto* (n = 1)	Main meal	377.0 ± 21.2	3168.7 (1 bowl)
7	Fried rice (n = 2)	Main meal	374.8 ± 38.5	749.5 (1 plate)
8	Vermicelli soup @ *mihun sup* (n = 3)	Main meal	360.0 ± 78.8	2162.6 (1 bowl)
9	*Nasi lemak* with fried chicken (n = 4)	Main meal	349.1 ± 76.2	1431.5 (1 plate)
10	*Nasi tomato* (n = 1)	Main meal	345.5 ± 2.1	1935.7 (1 plate)
11	Noodles with gravy (curry/soy sauce) (n = 4)	Main meal	336.6 ± 248.4	1830.8 (1 bowl)
12	*Nasi lemak* (n = 12)	Main meal	321.7 ± 104.7	643.4 (1 plate)
13	*Kuey teow* soup (n = 1)	Main meal	307.0 ± 2.8	2076.4 (1 bowl)
14	*Laksa* (Penang style) (n = 5)	Main meal	306.6 ± 93.1	1966.6 (1 bowl)
15	*Nasi minyak* (n = 2)	Main meal	304.0 ± 46.0	1128.2 (1 plate)
16	*Nasi kerabu* (n = 2)	Main meal	298.5 ± 64.3	916.1 (1 plate)
17	Chicken rice (n = 3)	Main meal	298.5 ± 25.1	746.3 (1 plate)
18	*Laksa* (Perak style) (n = 1)	Main meal	288.0 ± 0.0	1789.9 (1 bowl)
19	Glutinous rice with *rendang* (meat cooked with spices) (n = 1)	Main meal	285.5 ± 17.7	467.7 (1 set)
20	Chicken porridge (n = 2)	Main meal	272.5 ± 0.7	452.4 (1 bowl)
21	Rice porridge (n = 3)	Main meal	245.5 ± 49.7	407.5 (1 bowl)
22	Glutinous rice with fried fish (n = 1)	Main meal	216.0 ± 8.5	335.1 (1 pack)
23	Spaghetti bolognese (n = 1)	Main meal	215.5 ± 13.4	1131.4 (1 plate)
24	*Laksam* (n = 1)	Main meal	185.5 ± 0.7	519.7 (1 bowl)

Table 5. Other street foods with medium (120–599 mg/100 g sample) and low (<120 mg/100 g sample) sodium contents.

Street Foods with Medium Sodium Content (120–599 mg/100 g Sample)				
No.	**Name of Street Food (n = Number of States in Which the Street Food was Sampled)**	**Street Food Category**	**mg Sodium/100 g (Mean ± Std Dev)**	**mg Sodium/Serving (Household Measurement)**
1	*Apam balik* with cheese (n = 1)	Dessert	586.5 ± 12.0	1343.1 (1 piece)
2	Kebab (n = 2)	Main meal	531.5 ± 89.1	616.5 (1 piece)
3	*Kerepek* (n = 1)	Snack	512.5 ± 61.5	71.8 (1 piece)
4	Takoyaki (n = 4)	Snack	502.4 ± 95.3	926.0 (6 pieces)
5	Fried chicken (n = 10)	Snack	464.9 ± 118.3	715.9 (1 piece)
6	Fried chicken (non-meat parts) (n = 1)	Snack	461.5 ± 72.8	113.1 (4 small pieces)
7	*Roti john (n = 2)*	Main meal	412.0 ± 59.4	696.3 (3 pieces)
8	Satay (n = 2)	Snack	395.3 ± 177.8	296.4 (5 sticks)
9	*Satar* (n = 1)	Snack	390.5 ± 0.7	338.3 (3 pieces)
10	*Kuih kacang* (n = 1)	Dessert	384.5 ± 16.3	339.9 (3 pieces)
11	*Cakoi* (n = 2)	Snack	376.3 ± 223.1	508.4 (3 pieces)
12	Fried *popiah* (n = 1)	Snack	350.0 ± 7.1	147.0 (1 piece)
13	*Pulut panggang* (n = 1)	Snack	349.5 ± 3.5	174.8 (1 piece)
14	*Putu piring* (n = 1)	Dessert	334.5 ± 57.3	184.0 (1 piece)
15	*Kuih bom* (n = 1)	Dessert	327.0 ± 4.2	143.9 (1 piece)

Table 5. *Cont.*

Street Foods with Medium Sodium Content (120–599 mg/100 g Sample)				
No.	**Name of Street Food (*n* = Number of States in Which the Street Food was Sampled)**	**Street Food Category**	**mg Sodium/100 g (Mean ± Std Dev)**	**mg Sodium/Serving (Household Measurement)**
16	Curry puff (*n* = 8)	Snack	305.0 ± 87.0	146.5 (1 piece)
16	Banana fritters with cheese (*n* = 1)	Dessert	293.0 ± 4.2	193.4 (3 pieces)
17	*Murtabak* (*n* = 2)	Snack	292.3 ± 188.4	825.6 (1 piece)
18	*Jering rebus* (*n* = 1)	Snack	291.5 ± 0.7	145.8 (4 pieces)
19	*Kuih seri muka* (*n* = 2)	Dessert	271.3 ± 73.2	260.4 (1 piece)
20	French fries with sauce (*n* = 1)	Snack	262.0 ± 7.1	175.5 (1 small serving)
21	*Apam balik telur* (*n* = 1)	Snack	262.0 ± 2.8	600.0 (1 large piece)
22	*Roti canai* (flat bread) (*n* = 2)	Main meal	262.0 ± 154.9	220.1 (1 piece)
23	*Kuih akok* (*n* = 2)	Dessert	259.3 ± 20.2	375.3 (4 pieces)
24	Donut (*n* = 4)	Dessert	254.9 ± 158.1	188.6 (1 piece)
25	*Apam balik* (*n* = 4)	Dessert	253.9 ± 76.1	581.4 (1 large piece)
26	*Kuih cara berlauk ayam* (*n* = 1)	Snack	246.5 ± 3.5	310.6 (4 small pieces)
27	Net crepes *(Roti jala)* (*n* = 2)	Main meal	240.8 ± 1.1	584.9 (1 pack)
28	*Kuih tepung gomak* (*n* = 1)	Snack	235.0 ± 12.7	276.7 (4 pieces)
29	Grilled chicken (small pieces) (*n* = 1)	Snack	229.5 ± 4.9	581.0 (4 small pieces)
30	Corn (savory) (*n* = 2)	Snack	220.9 ± 242.0	1361.3 (1 sheaf)
31	Egg tart (*n* = 1)	Dessert	204.5 ± 9.2	242.9 (1 piece)
32	*Kuih puteri ayu* (*n* = 1)	Dessert	194.0 ± 2.8	276.2 (4 pieces)
33	Banana fritters (*n* = 4)	Dessert	160.3 ± 66.8	105.8 (3 pieces)
34	*Chee cheong fun* (*n* = 1)	Snack	146.0 ± 1.4	826.0 (1 plate)
35	*Beh hua chee* (*n* = 1)	Snack	138.5 ± 12.0	305.4 (3 pieces)
36	*Kuih sagu* (*n* = 1)	Dessert	138.0 ± 0.0	93.8 (1 piece)
37	*Kuih cek mek molek* (*n* = 1)	Dessert	125.5 ± 0.7	128.9 (4 pieces)
38	Popcorn (*n* = 1)	Dessert	121.5 ± 6.4	245.4 (1 container)
Street Foods with Low Sodium Content (<120 mg/100 g Sample)				
No.	**Name of Street Food (*n* = Number of States in Which the Street Food Was Sampled)**	**Street Food Category**	**mg Sodium/100 g (Mean ± Std Dev)**	**mg Sodium/Serving (Household Measurement)**
1	*Kuih keria* (*n* = 1)	Dessert	119.5 ± 2.1	37.0 (1 piece)
2	Cubed rice with peanut gravy *(nasi impit)* (*n* = 1)	Main meal	117.5 ± 0.7	58.8 (8 cubes)
3	*Kuih lepat* (*n* = 2)	Dessert	109.2 ± 44.3	126.6 (1 piece)
4	*Kuih calak kuda* (*n* = 1)	Dessert	104.0 ± 0.0	99.8 (4 pieces)
5	*Kuih buah Melaka* (*n* = 1)	Dessert	89.9 ± 0.6	13.5 (1 piece)
6	*Kuih lapis* (*n* = 2)	Dessert	89.6 ± 93.9	89.6 (1 piece)
7	*Cekodok* (*n* = 1)	Dessert	84.7 ± 33.0	25.4 (3 pieces)
8	Steamed baozi with sweet fillings (*n* = 2)	Dessert	69.1 ± 27.5	34.6 (1 piece)
9	*Peneram* (*n* = 1)	Dessert	59.1 ± 1.6	18.9 (5 small pieces)
10	*Cendol* (*n* = 2)	Dessert	56.9 ± 2.8	431.7 (1 bowl)
11	*Kuih apam* (*n* = 1)	Dessert	56.1 ± 1.3	22.4 (3 pieces)
12	*Kuih jelurut* (*n* = 1)	Dessert	49.8 ± 2.1	101.6 (4 pieces)
13	*Tau fu fa* (*n* = 1)	Dessert	6.4 ± 0.4	23.6 (1 container)
14	*Kuih penjaram* (*n* = 1)	Dessert	5.3 ± 0.1	9.1 (4 pieces)
15	*Kuih angku* (*n* = 1)	Dessert	5.2 ± 0.6	14.1 (4 pieces)
16	*Putu* (*n* = 1)	Main meal	4.1 ± 0.2	7.2 (1 piece)
17	*Sianglag* (*n* = 1)	Main meal	3.9 ± 0.7	4.9 (1 pack)

4. Discussion

The highest percentage of street food surveyed in this study was from Selangor (22%), followed by Kuala Lumpur (20%). This could be attributed to Selangor having the highest population in 2022 [12]. Furthermore, Selangor and Kuala Lumpur were surveyed before the first nationwide lockdown in March 2021, when most street food stalls were still operating as usual. Main meals (37%) and snacks (40%) were the most common street food categories available in this study. This was similar to another study in Harare, Zimbabwe, where main meals (70.8%) and snacks (20.8%) were the most common [20]. Almost half of the main meals sampled in this study consisted of rice or noodle dishes. Rice is the staple food for half the world's population, and it is cultivated predominantly in Asia [21].

The majority of street foods in this study were cooked foods (63%), and deep-frying was the most common street food preparation method. Deep-fat frying may be defined as immersing foodstuff in edible oil or fat at 150–200 °C [22]. Deep-fat frying is one of the

most well-accepted methods in both local and international food preparation because of its convenience and highly stimulating properties (e.g., aroma and taste) to consumers [23].

There was no difference in the sodium contents of main meals and snacks between the different coasts or parts of Malaysia. This could be due to the same preparation methods being used for the common street foods. In the present study, 41 types of street foods were found across Malaysia. *Nasi lemak*, fried vermicelli, fried *mihun*, and fried chicken were frequently available across 12, 11, and 10 of the surveyed states, respectively. This was in line with a previous study, where *nasi lemak* and fried *bihun* were among the top 10 most consumed breakfast foods among adults in Malaysia [24].

Street foods with high amounts of sodium (605–805 mg/100 g sample) included processed foods, such as sausages, fish balls, chicken balls, and crab meat. The highest sodium content was found in fish balls. Fish balls are made from processed fish and can be eaten alone (fried) or in soups (boiled). The high sodium content in commercial fish balls accounts for the additional salt or sodium polyphosphate in preservatives and flavor enhancers [25]. Processed foods made from fish and chicken have sodium levels in the range of 479–500 mg/100 g sample, as listed on the nutrition labels [26].

The other street food in the snack category (452–584 mg sodium/100 g sample) included processed foods, such as beef burgers, nuggets, and sausages with cheese. *Keropok lekor* contained the second-highest sodium content in the snack category. It is a traditional fish-based snack from Terengganu and is popular on the East Coast of Peninsular Malaysia. *Keropok lekor* is commonly sold in all states of Malaysia, especially at roadside stalls and night markets [27]. In Phase I of this study, *keropok lekor* was frequently available in seven states, such as Johor, Sabah, Sarawak, Federal Territory of Kuala Lumpur, Negeri Sembilan, Terengganu, and Pahang. It is made from a mixture of minced/processed fish, sago flour, salt, monosodium glutamate, sugar, and ice-cold water [28]. The main source of sodium in the making of *keropok lekor* are salt and monosodium glutamate [29].

Seaweed pickle is very popular among the Bajau, and it was only collected in Sabah. Since it is harvested from the sea, the sea salt resulted in the high sodium content of the seaweed pickle. Normally, the seaweed pickle is served as a side dish to be taken with rice. Fried chicken with cheese was the fourth highest street food with a sodium content in this range. The average sodium content in 112 cheese products sold in major supermarkets in Malaysia was reported to be relatively high (856.54 mg/100 g sample) [26].

Fried noodles contained a high sodium content due to the ingredients used in the preparation stage. Fried noodles are often cooked with many sauces, especially soy sauce, thick soy sauce, oyster sauce, and chili sauce which contribute to high sodium content [30–32]. The sauces were added to add more flavor to the food and to make it more palatable to eat [33,34]. Additionally, the noodles contain a relatively high amount of salt in themselves [35,36]. *Kolo mee* or *mi kolok* is a signature dish from Sarawak. It has a high sodium content, which may be attributed to the sweet and salty soy sauce used in the dish. In Malaysia, the addition of salt and salty sauces to foods has been identified as the major source of sodium in the Malaysian diet [37].

In this study, 35% of main meals contained sodium content exceeding the recommended intake of 2000 mg sodium/day. However, the percentage is lower compared to the 62.6% of main meal dishes in 192 restaurants in China [38]. *Soto* is mainly composed of broth, meat, and vegetables, with a high sodium content (>3000 mg) per serving. Other noodle-based dishes, such as *bakso* (noodles with meatballs), noodle soup, fried *kuey teow*, and *char kuey teow* also contained high amounts of sodium (2000–2500 mg) per serving. This could be linked to the sauces (e.g., soy sauce and oyster sauce) used in their preparation. Soy sauce has been reported as one of the main sources of sodium during the preparation of ready-to-eat dishes in China [38]. *Laksa* uses fish-based broth that contains 1800–1900 mg sodium per serving, which could also be due to the use of shrimp paste.

Other rice-based dishes, such as *nasi tomato* (rice cooked with tomato paste), contained the highest sodium content, followed by *nasi minyak* (rice cooked with ghee and other condiments) and *nasi lemak* (rice cooked with coconut milk) with fried chicken

(1400–1900 mg/serving). A normal set of *nasi lemak* (rice and condiment only) contained 643 mg sodium per serving. *Nasi kerabu*, a blue-colored rice dish that is eaten with dried fish or fried chicken (a source of sodium) and crackers (another source of sodium), contained 1000 mg of sodium per serving.

In the dessert category, *apam balik* contained medium sodium content. *Apam balik* is a popular Malaysian traditional cake and is included as a Malaysian Heritage Food [39,40]. *Apam balik*, *apam balik* with egg, and *apam balik* with cheese contained 581, 600, and 1343 mg sodium per serving, respectively. The sodium content is directly associated with the use of sodium bicarbonate in its preparation. The addition of cheese to the snack and dessert categories increased the sodium content to the range of 35–55%.

Street foods with a sodium content of less than 120 mg sodium/100 g sample were found mainly in the dessert category. Kamaruzaman et al. stated that as many as 70 types of traditional Malay cakes are still popularized in Malaysia [41]. The basic ingredients used to produce traditional sweet cakes are sugar, coconut milk, brown sugar, and Malacca sugar [42]. Thus, not as much salt is used in dessert preparation as compared to main meals and snacks. Snacks that are popular among the Chinese population, such as *chee cheong fun* (rice noodle roll) and *beh hua chee* (fried dough), also fall in this range (120 mg sodium/100 g sample).

The data on the sodium content in street food can be updated in the Malaysian Food Composition Database (MyFCD). This ensures that the nutrients contained in these street foods are known to the public, and that healthier food selections can be made. Apart from that, these findings justify the importance of salt reduction during the preparation of street foods and look into the use of salt substitutes, such as potassium chloride. Studies have demonstrated that replacing salt with sodium-reduced and potassium-enriched salt substitutes is better for the general population [43]. This study can serve as a foundation for future related studies.

5. Conclusions

Local street food in the snack and main meal categories contained significantly higher amounts of sodium than in dessert foods. Main meal street foods (e.g., noodle soup and fried noodles) and processed foods that were used in the preparation of street foods contain high amounts of sodium per serving, which exceeded the recommended daily sodium intake. *Keropok lekor* is one of the high-salt snack which is available in most states that should be targeted for reformulation and monitored over time. It is vital to disseminate information on the high sodium content of local street food to the public through advertisements and social media. The public needs to be informed about the sodium content in a single serving of street food to limit their sodium intake to 2000 mg daily. This practice will help reduce the sodium consumption in the Malaysian population and subsequently, will reduce the prevalence of NCDs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods11233791/s1, Figure S1: Flowchart of data collection in Phase I and Phase II, Table S1: Streetfood Survey Form, Table S2: Explanation of each street food.

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Data Availability Statement: Data is contained within the article or supplementary material.

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Article

Anti-Inflammatory and Antioxidant Effects Induced by *Allium sativum* L. Extracts on an Ex Vivo Experimental Model of Ulcerative Colitis

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Abstract: Inflammatory bowel diseases (IBDs) are chronic and multifactorial inflammatory conditions of the colonic mucosa (ulcerative colitis), characterized by increased and unbalanced immune response to external stimuli. Garlic and its bioactive constituents were reported to exert various biological effects, including anti-inflammatory, antioxidant and immunomodulatory activities. We aimed to evaluate the protective effects of a hydroalcoholic (GHE) and a water (GWE) extract from a Sicilian variety of garlic, known as Nubia red garlic, on an ex vivo experimental model of ulcerative colitis, involving isolated LPS-treated mouse colon specimens. Both extracts were able to counteract LPS-induced cyclooxygenase (COX)-2, tumor necrosis factor (TNF)-α, nuclear factor-kB (NF-kB), and interleukin (IL)-6 gene expression in mouse colon. Moreover, the same extracts inhibited prostaglandin (PG)E_2, 8-iso-PGF$_{2\alpha}$, and increased the 5-hydroxyindoleacetic acid/serotonin ratio following treatment with LPS. In particular, GHE showed a better anti-inflammatory profile. The anti-inflammatory and antioxidant effects induced by both extracts could be related, at least partially, to their polyphenolic composition, with particular regards to catechin. Concluding, our results showed that GHE and GWE exhibited protective effects in colon, thus suggesting their potential use in the prevention and management of ulcerative colitis.

Keywords: garlic; colon; multimethodological evaluation; CIEL*a*b*; HS–SPME/GC–MS; apoptosis; inflammatory bowel disease

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1. Introduction

Garlic (*Allium sativum* L.) is an herbaceous plant, belonging to the Amarillidaceae family, that is used all over the world as traditional medicine and spice [1]. Garlic contains a number of biologically active compounds, including phenolic compounds [2], saponins [3], polysaccharides [4], as well as organosulfur compounds [1] that contribute to its countless pharmacological properties. In particular, the health-promoting properties induced by garlic were suggested to be related to its bioactive compounds, including phenolic compounds, whose content in this plant is relatively high [5]. However, the content of phenolic compounds in garlic was reported to be dependent on environmental, genetic and agronomic

factors [6]. Preclinical and clinical studies demonstrated the anti-inflammatory, antioxidant, antiatherosclerotic, anticancer, immunomodulatory, antidiabetic, antiobesity, neuroprotective, as well as digestive system protective activities of both garlic and its phenolic constituents [1]. Interestingly, raw garlic showed higher antioxidant activity compared to cooked garlic [7], suggesting that the antioxidant property of garlic could be modified by the processing methods. Moreover, in vitro and in vivo studies showed that garlic could suppress inflammation mainly through inhibition of various inflammatory biomarkers, including nitric oxide (NO), tumor necrosis factor (TNF)-α, and interleukin (IL)-1 [1].

The beneficial effects induced by garlic were also be related to its organosulfur compounds, including diallyl thiosulfonate (allicin), diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), E/Z-ajoene, S-allyl-cysteine (SAC), as well as S-allyl-cysteine sulfoxide (alliin) [1]. In particular, allicin was reported to be among the most important bioactive constituents exerting antioxidant effects [8].

Inflammatory bowel diseases (IBDs) are chronic and multifactorial inflammatory conditions of the colonic mucosa (ulcerative colitis), characterized by increased and unbalanced immune response to external stimuli [9–11]. In this context, antioxidant/anti-inflammatory herbal extracts were found to contrast IBD-related symptoms [12,13] by reducing various pro-inflammatory and oxidative biomarkers, such as reactive oxygen/nitrogen (ROS/RNS) species, prostaglandins, and cytokines.

Interestingly, garlic was suggested to exert protective effects against ulcerative colitis [14]. In addition, garlic oil suppressed endotoxin-induced neutrophil infiltration in small intestine of rats [15]. Moreover, Balaha and collaborators (2016) [16] showed that garlic oil (GO) was able to inhibit ulcerative colitis induced by dextran sulfate sodium in rats. This effect was suggested to be related to antioxidant, anti-inflammatory as well as to immunomodulatory effects of GO. Accordingly, more recently, GO was reported to decrease inflammation and cellular damage in a rat model of colitis induced by acetic acid [17].

On the basis of these findings, in this study, we aimed to evaluate the potential protective effects of a hydroalcoholic extract (GHE) and a water extract (GWE) of garlic on isolated mouse colon specimens treated ex vivo with *E. coli* lipopolysaccharide (LPS), which represents an experimental model of ulcerative colitis [18]. In particular, our research focused on hydroalcoholic and water extracts from a Sicilian variety of garlic (Nubia red garlic), known for the intense red color of the robes of its bulbils [19]. This variant is a protected denomination of origin product (DOP), which is appreciated all over the world. We reported here in, to the best of our knowledge, the first potential beneficial effects of Nubia red garlic on colon inflammation.

2. Materials and Methods

2.1. Preparation of Garlic Extracts

Garlic cloves were supplied as dried powder by il Grappolo S.r.l. (Soliera, Modena, Italy). Plant sample (1 g) was mixed with either a solution of ethanol–water (20:80, *v/v*) or water (final concentration = 1 g/mL), as previously reported [20–22]. The supernatant was filtered and then dried (freeze-drying). The dry residue, a yellow sugary solid, was stored at 4 °C, until chemical analyses were performed.

2.2. High Performance Liquid Chromatography (HPLC)–Diode Array (DAD)–Mass Spectrometry (MS) Analysis

Selected phenolic compounds contained in the extracts were identified and quantified by HPLC–DAD–MS analysis. The HPLC apparatus consisted of a two PU-2080 PLUS chromatographic pump, a DG-2080-54 line degasser, a mix-2080-32 mixer, UV, diode array (DAD) and detectors, a mass spectrometer (MS) detector (expression compact mass spectrometer (CMS), Advion, Ithaca, NY, USA), an AS-2057 PLUS autosampler, and a CO-2060 PLUS column thermostat (all from Jasco, Tokyo, Japan). Integration was conducted through ChromNAV2 Chromatography software. The separation was performed on an Infinity lab Poroshell 120-SB reverse phase column (C18, 150 × 4.6 mm i.d., 2.7 μm) (Agilent,

Santa Clara, CA, USA). Column temperature was set at 30 °C. The separation was conducted within 60 min of the chromatographic run, starting from the following separation conditions: 97% water with 0.1% formic acid, 3% methanol with 0.1% formic acid, as previously described [23]. Quantitative determination of phenolic compounds was conducted through a DAD detector. Qualitative analysis of GHE and GWE was performed by an MS detector in the positive and negative ion modes. MS signal identification was performed by comparison with a standard solution and MS spectra available in the MassBank Europe database (https://massbank.eu/MassBank/ (accessed on 11 November 2021). All HPLC grade solvents were purchased from Merck Science Life S.r.l. (Milan, Italy). Each analysis was performed in triplicate. The detailed protocol is enclosed as supplementary materials.

2.3. Headspace Solid-Phase Microextraction-Gas Chromatography–Mass Spectrometry (HS–SPME–GC–MS) Analysis

The 4 mL vials were loaded with 50 mg of the garlic dry residue. According to the extraction parameters optimized in our previous screening study performed on the garlic powder [15], the sample was allowed to equilibrate at 80 °C for 20 min and then the SPME fiber DVB-CAR-PDMS (Sigma Aldrich, now Merck KGaA, Darmstadt, Germany) was exposed to the head space of the vial for 20 min at 80 °C.

After sampling, fiber was withdrawn in the needle and exposed into the GC inlet at 260 °C for 0.5 min. The desorbed analytes were introduced in a gas chromatograph (6850, Agilent Technologies, Santa Clara, CA, USA) coupled with a mass spectrometer (5975, Agilent Technologies, Santa Clara, CA, USA), equipped with the non-polar capillary column HP-5MS (30 m × 0.25 mm inner diameter, and film thickness 0.25 μm). The gas-chromatographic parameters were set as follows: inlet temperature, 260 °C; injection mode, splitless (splitter valve was opened after 0.2 min and split ratio = 20/1); flow rate of the helium carrier gas (99.995% purity), 1.0 mL/min; oven temperature starting from 40 °C, after 5 min raised to 200 °C at 5 °C/min, and kept at this final temperature for 60 min. Mass spectrometry parameters were set as follows: EI energy, 70 eV; source temperature, 230 °C; quadrupole temperature, 150 °C; and mass scan was carried out over the 50–350 m/z range. The analyses were performed in triplicate.

Two analytical criteria were used to allow the identification of the eluted compounds, namely the comparison between the EI experimental spectra and those collected in the commercial (FFNSC 3) and free access databases (NIST 11, Flavor2) and the Kovats index (KI) measured using a mixture of *n*-alkanes (C7–C40) with the same chromatographic conditions, and then compared with values reported in the FFNSC 3 and NIST 11 databases. A manual integration of chromatographic peaks with a S/N ratio above 3 was performed without any further modification.

2.4. Positive-Ion Direct Infusion–Electrospray Ionization–Mass Spectrometry (DI–ESI–MS) Analysis

The dry residue of garlic was dissolved in H_2O:MeOH (7:3) to a final concentration of 20 μg/mL and directly infused at 10 μL min^{-1} in the ESI source of a LTQ XL linear ion trap (Thermo Fisher Scientific, Waltman, MA, USA). The source parameters were set as follows: source voltage = 4.5 kV; capillary voltage = 15 V; capillary temperature = 300 °C; tube lens voltage 89.9 V, sheath gas flow rate 10 (arbitrary units). Each spectrum, acquired over the 170–2000 m/z range, was from averaging 10 full scans, each one consisting of 5 micro scans. The major peaks were isolated and submitted to MS tandem experiments to allow the compound identification by comparing their relevant MS/MS spectra with those reported in literature or collected in a free access database (https://massbank.eu/MassBank/Search (accessed on 19 May 2022). The precursor ion isolation width was 1–2 Da and the normalized collision energy was set to the value needed to reduce the intensity of the precursor ion to approximately 10%.

2.5. Colorimetric Analysis

Colorimetric analysis of garlic powder (GP) sample and GWE was performed by X-Rite MetaVueTM® (Prato, Italy) as previously described [15]. GP sample and GWE analysis was conducted at the time of delivery (t°) and after 12 months (t^{12m}) of storage in the darkness at room temperature (25 ± 2 °C). The detailed description of colorimetric analysis is reported in the Supplementary Materials section.

2.6. HPLC–DAD Analysis

GWE was weighed, dissolved in water and filtered before injection into a HPLC Perkin Elmer apparatus (Series 200 LC pump, Series 200 DAD and Series 200 autosampler, Milan, Italy). Chromatography was conducted on RP-18 column (3 μ) using a linear gradient consisting of acetonitrile and acidified water (5% formic acid), from 100% aqueous phase to 85% in 15 min, 85 to 55% in 30′ and 55 to 40% in 20′, at a flow rate of 0.8 mL/min, at 254 nm. Alliin was quantified as previously described [22] (y = 6.35x + 50.34; R2 = 0.9987, in the range between 2 and 400 μg/mL, LOD 0.6 μg/g and LOQ 2.0 μg/g extract in dry weight). Each analysis was performed in quadruplicate. The detailed protocol related to HPLC-DAD analysis is described in the Supplementary Materials section.

2.7. Cell Lines

Colorectal cancer cell line SW480 was cultured in RPMI1640 (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 1% Pen/Strep and 1% L-glutamine, and maintained in a humidified incubator at 37 °C, 5% CO_2.

2.8. Cell Viability Assay

Evaluation of cell viability was performed by MTT assay [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (Sigma, St. Louis, MO, USA) as previously reported [24]. SW480 cell line was seeded in 96-well plates (5×10^3 cells/well) and was treated the following day with GHE (1, 10, and 100 μg/mL), GWE (1, 10, and 100 μg/mL) or vehicle (culture medium). Each analysis was performed in triplicate.

2.9. Apoptosis Assay

To evaluate apoptosis, BD Pharmingen™ APC Annexin V antibody (BD, Becton-Dickinson Biosciences, San Jose, CA, USA) and propidium iodide (PI) (Sigma, St. Louis, MO, USA) were used according to the manufacturer's instructions, essentially as previously described [25]. Each analysis was performed in triplicate

2.10. Ex Vivo Studies

Adult C57/BL6 male mice (3-month-old, weight 20–25 g) were housed in Plexiglas cages (2–4 animals per cage; 55 cm × 33 cm × 19 cm) and maintained under standard laboratory conditions. Experimentation procedures were in agreement with the European Community ethical regulations (EU Directive no. 26/2014) on the care of animals for scientific research and approved by local ethical committee ('G. d'Annunzio' University, Chieti, Italy) and Italian Health Ministry (Project no. 885/2018-PR).

After collection, isolated colon specimens were maintained in a humidified incubator with 5% CO_2 at 37 °C for 4 h (incubation period) in RPMI buffer with added bacterial LPS (10 μg/mL) [26,27] and treated with either GHE or GWE (1, 10, and 100 μg/mL). Prostaglandin (PG) E_2 and 8-iso-$PGF_{2\alpha}$ levels (pg/mg wet tissue) were measured by radioimmunoassay (RIA) in tissue supernatants [28,29]. Each analysis was performed in triplicate.

Extraction of total RNA from colon specimens was performed using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (ThermoFischer Scientific, Waltman, MA, USA). Gene expression of cyclooxygenase (COX)-2, TNF-α, nuclear factor-*k*B (NF-*k*B), IL-6, and nuclear factor erythroid 2-related

factor 2 (Nrf2) was measured by quantitative real-time PCR using TaqMan probe-based chemistry [30,31]. The real-time PCR was performed in triplicate. Relative quantification of gene expression was conducted through the comparative $2^{-\Delta\Delta Ct}$ method [32]. The detailed description of real-time PCR is reported in the Supplementary Materials section.

Extraction of serotonin (5-HT), and 5-hydroxyindolacetic acid (5HIIA) was performed from individual colon specimens homogenized in perchloric acid solution (50 mM). Analysis of colon 5-HT, and 5HIIA levels was performed through high performance liquid chromatography coupled to electrochemical detection consisting of ESA Coulochem III detector equipped with ESA 5014B analytical cell [33,34]. Each analysis was performed in triplicate.

2.11. Statistical Analysis

Analysis of the data was performed by using the software GraphPad Prism version 6.0 (Graphpad Software Inc., San Diego, CA, USA). Means ± SEM were assessed for each experimental group and analyzed by one-way analysis of variance (ANOVA), followed by the Newman–Keuls multiple comparison post hoc test. As for quantification of the investigated phenolic compounds detected in GHE and GWE, analysis of the data was conducted by unpaired t test (two-tailed p value). The limit of statistically significant differences between mean values was set at p-value < 0.05. Calculation of the number of animals randomized for each experimental group was performed by using the "Resource Equation" $n = (E + T)/T$ ($10 \leq E \leq 20$) [35].

3. Results and Discussion

3.1. Phytochemical Analyses

3.1.1. HPLC–DAD–MS Analysis

Both garlic extracts were analyzed for the measurement of the levels of selected flavonoids and phenols by quantitative HPLC–DAD–MS analysis. The list of the polyphenolic compounds studied, as well as the retention time, wavelength, and the m/z ratio for their determination are reported in Table S2. Quantification was performed as previously reported [23]. The characterization of the investigated phenolic compounds is reported in Table 1. A variety of factors influence composition of garlic extracts, such as source of garlic strain, conditions of storage, processing type, and aging [36,37]. The biological activity of garlic components has also been reported to be dependent on extraction, temperature, preparation, and storage [38]. In addition, the data are not easily comparable with literature, being chiefly related to the garlic clove and garlic powder rather than to the separated parts [39,40]. The chromatographic analysis of GHE and GWE confirmed the presence of 19 and 18 phytochemicals, respectively (Figure 1 and Table 1). In the present study, the prominent compound found in GHE was catechin, while the prominent compounds found in GWE were catechin and benzoic acid. Gallic acid, chlorogenic acid, *p*-coumaric acid, *t*-ferulic acid, benzoic acid, resveratrol, naringenin, hesperetin, and flavone were present at concentrations ranging from 22.66 to 13.33 μg/mL in GHE, and from 12.73 to 21.51 μg/mL in GWE. On the other hand, concentrations of 3-hydroxytyrosol, caffeic acid, epicatechin, syringaldehyde, *p*-coumaric acid, *t*-cinnamic acid, quercetin, and 3-hydroxyflavone ranged from 8.94 to 11.83 μg/mL in GHE, and from 7.23 to 11.59 μg/mL in GWE. Caftaric acid was detected only in GHE, at very lower concentration (0.93 μg/mL). Interestingly, GHE was richer than GWE in catechin, epicatechin, *t*-ferulic acid, benzoic acid, resveratrol, quercetin, naringenin, and hesperetin. On the other hand, GWE showed higher levels of 3-hydroxytyrosol and 3-hyroxyflavone as compared to GHE.

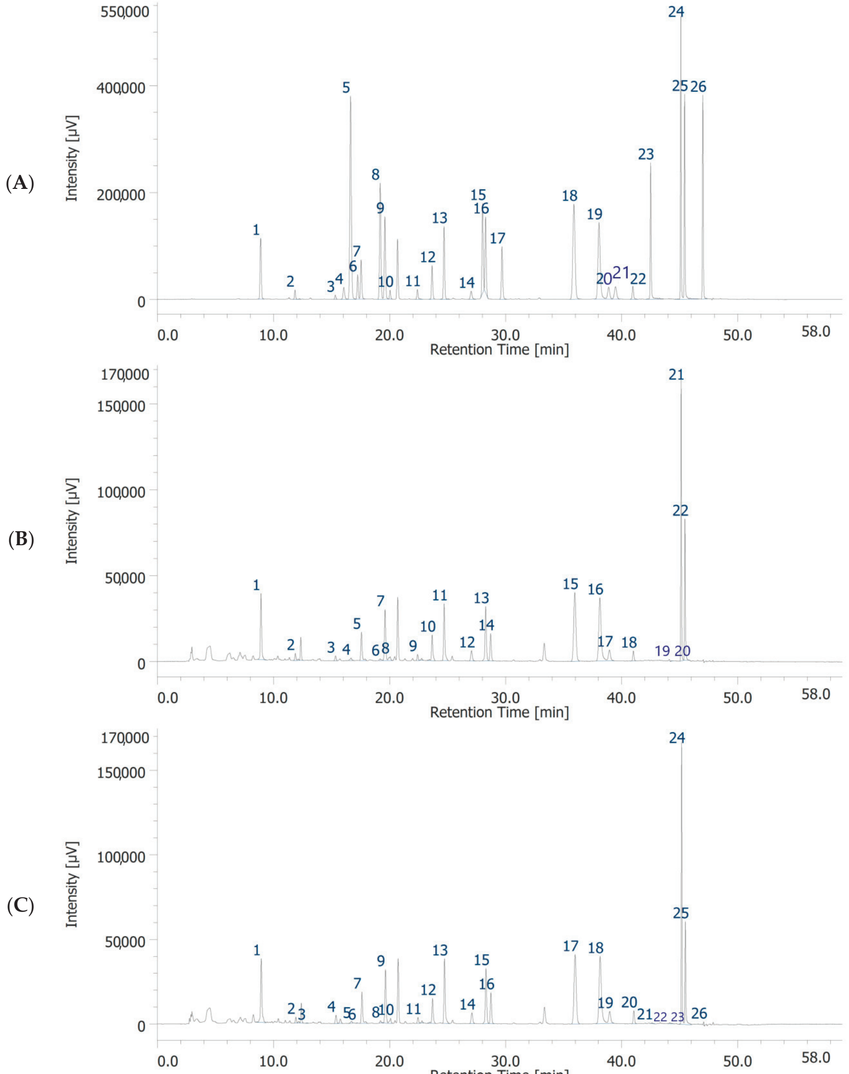

Figure 1. HPLC-DAD chromatograms of standards solution (**A**), garlic water extract (GWE) (**B**) and garlic hydroalcoholic extract (GHE) (**C**). Chromatographic analysis of GWE (**B**). The chromatographic analysis showed the presence of 18 phytochemicals: gallic acid (peak #1), 3-hydroxytyrosol (peak #2), catechin (peak #3), chlorogenic acid (peak #5), caffeic acid (peak #7), epicatechin (peak #8), syringaldehyde (peak #9), p-coumaric acid (peak #10), t-ferulic acid (peak #11), benzoic acid (peak #12), rutin (peak #13), resveratrol (peak #14), t-cinnamic acid (peak #15), quercetin (peak #16), naringenin (peak #17), hesperetin (peak #18), flavone (peak #21), 3-hydroxyflavone (peak #22). Chromatographic analysis of GHE (C). The chromatographic analysis showed the presence of 19 phytochemicals: gallic acid (peak #1), 3-hydroxytyrosol (peak #2), caftaric acid (peak #3), catechin (peak #4), chlorogenic acid (peak #7), caffeic acid (peak #9), epicatechin (peak #10), syringaldehyde (peak #11), p-coumaric acid (peak #12), t-ferulic acid (peak #13), benzoic acid (peak #14), rutin (peak #15), resveratrol (peak #16), t-cinnamic acid (peak #17), quercetin (peak #18), naringenin (peak #19), hesperetin (peak #20), flavone (peak #24), 3-hydroxyflavone (peak #25).

Table 1. Quantification of the investigated phenolic compounds detected in garlic hydroalcoholic extract (GHE) and garlic water extract (GWE) (μg/mL).

Compound	GHE	GWE
Gallic acid	17.21 ± 0.17	16.88 ± 0.12
3-Hydroxytyrosol	10.13 ± 0.15 ***	12.86 ± 0.22
Caftaric acid	0.93 ± 0.06	n.d.
Catechin	37.40 ± 1.25 ***	21.38 ± 0.72
4-Hydroxybenzoic acid	n.d.	n.d.
Loganic acid	n.d.	n.d.
Chlorogenic acid	13.54 ± 0.72	12.14 ± 0.53
Vanillic acid	n.d.	n.d.
Caffeic acid	10.91 ± 0.95	10.19 ± 0.58
Epicatechin	8.94 ± 0.23 *	7.23 ± 0.40
Syringaldehyde	9.53 ± 0.12	9.54 ± 0.23
p-Coumaric acid	13.33 ± 0.17	12.73 ± 0.15
t-Ferulic acid	15.82 ± 0.26 **	13.43 ± 0.21
Benzoic acid	22.66 ± 0.35 *	21.51 ± 0.18
Rutin	13.68 ± 0.22	13.08 ± 0.23
Resveratrol	16.18 ± 0.27 **	13.97 ± 0.15
t-Cinnamic acid	11.83 ± 0.09	11.59 ± 0.12
Quercetin	15.86 ± 0.20 *	14.76 ± 0.21
Naringenin	17.93 ± 0.21 *	16.02 ± 0.37
Hesperetin	17.06 ± 0.58 **	12.72 ± 0.31
Kaempferol	n.d.	n.d.
Carvacrol	n.d.	n.d.
Thymol	n.d.	n.d.
Flavone	20.43 ± 0.15	19.83 ± 0.19
3-Hydroxyflavone	10.27 ± 0.29 **	13.46 ± 0.25
Emodin	n.d.	n.d.

Values expressed are the means ± S.D. of three parallel measurements. n.d., not detected. *t*-test, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. vehicle.

3.1.2. HS–SPME–GC–MS and DI–ESI–MS Analysis

The compounds identified through the HS–SPME–GC–MS were clustered in three main classes (Table 2), namely the monoterpenes and their oxygen derivatives (61.2%), the sulfur-containing-compounds (SCC, 22.8%) and finally the aldehydes (3%). Thymol and carvacrol, two monoterpenoids, are the most abundant species (22.4% and 36.1%, respectively), followed by allyl disulfide (9.52%) and the 3-vinyl-1,2-dithiacyclohexene isomers (7.14%) belonging to the SSC class.

The DI–ESI–MS full spectrum was dominated by the regular repetition of two monocharged species differing by 16 mass units (Figure 2), that suggests the presence of species complexing Na^+ and K^+ cations. The 162 Da intervals correspond to the difference of one -$C_6H_{10}O_5$- unit, typical of a homologous series of polysaccharides. The MS/MS spectrum of the 1029 *m/z* ion, reported in Figure 3 as representative ion of the series, showed the sequential loss of 162 Da (=$C_6H_{10}O_5$) and 18 Da (=H_2O) according to the MS2 fragmentation of a polysaccharide. The detected oligosaccharides, most probably fructans [41], are characterized by a wide degree of polymerization (DP), namely in the 2-11 DP range (Table 3). The Na^+ and K^+ ions distribution emerging from the fructans series indicates a larger content of the latter, despite what one should expect considering the ubiquitous presence of Na^+ cation in the ESI–MS experiments. In addition to the fructans, the full scan showed two intense signals at m/z 175 and 214, corresponding to the protonated L-arginine and to N-butylbenzene sulfonamide (a plasticizer contaminant), respectively (Table 3).

Table 2. HS–SPME–GC–MS analysis of dry residue of garlic water extract (GWE).

Compound	Class	Area%	RI	RI_L [a]
2-Propenal [b]	Aldehyde	1.89		
Heptane [b]	Other	0.33		
Diallyl sulfide	SCC	0.43	864	857
Methyl allyl disulfide	SCC	1.47	927	919
Methoxy phenyl oxime [b]	Other	5.72	943	
p-cymene	Monoterpene	0.47	1041	1025
Allyl disulfide	SCC	9.52	1088	1090
3-Allyl-thio-propionic acid	SCC	1.70	1107	1093
Linalyl anthranilate	Monoterpenoid	0.95	1110	1104
Nonanal	Aldehyde	1.16	1113	1102
Methyl 2-propenyl trisulfide	SCC	2.51	1146	1142
Isoborneol	Monoterpenoid	0.44	1178	1165
Terpinen-4-ol	Monoterpenoid	0.55	1187	1184
3-Vinyl-1,2-dithiacyclohex-4-ene	SCC	3.18	1195	1191
Alpha-terpineol	Monoterpenoid	0.27	1203	1207
Decanal	Aldehyde	0.63	1214	1208
3-Vinyl-1,2-dithiacyclohex-5-ene	SCC	3.96	1222	1224
Thymol	Monoterpenoid	22.4	1310	1293
Carvacrol	Monoterpenoid	36.1	1319	1317
7 unknown compounds		6.32		
Class				
Monoterpene and derivatives		61.2		
SCC		22.8		
Aldehyde		3.68		
Other		6.05		

[a] RI reported in literature; [b] MS-only identification method.

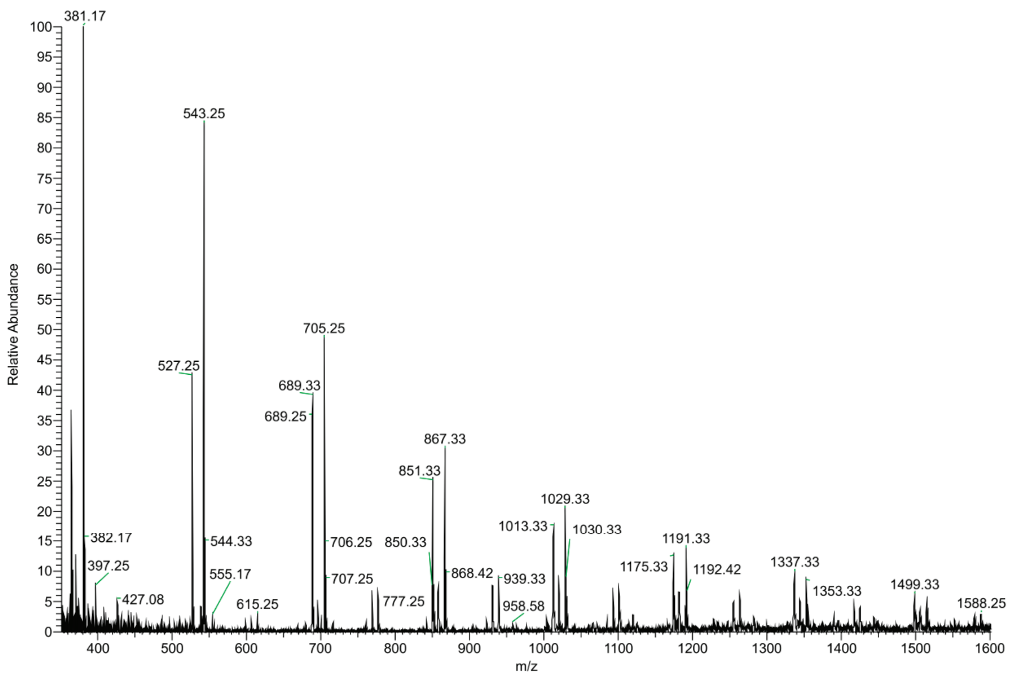

Figure 2. (+) ESI–MS full scan of the dry residue of garlic water extract (GWE).

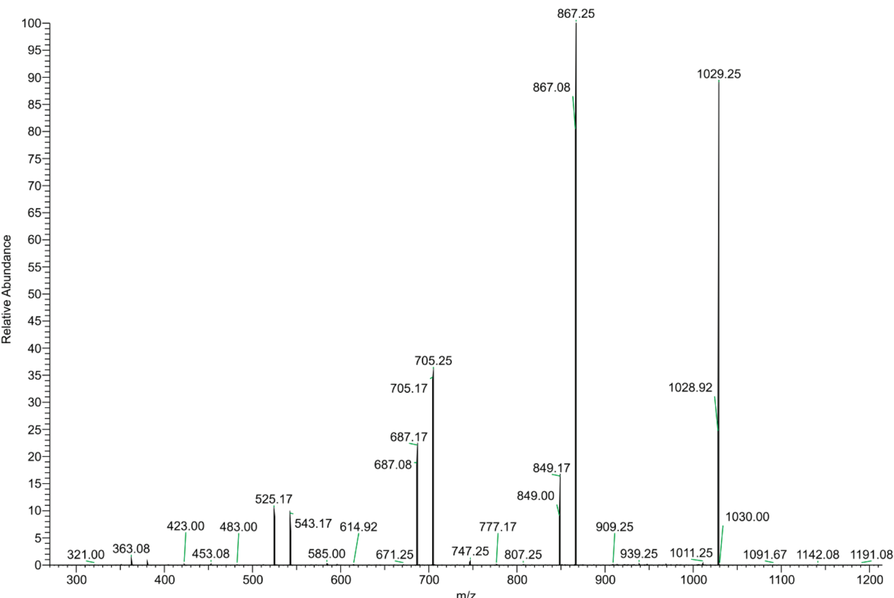

Figure 3. MS/MS spectrum of the *m/z* 1029 positively charged ion.

Table 3. DI–ESI–MS analysis of dry residue of garlic water extract (GWE).

m/z	Compound		Charge
	Positive mode		
163	Allicin		H^+
175	Arginine		H^+
214	N-butylbenzene sulfonamide		H^+
		DP	
365	Disaccharide		Na^+
381	Disaccharide		K^+
527	Trisaccharide	3	Na^+
543	Trisaccharide	3	K^+
689	Oligosaccharide	4	Na^+
705	Oligosaccharide	4	K^+
851	Oligosaccharide	5	Na^+
867	Oligosaccharide	5	K^+
1013	Oligosaccharide	6	Na^+
1029	Oligosaccharide	6	K^+
1175	Oligosaccharide	7	Na^+
1191	Oligosaccharide	7	K^+
1337	Oligosaccharide	8	Na^+
1353	Oligosaccharide	8	K^+
1449	Oligosaccharide	9	Na^+
1515	Oligosaccharide	9	K^+
1661	Oligosaccharide	10	Na^+
1677	Oligosaccharide	10	K^+
1823	Oligosaccharide	11	Na^+
1839	Oligosaccharide	11	K^+
	Negative mode		
191	Citric acid		$[M-H]^-$
289	Catechin		$[M-H]^-$

DP: degree of polymerization.

3.1.3. Colorimetric Analysis

The color parameters and relative reflectance curves of the analyzed samples are shown in Table 4 and Figure 4.

Table 4. Colorimetric CIEL*a*b* parameters of garlic powder (GP) and garlic water extract (GWE).

	L*	a*	b*	c*	h°	DE°
GP t°	90.15	0.47	16.02	16.03	88.32	-
GP t^{12m}	78.55	1.11	20.17	20.20	86.85	12.34 [a]
GWE t°	76.41	−1.10	16.83	16.87	93.74	13.85 [a]
GWE t^{12m}	65.00	−2.87	28.00	27.95	95.90	28.06 [a]–16.06 [b]

Reported values represent the mean of four measurements. Mean error < 2%. ΔE represents the overall color variation, [a] using GP t° as reference, and [b] using GWE t° as reference $\Delta E = [(L^*_2-L^*_1)^2 + (a^*_2-a^*_1)^2 + (b^*_2-b^*_1)^2]^{\frac{1}{2}}$.

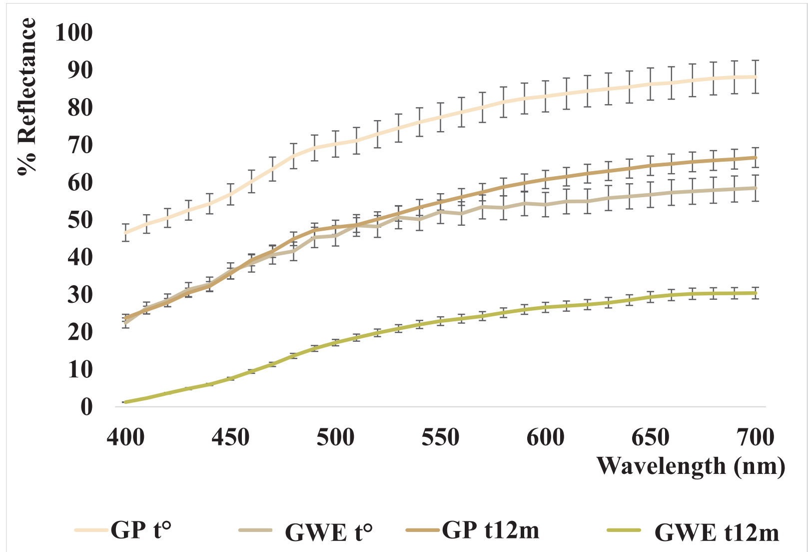

Figure 4. Reflectance curves of the analyzed garlic samples.

As reported in our previous work [22], the garlic powder analyzed at t° has a very bright color tending to pale yellow (L* = 90.15 and b* = 16.02) that, after eight months of storage, seemed to fade slightly (L* ≈ 94 vs. 90 at t^0, b* ≈ 14 vs. 16 at t^0, with a ΔE ≈ 2, data previously reported). In this study, the analyses performed at t^{12m}, showed a substantial change in color (ΔE = 12.34), characterized by a strong darkening of the powder (L* ≈ 78; b* ≈ 20), as also shown by the lowering of the reflectance curve reported in Figure 4. The initial light bleaching and subsequent browning were already described in milk powder samples in Cesa and collaborators (2015) [42], and interpreted as carotenoid degradation followed by Maillard reaction. In the case of garlic, bleaching was not previously described. Nevertheless, the presence of carotenoids in *Allium* spp. phytocomplex was reported in the literature [43] and the carotenoid bleaching seems the simplest hypothesis, despite all our attempts to extract a carotenoid fraction by organic solvents failed. Therefore, the slight bleaching should be attributed to other yellow, hydrosoluble, components. On the other hand, the powder darkening could be mainly due to the different sulfur components (the presence of many different sulfur compounds was confirmed by HS–SPME–GC–MS analysis (see Section 3.1.2)) which, modifying the pH and the activity of polyphenol oxidases, with a not completely known mechanism depending on the state of preservation and on the temperature, cause darkening of the matrix [44,45].

This darkening is also confirmed in the aqueous extract newly prepared and analyzed after 12 months (see Figure 4). In fact, the L* parameter of GWE t° decreases significantly from 76 to 65, as well as the b* parameter increases even more, from 17 to 28, denoting altogether a strong color change characterized by a substantial browning with respect to the starting points (ΔE = 28 respect to G_P t° and ΔE = 16 respect to GWE t°).

3.1.4. HPLC–DAD Analysis

The HPLC–DAD analysis was conducted at 254 nm for the identifying benzoic and hydroxycinnamic acids, flavanols and organosulfur compounds. The molecular profile is confirmed by literature even if no chromatograms of aqueous extracts are reported [46,47].

The chromatogram showed the presence of alliin and an its diastereoisomer (361.1 ± 17.3 μg/g dry extract) as reported by Dethier et al., 2012 [48]. Furthermore, chlorogenic acid, caffeic acid, and epicatechin (see Section 3.1.1 for the relevant quantification) were also shown.

These results are only partially comparable to those present in the literature, because the garlic phytocomplex and relative aqueous extracts are very variable according to the different cultivar, geographic area, and storage conditions. There are no references in the literature on quantitative data related to garlic aqueous extracts. Existing data refer to methanolic or hydroalcoholic extracts reporting alliin content in range of 0.5–33 expressed in mg/g dry weight [47,49]. Compared to the hydroalcoholic extract, reported in our previous work [22], alliin values found in GWE are significantly lower (approximately 360 vs. 1200 μg/g dry extract).

3.2. Toxicological and Pharmacological Studies

In a previous study of ours, GHE (1–100 μg/mL) was not able to modify cell viability of H9c2 cells (rat cardiomyoblasts), in basal conditions, confirming its good biocompatibility [22]. Moreover, the same extract (10–100 μg/mL) was effective in protecting cells from cytotoxicity induced by H_2O_2 (200 μM) [22]. In the present study, we investigated the effects of GHE and GWE, in the dose range 1–100 μg/mL, on colon cancer SW480 cell line viability, in basal conditions. Compared to the control group, GHE was not able to affect SW480 cell viability (Figure 5). However, GWE (100 μg/mL) significantly suppressed SW480 cell viability, even if the cell viability was not under the biocompatibility limits (70% viability compared to control, respectively) (Figure 5).

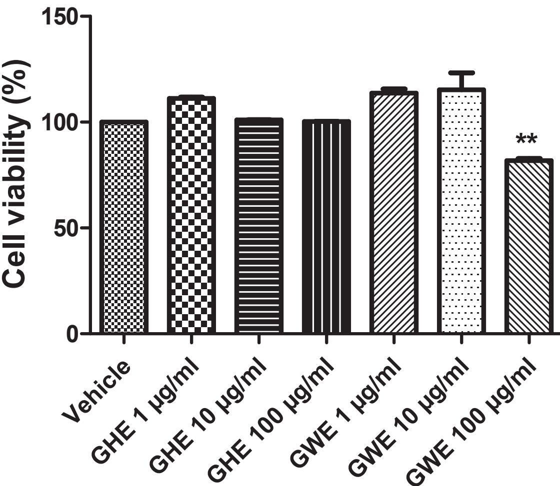

Figure 5. MTT assay of SW480 cell line treated with garlic hydroalcoholic extract (GHE) (1, 10, and 100 μg/mL), garlic water extract (GWE) (1, 10, and 100 μg/mL), and vehicle (RPMI) for 48 h, in basal conditions. Data are displayed as the means ± SEM. ANOVA, $p < 0.001$; ** $p < 0.01$ vs. vehicle.

Accordingly, both GHE and GWE were not able to modify apoptosis of SW480 cell lines following 48 h of treatment in basal conditions (Figure 6).

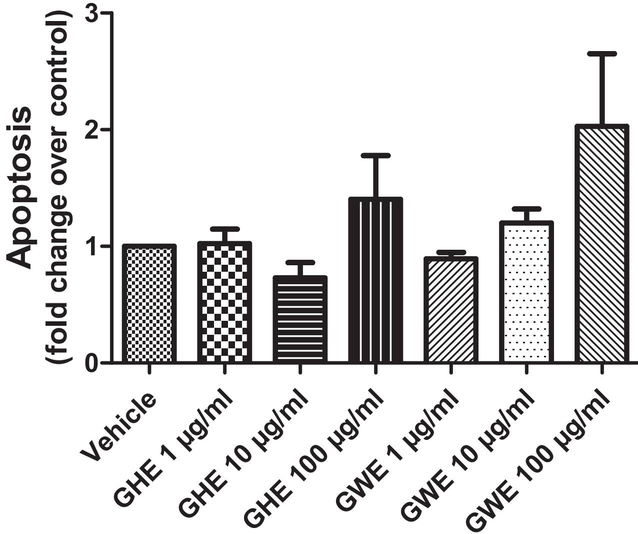

Figure 6. Apoptosis assay in SW480 cell line treated with garlic hydroalcoholic extract (GHE) (1, 10, and 100 µg/mL), garlic water extract (GWE) (1, 10, and 100 µg/mL), and vehicle (RPMI) for 48 h, in basal conditions.

GHE and GWE, in the dose range 1–100 µg/mL, were then tested to evaluate their potential protective activities on oxidative and inflammation pathways in mouse colon specimens treated with LPS. In particular, GHE, in the tested dose range, was effective in suppressing LPS-induced gene expression of pro-inflammatory markers strongly involved in colon inflammation, including COX-2, TNF-α, NF-κB, and IL-6 (Figure 7A–E) [50–53]. In the same experimental paradigm, GWE (1–100 µg/mL) inhibited LPS-induced TNF-α, NF-κB, and IL-6 (Figure 7A–E) gene expression. Moreover, the higher concentrations of GWE suppressed COX-2 (Figure 7A) gene expression induced by LPS treatment. On the other hand, GHE and GWE did not modify LPS-induced Nrf2 gene expression (Figure 7E), ruling out a possible role of this mediator in mediating the protective effects exerted by GWE and GHE in mouse colon.

Accordingly, Hodge and collaborators (2002) [54] showed that, in peripheral blood monocytes, production of various leukocyte pro-inflammatory cytokines, such as IL-6, IL-8 and TNF-α, was reduced by a garlic extract (≥10.0 µg/mL). On the other hand, it also stimulated IL-10 synthesis in the same experimental model. In this context, the same authors suggested a possible therapeutic use of garlic in the management of inflammatory conditions, including inflammatory bowel disease [54]. Our present findings also agree with those by Shin and collaborators (2013) [55], who observed inhibitory effects induced by fresh and heated raw garlic extracts (FRGE and HRGE) on LPS-induced release of pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-6, in RAW 264.7 macrophages. Moreover, we investigated the effects of the extracts on LPS-induced levels of PGE_2, a pro-inflammatory mediator generated by COX-2, which is strongly involved in the pathophysiology of inflammatory bowel disease [56]. Our present results showed that both GHE (1–100 µg/mL) and GWE (10–100 µg/mL) suppressed LPS-induced PGE_2 levels in isolated colon specimens (Figure 8), further supporting the anti-inflammatory effects exerted by these extracts.

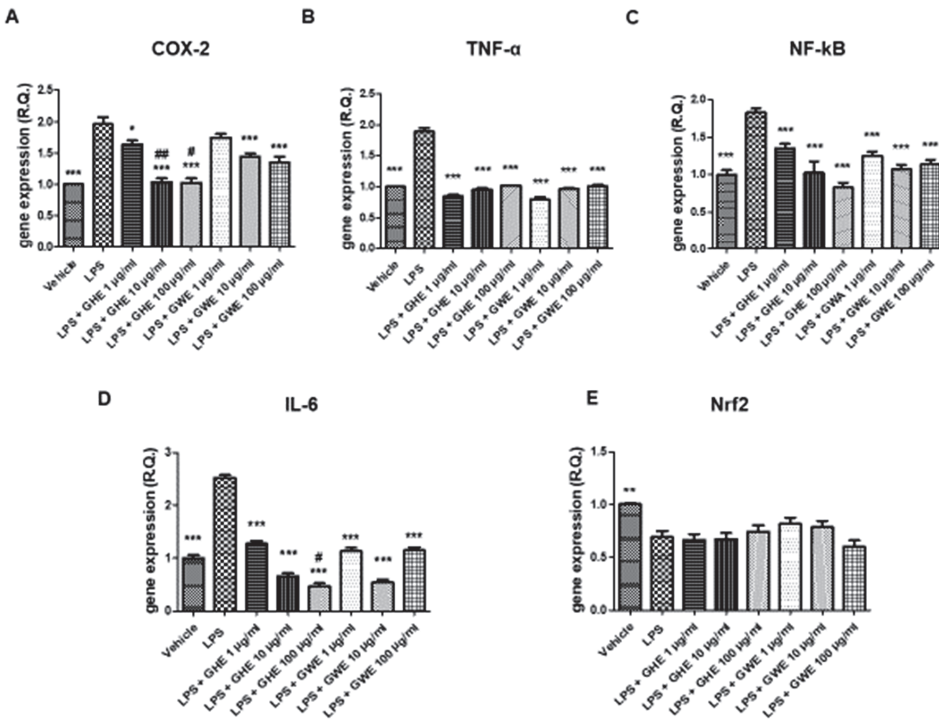

Figure 7. Effects of garlic hydroalcoholic extract (GHE) (1, 10, and 100 μg/mL), garlic water extract (GWE) (1, 10, and 100 μg/mL) and vehicle (RPMI) on LPS-induced cyclooxygenase (COX)-2 (**A**), tumor necrosis factor (TNF)-α (**B**) nuclear factor-kB (NF-kB (**C**)), interleukin (IL)-6 (**D**), nuclear factor erythroid 2-related factor 2 (Nrf2) (**E**) gene expression (RQ, relative quantification) in mouse colon specimens. Data are displayed as the means ± SEM. ANOVA, $p < 0.01$; * $p < 0.05$, ** $p < 0.01$ vs. LPS, and *** $p < 0.001$ vs. LPS; $^{\#}$ $p < 0.05$ vs. LPS+GWE 100 μg/mL; $^{\#\#}$ $p < 0.01$ vs. GWE 10 μg/mL.

Accordingly, we previously reported that GWE exerted cardioprotective activities, by suppressing LPS-induced COX-2, IL-6, and NF-kB gene expression, as well as PGE_2 levels, in heart specimens. To this regard, we hypothesized that the suppression of NF-*k*B gene expression could be involved in modulating the inhibitory effects induced by GWE on COX-2 gene expression and PGE_2 production [22]. Accordingly, ethyl linoleate from garlic was reported to suppress LPS-induced COX-2 mRNA and protein expression as well as PGE_2 production in RAW264.7 cells, by suppressing NF-*k*B activation [57]. Actually, the anti-inflammatory effects exerted by GHE and GWE could be related, at least in part, to their phenol and flavonoid content [58–67], with particular regards to catechin [58]. To this regard, catechin was suggested to act as a potential therapeutic agent in the prevention of inflammation. In particular, catechin exhibited anti-inflammatory properties by suppressing inducible nitric oxide synthase (iNOS), and COX-2 protein expression, as well as IL-6 and TNF-α mRNA levels in LPS-treated RAW264.7 cells [68]. Interestingly, GHE was shown more effective than GWE in decreasing gene expression of COX-2, and IL-6 (Figure 7A,D), as well as PGE_2 levels (Figure 9) in isolated mouse colon specimens following LPS challenge. This finding could be related to its major content in catechin [69], resveratrol [70], naringenin [71], and hesperetin [72]. Accumulating evidence showed that 5-HT is a pro-inflammatory mediator critically involved in the pathogenesis of intestinal disorders, such as IBD [73–75], irritable bowel syndrome [73,76,77], as well as DSS-induced experimental colitis [78]. In the present study, we also investigated the effects of the garlic extracts on the LPS-induced 5HIIA/5-HT ratio, which is known as a

useful index of 5-HT turnover, in vivo [79,80], deeply related to the activity of monoamine oxidase (MAO)-A [81].

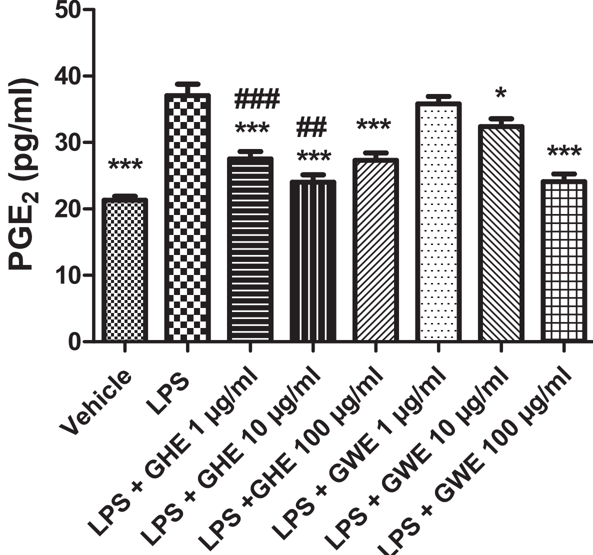

Figure 8. Effects of garlic hydroalcoholic extract (GHE) (1, 10, and 100 µg/mL), garlic water extract (GWE) (1, 10, and 100 µg/mL) and vehicle (RPMI) on LPS-induced prostaglandin E_2 (PGE_2) levels in mouse colon specimens. Data are shown as the means ± SEM. ANOVA, $p < 0.0001$; * $p < 0.05$, and *** $p < 0.001$ vs. LPS; ## $p < 0.01$, ### $p < 0.001$ vs. co-respective treatment with GWE.

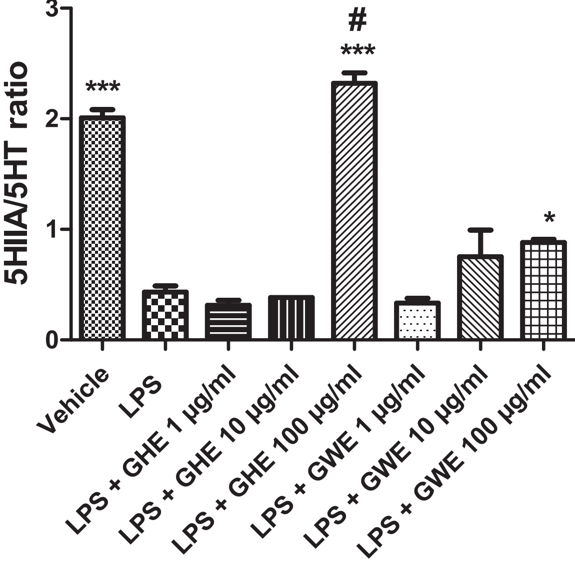

Figure 9. Effects of garlic hydroalcoholic extract (GHE) (1, 10, and 100 µg/mL), garlic water extract (GWE) (1, 10, and 100 µg/mL) and vehicle (RPMI) on the 5-HIIA/5-HT ratio in mouse colon specimens treated with vehicle. Data are displayed as the means ± SEM. ANOVA, $p < 0.0001$; * $p < 0.05$, and *** $p < 0.001$ vs. LPS; # $p < 0.001$ vs. LPS + GWE 100 µg/mL.

Consistently with the literature data [33], we found that LPS reduced the 5HIIA/5-HT ratio (Figure 9) in isolated mouse colon specimens.

However, GHE and GWE (100 μg/mL) prevented LPS-induced reduction in 5-HT turnover. The decreased levels of 5-HT, measured as the 5HIIA/5-HT ratio, could further account for the anti-inflammatory effects exerted by garlic extracts. To this regard, we have previously found that anti-inflammatory herbal extracts suppressed 5-HT levels in isolated rat colon treated with LPS [82,83]. In particular, GHE was found more effective than GWE in counteracting the LPS-induced decrease in the 5HIIA/5-HT ratio (Figure 9). Actually, the higher activity of GHE compared to GWE could be related to its higher content in benzoic acid and flavonoids, such as quercetin [84,85].

A wide body of evidence showed that imbalance between the oxidative reactions and antioxidant defense mechanisms played a key role in the initiation and progression of IBD. This imbalance generates oxidative stress resulting from either reactive oxygen species (ROS) overproduction or a reduction in antioxidant activity [86,87]. In particular, ROS overproduction was suggested to be involved in functional disruption of the enteric mucosa [52]. Increased production of ROS is known to damage cellular lipids, proteins, as well as nucleic acids, and finally disrupt gastrointestinal barrier integrity [88]. 8-iso-$PGF_{2\alpha}$ is an isomer of prostaglandins produced from membrane arachidonic acid by free radical-catalyzed peroxidation, which is regarded as a stable marker of lipid peroxidation and oxidative stress [89]. As shown in Figure 10, both GHE and GWE (1–100 μg/mL) were able to counteract 8-iso-$PGF_{2\alpha}$ levels induced by LPS treatment in isolated mouse colon specimens. In particular, GHE (1 μg/mL) showed higher efficacy in inhibiting 8-iso-$PGF_{2\alpha}$ levels (Figure 10).

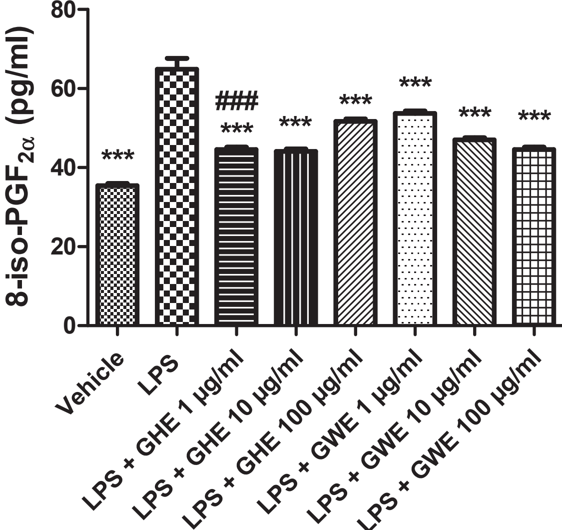

Figure 10. Effects of garlic hydroalcoholic extract (GHE) (1, 10, and 100 μg/mL), garlic water extract (GWE) (1, 10, and 100 μg/mL) and vehicle (RPMI) on LPS-induced 8-iso-prostaglandin (PG)$F_{2\alpha}$ levels in mouse colon specimens. Data are shown as the means ± SEM. ANOVA, $p < 0.0001$; *** $p < 0.001$ vs. LPS; ### $p < 0.001$ vs. co-respective treatment with GWE.

These results agreed with the antioxidant effects induced by GHE, tested in the same concentration range, in isolated mouse heart [22]. Accordingly, aqueous garlic extract was also shown to possess antioxidant properties by scavenging ROS and increasing cellular antioxidant enzymes including superoxide dismutase, catalase, and glutathione peroxidase [36]. The antioxidant effects induced by GHE and GWE are consistent with their polyphenol content [90], with particular regards to catechin [58]. In this context, catechins

were found able to reduce the colonic oxidative damages to the colon, by suppressing oxidative stress, via exerting direct or indirect antioxidant effects, and by enhancing the activity of various antioxidant enzymes including glutathione peroxidases [58].

4. Conclusions

Concluding, GHE and GWE, particularly GHE, showed protective effects, as confirmed by the inhibitory effects on selected pro-inflammatory and pro-oxidant markers, in LPS-stimulated colon, suggesting a potential role in the prevention and management of ulcerative colitis. The phytochemical analyses suggested these effects could be related, albeit partially, to their phenol and flavonoid content, with particular regards to catechin. Moreover, other components of nutraceutical and pharmaceutical interest were detected in these extracts. On the other hand, further studies are necessary to accurately evaluate the in vivo activity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods11223559/s1. Table S1: MS analysis conditions. Table S2: Retention times, wavelengths of quantification, mass to charge (m/z) ratios, and molecular weight of the investigated phenolic compounds. Table S3: Gradient elution conditions of the HPLC-DAD analyses for investigated polyphenolic compounds in garlic hydroalcoholic and water extracts (GHE and GWE).

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Article

Can Bioactive Compounds in Beetroot/Carrot Juice Have a Neuroprotective Effect? Morphological Studies of Neurons Immunoreactive to Calretinin of the Rat Hippocampus after Exposure to Cadmium

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Abstract: Cadmium ions (Cd^{2+}) penetrate the blood–brain barrier and can, among other effects, influence intracellular calcium metabolism, leading to neurodegeneration. In the presented work, we estimated the effect of Cd^{2+} on the expression of calretinin in the neurons of the rat hippocampus and analyzed the reverse effect of freshly pressed beetroot/carrot juice in this context. In the 12-week lasting experiment, 32 8-week-old male Wistar rats were divided into four experimental groups (n = 8): the control group (C) received pure tap water; the Cd group (Cd)—received Cd^{2+} dissolved in tap water (5 mg Cd^{2+}/kg b.w.); and two groups received beetroot/carrot juice: the BCJ group was administered only juice, and the Cd + BCJ group received juice with the addition of Cd^{2+} (5 mg Cd^{2+}/kg b.w.). The exposition to low doses of Cd^{2+} caused a significant decrease in calretinin-immunoreactive (Cr-IR) neurons compared to the non-exposed groups. Moreover, the addition of Cd^{2+} to tap water reduced the numbers and length of Cr-IR nerve fibers. The negative effect of Cd^{2+} was significantly attenuated by the simultaneous supplementation of beetroot/carrot juice (Cd + BCJ). The study showed that the bioactive compounds in the beetroot/carrot juice can modulate Ca^{2+} levels in neurons, and thus, potentially act as a neuroprotective factor against neuronal damage.

Keywords: cadmium; beetroot/carrot juice; calcium binding protein; calretinin; central nervous system; dementia

1. Introduction

Cadmium (Cd^{2+}) is a highly toxic heavy metal that is abundant in the environment. It enters the body through the respiratory or digestive tract, leading to excessive accumulation in internal organs, such as the kidneys, liver, or bones, increasing the risk of organ damage, osteoporosis, hypertension, or diabetes [1,2]. Cd^{2+} is also a known cause of neuroinflammation and dementia (including Alzheimer's disease), as essentially pointed out in several excellent reviews (e.g., by Huat et al. [3]; Zhang et al. [4]). It is estimated that a significant part of Cd^{2+} present in the bloodstream crosses the blood–brain barrier and accumulates in various structures of the central nervous system (CNS) [5]. Within the CNS, the hippocampus is the target structure for Cd^{2+} toxicity. The hippocampus proper is a part of the limbic system. It plays an important role in the consolidation of information from short-term memory to long-term memory and in spatial navigation. On the one hand, it is a specific center of the brain's memory and reasoning, and on the other, it is the area of the brain most affected by neurodegenerative diseases, of which dementia is the main

symptom [6]. Cd^{2+} can also influence intracellular calcium (Ca^{2+}) metabolism. Excessive accumulation of Ca^{2+} in nerve cells is responsible for the activation of processes that lead to neurodegenerative diseases, and ultimately to cell death [7]. Ca^{2+}-binding proteins (CaBPs) provide an important first line of defense due to their ability to buffer incoming calcium, allowing the neurons to rapidly attain homeostasis. Moreover, it is known that Cd^{2+} is a strong calcium channel blocker—it inhibits Ca^{2+} uptake by cells, which disrupts the transmission of neuronal signals [8]. Calretinin (Cr) is one of the three main types of CaBPs present in inhibitory GABAergic neurons. The important function of Cr is the regulation of Ca^{2+} flow; it participates in synaptic plasticity and also influences the excitability of interneurons in the hippocampus by regulating other GABAergic neurons [9,10]. Studies on another heavy metal, Pb^{2+}, have shown some promising effects in terms of the ability of beetroot juice to alleviate some of its toxic impact on the organism, particularly with regard to antioxidant and neurological functions [11–13].

The beet (*Beta vulgaris* L.) is a valuable source of unique natural compounds exerting antioxidant properties that may be beneficial to human health, as comprehensively presented in selected review papers, e.g., by [14] or [15]. It was confirmed that the consumption of beetroot may improve cerebral blood flow, and consequently, the cognitive function [16]. Beetroot extract showed anti-anxiety and antidepressant properties [17]. The carrot (*Daucus carota* L., *Apiaceae* family) is another highly appreciated, edible fleshy root, widely discussed in some excellent reviews, e.g., by Ahmad et al. [18] or Aćimović [19]. It is a source of α- and β-carotene, anthocyanins, lycopene, and phenolic acids [20], compounds known to have antioxidant properties (e.g., Mazewski et al. [21]). In our previous paper, we showed that beetroot/carrot juice was a rich source of bioactive compounds (mainly polyphenols, nitric pigments, and saponins). The juice revealed strong antioxidant activity, as confirmed using three experimental in vitro methods [22]. It has been reported before (e.g., in [3]), that Cd^{2+} can contribute to the development of Alzheimer's disease. Although the presented paper focused in the first place on the ability of the juice to reduce the general toxicity of Cd^{2+} in the brain, a possible relationship between Cd^{2+} toxicity and acetylcholine-related enzymes was assessed as markers of cognitive function.

Based on the above, the present study aimed to evaluate the potential neuroprotective properties of bioactive compounds (present in beetroot/carrot juice), as a protective factor against neurodegenerative diseases, and also determine the effect of Cd^{2+} on neurons in the CNS, sensitive to Cd^{2+}-induced memory disorders, with a particular focus on Cr immunoreactivity in rat hippocampal neurons.

2. Materials and Methods

2.1. Preparation of Beetroot/Carrot Juice (BCJ)

The beetroot/carrot juice (BCJ) was prepared essentially as described in our previous work [22]. In short, freshly pressed juices were obtained from thoroughly washed vegetables (Opolski beetroot and Nantejska carrot; in tap water), using an Angel 750 low-pressure screw press (Angel Co. Ltd., Naarden, The Netherlands). A single raw juice blend was prepared by mixing the beetroot and carrot juices at a ratio of 4:1 v/v (with pH corrected to 4.0 by the addition of 0.25 g of citric acid per 100 mL of juice). The final juice was freshly prepared and administered to rats within 2 days.

2.2. Qualitative Characteristics of Juice

The composition of the juice, as well as its total polyphenolic content and antioxidant activity were given in our previous work [22]. The anticholinesterase activity of BCJ (against acetyl- and butyrylcholinesterase) was studied as described by Studzińska-Sroka et al. [23] using juice prepared as described above and not otherwise processed in any way prior to analysis. To check the pro-cognitive efficiency of the juice, the ability to inhibit acetyl- and butyrylcholinesterase activity was tested as described earlier [23], expressed as the equivalent concentration of donepezil, a well-known cholinesterase inhibitor.

2.3. Animals

This experiment was approved by the 2nd Local Ethical Committee at the University of Life Sciences in Lublin, Poland (ref. no. 105/2015). Thirty-two (n = 32) male, 8-week-old Wistar rats were kept in single cages under a 12 h light–dark cycle with the light on at 06:00 a.m., at a room temperature of 21 ± 1 °C and a relative humidity of 55 ± 10%, with the cages ventilated every 4 min. The rats received the standard commercial rodent food (LSM; Agropol, Motycz, Poland; containing 12.06 MJ/kg ME (metabolic energy); crude protein: min. 16.00%; crude fat: min. 2.8%; crude ash: max. 7.00%; crude fiber: max. 5.00%; calcium min. 1.10%; phosphorus min. 0.70%; sodium max. 0.22 %; and vit. A: 8000 IU/kg; Vit. D3: 1000 IU/kg; Vit. E: 50 mg/kg) in the amount of 210 g per rat per week. Cadmium chloride of very high purity (99.999%) was used (Sigma-Aldrich, Poznań, Poland, cat. no 439800). The animals were acclimatized to the laboratory for at least one week before being used in the experiment. The rats were randomly divided into four experimental groups (n = 8): the control group (C) received 100 mL of tap water; the Cd group (Cd) received Cd^{2+} dissolved in 100 mL tap water/day (equivalent to 5 mg Cd^{2+}/kg b.w.); and two groups received beetroot/carrot juice: the BCJ group was administered only 100 mL of juice per day, and the Cd + BCJ group received juice with the addition of Cd^{2+} ions (Cd^{2+} dissolved in 100 mL juice/day (equivalent to 5 mg Cd^{2+}/kg b.w.). The experimental dose of Cd^{2+} was based on literature data [24–26]. The feed and fluid intake was controlled daily. The experiment lasted for 12 weeks.

2.4. Experimental Procedures and Analyses

After euthanasia (decapitation), the rats' brains were stored in 10% buffered formalin (pH = 7) for 12 h at 4 °C, hydrated in decreasing concentrations of ethyl alcohol, and embedded in paraffin blocks in accordance with the previously described method [27]. Briefly, the paraffin blocks were cut into 5 μm-thick sections which were placed on silanized glass-slides (SuperFrostPlus, Thermo Fisher Scientific, Braunschweig, Germany). To block the endogenous peroxidase activity, the sections were chilled and washed in 3% hydrogen peroxidase (20 min). The slides were then flushed twice with PBS (pH 7.4) (15 min each time) and incubated in 2.5% normal goat serum (ImPRESS™; MP-7451, Vector Labs, Burlingame, CA, USA) at room temperature (RT) for 20 min. The sections were incubated for 24 h at 4 °C with primary monoclonal mouse antibodies raised against Cr (1:2000; C7479, Sigma, Taufkirchen, Germany). The next day, the slides were washed in a washing buffer (2 × 15 min) and covered with anti-mouse/rabbit Ig (ImPRESS™; MP-7500 Vector Labs, Newark, CA, USA) for 1 h. The specificity of the antibodies used was verified using a negative control in which primary antibodies were replaced with the same concentrations of appropriate non-immune IgG. 3,3′-diaminobenzidine chromogen (ImmPACT™DAB, SK-4105, Vector Labs, Newark, CA, USA) was used to visualize the primary antisera. A working solution of DAB was applied onto the sections, and the process was monitored under a light microscope. Finally, the slides were rinsed with distilled water. Moreover, counterstaining (for 20 min) with Mayer's hematoxylin was performed. After washing in distilled water, the sections were dehydrated in increasing concentrations of ethyl alcohol, cleared in xylene, mounted in DPX (Sigma-Aldrich, St. Louis, MO, USA), and cover slipped. The slides were viewed under a light microscope (Axiolab, Zeiss, Jena, Germany) connected to a digital camera (Olympus Color View III, Tokio, Japan). From each animal, approx. 25–30 sections immunostained for Cr were examined. The Cr-IR neurons were assessed by analyzing and counting no less than one hundred neurons immunoreactive (IR) to Cr in the CA1 field of the hippocampus of each group (the control and experimental) using Cell D software (Olympus, Tokio, Japan). Image J software (ImageJ 1.53 k; National Institute of Health, Bethesda, MD, USA) was used to quantify and statistically compare the length of Cr-IR nerve fibers. At least two independent observers were involved in quantification analyses, and the results obtained by them were averaged.

2.5. Statistical Analysis

The collected data were analyzed with Statistica software ver. 13.1 ((StatSoft, Kraków, Poland). Normality was assessed using the Kolmogorov–Smirnov test, and Levene's homogeneity of variance test was applied to examine the equality of variances. One-way ANOVA and Tukey's post-hoc tests were performed to compare all the experimental groups individually, whereas the two-way ANOVA was used to determine the impact of experimental factors: Cd^{2+} exposure and beetroot/carrot juice treatment and their interaction. Significant differences between the groups were identified at $p \leq 0.05$ and $p \leq 0.01$.

3. Results

3.1. Anticholinesterase Activity of the Studied Juice

In our previous paper [22], we presented the composition of BCJ (using high-resolution, qualitative, and quantitative HPLC-ESIQTOF- MS) and identified the main bioactive components present (betanin, isobetanin, vulgaxanthin I and II, indicaxanthin, neobetanin, dexarboxyneobetanin, and decarboxybetanin). The juice showed significant total polyphenolic content, high antioxidant activity (tested using three experimental models), and positive effects towards human colon epithelial normal and cancer cells [22], so it was a promising research material in the context of the presented work. In the present work, we tested the ability of the juice to decrease the activity of acetyl- and butyrylcholinesterase and we report that the activity towards both enzymes was high (equal to donepezil applied at the concentration of 0.05 ± 0.00 µg/mL and 0.09 ± 0.01 µg/mL, respectively).

3.2. Cr Expression in the CA1 Field of the Hippocampus

The hippocampus consists of the Ammon's horn (CA1–CA3) and the dentate gyrus (DG), but in the present study, we focused on the CA1 field of the rat hippocampus due to the high sensitivity of the nerve cells in this area to damage, as compared to CA2 and CA3 fields of the hippocampus [28]. The dorsal portion of the hippocampus was examined. In all the studied groups, multiform (oval, round, triangular, and fusiform) Cr-IR neurons were observed, unevenly distributed in all layers (the marginal, pyramidal, and multiform) of the CA1 field of the rat hippocampus. The neurons were characterized by the presence of cytoplasmic and nuclear reactions (Figure 1). The average numbers of Cr-IR neurons in the CA1 field of the rat hippocampus were estimated at 6.62 ± 0.72 in the control group, while the analogous neuronal populations in the BCJ group were calculated at 6.85 ± 0.30 (Figure 2a). There were no statistically significant differences in the mean numbers of Cr-IR neurons between the control group receiving tap water and the BCJ group (Figure 2a). In both cases, the neurons showed intense (+++) or moderate (++) nuclear/cytoplasmic reactions to Cr (Figure 1 C and BCJ).

Although collectively in both groups exposed to Cd^{2+}, a decrease in the average number of Cr-IR neurons was stated in comparison to non-exposed groups ($p < 0.001$; Figure 2a; Table 1), and a statistically significant decrease ($p \leq 0.01$) in the average number of Cr-IR neurons was only observed in the group receiving Cd^{2+} with tap water (Cd) as compared to control group (6.62 ± 0.72 vs. 5.43 ± 0.33, respectively) (Figure 2a). On the other hand, a positive trend was stated in case of the BCJ treated group. The mean number of Cr-IR neurons was higher in the brains of rats receiving Cd^{2+} dissolved in beetroot/carrot juice (Cd + BCJ group) as compared to the Cd group, although the differences were not confirmed statistically (Figure 2a). BCJ treatment significantly affected the numbers of Cr-IR neurons as compared to non-treated groups (6.55 ± 0.47 vs. 6.03 ± 0.82; $p = 0.025$; Table 1). In the Cd^{2+}-treated group (Cd), the nuclear/cytoplasmic reaction was weak (+) and individual Cr-IR neurons were located only in the layer of pyramidal cells of the CA1 fields (Figure 1 Cd). In contrast, in the Cd^{2+} + BCJ group, weak (+) to moderate (++) nuclear/cytoplasmic reactions in Cr-IR neurons were recorded, and neurons IR to Cr were present in all layers of the hippocampal CA1 fields (Figure 1 Cd + BCJ).

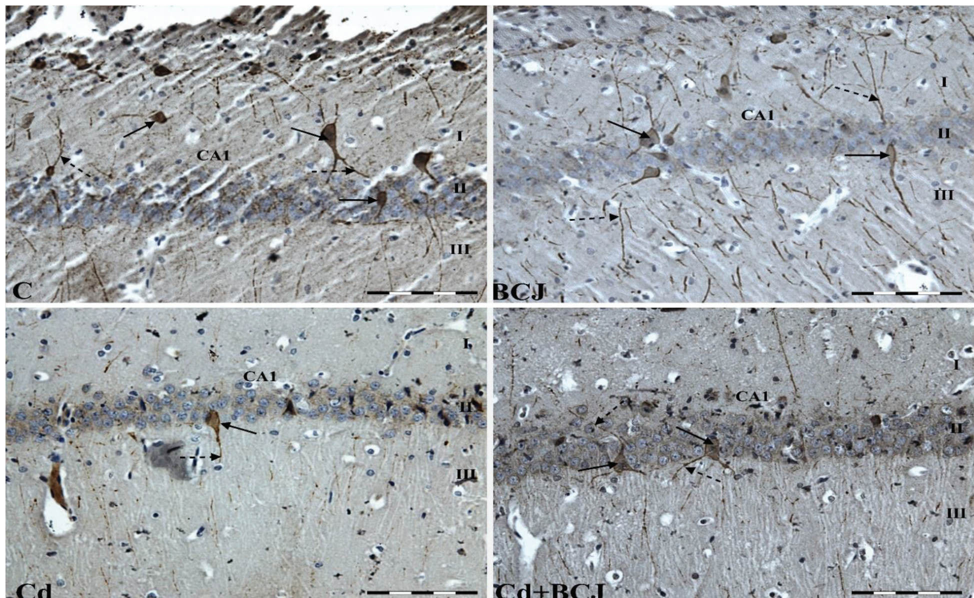

Figure 1. Cr-IR neurons and Cr-IR fibers in the hippocampal CA1 fields of experimental rats' brains in the respective groups: C—the control group received 100 mL of tap water; Cd group—received Cd^{2+} dissolved in 100 mL tap water/day (equivalent to 5 mg Cd^{2+}/kg b.w.); BCJ group—received 100 mL of juice per day; Cd+BCJ group—received juice with the addition of Cd^{2+} ions (Cd^{2+} dissolved in 100 mL juice/day (equivalent to 5 mg Cd^{2+}/kg b.w.); I: the marginal layer, II: the pyramidal layer, III: the multiform layer; the arrows indicate Cr-IR neurons (solid one) and Cr-IR fibers (dotted one) of the hippocampus. Scale bars = 20 μm.

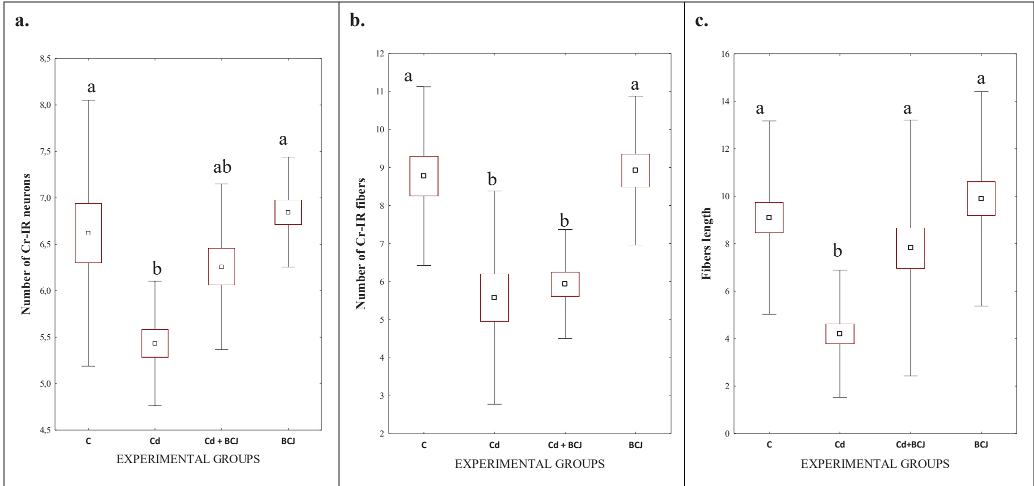

Figure 2. The figures present: the average numbers of Cr-IR neurons (**a**) and the average number of Cr-IR fibers (**b**) in the CA1 fields of rat hippocampus and the average length of nerve fibers (**c**). The data are expressed as means ± SEM (standard error of the mean; box) and standard deviation (whiskers); ^{a,b}—different letters indicate significant differences between the experimental groups at $p \leq 0.01$.

Table 1. The impact of experimental factors: Cd exposition and BCJ treatment on average numbers of Cr-IR neurons and Cr-IR nerve fibers and length of nerve fibers (μm) in the CA1 field of rat hippocampus in the experimental groups.

Parameter	Cd Exposition		BCJ Supplementation		Two-Way ANOVA Impact of		
	−	+	−	+	Cd Exposition	BCJ Treatment	Cd × BCJ
No of Cr-IR neurons	6.73 [a] ± 0.53	5.85 [b] ± 0.57	6.03 [B] ± 0.82	6.55 [A] ± 0.47	<0.001	0.025	0.178
No of Cr-IR fibers	8.85 [a] ± 1.02	5.76 [b] ± 1.06	7.18 ± 2.08	7.43 ± 1.77	<0.00001	0.620	0.835
Length of nerve fibers	9.50 [a] ± 2.13	6.01 [b] ± 2.78	6.65 [b] ± 3.02	8.86 [a] ± 2.64	<0.0001	0.002	0.044

Explanations: The data are expressed as means ± SD (standard deviation); [a,b]—values in the rows with different letters differ significantly at $p \leq 0.01$; [A,B]—values in the rows with different letters differ significantly at $p \leq 0.05$.

The immunoreactivity to Cr was also observed in the nerve fibers of the hippocampal CA1 field. In the control and BCJ groups, the reaction to Cr in the nerve fibers was intense (+++) or moderate (++) (Figure 1 C and BCJ). The Cr-IR nerve fibers were numerous (the average numbers of Cr-IR nerve fibers were 8.77 ± 1.17 in the control group and 8.92 ± 0.98 in the BCJ group; Figure 2b) and long (9.10 μm and 9.89 μm, respectively; Figure 2b). There were no statistically significant differences in terms of the mean number of nerve fibers between those groups (Figure 2b). This is in contrast to the Cd^{2+}-treated groups (Table 1). In the Cd^{2+}-treated group (Cd), receiving Cd^{2+} dissolved in tap water, Cr-IR nerve fibers were less numerous (5.58 ± 1.40; Figure 2b), singular, with a weak (+) Cr-IR reaction (Figure 1). Moreover, in many areas of the CA1 field, no Cr-IR nerve fibers were observed (Figure 1 Cd). Exposition to Cd^{2+} had a significant impact on both the number, as well on the length, of the nerve fibers ($p < 0.0001$; Table 1). The length of the nerve fibers in the Cd^{2+} group was the shortest among all of the experimental groups (Figure 2c; Table 1). The supplementation of the BCJ juice attenuated the negative impact of cadmium ions on the length of the nerve fibers (4.20 μm in Cd groups vs. 7.82 μm in Cd+BCJ group; Figure 2c; $p = 0.002$; Table 1). The statistical analysis confirmed a significant interaction between both experimental factors (Cd and BCJ addition) on the length of the nerve fibers ($p = 0.044$; Table 1).

4. Discussion

The presented results show that chronic exposure to even low doses of Cd^{2+} can be associated with neurodegenerative disorders characterized by memory impairment or dementia. As it reaches the brain, Cd^{2+} inhibits neurogenesis, including in the hippocampus [29], while also producing free radicals that can damage neurons [30]. Based on the findings reported by Lopez et al. [31], it can be argued that Cd^{2+} affects the morphology of nerve cells—the changes mainly concern nerve projections (axons and dendrites), which completely disappear after prolonged exposure to Cd^{2+}. Likewise, the results of the present study show that there is a relationship between the consumption of Cd^{2+} in tap water and a weak Cr immunoreactivity observed in the nerve fibers of the hippocampal CA1 field. As compared to the control group, the groups of rats receiving Cd^{2+} (Cd and Cd + BCJ) had significantly fewer nerve fibers. Not only was the number of nerve fibers lower, the analysis of images confirmed that the nerve fibers were also significantly shorter. There were no statistically significant differences in the average number of nerve fibers between the groups receiving Cd^{2+}, irrespective of the solvent used (Figure 2c; Table 1).

In the present study, Cr immunoreactivity in the CA1 field of the rat hippocampus was assessed to investigate the effect of beetroot/carrot juice on neurons present in the CNS structure sensitive to memory disorders due to administration of Cd^{2+}. Previously, it was shown that beetroot consumption may improve the cognitive functions of the brain by

facilitating better cerebral blood flow [32]. Olasehinde et al. [33] observed an improvement of cognitive functions (decreased by scopolamine) in rats receiving beetroot powder (2 and 4% in feed). In another study, beetroot extract (100 μg d.m. mL^{-1}) lowered the activity of acetylcholinesterase (by 93.3% as compared to 94.2% observed for the standard enzyme inhibitor donepezil at the same concentration) [34]. The consumption of raw beet (100 g a day for 8 weeks) significantly improved antioxidant activity as well as cognitive functions in a group of patients suffering from type II diabetes [35]. It was previously shown that beetroot can exert a positive action against toxic heavy metal ions in the context of neurodegeneration and cognitive deficits. The administration of lead (Pb acetate, 40 mg/kg b.w.) intensified lipid oxidation and reduced glutathione levels and antioxidant capacity in cerebral tissues of rats. However, the administration of beet juice (8 mL kg^{-1} b.w.) increased the level of glutathione levels (15 vs. 25 mg GSH/g cerebral tissue). Moreover, acetylcholinesterase activity in the cerebral tissue was elevated by approximately 15% as compared to the group receiving Pb only [12]. Similarly, acetylcholinesterase activity levels were reduced in the blood of farmers who consumed a beet-based beverage for two months (2 months, 2 × 500 mL daily) [13].

Calcium is an omnipresent intracellular ion that acts as a signaling mediator in many cellular processes, including proliferation, differentiation, and cell survival/death. It is also involved in long-term processes such as memory acquisition, which are mediated by the interaction of Ca^{2+} with intracellular CaBPs [36]. Cr is a good neuronal marker due to its intracellular Ca^{2+} buffering properties. It is probable that a change in the level of CaBPs or their modification may lead to an impairment of neuronal calcium homeostasis and, consequently, cause pathological reactions and even cell death. Such changes may result from diabetes mellitus, neurodegenerative diseases, but also from the toxic effects of heavy metals. According to Xu et al. [37] and Yuan et al. [38], exposure to cadmium disrupts intracellular Ca^{2+} homeostasis, thereby inducing apoptotic morphological changes in neurons. In the present study, Cr-IR neurons were observed in all layers of the CA1 field of the rat hippocampus. The mean number of Cr-IR neurons in the control group and the group receiving beetroot/carrot juice was similar; no statistically significant differences were found. In both groups receiving Cd^{2+}, a decrease in the average number of Cr-IR neurons was shown as compared to the control group; however, in the group receiving Cd^{2+} with tap water the number of Cr-IR neurons was lower than in the group receiving Cd^{2+} dissolved in beetroot/carrot juice, which confirms the protective capacity of bioactive components present in the beetroot. The study also confirmed that Cd^{2+} intake is associated with the weak response of Cr-expressing hippocampal neurons. Accordingly, exposure to Cd^{2+} reduces the number of Cr-expressing nerve cells in the hippocampus.

5. Conclusions

The obtained results may suggest that chronic exposure to even low doses of Cd^{2+} can be associated with neurodegenerative disorders characterized by memory impairment or dementia. Cd^{2+} had a significant impact on the number of neurons, and also on the morphology of nerve fibers, as well as the immunoreactivity to Cr in rat hippocampal neurons, which impaired intracellular Ca^{2+} homeostasis.

However, the negative impact of Cd^{2+} was probably reduced with the inclusion of beetroot/carrot juice. The beneficial effects of beetroot/carrot juice may be related to the action of bioactive compounds that induce an increase of intracellular Ca^{2+} in the hippocampal neurons, thus preventing the toxic influence of heavy metals on the CNS structures and a protective factor against neurodegenerative diseases. The above data may suggest that the regulation of Cd-induced Ca^{2+} homeostasis may be a good strategy in the prevention of diseases affecting the CNS structures and provide the basis for further research into the possible neuroprotective role of bioactive compounds.

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Article

Is Better Knowledge about Health Benefits of Dietary Fiber Related to Food Labels Reading Habits? A Croatian Overview

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Abstract: The aim of this cross-sectional study was to determine the associations between health dietary patterns, knowledge, and consumption of dietary fiber (DF) with frequency of food label reading on food products with special reference to DF. The study was conducted in 2536 Croatian adults using an original questionnaire. Multiple linear regression models were used to assess associations between food label reading habits and predictor variables. Our study confirms the association between habits regarding the reading of labels on food products, especially in relation to information about DF with the sociodemographic factors of respondents, dietary food patterns and DF consumption, as well as knowledge and sources of information about DF. Women, individuals with a university-level education, and those living in an urban environment had more frequent labels used. Food habits as well as eating outside of the home were positive predictors while eating fast food was a negative predictor of food label reading. Knowledge about DF, especially about its health benefits, was also associated with food label reading. The interpretation of associations could help with the design of effective public health programs. Targeted education campaigns to educate and sensitize the population about food labeling and monitoring may improve general knowledge about healthy food and its benefits, which include indirect effects on the prevention of non-communicable chronic diseases.

Keywords: food labels reading; dietary fiber; knowledge; consumption; health benefits

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1. Introduction

The prevalence of nutrition-related health problems, such as chronic non-communicable diseases, has been progressively increasing [1,2]. It is estimated that, by 2030, 52 million people will be dying from these diseases each year [3]. Achieving optimal health for the population and target groups is a key aspect of public health programs aimed at achieving the Millennium Development Goals [4,5]. In achieving these goals, the promotion of healthy lifestyles and nutrition practices are two of the most challenging goals [2]. In order for people to realize the importance of a healthy and balanced diet with qualitatively and quantitatively balanced intakes of macro and micronutrients, raising knowledge in this area is important and is needed constantly. For example, it is well known that dietary fiber (DF) has a positive impact on health and the prevention of chronic diseases [6]. The consumption of DF is associated with many health benefits and contributes to the maintenance of good human health. Previous studies have confirmed that the consumption of DF is associated with lower cholesterol levels; the prevention of heart disease; the lowering and stabilization of blood glucose levels; and the prevention of obesity, diabetes, constipation, diverticulosis, and colon cancer, with some studies also reporting an impact on the prevention of breast cancer [7–12]. However, knowledge about DF sources in products and possible effects on

health are lacking in the general population [13–16]. Additionally, studies have shown a certain level of disparity between knowledge and attitude towards the intake of DF. This means that people are aware of the role of fiber-rich foods in health conditions, but consumption of these foods is low [17]. These behaviors may limit the health benefits of DF [17]. On the other hand, many previous studies have confirmed the relationships among consumer awareness, knowledge, and the frequency of consumption of foods containing DF [13,14,16,18–23]. DF may be obtained from different dietary sources. However, DF representation varies from food to food. Studies have confirmed that whole grains, fruits, and vegetables are the richest DF sources. For example, whole cereals are an important source of DF, representing about 50% of the total DF intake. Vegetables represent about 30–49%, while fruits represent about 16% of the daily DF intake [24]. Despite this, the consumption of these dietary-rich foods is below the recommended level [19].

One possible way to improve this is to improve the knowledge of consumers about the consumption of these foods through the reading of food labels. Nutrition labeling literature suggests that the effectiveness of food labels is influenced by the consumers' motivations, health or diet goals, nutritional knowledge, and time pressure. The available nutrition labeling literature details the dual-process theory that exists in food decision making. This depends on the consumer's ability to quickly and automatically or slowly and deliberately choose food products based on the information on food labels [25]. Additionally, the hierarchy of effects model emphasizes the fact that consumers should be exposed to food labels and be able to understand them and that the ability to do this will be affected by consumers' knowledge about nutrition [26]. Labels are important to consumers who are aware of the health benefits of certain food compounds, such as DF, and have enough knowledge about them. In addition to the fact that reading declarations may be determined by these personal characteristics and prior knowledge, it is very important to have distinct and convenient information on the food label [27].

Food labels provide information about the food product, such as its energy value, the contents of certain nutrients it contains (fats, saturated fatty acids, carbohydrates, sugars, proteins, salts, vitamins, minerals, DF, and other nutrients), and some mandatory components [28–31]. Therefore, food labels carry useful information about products and can be thought of as the identity card of a food product. They give the consumer the opportunity to consciously choose what to buy and allow them to make a final decision [28,30]. A potential problem is that legal regulation does not oblige the labeling of raw whole cereals, fruits, or vegetables, which are actually the richest categories, while all products made from these ingredients must contain a label [32]. Nevertheless, consumers who have better knowledge about fruits and vegetables or whole cereals as good sources of DF and their health benefit may be more inclined to choose and consume such foods or their products [19]. Additionally, studies confirm that higher scores for understanding labels were associated with consuming more vegetables [33]. Also, people who never sought information on health reported a significantly lower intake of fruit and vegetables, which is strongly linked to all of the major lifestyle diseases [23,34]. Knowledge about this potentially plays an important role in dietary behavior [35]. Less likely people read food labels if they have weaker knowledge and do not consume such healthy food. According to that, the knowledge and frequency of consumption of these rich dietary food products may be useful indicators in the analysis of food label reading. Additionally, sociodemographic characteristics and food habits may be associated with consumers' habits of food labels [33,35].

Food labels may contain nutritional and health claims, which are two very important types of information [30]. A nutritional claim is any claim that states, suggests, or implies that a food has particular beneficial nutritional properties, e.g., low/"light-lite"/reduced content of fats, saturated fats, sugar, or sodium; high contents of DF, protein, vitamins, or unsaturated (monounsaturated or polyunsaturated fat) fats; or naturality [36,37]. A nutritional claim is only permitted if it is listed in the Annex of Regulation (EC) No 1924/2006, lastly amended by Regulation (EU) No 1047/2012 [37]. A health claim states, suggests, or implies that there is a link between a precisely determined food or precisely determined

food ingredients and health [30]. In food labeling, health claims are strongly reviewed by organizations like the Food and Drug Administration (FDA) and can be put on specific food products to show that this food or food component may reduce the risk of a particular disease or could help with certain health-related conditions.

Nutritional and health claims are not mandatory on food products, and legal norms strictly prohibit the use of information that could mislead the purchaser or that attributes medicinal properties to food [29,37,38]. However, the growing link between diet and health has prompted the need for a clearer emphasis on nutrition labeling [6,39] in terms of highlighting nutritional and health benefits. According to the World Health Organization (WHO), the Food and Agriculture Administration (FAO), and the Codex Committee on Food Labeling, all food products should be marked in accordance with legal norms and in the language of the country in which they are offered to consumers [30,40]. Food labelling, consumer awareness, and comprehension of label information are crucial for determining, maintaining, and communicating food value [41]. It is also recommended that DF, due to its positive health effect, should be included in the supplemented mandatory food nutrition label [41]. Studies have confirmed that the use of food nutrition labels is associated with lower fat intake and belief of an association between diet and diseases [20]. Subjective norms and dietary health concerns are associated with the intention to use food labels [42].

Despite this, the reading of food labels is not widely carried out. In order to prevent chronic diseases related to unbalanced and unhealthy eating habits, public health organizations need to strongly support the development of healthy lifestyle habits, including healthy dietary patterns and the use of food-labels to promote the choice of healthier products [2]. This is important because food labels can assist consumers in the appraisal of a product. Furthermore, they could encourage the producer to enhance and adapt their manufacturing systems to meet their own expectations and needs for maintaining health and preventing chronic diseases. Consumers are very interested in how their diet can impact their health, prevent diseases, or aid in the management of diseases that they are already suffering from [6,29]. These factors may play crucial roles when making decisions on nutrition consumption. Scientists who emphasize the importance of labeling on consumers' selections and future behaviors consider there to be links among choosing and consuming healthy foods, having knowledge about the relationship between diet and diseases, and improving eating habits through the reviewing and reading of food labels [2,28,40,43–46].

Despite these factors, little is known about associations between general and health knowledge about DF and food label reading. By assessing the knowledge of consumers about the health benefits of DF, the frequency of DF consumption, and the frequency of reading declarations on foodstuffs, it will be possible to assess the need for public health program activities that can act to improve health in a very simple way. Therefore, well-designed assessment studies are necessary to clarify the associations between knowledge about the benefits of DF and consumers' awareness about the importance of reading food labels. Additionally, the lack of evidence on label reading and its association with consumer knowledge warrants future studies. Our assumption is that consumers do not pay enough attention to food labels, especially not to the extent that can change behaviors and thus affect health. The reason for this may be a lack of knowledge on this topic. Consequently, we assume that the lack of knowledge about DF is associated with a lower frequency of reading food labels about it. Our hypothesis is that an association exists between the reading of food labels, the frequency of DF consumption, food habits, and understanding the health benefits associated with DF consumption. If our assumptions are accurate, this study will directly confirm the necessary implementation of public health activities, which may improve knowledge and awareness among consumers about foods that are rich sources of DF, the reading of food labels, and the need to change negative food habits as well as other lifestyle habits. This may be crucial to prevent numerous chronic diseases and to preserve the population's health.

The aim of this study was to determine the associations between sociodemographic factors, sources of information, and acquired knowledge, and the frequency of reading labels on food products with special reference to DF.

2. Materials and Methods

This descriptive study was based on a cross-sectional questionnaire, which was conducted on a non-probabilistic convenience sample of 2536 respondents from all counties of the Republic of Croatia, although participants from Dalmatia predominated. Ethical approval was granted by the Human Research Ethics Committee of Zadar General Hospital (No. 01-178-3/15). The entire data collection and analysis were conducted in accordance with the ethical standards of the Declaration of Helsinki.

2.1. Study Population

We recruited respondents from the general population by word-of-mouth; advertisements; and contact in universities, shopping centers, and downtown areas. This ensured wide demographic and socio-economic distributions. After signing the consent form, the respondents started filling in the questionnaire using the paper and pencil method, and each respondent was given privacy while filling out the questionnaire. The inclusion criteria were adult volunteers (i.e., 18 years or older) who fully completed the questionnaire and were taken into consideration, while respecting protocols regarding their informed consent and anonymity. The exclusion criteria were respondents who did not complete the entire questionnaire, tourists, and students from other countries.

2.2. Methods

The original questionnaire was written and validated in English by the CI&DETS Research Center of the Polytechnic Institute in Viseu, Portugal [47]. This English survey was translated into Croatian by two native Croatian speakers with experience in public health and nutrition studies. No questions were modified, removed, or added during the translation. In this survey, respondents were asked about demographic characteristics, knowledge, and sources of information about DF in foods or its importance for human health. They were also asked about habits regarding the reading of food labels and their awareness of concepts, definitions, and health effects of nutritional information with special reference to DF.

In the analysis of sources of information about DF, health institutions (health centers and hospitals), educational institutions (schools), media (radio, television, Internet), and educational materials (magazines, books) were taken into account. The entry of ordinal values from 1 to 6 was used to estimate how often sources of information about nutrition and DF are used (health centers and hospitals, radio, television, school, magazine and books, internet). Respondents ordered these sources from most important (1st) to least important (6th).

To assess the knowledge about DF, the questions were grouped into three categories: general knowledge about food fiber (6 items), specific knowledge about the relationship between DF and food (6 items), and health knowledge concerning the relationship between DF and health benefits related to the consumption of DF and the prevention of chronic diseases, such as cardiovascular diseases, hypercholesterolemia, colon cancer, breast cancer, and diabetes (10 items). The items were rated on a Likert scale from 1 to 5, where 1—completely disagree; 2—disagree; 3—neither disagree nor agree; 4–agree; and 5—completely agree. Responses to questions formulated in a negative way were scored using the same 5-point scale in reverse, so that higher scores always corresponded to greater knowledge. We summed the 6 items used to assess general knowledge and the 6 items used to assess specific knowledge using the minimum (1—never) answer to all questions and the maximal score (5—always) for all questions and for general and specific knowledge separately. For health knowledge, we summed each of the 10 items using the minimum score (1—never) and the maximal response (5—always) to all questions. Potential scores for general and

specific knowledge ranged from 6 to 30, while the score range for health knowledge was 10 to 50.

To estimate the frequency of reading food labels, the Likert rating scale was used. This allows answers on a scale from 1 to 5 (1—never; 2—rare; 3—sometimes; 4—often; 5—always). The questions were grouped into two categories: reading labels about food products (2 items, with potential score of 2–10) and reading information on labels about DF (3 items, with potential score of 3–15). Overall label reading was computed as the sum of all answers on the food label scale (sum of all 5 items; the potential range of the summed score was 5–25). The minimum score of 5 points meant that respondents never read food labels, and the maximum score of 25 points meant that respondents always read food labels.

The Cronbach's alpha scores, $\alpha = 0.77$ for knowledge and $\alpha = 0.86$ for reading food labels, were acceptable.

2.3. Statistical Analysis

The data were processed using SPSS 26.0 (IBM, New York, NY, USA). We used the Kolmogorov–Smirnov test to examine the normality of the distribution. As data were asymmetrically distributed, in the presentation of descriptive statistics, the median and interquartile range were used. The Mann–Whitney U test (for two variables) and the Kruskal–Wallis test (for comparisons of three or more variables) were used to examine the differences between variables. Linear regression was used to identify predictors affecting the prediction of food label reading or DF content checking on products as a consequence. The regression model included the following predictor variables: gender, level of education, living environment, sources of information, levels of general and specific knowledge about DF, and knowledge about health benefits. The reliability of the questionnaire was evaluated by the Cronbach alpha coefficient. In the mentioned statistical analysis, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Sociodemographic Characteristics and Consumption of Fiber-Rich Foods in the Study Group

The average age of respondents was 30 (Mdn = 3.0; IQR = 24). The dominant group in the sample was females and younger respondents who were mostly living in urban areas and who had a university-level education (Table 1). The characteristics of the study population (age group, gender, living environment and education) are presented in Table 1. On average, the respondents stated that they consumed seven pieces of fruit per week (Mdn = 7; IQR = 6), corresponding to only one piece per day. The same results were found for weekly vegetable consumption (Mdn = 7; IQR = 4). Respondents stated that they consume whole cereals only twice per week (Mdn = 2; IQR = 4). Respondents stated that they eat outside the home twice per week (Mdn = 2.0; IQR = 5), and they eat fast food once per week (Mdn = 1; IQR = 1; Table 1).

Table 1. Sociodemographic characteristics, lifestyle, and knowledge about dietary fiber and frequency of food label reading in the study group (N = 2536).

Age (Years), Mdn (IQR)	**30.0 (24.0)**
Age groups, N (%)	
under 25	1010 (39.9)
26–35	488 (19.3)
36–45	385 (15.2)
46–55	480 (19.0)
56–65	143 (5.7)
66 and older	24 (0.9)
Gender, N (%)	
Male	826 (32.6)
Female	1704 (67.4)

Table 1. *Cont.*

Age (Years), Mdn (IQR)			**30.0 (24.0)**			
Education, N (%)						
Primary school						54 (2.1)
High school						1221 (48.3)
University degree						1250 (49.4)
Frequency of food consumption, Mdn (IQR)						
Fruit (pieces per week)						7.0 (6.0)
Salads (meals per week)						7.0 (4.0)
Whole cereals (meals per week)						2.0 (4.0)
Frequency of eating outside the home (times per week), Mdn (IQR)						2.0 (5.0)
Frequency of eating fast food (times per week), Mdn (IQR)						1.0 (1.0)
Sources of information about dietary fiber, N (%)						
	1st source	2nd source	3rd source	4th source	5th source	6th source
Health centers, hospitals	303 (12.8)	251 (10.6)	242 (10.2)	328 (13.8)	471 (19.9)	774 (32.7)
Radio	177 (7.5)	216 (9.1)	283 (12.0)	406 (17.1)	560 (23.6)	726 (30.7)
Television	331 (13.7)	467 (19.3)	552 (22.8)	533 (22.0)	342 (14.1)	196 (8.1)
School	306 (13.0)	314 (13.3)	451 (19.1)	487 (20.6)	391 (16.6)	411 (17.4)
Magazines, books	369 (15.2)	634 (26.2)	498 (20.5)	413 (17.0)	316 (13.0)	194 (8.0)
Internet	1120 (45.6)	384 (15.6)	246 (10.0)	215 (8.8)	173 (7.0)	317 (12.9)
Knowledge about DF, Mdn (IQR)						
General knowledge about food fiber						17.0 (4.0)
Specific knowledge about the relationship between dietary fiber and food						18.0 (5.0)
Knowledge about the health benefits of dietary fiber						35.0 (7.0)
Food label reading, Mdn (IQR)						
When I buy a food product, I usually consult the information on the label.						3.0 (2.0)
On the label, I usually look at the nutritional information related to the food.						3.0 (2.0)
In the nutritional table, I usually look at the fiber content of the food.						2.0 (2.0)
The amount of fiber is a factor to consider when choosing among similar foods.						3.0 (2.0)
If I buy a food that is referred to as "fiber rich" on the pack, I check the label to see the amount of fiber present.						2.0 (2.0)
Read labels about food products						6.0 (4.0)
Read information on labels about dietary fiber						7.0 (5.0)
Overall label reading						14.0 (7.0)

Note: Mdn (IQR)—Median (Interquartile range); N (%)—absolute number (percentage number); General knowledge of food fibers—variable range between 6 and 30; Specific knowledge about the relationship between dietary fiber and food—variable range between 6 and 30; Knowledge about the health benefits of dietary fiber—variable range is between 10 and 50; Items about food label reading were rated on a Likert scale from 1 to 5 (1—never; 2—rarely; 3—sometimes; 4—often; 5—always); Overall label reading—sum of all 5 items, variable range between 5 and 25.

3.2. Level of Knowledge and Sources of Information about DF

In the range from complete knowledge to complete ignorance, the level of knowledge about DF was Mdn = 17 (IQR = 4) for general knowledge about food fiber, Mdn = 18 (IQR = 5) for specific knowledge about the relationship between DF and food, and Mdn = 35 (IQR = 7) for health knowledge regarding the relationship between DF and health benefits in terms of the consumption of DF and the prevention of chronic diseases, (Table 1).

In the analysis of sources of information used for the acquisition of knowledge on DF, the results show that the internet is the primary source for respondents (45.6%), followed by magazine and books (15.2%), school (13.0%), television (13.7%), health centers and hospitals (12.8%), and then radio (7.5%) (Table 1).

3.3. Frequency of Reading Labels on Food Products

The average score for reading food labels was between rarely (Mdn = 2; IQR = 2) and sometimes (Mdn = 3; IQR = 2). The average frequency of reading labels about food products was Mdn = 6.0 (IQR = 4.0). The score for reading information about DF on labels was 7.0

(IQR = 5.0), while that for overall label reading was 14.0 (IQR = 7.0) (Table 1). Women, older respondents, participants from urban areas, and those with a university-level education were found to read food labels more frequently (Table 2). The frequency of reading food labels according to sociodemographic factors is shown in Table S1.

Table 2. Food label reading habits presented by gender, living environment, education, and age for a sample of residents from Croatia (N = 2536).

	RFL 1 †	RFL 2 †	RFL 3 †	RFL 4 †	RFL 5 †
			Gender		
Female	1336.58	1349.94	1342.02	1319.50	1321.61
Male	1094.69	1064.42	1069.50	1123.60	1129.20
p *	<0.001	<0.001	<0.001	<0.001	<0.001
			Living environment		
Rural	1122.82	1132.74	1210.12	1201.02	1255.90
Urban	1282.40	1278.24	1251.75	1257.50	1245.76
p *	<0.001	<0.001	0.208	0.092	0.763
			Education		
Primary school	826.75	880.91	1066.12	1088.51	1089.66
Secondary school	1193.64	1174.69	1210.61	1178.01	1233.06
University degree	1334.27	1348.06	1298.29	1332.70	1286.38
p *	<0.001	<0.001	0.001	<0.001	0.037
			Age (years)		
<25	1214.14	1311.75	1187.50	1260.93	1234.09
26–35	1230.75	1250.29	1262.04	1197.17	1216.12
36–45	1281.58	1223.20	1301.96	1266.81	1303.13
46–55	1356.99	1217.87	1328.78	1290.43	1296.33
56–65	1269.19	1126.65	1310.44	1293.59	1334.40
>66	1238.00	1155.39	1269.75	1100.91	1289.33
p *	0.013	0.019	0.003	0.284	0.182

* $p < 0.05$; Parameter estimates in each column are shown as the mean rank of the Mann–Whitney U test for gender and living environment or the mean rank of the Kruskal–Wallis test for education and age. † RFL = reading food labeling; RFL 1 = When I buy a food product, I usually consult the information on the label; RFL 2 = On the label, I usually look at the nutritional information relevant to the food; RFL 3 = On the nutritional table, I usually look at the fiber content of the food; RFL 4 = The amount of fiber is a factor to consider when choosing similar foods; RFL 5 = If I buy a food that referred to as having a "high fiber content" or being "fiber-rich" on the pack, I check on the label to see the amount of fiber present.

3.4. Associations among Reading Food Labels, Knowledge of DF, and Sociodemographic Characteristics in the Study Group

The multivariate linear regression confirmed the associations between reading food product labels, reading information on labels about DF, and overall label reading and sociodemographic characteristics, dietary food consumption, food habits, and knowledge about DF (Table 3). Female gender was associated with reading food product labels ($\beta = 0.12$, $t = 6.31$, $p < 0.001$), reading information on labels about DF ($\beta = 0.11$, $t = 5.55$, $p < 0.001$), and overall label reading ($\beta = 0.13$, $t = 6.61$, $p < 0.001$). Having a university-level education ($p = 0.002$; $p = 0.008$; $p = 0.002$) was a positive predictor for reading labels in general, while living in an urban environment was associated with reading food product labels ($p = 0.023$) (Table 3).

The frequency of dietary food consumption was also a positive predictor of label reading. For example, the frequency of whole cereal consumption was associated with reading food product labels ($\beta = 0.21$, $t = 10.54$, $p < 0.001$), reading information on labels about DF ($\beta = 0.21$, $t = 10.16$ $p < 0.001$), and overall label reading ($\beta = 0.23$, $t = 11.48$, $p < 0.001$). On the other hand, the frequency of fruit consumption was also associated with reading food product labels ($p < 0.001$) and overall label reading ($p = 0.014$), but there was no association with reading information on labels about DF ($p = 0.233$) (Table 3).

Table 3. Associations between lifestyle habits, knowledge about dietary fiber, and reading food labels in the study group determined using linear regression models.

	Reading Labels on Food Products			Reading Information on Labels about DF			Overall Label Reading		
	β	t	*p*	β	t	*p*	β	t	*p*
Sociodemographic characteristics									
Age	−0.04	−1.79	0.073	0.01	0.36	0.720	−0.01	−0.65	0.519
Women	0.12	6.31	<0.001	0.11	5.55	<0.001	0.13	6.61	<0.001
University-level education	0.06	3.08	0.002	0.05	2.65	0.008	0.06	3.12	0.002
Urban environment	0.04	2.28	0.023	−0.03	−1.78	0.075	0.00	−0.02	0.980
Dietary food consumption (frequency)									
Fruit (pieces per week)	0.07	3.62	<0.001	0.02	1.19	0.233	0.05	2.47	0.014
Salads (meals per week)	0.09	4.21	<0.001	0.08	3.70	<0.001	0.09	4.41	<0.001
Whole cereals (meals per week)	0.21	10.54	<0.001	0.21	10.16	<0.001	0.23	11.48	<0.001
Food habits									
Eating outside the home (times per week)	0.05	2.49	0.013	0.01	0.64	0.524	0.04	1.63	0.104
Eating fast food (times per week)	−0.13	−6.16	<0.001	−0.07	−3.10	0.002	−0.11	−4.94	<0.001
Source of information about DF (the least important)									
Health centers, hospitals	0.03	1.65	0.099	0.00	0.18	0.855	0.02	0.96	0.339
Radio	0.03	1.33	0.182	−0.01	−0.47	0.640	0.01	0.39	0.700
Television	0.02	1.33	0.185	0.01	0.30	0.765	0.02	0.85	0.398
School	−0.01	−0.66	0.512	−0.01	−0.71	0.475	−0.01	−0.74	0.460
Magazines, books	−0.02	−1.20	0.228	−0.05	−2.41	0.016	−0.04	−2.08	0.038
Internet	−0.03	−1.59	0.113	−0.04	−1.90	0.057	−0.04	−1.93	0.054
Knowledge about DF									
General knowledge about food fiber	0.09	3.83	<0.001	0.10	4.21	<0.001	0.11	4.48	<0.001
Specific knowledge about the relationship between DF and food	0.06	2.42	0.016	0.05	2.28	0.023	0.06	2.65	0.008
Knowledge about the health benefits of DF	0.09	4.62	<0.001	0.16	8.41	<0.001	0.14	7.51	<0.001

Note: DF—dietary fiber, β-beta standardized coefficient, t-statistic, *p*-*p* value.

Eating outside of the home was only positively associated with reading labels on food products (β = 0.05, t = 2.49, $p = 0.013$), while eating fast food was negatively associated with reading labels on food products (β = −0.13, t = −6.16, $p < 0.001$), reading information on labels about DF (β = −0.07, t = −3.10, $p = 0.002$), and overall label reading (β = −0.11, t = −4.94, $p < 0.001$) (Table 3).

We did not find an association between reading food labels and the primary source of information about DF. There was a negative association when the source was magazines or books, but this association was weak (β = −0.05, t = −2.41; $p = 0.016$ for reading information on labels about DF; and β = −0.04, t = −2.08, $p = 0.038$ for overall label reading) (Table 3).

General knowledge about food fiber, specific knowledge about the relationship between DF and food, and knowledge about the health benefits of DF were associated with all categories of label reading. We found that knowledge about the health benefits of DF was more highly associated with reading information on labels about DF than other types of knowledge (β = 0.16; t = 8.41; $p < 0.001$), (Table 3).

4. Discussion

The aim of this cross-sectional study was to assess associations between food label reading, the consumption of food rich in DF, the general eating patterns of the examined population, and knowledge about DF. This study shows that the reading and analysis of food labels in the general population are associated with general and specific knowledge about DF as well as knowledge about the relationship between DF and health.

In our study, we found that general knowledge about DF, specific knowledge about the relationship between DF and food items, and knowledge about the health benefits of DF were associated with reading labels on food products, reading information about DF, and overall label reading. In addition, in our study, we found positive associations between

the frequency of dietary food consumption and label reading, with special emphasis on whole cereals. Similar results were mentioned in other studies [15,46,48]. It is possible that people who have positive eating habits are more likely to read food labels. Additionally, it is possible that extensive promotion of healthy foods and greater knowledge increases a person's awareness about healthy nutrition with special reference to DF. This may have a positive impact by promoting a healthy lifestyle [15,49,50]. Despite these factors, it appears that the typical Croatian consumer is unconcerned about the DF data on labels. It seems they have a low level of interest in food labels and they read food labels only rarely or sometimes. A Portuguese study about attitudes towards food labels presented similar results, showing that Portuguese consumers also have a low frequency of food label reading [47]. Similar results were found in many other studies [41,42,51].

In our study, the frequency of dietary food consumption was also a positive predictor of label reading. For example, the frequency of consumption of whole cereals was associated with food label reading, reading information about DF, and overall label reading. It is possible that respondents who consume DF-rich foods more often read declarations more frequently, because they have a greater need to look for the presence of DF. Consequently, they are more inclined to buy foods that are richer in DF. It is also possible that those who frequently consume foods rich in DF take more care of their own health, and therefore their knowledge about it is greater. Despite the statistically significant association, the relationship between reading labels and the consumption of food items rich in DF is relatively weak, which may indicate that the consumption of DF is below the recommended level. Despite the recommended daily consumption levels being three to five pieces of fruit, three meals containing vegetables, and three meals containing whole cereals, studies have shown insufficient consumption of DF, and this was confirmed in our study. An increase in knowledge about the health benefits of DF and improved labeling and reading about the presence of DF in food could contribute to better consumption of DF in food. In our study, the level of knowledge about DF was found to be unsatisfactory, and we suggest that this may have a negative effect on the reading of food labels and or asking for information about DF on food products.

Eating outside the home is often a key component of a modern and fast-paced lifestyle and includes eating "on the go" [52]. This depends on personal attitudes and usual eating habits. Some studies have confirmed that a high rate of eating outside the home is related to a poorer diet quality; a higher intake of energy, sugar, and fats; and a lower intake of fiber, fruit, and vegetables [53]. Additionally, studies have confirmed that eating outside the home is occurring more frequently due to lifestyle changes, but the effect of this may vary depending on the country and eating out is not always necessarily unhealthy. In some countries, this may be an indicator of a higher socioeconomic status, while in other countries, this is a cheaper way of eating, usually marked by frequent fast food consumption and poor lifestyle habits [54]. For example, in our study, reading labels on food products showed a weak positive association with eating outside the home. On the other hand, the negative associations between eating fast food and reading food labels may indicate that fast food consumption may negatively affect the reading of food labels, neglecting their significance.

Many studies have shown that reading labels may be affected by age, gender, marital status, socioeconomic status, education level, living environment, food-related motivations, nutrition knowledge, lifestyle habits, special dietary needs, being concerned about health, and other factors [53]. The nutritional information on food labels is very rarely or only occasionally taken into account, and mostly, connectivity is achieved due to sociodemographic factors such as gender, level of education, and the living environment. Our results are similar to the results of other studies in which the frequency of label reading was also found to be significantly higher in women and highly educated respondents in urban areas. Other studies have also confirmed that women read food labels more frequently than men. The reason for this may be that women have a greater interest in healthy eating because of its impact on body satisfaction and self-image [55,56]. Some studies found that women

report more negatively regarding their perceptions of unhealthy foods such as sugar, red meat, white flour, and additives, which suggests that women eat more healthily than men but also have more body shape concerns and food and health anxieties [57].

Our bivariate analysis showed that, when buying food products, middle-aged respondents usually consult the food labels and pay more attention to health characteristics and, thus, the DF content in foods. Younger people pay more attention to nutritional information in general. This differs from the results of Satia et al., who found that the older sector of the population uses nutritional labels more in general [58]. It can be assumed that the incidence of diseases gets higher with age, which may be why we found that the middle-aged respondents check the food labels and content of DF in products more often than the younger sector of the population. It is possible that middle-aged and elderly respondents pay more attention to the need for a diet rich in DF because of the higher risk of developing certain diseases in these age groups. However, this was not confirmed in our regression models, because the predictor of age was not associated with the reading of food labels. Additionally, disease risk factors and their prevalence were not examined in this study. However, due to the focus on disease prevention or the possible deterioration of their own health and the alleviation of existing symptoms of diseases, some consumers need a certain diet and must consume foods with a specific dietary composition.

Various reasons exist to explain why consumers use or do not use nutritional labels. These may include consumer perceptions of positive impacts on their own health, such as the prevention of or therapy for chronic diseases like diabetes, cardiovascular diseases, and obesity. However, variables may also include rising health literacy levels and motivational factors for future positive health behaviors [51,53,59–61]. In addition, Kim et al. found that nutrition label reading is associated with higher intakes of calcium and vitamin C, a lower intake of calories, and a higher energy ratio for protein intake [62]. Kessler and Wunderlich noted that people with diabetes read food label information more often than general consumers [59]. However, the same authors also noted that the use of nutritional labels has a limited effect on nutrition knowledge gain, although some studies have confirmed that the reading of nutritional labels is associated with a lower prevalence of metabolic syndrome and a lower fat intake [20,59,63]. Additionally, a longitudinal American study found that nutrition label users have a decreased risk of getting a diabetes diagnosis [60]. Our observations confirm that there is a correlation between the consumption of fruits, vegetables, salads, and whole cereals, and label reading. However, some studies have not found an association between label use and fruit and vegetable consumption or have confirmed that people are least likely to view labels on fruits and vegetables [20,64].

However, studies have confirmed that levels of knowledge and education, motivation for health behaviors, lifestyle, and environment can influence eating habits and food consumption [19,23,37,61]. Studies have also confirmed that having a university-level education is associated with having greater knowledge and paying more attention to food labels. It is possible that those with a higher level of education have a greater level of interest in obtaining new knowledge and having a healthy lifestyle [22]. Despite this, in parts of the living environment, the awareness of food labels is still debatable, as is their influence on consumers' attitudes, preferences, and quality perceptions. Some studies have suggested a need for the strengthening of research base systems used on labels and their influences on consumer behaviors to obtain more valid and compliant evidence-based policies in this area [65,66]. Still, findings from prior research on the role of labels are particularly controversial, with some authors considering them a critical antecedent of consumers' behavior and others finding them a factor of minor influence [41,53].

Although there are some differences in food labeling among individual countries, consumers generally consider food labels on products, particularly information related to claims, as beneficial for health maintenance or improvement. They could also be important for consumers' analysis and judgmental systems and, ultimately, could affect consumers' behavior and promote and satisfy socioeconomic objectives, such as improving human health and safety as well as environmental factors associated with food production and

consumption [44,67]. Therefore, food information in terms of food labels, other than factors that legally have to be present on products, should be legible and scientifically updated in order to better educate and inform the consumer.

On the other hand, studies have confirmed that a significant proportion of consumers do not have sufficient knowledge to allow them to understand the values stated on food labels and do not clearly distinguish between nutrient content, structure function, and health or nutrition claims. Thus, there is a need to increase the level of consumer knowledge [44,45,67]. Consumer confidence is most often based on trust in certain brands, labels, and traditions regarding certain foods [22]. These results are in line with our knowledge that most consumers only review food labels sometimes. It is possible that they read food labels briefly or review only the origin and shelf life of the food and have a certain level of indecision and partial knowledge regarding the importance of reading food labels. This may be due to the time required to analyze data as well as knowledge on where the information collection and analysis took place. A number of studies have confirmed that, for consumers, it is best to keep product information short and use both sides of the package, for example, present short claims on the front and more detailed information on the back [44,68]. Clearly, consumers prefer short, concise information that creates a more convincing and positive picture without having to read a lot of text [44,68]. The reason for this is that information overload can occur due to exposure to too much information, especially at the point of purchase where consumers generally have limited time or are exposed to various distractors that can interfere with the analysis of read information [31,43,67]. However, although an excessive amount of information increases the risk of consumer overload, there is a growing need to provide more product information to promote the consumption of a balanced and healthy diet [31,43]. It is possible that the implementation of front-of-pack nutritional labels, like warning labels, could increase label reading and could warn consumers about critical nutrients that could represent a health risk, such as sugar, salt, or saturated fats [69].

The nutritional content information presented on labels has an effect on choice of food and stimulates the consumption of healthy food, but labels can also have an inadvertent effect by promoting excessive intake of certain nutrients or products [30]. Although having a food label on the product is mandatory, for some consumers, the presence of it leaves an impression that the food is healthy, and this "halo" effect may deter them from seeking further information about the food and its composition [44]. Additionally, food labels can create confusion if they are not presented in a way that is easily understood by consumers.

Even though consumers may be shopping with a health or diet goal in mind, particular distractions, such as time pressure, may have an impact on their attention to food labels and their nutrition or health claims. Consumers may not use nutrition labels if they are complicated and time-consuming [27]. Reading food labels may be related to different factors, for example, sociodemographic characteristics, food-related motivation, health orientation, point-of-sale, repeat purchase factors, and time and daily segments of food shopping [53,70]. Additionally, it is possible that respondents who shop in supermarkets and large shopping centers have more time to analyze food products when they wait in line compared with those who go to small shops. However, we did not examine the sales point at which they buy fiber-rich foods (supermarket, small shops), how long purchases last, or when consumers shop.

However, the health benefits of reading food labels are much greater than these findings, and this paper shows that there is a weak connection between consuming DF and reading food labels, with the largest share of impact being nutritional information and the analysis of the food label itself, rather than the DF itself. Additionally, in this study, we found a relatively weak correlation between general and health knowledge about DF and the frequency of reading labels. We did not find an association between sources of information about DF and label reading, despite other studies showing that nutritional knowledge provides support for food label use [45]. Accordingly, greater knowledge about the health benefits of DF is needed in the general population, because this may

have a positive impact on the consumption of foods containing fiber, which may improve people's health.

The strengths of this study are the relatively large sample size and the inclusion of questions on both general and health-related knowledge about DF and reading food product labels and information about DF on labels. Despite these factors, our results should be interpreted with the consideration of some limitations. First, this was a cross-sectional study, which means that it cannot conclude that there is a causal relationship between the use of nutritional labels and actual nutrient intake. Likewise, the labels for different food categories are quite different, so it is quite difficult to carry out comparisons, especially knowing that some raw produce with high DF do not need labels and that the variables of frequent consumption of whole grain, fruits, and vegetables are more like identifiers of the consumer food habits. Additionally, this study lacked data on the health statuses of respondents, possible bad habits in terms of smoking and physical activity, and the BMI data of respondents.

Hence, because food labels include information relating to both safety and nutritional content, they have been shown to be important for consumer protection [71]. Thus, it is very important that consumers are instructed to understand and have confidence in the information on food labels to allow them to correctly select and consume foods. Public health programs urgently need to raise public awareness about the importance of understanding nutritional labels to achieve healthy food consumption. Hence, emergency actions must be undertaken to increase attitudes towards the importance of food label reading, because it has been observed that many people do not pay attention to the nutritional information present on food labels that might help them with better food selections.

5. Conclusions

Monitoring dietary fiber on food labels was chosen as a topic, because it provides an instructive example for consumers in terms of understanding and reading different types of labels and claims known and distinguished by the law. This detailed analysis of the level of reading of nutritional labels and information about dietary fiber may help with the design of effective public health programs in Croatia and potentially other countries. Thus, targeted education campaigns should be developed to educate and sensitize the population about food labeling, monitor dietary fiber through food labels, and improve knowledge about healthy foods and their health benefits. The use of these very simple steps in public programs may increase and/or sensitize the population to consume healthy foods and be active in the prevention of chronic non-communicable diseases. Public health efforts should be made to help consumers to understand the nutritional information on food labels in order to carry out more effective decision making.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods11152347/s1, Table S1: Frequency of food label reading in accordance with sociodemographic factors of subjects; N (%).

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Phenolic Acid Profiling of *Lactarius hatsudake* Extracts, Anti-Cancer Function and Its Molecular Mechanisms

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Abstract: Cancer is still the leading cause of death across the world, and there is a lack of efficient therapies. *Lactarius hatsudake* is a mushroom with a food and medicine homology that contains numerous biologically active substances. This study aimed to investigate the composition of extracts from *Lactarius hatsudake* (*L. hatsudake*) and their anti-cancer function and molecular mechanisms. Our results showed that the total phenolic content of *L. hatsudake* extracts was 139.46 ± 5.42 mg/g. The following six phenolic compounds were identified from *L. hatsudake* extracts by HPLC and UPLC-QTOF/MS: gallic acid, pyrogallol, chlorogenic acid, ferulic acid, myricetin, and cinnamic acid. Colorectal cancer cell HCT116 and hepatic cancer cell HepG2 were used to evaluate the anti-cancer function of the *L. hatsudake* extracts. Compared with HepG2 cells, the *L. hatsudake* extracts showed stronger anti-cancer activity against HCT116 cells and these were used to study molecular mechanisms. The results indicated that the *L. hatsudake* extracts could arrest the cancer cell cycle and inhibit cancer cell proliferation, which may be mediated by the MAPK/NFκB/AP-1 signalling pathway; the *L. hatsudake* extracts also promoted cancer cell apoptosis through a mitochondrial-dependent pathway. Taken together, these findings demonstrate that *L. hatsudake* ethanol extracts contain six main phenolics and illustrate the remarkable potentiality of *L. hatsudake* as a source of natural phenolics for cancer prevention and as an adjuvant in the treatment of functional foods.

Keywords: *Lactarius hatsudake*; phenolic acids; anti-cancer; molecular mechanism

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1. Introduction

Cancer is still the main cause of death across world. According to the estimation of the World Health Organization (WHO) [1], there were about 19.29 million new cancer cases and 9.96 million deaths in 2020 worldwide. Cancer is a kind of sporadic disease caused by abnormal mechanisms of cell proliferation and apoptosis, which are induced by a variety of factors such as environmental, pathological, and biological mechanisms. The current anti-cancer drugs in clinical therapy show side effects and complications compared with natural bioactive products [2]. Therefore, developing natural compounds to reduce the major cancer risk factors and prevent carcinogenesis becomes necessary. Recently, mushroom-derived secondary metabolites have attracted greater attention in chemoprevention. The compounds can kill or inhibit tumor cells as cytotoxic anti-tumor drugs [3]. For example, 200 and 300 mg/kg of the *Ganoderma lucidum* polysaccharide decreased AOM/DSS-induced tumorigenesis in a dose-dependent manner [4]. Their anti-cancer activities are closely related to carcinogenesis-related signal pathways such as PI3K/Akt pathway, NFκB pathway, MAPK pathway, etc. Further investigations of the anti-cancer effects of mushrooms or their active compounds has been conducted, and indicates its great potential market value in the future [5,6].

Lactarius hatsudake (*L. hatsudake*), a great wild endemic fungus belonging to the genus *Lactarius* family *Russulaceae* with a food and medicine homology, is widely distributed in Asia, Europe, and North America [7]. It is known for its abundance of major nutrients and its high content of bioactive compounds, e.g., polyphenols, polysaccharides, and ergosterols [8]. Several studies have indicated that mushroom polysaccharides have anti-tumor activities, ergosterols can promote calcium absorption and polyphenols have an antioxidation effect. *Lactarius* extracts showed the greatest anti-glioma potential compared with other mushrooms, but there are very few studies focusing on elucidating the key ingredients of its anti-tumor activity. Polyphenols are comprised of a variety of bioactive compounds that are usually divided into several classes such as flavonoids, phenolic acids, and lignans [9]. The phenolic acids can reduce the adhesion of various microbial cells, which ultimately leads to reduced causes of diseases [10]. It has been reported that polyphenols composed of hydroxycinnamic acid derivatives, flavan-3-ols, and flavonols could inhibit the progress of glioblastoma, colon cancer, and lung carcinoma in a significant dose-dependent manner [5,11]. Other studies showed that geraniin and isocorilagin exhibited higher cytotoxicity against MCF-7 with an IC50 of 13.2 and 80.9 μg/mL [12]. Those investigations suggest that extracts including phenolic from *L. hatsudake* may be the important compounds exerting anti-cancer effect.

In this study, the aim was to evaluate the anti-tumor activity of *L. hatsudake* extracts. *L. hatsudake* extracts were obtained with 60% ethanol under ultrasonic conditions, and then purified with AB-8 macroporous resin. Six phenolics were identified by using both HPLC and UPLC-QTOF/MS [13]. Their anti-cancer activities were determined using in vitro assay with HCT116 cells and HepG2 cells. The effects of *L. hatsudake* extracts on cell proliferation and apoptosis were assessed and the anti-cancer mechanisms were explored, as shown in Figure 1.

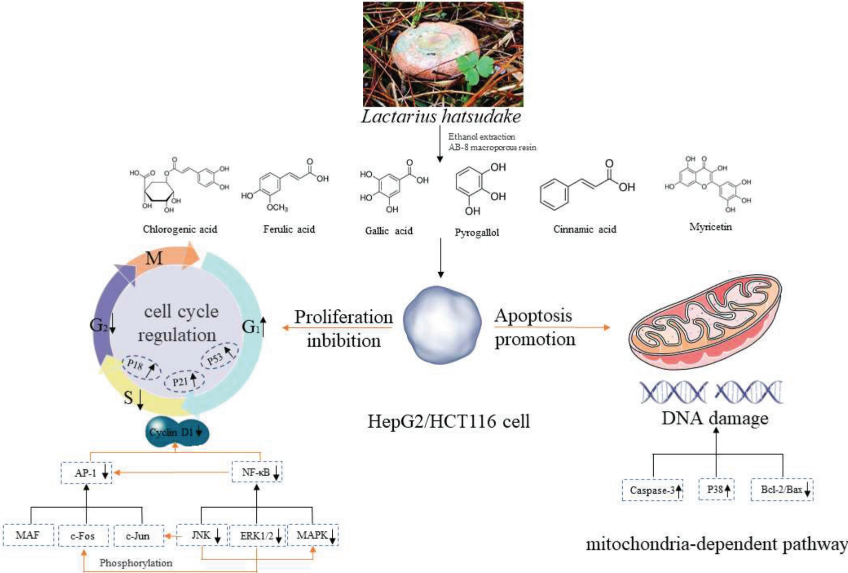

Figure 1. Composition and anticancer mechanism of *L. hatsudake* extracts.

2. Materials and Methods

2.1. Chemicals

The fresh *L. hatsudake* fruit bodies were purchased from Hunan, Changsha Province, China. The standards of myricetin, cinnamic acid, and pyrogallol were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Caffeic acid,

chlorogenic acid, ferulic acid, and gallic acid were purchased from Sigma-Aldrich (Shanghai, China) Trading Co., Ltd. Acetonitrile, methanol, and formic acid were of HPLC grade and purchased from Sigma-Aldrich Trading Co., Ltd. HCT116 and HepG2 cells were obtained from the Cell Culture Center of Shanghai Institute for Biological Sciences (Shanghai, China). Additionally, 0.25% trypsin-EDTA, fetal bovine serum (PBS), 1640 medium, penicillin-streptomycin, and trypsin were purchased from Gibco (Waltham, MA, USA). BSA was purchased from Beijing Solaibao Biotech Water Co., Ltd. (Beijing, China). MTS was purchased from Millipore and SYBR Green was purchased from the Beijing Quansi Gold Biotechnology Co., Ltd. (Beijing, China).

Other chemicals not mentioned above were of HPLC or analytical grade. Aqueous solutions were prepared with deionized water (18.2 MΩ·cm; Simplicity 185, Millipore Corp, Billerica, MA, USA).

2.2. Extraction and Purification

The protocols of this study are similar to those of reference [14], with some modifications. The clean fresh *L. hatsudake* fruit bodies were dried under freeze-drying, and then processed through a 100 mesh sieve after being ground to a powder. The powders were extracted using 60% ethanol in the ratio of 1:25 (*w*/*v*) and ultrasonic assisted for 80 min at 30 °C. The mixture was centrifuged at 3000 rpm for 15 min. The residues were re-extracted twice under the same conditions and all the ethanolic extracts were combined. The organic solvents in the extracts were removed with a rotary evaporator (N-1300V-W, Tokyo Rikakikai Co., Ltd. (Tokyo, Japan)) and then the residues were vacuum freeze-dried.

The *L. hatsudake* extracts were purified with AB-8 macroporous resin and the obtained compounds were condensed with a rotary evaporator and dried with a vacuum freeze dryer (Ytlg-12A, Shanghai Yetuo Co., Ltd. (Shanghai, China)).

2.3. Total Phenolic Content

The total phenolic contents with *L. hatsudake* ethanolic extracts was measured by means of the Folin–Ciocalteu assay [15]. Gallic acid was used to calculate the standard curve. Estimation of the total phenolic acids was carried out in six duplicates ($n = 6$). The result was expressed as mg of gallic acid per gram of the extracts.

2.4. Analysis of Phenolic Profiles

The composition of *L. hatsudake* extracts was analyzed using both the HPLC and UPLC-QTOF/MS methods described by Palacios [16]. An LC-20A HPLC (Shimadzu, Japan) equipped with a UV-vis detector was used for profiling. Separation was achieved on an ODS-C_{18} column (5 μm, 250 mm, 4.6 mm i.d.) at 30 °C by using the specific solvent system of 0.1% acetic acid in water (solvent A) and acetonitrile (solvent B) under the optimum gradient conditions (0–2 min, 10% B; 2–10 min, 20% B; 10–30 min, 80% B; 30–40 min, 100% B, 40–43 min, 8% B). The standard phenolic compounds (gallic acid, pyrogallol, chlorogenic acid, caffeic acid, ferulic acid, myricetin, and cinnamic acid) were used. A Waters ACQUITY UPLC IClass/Xevo in line with a Waters Xevo G2 Q-TOF mass spectrometer (Milford, MA, USA) was also used to detect the *L. hatsudake* extracts. Chromatographic separation was performed with a Luna C_{18} (2) column (250 mm × 4.6 mm, 5 μm; Phenomenex (Aschaffenburg, Germany)), with the temperature kept at 40 °C. Electrospray ionization (ESI) was performed in the negative mode (capillary temperature: 350 °C; capillary voltage: −5 kV; spray voltage: −4 kV).

2.5. Cell Culture and Polyphenol Treatments

The HCT116 cells were cultured in an RPMI-1640 medium including 10% (*v*/*v*) fetal bovine serum (FBS) and 1% penicillin/streptomycin (100 U/mL penicillin and 100 mg/mL streptomycin). *L. hatsudake* extracts were dissolved in ethanol and then diluted with ultrapure water to achieve the desired ratio of alcohol. The final concentration of *L. hatsudake* extracts was 10 mg/mL and they were stored in a 4 °C refrigerator. The stock solution was

diluted with the basal medium to prepare the required working fluid concentration. The final concentration of ethanol was less than 0.1%, which did not affect cell viability.

2.6. Cell Viability Assay

The cytotoxicity of *L. hatsudake* extracts was detected using an MTS assay. Briefly, HCT116 cells were seeded in 96-well culture plates at a density of 4×10^4 cells per well at 37 °C for 8–10 h. Next, cells were treated with different concentrations (0, 6.25, 12.5, 25, 50, 100, 200 μg/mL) of *L. hatsudake* extracts in culture medium for 24 h. Subsequently, 20 μL MTS solution (dissolved in PBS) was added to the well of plates, and the plates were incubated at 37 °C for 4 h. The absorbance was measured at 490 nm with a microplate reader to assess the optical density (OD), from which cell counts were determined. The inhibition rate of *L. hatsudake* extracts was calculated as follows:

$$\text{Inhibition rate (\%)} = (\text{1-experimental group OD/control group OD}) \times 100\%.$$

2.7. Hoechst 33258 Fluorescent Staining

HCT116 was digested into individual cells and then prepared into cell-like slices. When the cells adhered to the wall, *L. hatsudake* extracts of different concentrations (0, 25, 50, 100 μg/mL) were added. Afterwards, cells were treated at 4 °C for 5 min by using cell fixative (methanol: ice acetic acid = 3:1). After the PBS was gently cleaned, a solution of 5 g/mL Hochest 33258 was added for 10 min, then washed three times with PBS, each time for 5 min. After drying, the cellulate side of the slide was covered downward on the slide, and the slide was closed with transparent nail polish and observed under a fluorescence microscope.

2.8. SDS–PAGE and Western Blot Analysis

HCT116 cells were cultured at a density of 1×10^6 cells/mL in a Petri dish with a diameter of 10 cm. After 12 h, fresh medium was added at 8 mL per dish and *L. hatsudake* extracts were added for a final concentration of 0, 25, 50, and 100 ug/mL. After continued culturing for 16–18 h, the culture solution was discarded, washed twice with ice-cold PBS, and the PBS in the Petri dish was aspirated with a pipette. A total of 300 μL of the RIPA lysate containing phosphatase and protease inhibitors was added to each well. The cells were scraped, and the lysate was pipetted into a 1.5 mL centrifuge tube and centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant was aspirated to obtain the total protein. The protein concentration was determined using a BCA kit to determine the amount of the sample. After the 5 × SDS buffer (10% SDS, 50% glycerol, Tris-HCl pH6.8 125 mM) was added to the above supernatant, it was immediately heated at 95 °C for 5 min. The equivalent protein sample was subjected to SDS-PAGE and electrophoresis transfer to the PVDF membranes semidry transfer device (Bio-rad, Hercules, CA, USA). Nonspecific binding sites in PVDF membranes were sealed with 5% bovine serum albumin in TBST (20 mM Tris, 166 mM NaCl, and 0.05% Tween 20, pH 7.5) for 1 h. The PVDF membrane with an appropriate primary antibody was stored at 4 °C overnight, and then incubated along with their respective horseradish-peroxidase conjugated secondary antibody at room temperature. Finally, the PVDF membrane was washed three times with TBST. It was detected using the chemiluminescent substrate (Thermo Fisher, Waltham, MA, USA) and visualized with the Molecular Image chemdoc XRS system (Bio-rad, Hercules, CA, USA).

2.9. Reverse Transcription-Quantitative PCR (RT-qPCR) Analysis

HCT116 cells were seeded at a density of 1×10^6/well in a 6-well culture dish and placed in a carbon dioxide incubator for 24 h. Cells were treated with *L. hatsudake* extracts (0, 25, 50, and 100 μg/mL) for 12 h. Extraction of total RNA from cells was performed using the Transzol-Up kit according to the manufacturer's instructions. The mass, purity, and concentration of the RNA samples were analyzed using a Nanodrop ultra-differential photometer instrument. A total of 2 μg of RNA was added to a reverse transcription

system of 20 μL, and cDNA synthesis was performed using a high-efficiency cDNA reverse transcription kit. qPCR reactions were observed using SYBR Green I (Trans Gen Biotech Co., Ltd.; Beijing, China) in accordance with the manufacturer's protocol. The amplification conditions were as follows: 94 °C, initial denaturation for 3 min; 94 °C, denaturation for 30 s; 60 °C, annealing for 40 s; and 72 °C, extension for 1 min, 40 cycles. Relative expression levels of the target genes were calculated by using $2-\triangle\triangle Ct(RQ)$method. For PCR primers, see Table 1.

Table 1. Primers sequences used for quantitative PCR analysis of gene expression.

Primer	Forward	Reverse
p15	5′ GTT GTT TGG TTA TTG TAT GGG 3′	5′ CCC TTA TTC TCC TCA CAC AT 3′
p16	5′ CCC AAC GCA CCG AAT AGT TAC 3′	5′ GTT CTT TCA ATC GGG GAT GTC 3′
p18	5′ GGG GAC CTA GAG CAA CTT ACT AGT TT 3′	5′ AAA TCG GGA TTA GCA CCT CTA AGT A 3′
p21	5′ ATG TGG ACC TGT CAC TGT CTT GTA 3′	5′ GTT GGA GTG GTA GAA ATC TGT CAT 3′
p27	5′ AGT GGA TGA TGA GAT TGT GGA GTT 3′	5′ AAC AAG TCT AAG CTG GTG TTT TTC C 3′
p53	5′ CCC AAG CAA TGG ATG ATT TGA 3′	5′ GGC ATT CTG GGA GCT TCA TCT 3′
β-actin	5′ CAT GTA CGT TGC TAT CCA GGC 3′	5′ CTC CTT AAT GTC ACG CAC GAT 3′

2.10. Cytometry Analysis

HCT116 cells were treated with different concentrations of *L. hatsudake* extracts for 48 h. Cells were washed twice with D-Hanks at 4 °C and the supernatant was discarded. They were fixed for 12 h with 70% ethanol in ice-cold PBS at −20 °C, centrifuge and ethanol was discarded, they were washed twice with PBS, and 100 μL was added. The FITC annexin V apoptosis detection kit (BD Biosciences, San Jose, CA, USA) was used for DNA extraction. A DNA extraction buffer reaction occurred at room temperature for 30 min. Then, 1 × PBS was used for washing the cells, the cells were resuspended using 100 μL 1 × PBS, 10 μL 10 mg/mL RNase and 10 μL 1 mg/mL PI solution were added, and they were incubated at room temperature in the dark for 30 min. An FACS Caliber Flow Cytometer (BD Biosciences, San Jose, CA, USA) was used to detect the cell cycle.

2.11. Apoptosis Detection

The HCT116 cells were up to 90% full, monolayer adherent cells were digested with 0.05% trypsin without EDTA, and the cell concentration was diluted to 5×10^5 cells/mL with medium and inoculated into a six-well plate in an amount of 1 mL per well. Then, they were incubated overnight, the medium was discarded, and cells were washed twice with PBS, after which 1 mL of fresh medium was added alongside *L. hatsudake* extracts to a final concentration of 0, 25 μmol/L, 50 μmol/L, and 100 μmol. After 12 h, the HCT116 cells were digested and collected, and then centrifuged at 1000 rpm for 4 min at 5 °C, the supernatant medium was removed, resuspended in PBS, centrifuged again, and the supernatant was repeated once, and placed on a filter paper. After about 1 min, the PBS was removed. A total of 400 μL of the 1 × Annexin Binding buffer was added and cells were gently resuspended. Then, 5 μL of Annexin V-FTTC and 5 μL of PI were added to each of the groups, which were incubated at room temperature for 10–15 min in the dark, and checked on the machine within 1 h.

2.12. Luciferase Reporter Activity Assay

HCT116 cells in the logarithmic growth phase were obtained, the culture solution was drained, cells were washed twice with D-Hanks buffer, the residual liquid was aspirated, and the cells were digested by adding the appropriate amount of trypsin. The flask was removed and to stop digestion 1 ml of the 1640 medium was added and gently pipetted to make a cell suspension. After counting using the cytometer, the medium was diluted to a concentration of 4×10^6 /mL. The cell suspension was inoculated into a 24-well plate at 500 μL per well. After incubation overnight, the cells were grown by approximately 70–80%, the medium was aspirated, cells were washed twice with ice-cold PBS, and the

remaining PBS was blotted in the six-well plate with a pipette. With serum-free medium, the cells were washed, which was then replaced with 500 μL per well of fresh serum-free medium. A total of 1.5 μL Lipo2000 was added to each well, and 1.0 μg of AP-1 or NF-κB plasmid was added, mixed gently, and absorbed for 3 h. After removing the medium and washing once with PBS, the normal medium was added, incubated for 5 h at 37 °C, 5% CO_2 was added and *L. hatsudake* extracts at 25 μg/mL, 50 μg/mL and 100 μg/mL were added. Culturing continued for 12 h, the culture medium was discarded, 200 μL of the cell lysate was added, then cells were incubated at room temperature for 5 min, and after lysis, transferred to a 1.5 mL EP tube, centrifuged at 10,000 rpm for 5 min, and the supernatant was aspirated. A total of 50 μL of the treated supernatant was added to the assay plate for 3 replicates at each concentration. A total of 80 μL of luciferase was added before the assay and detected by the machine.

2.13. Statistical Analysis

The phenolic compound content of *L. hatsudake* extracts was expressed as the mean ± SD (standard deviation) of the six replicates. Statistical analysis was conducted using SPSS19 software (SPSS, Inc., Chicago, IL, USA) with a one-way analysis of variance (ANOVA), and a value of $p < 0.05$ was taken as the level of significance.

3. Results and Discussion

3.1. The Contents and Composition of Total Phenols

The total phenolic content obtained from the crude extract and the purified extract from the *L. hatsudake* samples ($n = 6$) was found. The total phenolic content of the crude extract was 5.05 ± 0.15 mg/g as equivalents of gallic acid (Figure S1) under the optimal conditions which were as follows: ethanol concentration 60%, solid–liquid ratio 1:25 (*w*/*v*), and extraction time of 80 min, as shown in Figures S2–S4. After purification by AB-8 macroporous resin, the phenolic content increased to 139.46 ± 5.42 mg/g as equivalents of gallic acid. The total phenolic content in *L. hatsudake* samples was calculated from the crude extract and 0.72 ± 0.02 mg/g was obtained.

The phenolic profiles of the purified extract in *L. hatsudake* were analyzed by both HPLC and UPLC-QTOF/MS. As shown in Figure 2, the extracts from *L. hatsudake* mainly contained gallic acid, pyrogallol, chlorogenic acid, ferulic acid, myricetin, and cinnamic acid compared with standard substances [17]. Interestingly, all phenolic compounds from *L. hatsudake* extracts belonged to phenolic acids from HPLC detection, shown in Figure 2B. However, the sample and standard substances have some deviations in retention time due to bad parallelism with HPLC. To further verify whether the results are reliable and to explore the specific composition, the results were observed using UPLC-QTOF/MS with standard substances. According to Table 2, gallic acid, pyrogallol, chlorogenic acid, ferulic acid, myricetin, and cinnamic acid were detected at similar rates as for the HPLC results, and the results are also consistent with previous articles [18–20]. Besides that, there are another seven unknown substances which appeared that required further exploration. Previous studies have shown that phenolic acids exhibit obvious bioactivities to reduce the occurrence of disease [21]. Some groups found that chlorogenic acid decreased colon cancer cell proliferation with major colonic microbial metabolites as a result of anti-proliferative effects, S-phase cell-cycle arrest and apoptosis in human colon cancer Caco-2 cells [22]. Another study found that gallic acids could induce apoptosis in HL60 cells due to the production of H_2O_2 [23]. The results indicated that the phenolic content in *L. hatsudake* extracts promoted the potential for anti-cancer activities.

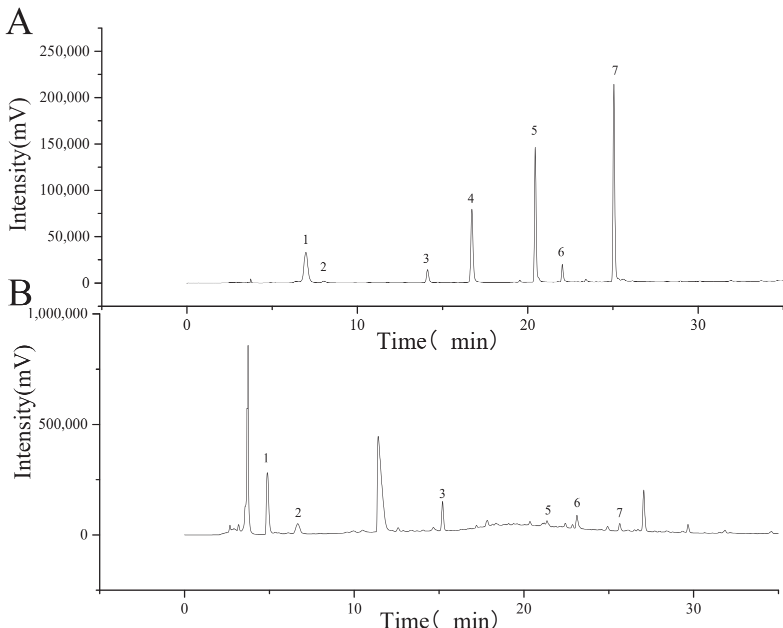

Figure 2. The chromatogram of *L. hatsudake* extracts analyzed by HPLC. (**A**) Standard mixture of phenolic; (**B**) phenolic profiles extracted from *L. hatsudake*. 1 gallic acid; 2 pyrogallol; 3 chlorogenic acid; 4 caffeic acid; 5 ferulic acid; 6 myricetin; and 7 cinnamic acid.

Table 2. Mass spectrometric results of substances in purified products.

	Parent Ion	Sub-Ion Ion
Chlorogenic acid	353.15	96.70
Ferulic acid	193.04	148.83
Caffeic acid	No detected	No detected
Gallic acid	187.08	124.82
Pyrogallol	125.08	97.62
Cinnamic acid	147.11	120.78
Myricetin	317.08	272.76
Substance-1	264.97	96.74, 79.72
Substance-2	187.04	124.69
Substance-3	278.77	261.09
Substance-4	309.13	96.89, 290.78, 208.70
Substance-5	433.18	152.77, 78.77, 170.47
Substance-6	295.02	276.85, 156.70, 182.72
Substance-7	149.87	95.69, 107.38, 122.78

3.2. L. hatsudake Extracts Inhibited Cancer Cell Proliferation

The notion of chemoprevention by natural bioactivities compounds has received much attention in the past decade, especially in anti-cancer activity with mushroom extracts [24]. To detect the mechanism of the anti-cancer function of *L. hatsudake* extracts, colorectal cancer HCT116 and hepatic cancer cell HepG2 were tested in vitro. The two kinds of cells were added at various concentrations (0, 6.25, 12.5, 25, 50, 100, and 200 μg/mL) of *L. hatsudake* extracts for 24 h, and cell viability was analyzed using the MTS assay. Under an optical microscope, the morphology of two cancer cells ruptured and died, and the number of cancer cells decreased (Figure 3A,B). The MTS experiment results showed that *L. hatsudake* extracts inhibited the cell proliferation of HCT116 with 72% cell viability and HepG2 with 75% cell viability at 200 μg/mL. With the increase in the *L. hatsudake*

extracts concentration, cell viability decreased. Compared with the phenolic extracted from Sorghum, Bran had a significant inhibitory effect at 1.5 mg/mL, and *L. hatsudake* extracts inhibited cell proliferation at a quantity of 25 μg/mL [11] (Figure 3C,D). Additionally, the anti-cancer effect of *L. hatsudake* extracts is a little more sensitive to HCT116 cells and we used the cell line to study further experiments. Our results suggest that *L. hatsudake* extracts can prevent the proliferation of cancer cells.

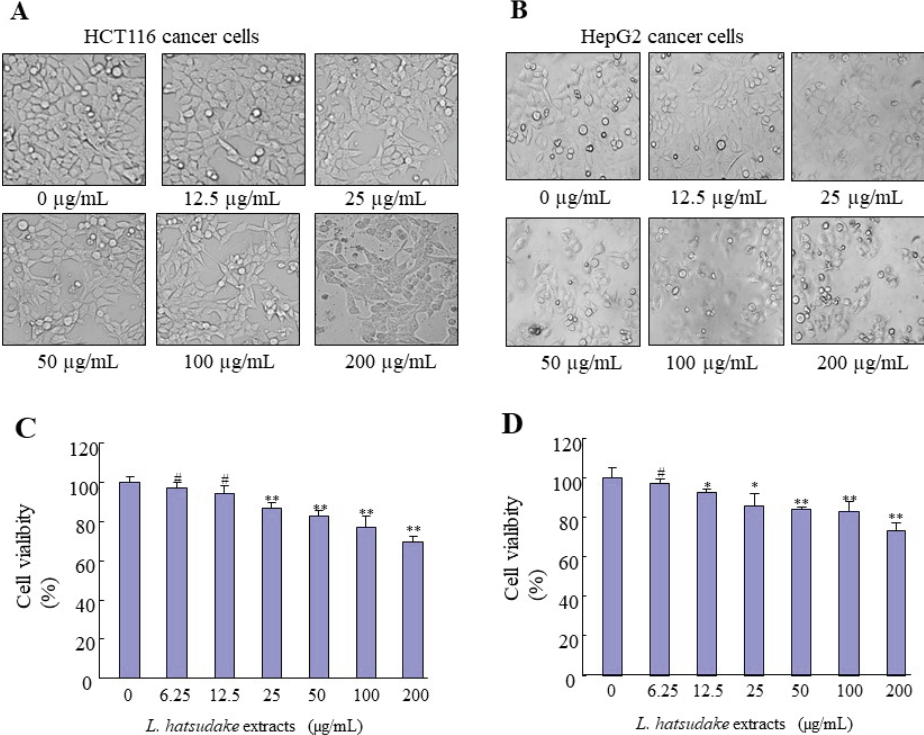

Figure 3. The effect of *L. hatsudake* extracts on the cell morphology proliferation of HCT116 and HepG2 cancer cells. (**A**) The cell morphology of HCT116 cells. (**B**) The cell morphology of HepG2 cells. (**C**) The cell viability of HCT116 cells by MTS analysis. (**D**) The cell viability of HepG2 cells by MTS analysis. *: $p < 0.05$; **: $p < 0.01$; #: $p > 0.05$.

3.3. *L. hatsudake* Extracts Arrest Cell Cycle of Cancer Cells

To better understand the bioactivity of *L. hatsudake* extracts, a study on the mechanism of its antiproliferative activity against HCT116 cells is currently underway. One of the mechanisms of the prevention of cancer is regulating the cell cycle, which can arrest the cell cycle at the G0/G1, S, or G2/M phase. To assess the effect of *L. hatsudake* extracts on the cell cycle, colorectal cancer HCT116 cells were added to 0, 25, 50, and 100 μg/mL *L. hatsudake* extracts for 24 h. The cytometry results showed that *L. hatsudake* extract treatments caused the percentage of the G1-phase to increase from 34.2% to 41.7%, 45.1%, and 49.0%, respectively. On the contrary, the percentages of the S-phase and G2/M-phase also reduced in a dosage-dependent manner (Figure 4A). The data suggest that *L. hatsudake* extracts can increase the percentage of resting cancer cells and decrease the percentage of mitotic cancer cells, which cause cell cycle arrest. The most important feature of cancer cells is that the proliferation of cells is out of control, and *L. hatsudake* extracts can inhibit the proliferation of cancer cells, meaning that it has an anti-cancer effect.

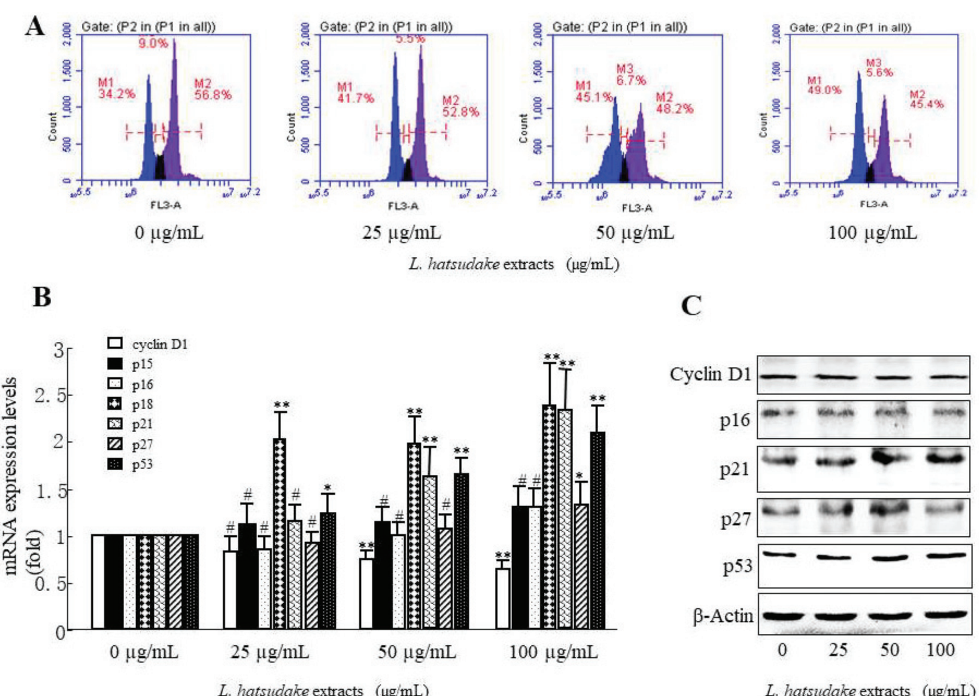

Figure 4. The effect of *L. hatsudake* extracts on the cell cycle, CDKIs and cyclin D1 in HCT116 cancer cells. (**A**) The effect of phenolic acids on the cell cycle of HCT116 cancer cells. (**B**) cyclin D1 and CDKI mRNA expressions; (**C**) cyclin D1 and CDKI protein expressions; *: $p < 0.05$; **: $p < 0.01$; #: $p > 0.05$.

3.4. *L. hatsudake* Extracts Decreased CDKIs and Cyclin D1 Expressions

The cell cycle is regulated by the Cyclin/CDK/CDKI complex system. Our data indicated that *L. hatsudake* extracts increased the CDKI mRNA expression levels of p18, p21, and p53 in a dosage-dependent manner (Figure 3B). The Western blotting analysis further proved that *L. hatsudake* extracts reduced cyclin D1 protein expression levels and increased p21 and p53 protein expression (Figure 4C). p18 can bind with CDK4 and CDK6 and inhibits their activities [25], and p21 can bind CDK2, CDK4, and CDK6 and reduce their activities, which promote down-regulated activities and cell cycle arrest [26]. Wild-type p53 is a tumor suppressor gene, and up-regulated p53 expression can promote p21 expression, which inhibits CDKs and results in cell cycle arrest. Meanwhile, up-regulated p53 expression can promote cancer cell apoptosis [27]. *L. hatsudake* extracts could increase expressions of p18, p21, and p53, suggesting that *L. hatsudake* extracts can inhibit cancer cell proliferation by increasing the expression of CDKIs and inhibiting CDK activities. Our data showed that *L. hatsudake* extracts downregulated cyclin D1 mRNA expression due to RT-qPCR assay in HCT116 cancer cells, and the decreased expression of cyclin D1 reduced the binds to CDK4 and CDK6, which inhibited RB in releasing transcription factor E2F and reduced proliferation-related gene expressions [28]. Taken together, the results demonstrate that *L. hatsudake* extracts may prevent the cell cycle by decreasing cyclin D1 expression and increasing CDKI expressions.

3.5. *L. hatsudake* Extracts Inhibited Transcript Activities of NFκB and AP-1

Transcription factor activator protein 1 (AP-1) is a dimeric complex and its members include Jun, Fos, ATF, and MAF. AP-1 activation is closely related to carcinogenesis and is mostly activated in cancer. In the promoter of cyclin D1, AP-1 binding sites exist and AP-1 regulates the expression of cyclin D1. In this study, *L. hatsudake* extracts inhibited

the transcript activities of AP-1, as found in the luciferase reporter gene analysis, in a dosage-dependent manner (Figure 5A) and *L. hatsudake* extracts decreased the protein expression of cyclin D1 (Figure 4C), suggesting that *L. hatsudake* extracts may decrease cyclin D1 expression by inhibiting AP-1 activation. Transcript factor NFκB has long been considered as a prototypical proinflammatory signaling pathway, largely based on the activation of NFκB by proinflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor α (TNFα). NFκB activation also participates in carcinogenesis. In this study, *L. hatsudake* extracts inhibited the transcript activities of NFκB, as found in the luciferase reporter gene analysis, in a dosage-dependent manner (Figure 5B). This indicates that *L. hatsudake* extracts may decrease cyclin D1 expression by inhibiting NFκB activation. Taken together, *L. hatsudake* extracts may downregulate the activities of NFκB and AP-1 and inhibit cyclin D1, which results in the cell cycle arrest of cancer cells.

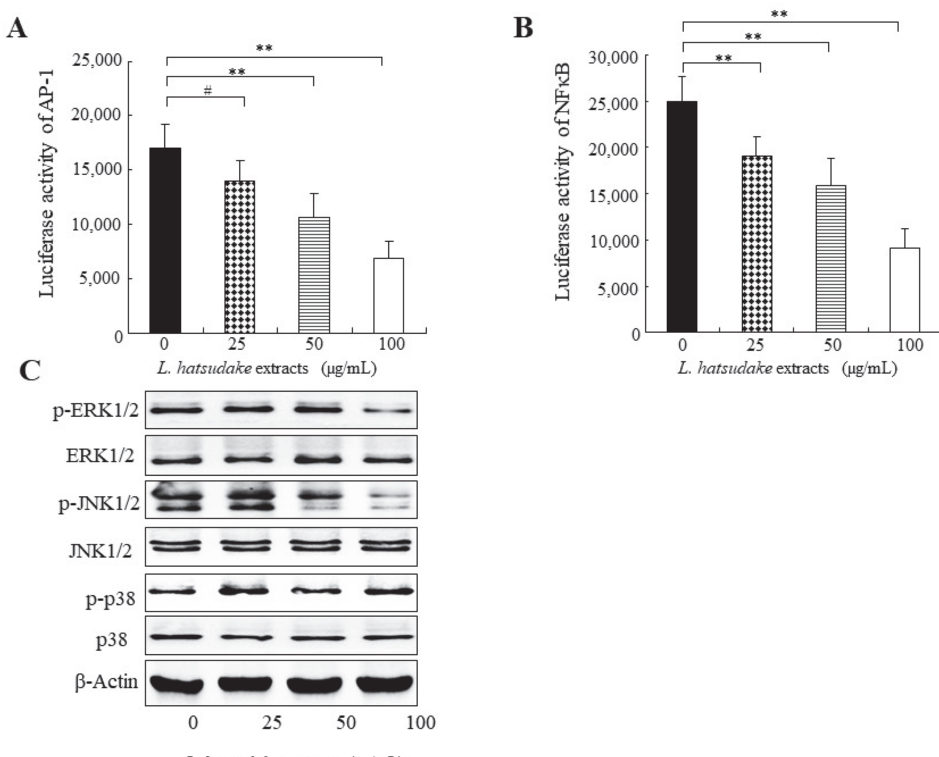

Figure 5. The effect of *L. hatsudake* extracts on the activities of AP-1, NFκB and MAPK signal pathway in HCT116 cancer cells. (**A**) *L. hatsudake* extracts inhibited the transcript activity of AP-1 in HCT116 cancer cells. (**B**) *L. hatsudake* extracts inhibited the transcript activity of NFκB in HCT116 cancer cells. **: $p < 0.01$; #: $p > 0.05$. (**C**) The effect of *L. hatsudake* extracts on MAPK signal pathway in HCT116 cancer cells.

3.6. *L. hatsudake* Extracts Inhibited MAPK Signal Pathway

MAPKs are always activated in cancer cells and uncontrolled cancer cell proliferation is closely related to MAPK activation. Our data indicated that *L. hatsudake* extracts decreased the phosphorylated proteins of ERK1/2 and JNK, as observed in the Western blotting analysis (Figure 5C), which means *L. hatsudake* extracts can prevent MAPK activation. Interestingly, ERK1/2 can cause the phosphorylation of c-Fos (one member of AP-1) and JNK can cause the phosphorylation of c-Jun (another member of AP-1) [29], which suggests

that *L. hatsudake* extracts inhibit the transcript activation of AP-1 by decreasing MAPK activities. ERK1/2 also can cause NFκB activation [30]. Our results showed that *L. hatsudake* extracts inhibit the transcript activation of NFκB, proving that *L. hatsudake* extracts inhibit the transcript activation of NFκB by decreasing MAPK activities. Taken together, our data indicate that *L. hatsudake* extracts block MAPK activation and downregulate the activities of NFκB and AP-1, which further inhibit cyclin D1 and the proliferation of cancer cells.

3.7. Pro-Apoptosis Effect and Mechanism of L. hatsudake Extracts

Apoptosis is a physiological and crucial process that is regarded as the preferred way to eliminate cancer cells [31]. Colorectal cancer cell HCT116 was treated with *L. hatsudake* extracts for 24 h, cells were fixed with a mixture solution of methanol: glacial acetic acid. Then, HCT116 cells were stained using Hoechst33258 fluorescent dye and the cell morphology was observed with a fluorescent microscope. We found that *L. hatsudake* extracts treatment resulted in nuclear condensation and nuclear fragmentation, and apoptotic bodies appeared (Figure 6A), as indicated by the arrow.

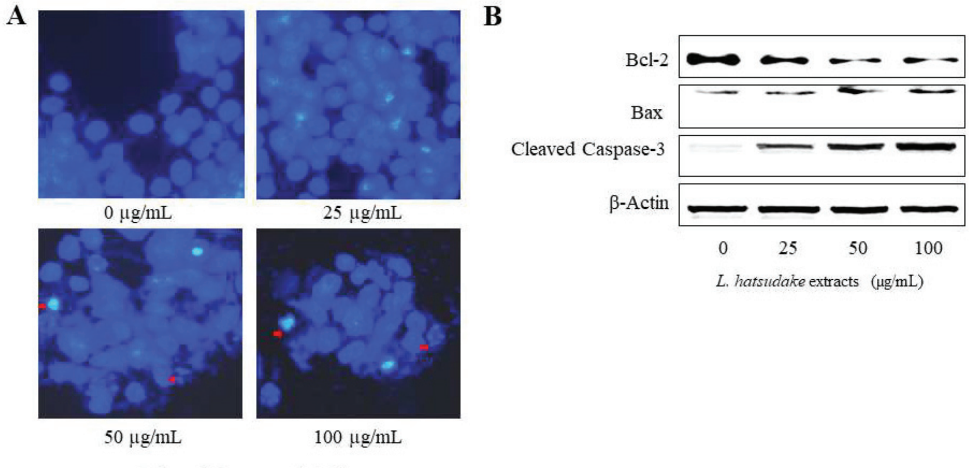

Figure 6. *L. hatsudake* extracts promoted HCT116 cell apoptosis and its molecular mechanism. (**A**) *L. hatsudake* extracts induced the apoptosis by Hoechst33258 fluorescent staining; (**B**) *L. hatsudake* extracts regulated apoptosis-related gene expressions by Western blotting in HCT116 cells.

To explore the molecular mechanism of the promoting apoptosis of *L. hatsudake* extracts, various concentrations of *L. hatsudake* extracts were added to HCT116 cells. The Western blotting analysis found that *L. hatsudake* extracts increased cleaved Caspase-3 protein and promoted cancer cell apoptosis. *L. hatsudake* extracts also increased the expression of promoting apoptosis proteins Bax in a dosage-dependent manner; on the contrary, anti-apoptotic protein Bcl-2 expression was reduced. All of these proteins involved in the downstream events of mitochondria and Bcl-2, depended on the mitochondrial apoptosis pathway [32] (Figure 6B). These findings suggests that *L. hatsudake* extracts may promote cancer cell apoptosis by altering Caspase-3 and the ratio of Bcl-2/Bax [33]. Meanwhile, *L. hatsudake* extracts activate p38 and may also promote the apoptosis of cancer cell HCT116 (Figure 6). Aside from altering apoptosis gene expression, DNA damage is also an important factor in HCT116-cell apoptosis, which is an important molecular target in the killing of tumor cells [34,35]. Importantly, the decreased phosphorylated proteins of ERK1/2 and JNK, were found to be downstream target kinases of DNA damage-induced apoptosis [36]. These data suggest that *L. hatsudake* extracts can promote HCT116-cell apoptosis via the mitochondria-dependent pathway.

4. Conclusions

In conclusion, the extracts of *L. hatsudake* mainly consisted of six phenolic compounds, which we found by conducting an HPLC/HPLC-MS analysis. *L. hatsudake* extracts can inhibit the MAPK pathway and the transcript activities of NFκB and AP-1, which modulate the expressions of cyclin D1. The down-regulated expression of cyclin D1 and up-regulated expression of CDKIs can reduce CDKs' activities and cause cell cycle arrest, which prevents cancer cell proliferation. Meanwhile, *L. hatsudake* extracts can induce the apoptosis of cancer cells. *L. hatsudake* extracts can activate apoptotic effector protein kinase Caspase-3, change the ratio of Bcl-2/Bax and cause mitochondrial damage, which means *L. hatsudake* extracts exert an apoptotic effect through the mitochondrial-dependent pathway. Taken together, our data demonstrated that *L. hatsudake* extracts exhibited an anti-cancer function via inhibiting cancer cell proliferation and promoting the apoptosis of cancer cells. This research suggests that *L. hatsudake* may be a potential anti-cancer function food. Further investigating monomers from *L. hatsudake* extract will promote our understanding of active compounds in *L. hatsudake*, which will contribute to the development of *L. hatsudake* as a novel ani-cancer function food and reduce the occurrence of cancers.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods11131839/s1, Figure S1: Standard curve of gallic acid; Figure S2: Effects of solid-liquid ratio on the extraction of phenolics from Lactarius hatsudake; Figure S3: Effects of solvents and concentration on the extraction of phenolics from Lactarius hatsudake; Figure S4: Effects of extraction time on the extraction of phenolics from Lactarius hatsudake.

Author Contributions: Conceptualization, J.R.; data curation, X.Z. and H.Q.; formal analysis, F.L. and Q.Y.; funding acquisition, J.R.; investigation, J.R. and X.Z.; methodology, X.Z.; project administration, J.R.; resources, J.R.; software, F.L. and Q.Y.; supervision, J.R.; validation, Q.Y. and H.Q.; visualization, Q.Y. and X.Z.; writing—original draft, F.L. and Q.Y.; writing—review and editing, J.R., F.L. and Q.Y. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Our study did not require ethical approval.

Informed Consent Statement: Our study did not involve humans.

Data Availability Statement: Data is contained within the article or supplementary material.

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

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Article

Aronia melanocarpa Fruit Juice Modulates ACE2 Immunoexpression and Diminishes Age-Related Remodeling of Coronary Arteries in Rats

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Abstract: The aim of the study is to evaluate the effect of *Aronia melanocarpa* fruit juice (AMJ) supplementation on age-related coronary arteries remodeled in aged rat hearts. Male Wistar rats (n = 24) were divided into three groups: (1) young controls (CY), aged 2 months, without AMJ supplementation; (2) old controls (CO), aged 27 months, without AMJ supplementation; and (3) the AMJ group (A), which used 27-month old animals, supplemented orally with AMJ for 105 days. AMJ supplementation did not influence the wall-to-diameter parameter (Kernohan index) of the coronary arteries of test animals. Aged rats supplemented with AMJ showed a significant decrease in the amount of collagen fibers in their coronary tunica media, as compared with the old controls. The intensity of the immunoreaction for alpha smooth muscle actin (αSMA) in the coronary tunica media was significantly lower in the supplemented group than in the old controls. The intensity of the angiotensin-converting enzyme 2 (ACE2) immunoreaction in the coronary tunica media of the supplemented group was significantly higher than the one observed in the old controls. These results indicate the positive effects of AMJ supplementation on the age-dependent remodeling of coronary arteries and support for the preventive potential of antioxidant-rich functional food supplementation in age-related diseases.

Keywords: ageing arteries; black chokeberry (*Aronia melanocarpa*); ACE2; functional foods

1. Introduction

The incidence and spread of cardiovascular and cerebrovascular diseases, such as hypertension, coronary heart disease, congestive heart failure, and stroke increase with advancing age. The greater part of research in this field focuses on developing interventions directed to the "traditional" risk factors for coronary heart disease (e.g., hypertension, hypercholesterolemia, etc.), whereas ageing remains outside the focus of attention [1]. With age, the blood vessels undergo structural and functional changes, even in visibly healthy individuals. All three layers of the arterial wall are involved—tunica intima, tunica media, and tunica adventitia. Age-related alterations in the arterial wall and function have been investigated in rodents, rabbits, and primates, and are rather similar to those observed in humans [1,2]. Age-related changes in the cells building up the three layers of the vascular wall underlie the tissue alterations observed. The pathophysiological processes associated

with age are triggered by oxidative stress and pro-inflammatory microenvironment mediated by mechanical and humoral factors [3]. The changes in the function and redox status of vascular smooth muscle cells (VSMCs) underlie age-related vascular remodeling. In the process of ageing, the VSMCs alter their phenotype from contractile to a synthesizing one. They cease to interact normally with the extracellular matrix due to the alterations in integrin expression or the alterations in its composition [4,5]. In the ageing process, even when there is no vascular disease present, VSMCs' migration from the media, and their proliferation, cause intimal thickening in the arterial walls [6]. The aged smooth muscle cells produce a larger quantity of matrix metalloproteinase 9 (MMP 9), thus contributing to the disintegration of extracellular matrix (ECM) [7]. Age-related remodeling of the vascular wall is characterized by the deposition of an excessive amount of collagen, proliferation of VSMCs, accumulation of the extracellular matrix, and suppression of its disintegration. This is a dynamic process, initially occurring as a reversible adaptive reparative response; however, the fibrogenic process progresses, resulting in further deterioration of arterial rigidity. The accumulation of collagenous connective tissue occurs in the large, as well as small, arteries [8]. The combination of ageing and factors favorable for the development of hypertension, as well as the activation of the renin-angiotensin-aldosterone system (RAAS), inflammation, oxidative stress, salt consumption, and genetic factors, result in alterations in delineating the border of pathology [8].

The renin-angiotensin-aldosterone system is a key factor in cardiovascular physiology and pathophysiology. It plays a central role in the structural and mechanical alterations in the vessels. Angiotensin II (Ang II) is the main effect-producing RAAS substance, possessing powerful vasoconstricting, pro-inflammatory, and pro-fibrotic properties [9]. The arterial components of the Ang II-signaling cascade increase with advancing age in rats, non-humanoid primates, and humans [1]. All components of RAAS are present in the cardiomyocytes and the vascular wall. Key elements in the cascade of the RAAS system are the angiotensin-converting enzyme (ACE) and its human homologue, the angiotensin-converting enzyme 2 (ACE2). Apart from being an important regulator of RAAS, ACE2 is a main factor in cardiac function, hypertension, and diabetes [9]. ACE2 has been found to act antagonistically with regard to ACE, so it is the functional mechanism eliminating Ang II and reducing its effects, respectively [10]. Expression of ACE2 is found in the cytoplasm, cell membrane, and cell nucleus of endothelial cells, smooth muscle cells, and cardiomyocytes. ACE2 is involved in regulating the process of proliferation, fibrosis, apoptosis, as well as vascular tone, and endothelial function. The expression of this protein is most marked in embryonic undifferentiated cells, and cells of young individuals, but it decreases with age [11]. Until recently, the processes of age-related remodeling of blood vessels were considered to be definitive and irreversible; however, there is evidence showing that they can be slowed down with the help of medications and non-drug management. The concepts of successful and unsuccessful vascular ageing have been coined, and when no signs have been manifested clinically, the most adequate intervention is the prophylactic non-drug management. Changes to one's way of life and dietary regimen, are strategies of immense potential.

An ever-increasing amount of evidence has shown that consumption of plant food is associated with a lowered risk of developing arteriosclerosis and diseases associated with oxidative stress [12,13]. Most antioxidants ingested with food are of plant origin. Polyphenolic compounds are found in all plants, and quantitatively, they are the most significant antioxidants taken with food [14]. The fruits of black chokeberry (*Aronia melanocarpa* (Michx). Elliot) are among the richest sources of polyphenols and especially anthocyanins in the plant kingdom [14]. Anthocyanins are known to exhibit a wide range of medicinal properties, such as capillary stabilizing, anti-inflammatory, hypotonic, and collagen stabilizing properties [14]. They play an important role in collagen crosslinking, and they inhibit its degradation by enzymes in the presence of inflammatory processes. In model systems, anthocyanin extracts have shown a cardioprotective effect and an ability to inhibit cancer cell growth [15], and aronia anthocyanins inhibit colon cancer by regulating glutamine

metabolism [16]. A number of in vitro and in vivo studies have demonstrated a wide range of applications of black chokeberry juice, extracts, and functional drinks, because of their anti-inflammatory, antimutagenic, anticarcinogenic, lipid lowering, antidiabetic, antihypertensive action, as well as hepatoprotective and immunomodulating effects [14,17–20]. Evidence of the effect of black chokeberry supplementation on the processes of age-related remodeling of the vascular wall is scarce in the available literature; therefore, the aim of the present study was to determine the effect of *Aronia melanocarpa* juice (AMJ) on age-related coronary artery remodeling in the hearts of aged rats.

2. Materials and Methods

2.1. *Aronia melanocarpa Juice*

Aronia melanocarpa berries were supplied by the licensed farmer Todor Petkov (Kazanlak, Stara Zagora district, Bulgaria) in the stage of full maturity. The fresh berries were placed in polyethylene bags, frozen immediately and stored at −18 °C until juice extraction. *Aronia melanocarpa* juice (dry solids content 18.8 °Bx) was obtained as described in Daskalova et al. [21] Briefly, five kilograms of frozen fruit were defrosted at room temperature and homogenized in a laboratory blender. The homogenate was transferred into a brown-glass bottle and incubated in a thermostatic shaking water bath at 60 °C for 1 h. After that, the pulp was filtered through a cheesecloth and the liquid fraction obtained was centrifuged and used for the study. The total polyphenol content and the major phenolic components, determined by high performance liquid chromatography (HPLC) of the juice, are shown in Table 1. HPLC analysis were preformed according to [21,22] as follows:

Table 1. Content of anthocyanins and other phenolic compounds in *A. melanocarpa* fruit juice [21].

Total Polyphenols (mg/L)	11,237.4 ± 456.2
Flavonoids (mg/L)	
Quercetin	49.6 ± 3.2
Quercetin-3-β-glucoside	228.8 ± 11.0
Rutin	446.5 ± 12.5
Epicatechin	408.2 ± 25.6
Anthocyanins (mg/L)	
Cyanidin-3-galactoside	1498.4 ± 102.3
Cyanidin-3-glucoside	120.1 ± 8.7
Cyanidin-3-arabinoside	501.9 ± 31.8
Cyanidin-3-xyloside	4.59 ± 0.2
Phenolic acids, mg/L	
Chlorogenic acid	1375.6 ± 80.3
Neochlorogenic acid	1543.1 ± 111.2

2.1.1. HPLC Analysis of Phenolic Compounds

HPLC analysis of phenolic components was performed on the HPLC system Agilent 1220 (Agilent Technology, Santa Clara, CA, USA), with a binary pump and UV-Vis detector (Agilent Technology, Santa Clara, CA, USA). Separation was performed on the Agilent TC-C18 column (5 µm, 4.6 mm × 250 mm) at 25 °C, and a wavelength of 280 nm was used. The following mobile phases were used: 0.5% acetic acid (A) and 100% acetonitrile (B) at a flow rate 0.8 mL/min. The gradient elution started with 14% B, between 6 min and 30 min, which was linearly increased to 25% B, then to 50% B at 40 min. Results were expressed as mg/100 g FW or per liter juice or nectar.

2.1.2. HPLC Determination of Anthocyanins

Anthocyanins were determined using the HPLC system Agilent 1220 (Agilent Technology, Palo Alto, CA, USA), with a binary pump and UV-Vis detector (Agilent Technology, Palo Alto, CA, USA). A wavelength of 520 nm was used. Anthocyanins were separated using an Agilent TC-C18 column (5 μm, 4.6 mm × 250 mm) at 25 °C. The following mobile phases were used: 5% formic acid (A) and 100% methanol (B) at a flow rate of 1.0 mL/min. The gradient condition started with 15% B, and linearly increased to 30% B at 20 min. Results were expressed as mg/100 g FW or per liter juice or nectar.

2.2. Animals

The study included 24 male Wistar rats provided by the Vivarium of Medical University-Plovdiv where they were maintained under standard laboratory conditions (housed in polypropylene cages in a controlled clean air environment at a temperature of 22 ± 3 °C, a 12-h light/dark cycle, and relative humidity of 60 ± 5%). Taking into account the hormonal influences in females, we used only male rats. The rats were divided into 3 groups: (1) young controls (CY), aged 2 months, with no AMJ supplementation; (2) old controls (CO), aged 27 months, with no AMJ supplementation; and (3) AMJ A group (A), which used 27-month old animals, supplemented orally with AMJ (10 $mL \cdot kg^{-1}$) using drinking water for 105 days. The duration of supplementation was based on our previous studies [21]. Rats were on a standard rodent chow (containing 13.45% protein, 51.6% carbohydrate, 3.40% fat) and tap water ad libitum. The animals of the third experimental group (A) received chokeberry juice diluted 1:1 in the drinking water. The daily dose of juice was calculated for every animal after body weight measurement (twice a month). The animals received clear water after ingesting the daily dose of diluted juice. For the whole experimental period, every animal consumed approx. 440 mL fruit juice. At the end of the experimental period, the animals were anesthetized with i.m. Ketamin/Xilazine (90 mg/kg/10 mg/kg) and euthanized by cervical decapitation. Body weight, body mass index (body weight (g)/nasoanal length^2 (cm^2), heart weight, and the heart weight index of the rats of all groups were measured and calculated. The hearts of the animals were fixed in 10% neutral formalin and paraffin-embedded, after which histochemical, immunohistochemical, morphometric, and statistical analyses were performed. The experimental protocol was approved by the Committee on Ethical Treatment of Animals of the Bulgarian Agency for Food Safety (No. 193/2018). All animals were treated in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Institute of Health (NIH publication No. 86–23, revised 1996).

2.3. Light Microscopy

After washing with a physiological serum, the hearts were cut immediately below the coronary sulcus. After that they were fixed with 10% neutral formalin. After conventional paraffin wax embedding, 5 μm serial sections were cut in order to observe the collagen fibers and smooth muscle cells. The sections were stained with azan (according to Heidenhain; the collagen fibers were stained blue, and the smooth muscle cells were stained red). All investigations were performed on sections of the left ventricle of heart.

2.4. Immunohistochemistry

The sections (5 μm-thick) obtained from rat heart were deparaffinized, then subjected to the following procedures: detection of antigenic epitopes with citrate buffer, blocking endogenous peroxidase with 3% hydrogen peroxide, blocking endogenous biotin using a kit (ref: № BBK 120, Scy Tek, Lab. Inc., Logan, UT, USA), blocking non-specific binding using a reagent (Superblock, Scy Tek, Lab. Inc., Logan, UT, USA), followed by incubation for 24 h (at 4 °C) with ACE2 polyclonal antibody 1:200 (E-AB-12224, Elabscience Biotechnology Inc., Houston, TX, USA), after which a second 10 min incubation followed, with a biotinylated secondary antibody (№ AGL015 Scy Tek Lab. Inc., Logan, UT, USA). The reaction was

visualized by 3,3′-diaminobenzidine tetrachloride (DAB, Scy Tek Lab. Inc., Logan, UT, USA), and the slices were counterstained with Mayer's hematoxylin. The same protocol was used for the immunohistochemical analysis of αSMA monoclonal antibody 1:5000 (A-2547, Sigma Chemical, St. Louis, MO, USA). All microphotographs were taken using a Nikon Microphot SA microscope (Olimpus, Japan), combined with Camedia-5050Z digital camera (Olympus, Japan).

2.5. Morphometric Analysis

The morphometric analysis involved tissue slices 5μm in thickness, obtained from rat hearts immediately below the coronary sulcus. Quantification of collagen was performed on the azan-stained slices by measuring the percentage of collagen fiber distribution in the tunica media of coronary arteries. The Kernohan index was calculated by measuring in micrometers the wall thickness and the diameter of all cross-sections of coronary arteries (muscular type) present in the slice. Every parameter was measured at least three times for each vessel. The intensity of the immune reaction in the coronary tunica media was measured in arbitrary units (AU) on the slices immunostained for αSMA and ACE2. Using a software, the average intensity of pixels was recorded in arbitrary units in the range 0–256 on microphotographs of the blood vessels, 0 being black, and 256 being white. A minimum of 50 points were measured in the tunica media of each blood vessel at magnification ×400. All measurements involved five slices per animal and an examination of all cross-sections of the coronary arteries present. The measurements were performed using the DP–Soft ver. 3.2 software, Olympus, Japan.

2.6. Statistical Analysis

The intergroup comparison was made by means of one-way ANOVA followed by Tukey's test. The differences were considered significant at $p < 0.05$. The data were processed by SPSS software (version 17.0) and presented as means ± SEM.

3. Results

3.1. Somatometric Parameters

Table 2 represents the results of the somatometric parameters of the groups studied. Our data showed that black chokeberry juice supplementation did not significantly affect the somatometric parameters and the body mass index.

Table 2. Somatometric parameters of test animals.

	Body Weight (g)	$p < 0.05$	BMI	$p < 0.05$	Heart Weight (g)	$p < 0.05$	Heart Weight Index
CY	158.57		0.52		0.50		0.31
CO	402.14	* CY	0.70	* CY	1.06	* CY	0.26
A	417.86	# CY	0.69	* CY	1.06	# CY	0.25

Body weight (g) * CO vs. CY, $p < 0.05$; # A vs. CY, $p < 0.05$; Body mass index * CO vs. CY, $p < 0.05$; # A vs. CY, $p < 0.05$. Heart weight (g) * CO vs. CY, $p < 0.05$; # A vs. CY, $p < 0.05$.

3.2. Chemical Composition of AMJ

As is evident from Table 1, the black chokeberry juice used is a very rich source of several classes of phenolic compounds–anthocyanins, hydroxycinnamic acids, and flavonols. Anthocyanins are represented by Cyanidin-3-galactoside, Cyanidin-3-glucoside, Cyanidin-3-arabinoside, and Cyanidin-3-xyloside, and their cumulative content in the juice exceeded 2100 mg/L.

3.3. AMJ Supplementation Did Not Influence Kernohan Index

Figure 1 shows the results obtained for the Kernohan index of coronary arteries, calculated on the basis of the data from the morphometric analysis. AMJ supplementation

did not influence wall-to-diameter parameter (Kernohan index) of coronary arteries of aged rats, and no significant differences were observed between young and old controls.

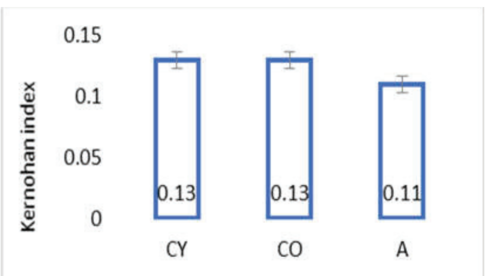

Figure 1. Kernohan index of coronary arteries of the studied groups.

3.4. Effect of AMJ Supplementation on the Amount of Connective Tissue

Figure 2 shows the photomicrographs of the azan-stained slices of rat heart. The blue collagen fibers in the tunica media of the coronary arteries can be visually distinguished and quantified owing to the histochemical technique used.

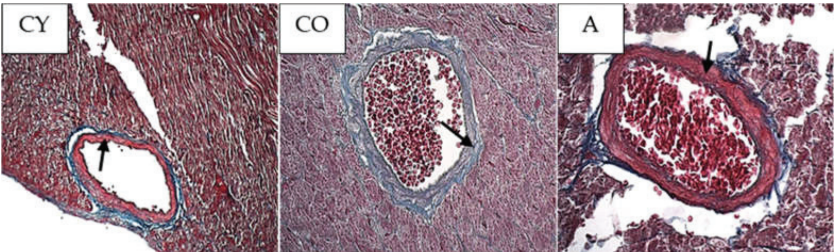

Figure 2. Myocardium. Coronary arteries of the left ventricle of the rat heart, azan staining, magnification × 200; black arrows indicate tunica media.

The data from the morphometric analysis of collagen fiber distribution in the coronary tunica media are presented in Figure 3.

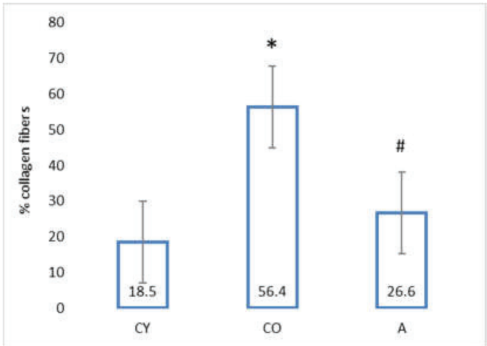

Figure 3. Percentage of collagen fiber distribution in the coronary tunica media of rat heart. * CO vs. CY, $p < 0.05$, # A vs. CO, $p < 0.05$.

The amount of collagen fibers in the coronary tunica media of old controls (CO) was significantly increased, as compared with that of young controls (CY), which could be

attributed to the natural ageing process; however, supplementation with AMJ resulted in a significant decrease in the amount of collagen fibers in the tunica media of the aronia-supplemented group (A) in comparison with the old controls (CO).

3.5. Effect of AMJ Supplementation on αSMA Immunoexpression

Figure 4 shows the photomicrographs of the αSMA immunoreaction in the tunica media of coronary arteries of rat hearts.

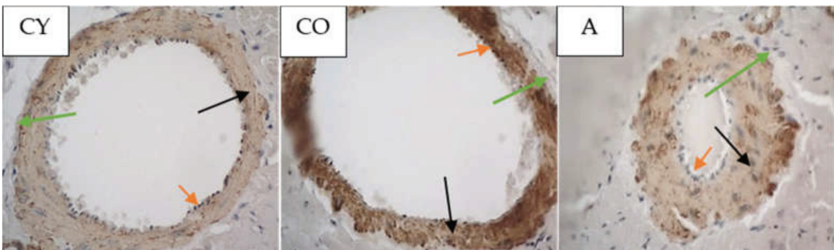

Figure 4. Myocardium. Coronary arteries of left ventricle of rat heart, αSMA immunoreaction in tunica media, magnification ×400. Orange arrows indicate tunica intima, black arrows—tunica media, green arrows—tunica adventitia.

The data from the morphometric analysis of the intensity of αSMA immunoreaction in tunica media of coronary arteries of rat heart are presented in Figure 5.

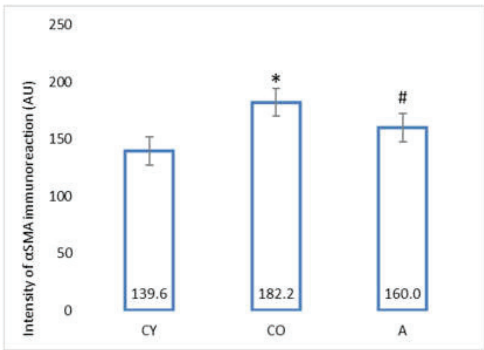

Figure 5. Intensity of αSMA immunoreaction (in arbitrary units) in tunica media of coronary arteries of rat heart. * CO vs. CY, $p < 0.05$, # A vs. CO, $p < 0.05$.

The old control (CO) group showed a significantly higher intensity of the αSMA immunoreaction, as compared with the young controls (CY), $p < 0.05$, which could be attributed to the natural ageing process. The immunohistochemical reaction for αSMA in the coronaries was significantly intensified in the supplemented animals (A), as compared with the old controls (CO), $p < 0.05$.

3.6. Effect of AMJ Supplementation on ACE2 Immunoexpression

Figure 6 shows the photomicrographs of the ACE2 immunoreaction in the tunica media of coronary arteries of rat hearts, whereas Figure 7 presents the data from the morphometric analysis of the immunoreaction intensity for ACE2 in the coronary tunica media.

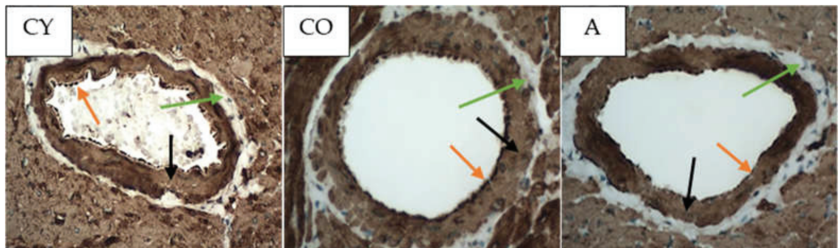

Figure 6. Myocardium. Coronary arteries of left ventricle of rat heart, ACE2 immunoexpression, magnification ×400. Orange arrows indicate tunica intima, black arrows—tunica media, green arrows—tunica adventitia.

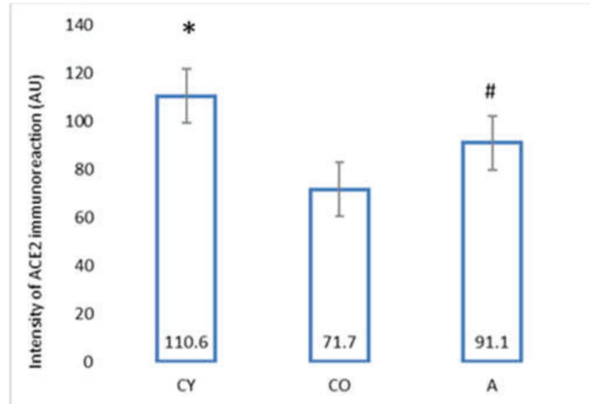

Figure 7. Intensity of ACE2 immunoreaction (in arbitrary units) in tunica media of coronary arteries of rat heart. * CO vs. CY, $p < 0.05$, # A vs. CO, $p < 0.05$.

In comparison with the young controls (CY), the intensity of the ACE2 immunoreaction of the old controls (CO) was significantly lower ($p < 0.05$), which could be attributed to the natural ageing process. The morphometric analysis revealed that the intensity of the ACE2 immunoreaction in the tunica media of the supplemented group (A) was significantly increased, as compared with the one observed in the group of old controls (CO), $p < 0.05$.

4. Discussion

Body weight and body mass index change significantly in the process of ageing due to the increased adipose tissue in the body on one hand, and changes in lean mass, on the other [23,24]; however, our results showed that aronia juice supplementation did not significantly affect the somatometric parameters and body mass index, which are the criteria for normal body structure. In our study, the Kernohan index remained unchanged in all experimental groups—a fact that indicates a lack of hypertrophy or any other vascular wall pathology and confirms the model of physiological ageing. The wall of the coronary arteries of the aged animals had an unaltered thickness, in spite of the reorganization of the vascular structural elements, which had occurred with ageing. This has been described as eutrophic remodeling [25]. The results obtained from the comparison of young and old controls in our investigation of the amount of collagenous connective tissue (CT) in the tunica media confirmed the results of other authors [8]. Our results showed that the amount of CT in the vascular wall of the aged animals from the supplemented group was reduced, as a result of which, the wall acquired a phenotype characteristic of young animals. Similar results—CT reduction following administration of *Aronia melanocarpa*—have been obtained in other

investigations of ours involving the aorta and thymus of adult rats, a fact which supports the results of the present study [26,27]. αSMA is a marker for smooth muscle cells and myofibroblasts in the vascular wall [28–30]. A significant increase in αSMA intensity was found in the old controls, as compared with the young controls ($p < 0.05$), which is likely to be a manifestation of the increased number of activated myofibroblasts in the tunica media. This finding also correlates with the increased amount of collagen fibers in the tunica media when comparing these two experimental groups. The myofibroblast transformation of VSMCs is a physiological process of gradual transformation of the contractile phenotype of these cells into a synthetic one under the influence of local and general factors [28]. AMJ administration resulted in a significant reduction of αSMA expression in the supplemented animals, as compared with the old controls ($p < 0.05$). This is a morphological manifestation of slowing down of the ageing process, probably associated with reduced smooth muscle cell activity in the tunica media. The result is a suppressed αSMA expression, with a subsequent reduction of collagenous connective tissue in the vascular wall. The increased collagen synthesis in the vascular wall correlates with the transition of smooth muscle cells in the tunica media from a contractile to a synthetic phenotype. This phenomenon has been described as a feature of ageing smooth muscle cells [4,5].

Harikrishnan et al. conducted a study in which they revealed that αSMA plays a part, not known to scientists up to that moment, in the regulation of type I collagen expression in cardiac fibroblasts treated with Ang II [29]. The increased collagen synthesis correlates with the transition of myocardial fibroblasts to an αSMA-positive phenotype. The expression of αSMA is a sign of the differentiation of myofibroblasts toward a synthetic phenotype. These results clarify the relation between the increased amount of type I collagen and the phenotype transformation of cardiac fibroblasts into producing myofibroblasts [29].

The comparison between young and aged animals revealed a reduced intensity of the ACE2 immunoreaction in the aged animals, a fact that has been found by other authors as well [9,31]. We observed two findings, which parallel the manifestation of the anti-ageing properties of black chokeberry juice. The significantly reduced amount of connective tissue and the increased ACE2 immune expression in the tunica media of the supplemented animals are indicative of a possible influence on the RAAS system. In another study of ours, we found a similar increase in ACE2 immunoexpression in cardiomyocytes, parallel to a reduced amount of collagen fibers in the myocardial interstitium, following AMJ supplementation in aged rats (unpublished data). Activation of RAAS, endothelin, and RAGE (receptor for advanced glycation end products) are different ways that organisms have at their disposal to react to chronic stress. According to Lakatta, the activation of these signaling cascades brings about a chronic inflammatory response, which, on its part, generates an additional oxidative stress contributing to the progression of age-related structural and functional arterial remodeling [31].

Ang II is one of the factors influencing the phenotype change of the VSMCs from a contractile type to a synthetic one [32]. Ang II is not only a powerful vasoconstrictor, but also a pro-inflammatory molecule stimulating cell growth and matrix deposition during arterial remodeling [25]. According to Heeneman et al. ACE and Ang II take part in the remodeling of large and small arteries in hypertension [33]. ACE2 antagonizes the vasoactive and proliferative action of Ang II. Our investigation showed that black chokeberry juice is likely to counteract Ang II pro-inflammatory effects, which is proven by the significantly increased intensity of the ACE2 immunoreaction in the wall of the coronary arteries. The polyphenol resveratrol increases ACE2 expression and decreases the profibrotic protein expression in Ang II-stimulated vascular smooth muscle cells [34]. The favorable effects of polyphenols are attributed mainly to their antioxidant capacity and ability to modulate the cellular antioxidant defense mechanisms by inducing synthesis of detoxication enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPx), NAD(P)H: quinone oxidoreductase 1 (NQO1), side by side with others [35,36]. Apart from that, recent studies have provided evidence of the effect of polyphenols as modulators of signaling pathways, such as TNFα,

IL-12p40 (a component of the cytokines interleukin IL-12 and IL-23), and p38 MAPK (a class of mitogen-activated protein kinases) [37,38].

Scientific interest in black chokeberry juice has grown significantly in the recent years. The mechanisms of its anti-aging effects have already been described in detail [39–41]. The review of Kasprzak-Drozd, (2021) summarizes the evidence of the efficacy of black chokeberry juice and lists the mechanisms of its action with regard to cardiovascular diseases. Examples of these mechanisms are increasing the activity of endothelial nitric oxide synthase (eNOS), glutathione peroxidase (GSHPx), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), nitric oxide (NO), prostaglandin E2 (PGE2), and so on. Moreover, it reduces the activity of the angiotensin converting enzyme (ACE), C-reactive protein, intercellular adhesion molecule (ICAM), IL-6, IL-8, IL-10, reactive oxygen species (ROS), substances reacting with thiobarbituric acid (TBARS), tumor necrosis factor-α (TNF-α), and the vascular cell adhesion molecule (VCAM) and so on [40]. In an experimental model of aging in mice, Zhao et.al. found that chokeberry polysaccharides reduced inflammation and oxidative stress in brain tissue by inhibiting the NLRP3 inflammasome by the AMPK/SIRT1/NF-κB signaling pathway. Activation of the key antioxidant enzymes SOD and CAT has been shown, as well as lowering malondialdehyde (MDA) levels in brain tissue. An improved composition of the intestinal flora has also been found, which reveals an interesting new perspective for elucidating the effects of chokeberry [39].

A number of studies including clinical tests, epidemiological data, as well as in vitro and in vivo investigations with animals have found a cause–effect relationship between a diet rich in polyphenols and its favorable impact on health [42,43]. At present, polyphenols are considered potential therapeutic agents possessing antioxidant properties, which can be used in the management of cardiovascular diseases [44–47]. Due to their treatment efficacy, they have been designated as anti-ageing molecules [48]. The greater part of traditional medications is directed to act upon the negative health consequences of vascular ageing, rather than its pathophysiology. For instance, the classical antihypertensive therapies reduce peripheral vascular resistance and influence only part of the arterial remodeling directly associated with ageing [25]. The results obtained in the current study are informative, but they cannot be generalized, since the investigation had several limitations. It involves only experimental animals and the study was performed on a tissue level without being supported by serum analyses. Furthermore, only morphological and no physiological indices were studied, and the indices studied provided indirect information on influencing inflammation and oxidative stress. Nevertheless, these results give us grounds to suppose that non-drug agents can influence age-related vascular remodeling, both as primary prevention (prior to the occurrence of pathology) and as secondary prevention (following the occurrence of pathological alterations). This is one of the possible ways to ensure successful vascular ageing. That is why it is justified to study in greater detail the mechanisms underlying the effect of black chokeberry juice supplementation on vascular structures.

Our results provoke interest in another way as well. At present, ACE2 is the focus of attention because of its role in the process of SARS-CoV-2 penetrating and infecting cells. Little is known about the effect of the binding of SARS-CoV-2 virus to ACE2, and the way in which this binding can modulate ACE2 enzyme activity [49]. The experimental data of Bartova et al. [11] suggest that the higher levels of ACE2 in the tissues provide a barrier against SARS-CoV-2 infection.

5. Conclusions

The current study demonstrates that supplementation with black chokeberry fruit juice reduces the CT amount and αSMA expression, and increases ACE2 expression in the tunica media of the coronary arteries of aged rats. The positive impact of age-related coronary artery remodeling in rats is a new aspect of the anti-aging action of black chokeberry juice that provides indirect evidence for its anti-inflammatory and antioxidant properties. Even though the obtained results are convincing, our study would be stronger if

the morphological tests had been supported by serum and physiological tests. It should be noted that conducting animal experiments have their drawbacks and limitations, but are still indicative enough to support the use of black chokeberry products in humans. Our data support the preventive potential of antioxidant-rich foods in age-related diseases; therefore, *Aronia melanicarpa* fruit juice may prove to be a means for successful vascular ageing.

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Article

Fermented Oyster (*Crassostrea gigas*) Extract Cures and Prevents Prednisolone-Induced Bone Resorption by Activating Osteoblast Differentiation

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Abstract: Osteoporosis is a bone resorptive disease characterized by the loss of bone density, causing an increase in bone fragility. In our previous study, we demonstrated that gamma aminobutyric acid-enriched fermented oyster (*Crassostrea gigas*) extract (FO) stimulated osteogenesis in MC3T3-E1 preosteoblast cells and vertebral formation in zebrafish. However, the efficacy of FO in prednisolone (PDS)-induced bone resorption remains unclear. In this study, we evaluated the osteogenic potential of FO in MC3T3-E1 preosteoblast cells and zebrafish larvae under both PDS-pretreated and PDS-post-treated conditions. We found that FO recovered osteogenic activity by up-regulating osteoblast markers, such as alkaline phosphatase (*ALP*), runt-related transcription factor 2, and osterix, in both PDS-pretreated and post-treated MC3T3-E1 osteoblast cells and zebrafish larvae. In both conditions, PDS-induced decrease in calcification and *ALP* activity was recovered in the presence of FO. Furthermore, vertebral resorption in zebrafish larvae induced by pretreatment and post-treatment with PDS was restored by treatment with FO, along with the recovery of osteogenic markers and downregulation of osteoclastogenic markers. Finally, whether FO disturbs the endocrine system was confirmed according to the Organization for Economic Cooperation and Development guideline 455. We found that FO did not stimulate estrogen response element-luciferase activity or proliferation in MCF7 cells. Additionally, in ovariectomized mice, no change in uterine weight was observed during FO feeding. These results indicate that FO effectively prevents and treats PDS-induced osteoporosis without endocrine disturbances.

Keywords: fermented oyster; prednisolone; osteoblast; osteoclast

1. Introduction

Glucocorticoids, which are synthetic adrenal corticosteroids, are considered a common etiology of drug-induced osteoporosis [1]. Glucocorticoids are generally prescribed as anti-inflammatory [2] and analgesic agents [3], which are known to interact with glucocorticoid receptors [4]. The anti-inflammatory properties of glucocorticoids are associated with the inhibition of immune cell function and consequent suppression of the secretion of inflammatory cytokines, such as interleukin (IL)-1β [5]. Nevertheless, clinical investigations have revealed that 20% of the prevalence in osteoporosis patients is attributed to glucocorticoid medication, and the incidents are higher among patients over 65 years of

age and who received medication with glucocorticoids over 3-month periods [6]. Direct activity of glucocorticoids on osteoblasts and the bone micro-environment are mediated by the downregulation of the expression and release of bone matrix regulators such as collagen 1 alpha 1 (*Col1α1*) and osteocalcin [7]. In addition, glucocorticoid medication is associated with vitamin D resistance, which consequently decreases calcium absorption from the gastrointestinal tract, causing increased renal excretion of calcium [8,9]. Estrogen or androgen deficiencies as a result of the hypogonadism caused by glucocorticoids also deteriorate the differentiation of mesenchymal stem cells toward the osteoblast precursor cells and prevent the terminal differentiation of osteoblasts, resulting in a decrease in mature osteoblasts [10]. Furthermore, shifting the differentiation of stromal bone marrow cells toward the adipocytic lineage has also been recorded in response to glucocorticoid treatment [11]. This indicates that overexposure to glucocorticoids leads to severe bone resorptive diseases such as osteoporosis in humans.

Prednisolone (PDS), the active form of prednisone, is a glucocorticoid and is basically prescribed as an anti-inflammatory, anti-allergy, and anti-autoimmune disorder agent [12,13]. However, long-term administration of PDS results in fluid and electrolyte disturbances, gastrointestinal problems, endocrine disruptions, metabolic disorders, and bone diseases such as osteoporosis [14]. Among them, PDS-mediated osteoporosis is implicated with increased apoptosis, inhibition of anabolic activities of osteocytes, and decreased secretion of osteoblast matrix, causing osteonecrosis and fractures in bone [15]. Additionally, long-term use of PDS impairs the expression of master osteoblast differentiation regulators, including runt-related transcription factor 2 (*RUNX2*), osterix (*OSX*), and alkaline phosphatase (*ALP*), while increasing the expression of osteoclast activators such as cathepsin K (CTSK), nuclear factor of activated T-cells 1 (NFATc-1), receptor activator of nuclear factor κB (RANK), and acid phosphatase (ACP) [16]. Bisphosphonates are commonly used to treat glucocorticoid-induced osteoporosis and reduce the risk of bone fractures [17]. However, approximately 50–60% people administered with bisphosphonates excrete them from the kidneys, indicating that the efficacy of bisphosphonates is low. Moreover, renal esophageal and acute phase complications have been recorded in the clinical trials medicated by bisphosphonates [18]. Therefore, it is important to identify effective and natural alternatives for the treatment and prevention of osteoporosis caused by glucocorticoids.

Fermented products have recently received potent attention because of their powerful pharmaceutic activities and low side-effects [19,20]. In our previous studies, we demonstrated that fermented oyster *Crassostrea gigas* (FO) extract promoted osteoblast differentiation and bone formation by activating the Wnt/β-catenin signaling pathway [21], concomitant with an increase in growth performance by upregulating insulin-like growth factor-1 (IGF-1) [22]. Additionally, we found that FO prevented ovariectomy (OVX)-induced bone loss [23,24]. Nevertheless, the therapeutic and preventive effects of FO on glucocorticoid-induced bone resorption such as osteoporosis have not been elucidated. In this study, we evaluated the potential of FO under both PDS-pretreated (therapeutic effect) and PDS-post-treated (preventive effect) conditions in preosteoblast MC3T3-E1 cells and zebrafish larvae. Additionally, the safety of FO on estrogen disturbance was tested in accordance with the Organization for Economic Cooperation and Development (OECD) guideline 455 (TG455).

2. Materials and Methods

2.1. Reagent and Antibody

Calcein, alizarin red, PDS, tricaine methanesulfonate, methylcellulose, β-glycerophosphate (GP), 17β-estradiol (E2), and ICI 182,780 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). A TRACP & *ALP* double-staining kit was purchased from Takara Bio Inc. (Kusatsu, Shiga, Japan). Minimum essential medium alpha modification (α-MEM), fetal bovine serum (FBS), and penicillin–streptomycin solution (100×) were purchased from WELGENE (Gyeongsan, Gyeongsangbukdo, Republic of Korea). Antibodies for *ALP* (sc-398461), *RUNX2* (sc-101145), *OSX* (sc-393325), and β-actin (sc-8432) were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA, USA). FO (product name: FO100) was obtained from Marine Bioprocess Co. (Busan, Korea) [21].

2.2. Cell Culture and Osteoblast Differentiation

Mouse MC3T3-E1 preosteoblast cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in α-MEM containing 10% FBS and penicillin–streptomycin solution. The cells were seeded at a density of 1×10^4 cells/mL in 6-well plates, and FO was used to evaluate both the therapeutic and preventive effects on PDS-induced bone resorption. For the therapeutic effect, 10 μM PDS was pretreated to MC3T3-E1 cells for two days, followed by treatment with various concentrations of FO (0–200 μg/mL) for five days. To evaluate the preventive effect, FO (0–200 μg/mL) was administered 2 h before 10 μM PDS exposure for seven days. Fresh media were replenished with FO and PDS every two days.

2.3. Alizarin Red Staining

To evaluate calcium deposition, MC3T3-E1 cells (1×10^4 cells/mL) were seeded and treated with 10 μM PDS and FO (0–200 μg/mL) for seven days. Afterward, the cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min at 37 °C. Then the fixed cells were stained with 2% alizarin red for 30 min. Images were obtained using phase-contrast microscopy (Macrotech, Goyang, Gyeonggido, Korea).

2.4. ALP Staining

MC3T3-E1 cells (1×10^4 cells/mL) were seeded and then treated with 0–200 μg/mL FO and 10 μM PDS. At seven days, *ALP* activity was measured using TRACP & *ALP* double-staining kit (Takara Bio Inc., Kusatsu, Shiga, Japan). According to the manufacturer's instructions, the cells were rinsed with PBS and fixed for 5 min. Afterward, the *ALP* substrate was added and incubated at 37 °C. The stained cell images were obtained using phase-contrast microscopy (Macrotech).

2.5. Reverse Transcription–Polymerase Chain Reaction (RT-PCR) Using MC3T3E-1 Cells

MC3T3-E1 cells were treated with 0–200 μg/mL FO and 10 μM PDS for seven days, and total RNA was extracted using the Easy-BLUE Total RNA Extraction Kit (iNtRON Biotechnology, Sungnam, Gyeonggi, Korea). RNA was reverse-transcribed using MMLV reverse transcriptase (iNtRON Biotechnology), and gene amplification was performed. All mouse primers used in this study are listed in Table 1 [21].

Table 1. Mouse primers and PCR conditions used in this study.

Gene *	Primer Sequence (5′-3′)	Size	T_m
ALP	F: 5′-TTGTGGCCCTCTCCAAGACA-3′ R: 5′-GACTTCCCAGCATCCTTGGC-3′	198 bp	60 °C
RUNX2	F: 5′-CATGGTGGAGATCATCGCGG-3′ R: 5′-GGCCATGACGGTAACCACAG-3′	171 bp	60 °C
OSX	F: 5′-AAGGCGGTTGGCAATAGTGG-3′ R: 5′-GCAGCTGTGAATGGGCTTCT-3′	194 bp	60 °C
GAPDH	F: 5′-ACCACAGTCCATGCCATCAC-3′ R: 5′-CACCACCCTGTTGCTGTAGC-3′	480 bp	63 °C

* *ALP*, alkaline phosphatase; *RUNX2*, runt-related transcription factor 2; *OSX*, osterix; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; F, forward; R, reverse; bp, base pairs; T_m, melting temperature.

2.6. Protein Extraction and Western Blotting

MC3T3-E1 cells were treated with 0–200 μg/mL FO and 10 μM PDS for seven days. Total proteins were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (iNtRON Biotechnology) with protease inhibitors (Sigma-Aldrich). Bio-Rad Protein Assay

Reagent (Bio-Rad, Hercules, CA, USA) was used to quantify the proteins. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene fluoride (PVDF) membrane (Thermo Fisher Scientific, Waltham, MA, USA), and then immunoblotted with the indicated antibodies. For the visualization, an Enhanced Chemiluminescence Plus Kit (Thermo Fisher Scientific) was used and the images were obtained using ImageQuant LAS 500 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). β-Actin was used as the house keeping protein.

2.7. Zebrafish Maintenance and Vertebral Staining

All the zebrafish experiments were conducted as described by the standard guidelines of the Animal Care and Use Committee of Jeju National University (Jeju Special Self-Governing Province, Republic of Korea; approval no.: 2021-0066). To evaluate the therapeutic effect of FO, zebrafish larvae at 5 days post fertilization (dpf) were pretreated with 20 μM PDS for two days, followed by treatment with FO (0–100 μg/mL) for another two days in the presence and absence of PDS. For the preventive effect, FO was pretreated for 2 h, followed by treatment with PDS for four days. The media were replenished with FO and PDS every two days. At 9 dpf, vertebrae were visualized using 0.3% calcein green fluorescent staining. After extensive rinsing, the larvae were anesthetized in 0.04% tricaine methanesulfonate solution and mounted on depression slides using 3% methylcellulose. Fluorescent images were obtained using a CELENA S digital imaging system (Logos Biosystems, Anyang, Gyeonggido, Korea).

2.8. RT-PCR Using Zebrafish Larvae

Zebrafish larvae at 5 dpf were treated with 0–100 μg/mL FO and 10 μM PDS, and mRNA was extracted using an Easy-BLUE Kit (iNtRON Biotechnology) at 9 dpf. Briefly, RNA were reverse-transcribed using MMLV reverse transcriptase (iNtRON Biotechnology), and PCR was performed using specific primers shown in Table 2 [25,26].

Table 2. Zebrafish primers and PCR conditions used in this experiment.

Gene *	Primer Sequence (5′-3′)	Size	T_m
zRUNX2a	F: 5′-GACGGTGGTGACGGTAATGG-3′ R: 5′-TGCGGTGGGTTCGTGAATA-3′	173 bp	58 °C
zRUNX2b	F: 5′-CGGCTCCTACCAGTTCTCCA-3′ R: 5′-CCATCTCCCTCCACTCCTCC-3′	149 bp	59 °C
zOSX	F: 5′-GGCTATGCTAACTGCGACCTG-3′ R: 5′-GCTTTCATTGCGTCCGTTTT-3′	153 bp	56 °C
zALP	F: 5′-CAAGAACTCAACAAGAAC-3′ R: 5′-TGAGCATTGGTGTTATAC-3′	170 bp	48 °C
zCTSK	F: 5′-GGACTCAATCACTATCACT-3′ R: 5′-AGAACAAGACATCTAAGACA-3′	117 bp	56 °C
zNFATc-1	F: 5′-AACCTTCCTCGTTCCCTCAA-3′ R: 5′-CGCTGTTATCCTCCACCTCA-3′	152 bp	57 °C
zRANK	F: 5′-GCACGGTTATTGTTGTTA-3′ R: 5′-TATTCAGAGGTGGTGTTAT-3′	109 bp	49 °C
zACP5b	F: 5′-GCTGCTGCTAACAAACAAT-3′ R: 5′-GACCAACCACGATGACAA-3′	76 bp	52 °C
zβ-actin	F: 5′-CGAGCGTGGCTACAGCTTCA-3′ R: 5′-GACCGTCAGGCAGCTCATAG-3′	155 bp	60 °C

* *zRUNX2a/b*, runt-related transcription factor 2a/b; *zOSX*, osterix; *zALP*, alkaline phosphatase; *zCTSK*, cathepsinK; *zNFATc-1*, nuclear factor of activated T-cells cytoplasmic 1; *zRANK*, receptor activator of nuclear factor κB; *zACP5b*, acid phosphatase 5b; F, forward; R, reverse; bp, base pairs; T_m, melting temperature.

2.9. Relative Uterine Weight/Body Weight in OVX Mice

In our previous study [23], OVX-induced osteoporosis mice were prepared according to the guidelines for the care and use of laboratory animals of Kyungpook National University (approval no.: 2017-57). In brief, OVX mice were randomly separated into five groups: sham-operated with vehicle (distilled water, shame), OVX with vehicle (OVX + V), OVX with E2 (OVX + E2), OVX with 100 mg/kg FO, and OVX with 200 mg/kg FO. FO was dissolved in distilled water and orally fed for four weeks. E2 (10 μg/kg) was intraperitoneally administered daily. Upon termination, uteri were quickly removed from the connective tissues, blotted, and weighed. The relative uterine weight was determined by calculating the uterine weight in terms of body weight.

2.10. ERE-Luciferase Activity in MCF-7 Cells

MCF-7 cells (ATCC, Manassas, MD, USA) were stably transfected with 1 μg human ERE-luciferase reporter plasmid (Addgene plasmid no. 11354) and 0.2 μg pCMV-β-gal (Addgene plasmid no. 155) using LipofectamineTM 2000 (Invitrogen, Paisley, UK). After 6 h of transfection, the cells were maintained for 24 h in fresh DMEM in the abscense of phenol red (supplemented with 10% charcoal dextran-treated FBS), followed by incubation with FO (2×10^{-4}–2×10^{2} μg/mL), corticosterone (10^{-11}–10^{-5} M), genistein (10^{-10}–10^{-4} M), and E2 (10^{-14}–10^{-8} M). In a parallel experiment, E2 (10^{-9} M) was treated in the presence of ICI 182,780 (10 μM, an ER antagonist) for 24 h. The luciferase and β-galactosidase activities were measured according to the manufacturer's specifications (Promega, Madison, WI, USA).

2.11. Cell Proliferation Assay

The viability was evaluated using a Cellrix Viability Assay Kit (MediFab, Seoul, Republic of Korea) based on water-soluble tetrazolium (WST)-1. Briefly, MCF-7 cells were seeded at a density of 1×10^{4} cells/mL for 24 h, followed by treatment with 200 μg/mL FO, 10^{-9} M E2, 10 μM ICI 182,780, or E2 + ICI 182,780 for three days. According to the manufacturer's specifications, the WST-1 cell proliferation reagent (10 μL) was added to each well, the plates were incubated at 37 °C for 2 h, and the cell viability was quantified at 450 nm using a microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA).

2.12. Statistical Analysis

All data represent in this study refer to at least three independent experiments, and the mean ± SEM is indicated. Significant differences were determined using Student's *t*-test and an unpaired one-way ANOVA test with Bonferroni correction. Statistical significance was set at *** and ### $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

3. Results

3.1. PDS-Induced Anti-Osteogenic Activity Decreases in MC3T3-E1 Cells by Post-Treatment with FO

To evaluate the therapeutic activity of FO on PDS-induced anti-osteogenic activity in MC3T3-E1 cells, the cells were pretreated with PDS for two days prior to exposure to FO for another five days. As expected, FO itself considerably increased bone mineralization (Figure 1A) and *ALP* activity (Figure 1B) in the absence of PDS. However, PDS conspicuously inhibited the activity of MC3T3-E1 cells. FO also restored the anti-osteogenic activity of PDS-treated MC3T3-E1 cells in a concentration-dependent manner. Furthermore, the osteoblastic marker genes, including *ALP*, *RUNX2*, and *OSX*, were evaluated under PDS-treated conditions on day 7. Pretreatment with PDS significantly lowered the expression of osteoblast marker genes compared to that in the untreated cells, whereas FO restored gene expression in a concentration-dependent manner (Figure 1C). Consistent with the transcriptional expression of the osteoblast marker genes, Western blot analysis revealed that FO remarkably restored PDS-induced inhibition of the expression levels of *ALP*, *RUNX2*, and *OSX* (Figure 1D). These results indicate that FO effectively restores PDS-induced anti-osteogenic activity in MC3T3-E1 cells.

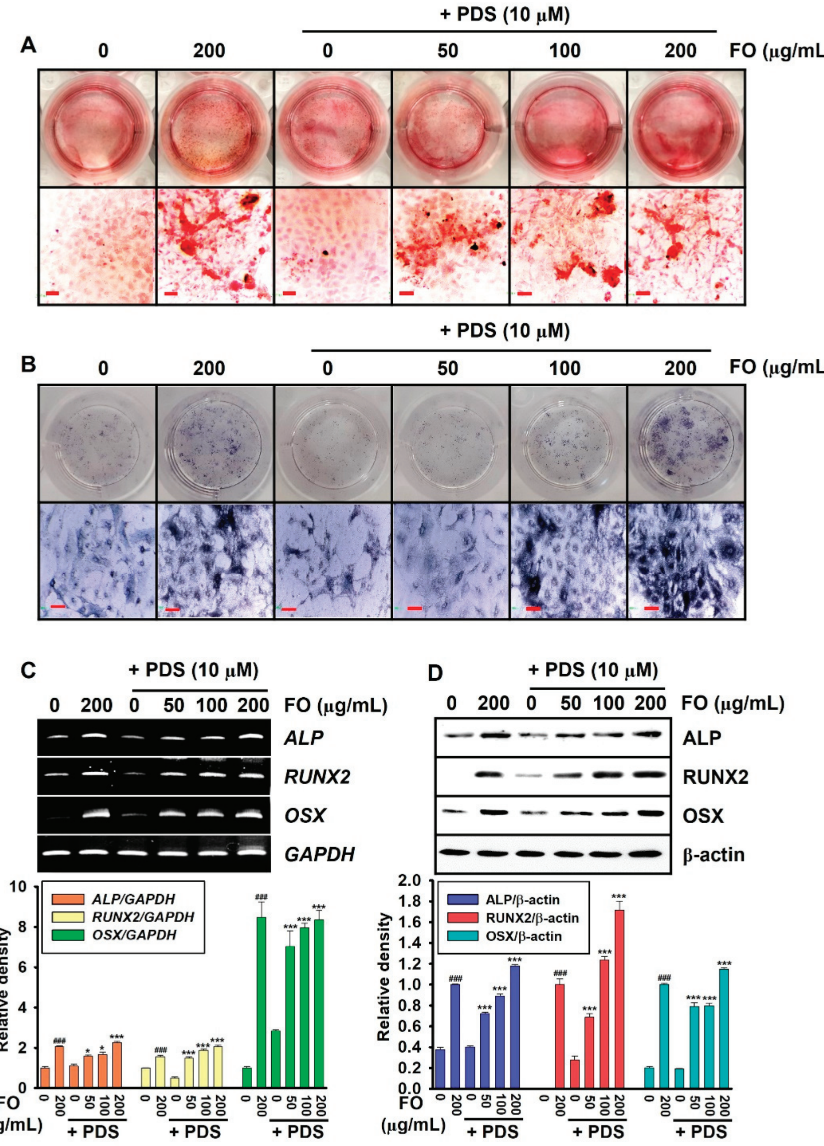

Figure 1. FO promotes osteogenic activity in prednisolone (PDS)-pretreated MC3T3-E1 cells. Mouse preosteoblast MC3T3-E1 cells (1×10^4 cells/mL) were pretreated with 10 μM PDS for two days prior to treatment with FO (0–200 μg/mL) for five days. Fresh media with FO and/or PDS were replenished every two days. At day 7, (**A**) bone mineralization was evaluated by alizarin red staining, and (**B**) *ALP* activity was evaluated using a TRACP & *ALP* Double-Staining Kit. (**C**) In a parallel experiment, total mRNA was extracted, and RT-PCR was performed to evaluate the gene expressions of *ALP*, *RUNX2*, and *OSX*. *GAPDH* was used as the internal control. (**D**) Total proteins were extracted, and Western blotting was performed to evaluate the expression of *ALP*, *RUNX2* and *OSX* proteins. β-Actin was used as the internal control. All data are presented as means ± standard error of the mean (### $p < 0.001$ vs. untreated MC3T3-E1 cells; * $p < 0.05$ and *** $p < 0.001$ vs. PDS-treated MC3T3-E1 cells). *ALP*: alkaline phosphatase; *RUNX2*: runt-related transcription factor 2; *OSX*: osterix; *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase.

3.2. Post-Treatment with FO Overcomes PDS-Induced Delay of Vertebral Formation in Zebrafish Larvae

Zebrafish larvae at 5 dpf were pretreated with PDS for two days prior to treatment with 0–100 μg/mL FO for another two days. As shown in Figure 2A, treatment with 100 μg/mL FO increased vertebral formation in zebrafish larvae at 9 dpf, and in the PDS-pretreated condition, FO restored vertebral formation attenuated by PDS. FO promoted the vertebrae number to 9.80 ± 0.53 in accordance with increased bone area (100.00 ± 8.67%) and relative bone density (100.00 ± 7.18%). However, PDS moderately decreased vertebral number from 5.72 ± 0.42 to 3.56 ± 0.22 (Figure 2B), bone area from 48.80 ± 5.65% to 28.58 ± 5.12% (Figure 2C), and relative bone density (Figure 2D) from 47.95 ± 5.61% to 26.41 ± 7.39%, compared with those in the untreated zebrafish larvae. Furthermore, treatment with FO in the presence of PDS reversed the PDS-induced bone resorption in a dose-dependent manner as 3.93 ± 0.28, 5.53 ± 0.58, and 6.86 ± 0.49 in vertebral number; 37.44 ± 4.95%, 51.59 ± 7.33%, 70.64 ± 12.06% in relative bone area; and 53.46 ± 7.81%, 57.69 ± 11.32%, and 76.90 ± 18.25% in relative bone density at 25, 50, and 100 μg/mL FO, respectively. Additionally, whether FO regulates the expression of osteogenic and osteoclastogenic genes was evaluated in PDS-pretreated zebrafish larvae at 9 dpf. As shown in Figure 2E, the expression of z*RUNX2a*, *zRUNX2b*, *zOSX*, and *zALP* was markedly increased in the presence of 100 μg/mL FO. However, they were completely downregulated in PDS-treated zebrafish larvae. In the PDS-pretreated condition, FO significantly restored the expression of osteogenic marker genes in a dose-dependent manner (Figure 2E). Furthermore, PDS remarkably increased the expression of osteoclastogenic genes, including z*CTSK*, *zNFATc-1*, *zRANK*, and *zACP5b*, in zebrafish larvae at 9 dpf (Figure 2F). However, FO mitigated PDS-induced osteoclastogenic gene expression in a dose-dependent manner. Overall, these results indicate that FO stimulates osteogenesis and inhibits osteoclastogenesis in PDS-pretreated zebrafish larvae, leading to the stimulation of vertebral formation.

3.3. Pretreatment with FO Prevents PDS-Induced Anti-Osteogenic Activity in MC3T3-E1 Cells

As we confirmed that post-treatment with FO restored osteoblastogenesis and consequent vertebral formation in PDS-pretreated MC3T3-E1 cells and zebrafish larvae, we pretreated the cells with FO for 2 h prior to exposure to 10 μM PDS. Alizarin red staining of bone mineralization (Figure 3A) and *ALP* staining (Figure 3B) revealed that pretreatment with FO dose-dependently prevented the PDS-mediated inhibitory effect on bone mineralization and *ALP* activity. In addition, both RT-PCR (Figure 3C) and Western blot analysis (Figure 3D) revealed that FO, in PDS-pretreated MC3T3-E1 cells, restored the expression of osteogenic markers, including *ALP*, *RUNX2*, and *OSX* in a dose-dependent manner. These results indicated that pretreatment with FO prevented PDS-induced anti-osteoblastic activity.

3.4. Pretreatment with FO Prevents PDS-Induced Vertebral Resorption in Zebrafish Larvae

To evaluate the preventive effect of FO on PDS-induced bone resorption, FO was treated in 5 dpf zebrafish larvae for 2 h prior to exposure to PDS to 9 dpf (Figure 4A). Treatment with 100 μg/mL FO increased vertebral number from 6.06 ± 0.34 to 13.11 ± 0.69 (Figure 4A,B) accompanied with the increased bone area (100.00 ± 12.24%, Figure 4C) and relative bone density (100.00 ± 14.67%, Figure 4D) compared with those in untreated zebrafish larvae. As expected, treatment with PDS significantly inhibited vertebral number (2.32 ± 0.46), relative bone area (28.46 ± 9.81%), and relative bone density (26.99 ± 10.81%). However, pretreatment with FO enhanced vertebral number (3.20 ± 0.24, 6.85 ± 0.37, and 9.31 ± 0.72 at 25, 50 and 100 μg/mL), relative bone area (44.56 ± 3.91%, 60.15 ± 7.01%, and 90.11 ± 13.39% at 25, 50 and 100 μg/mL), and relative bone density (41.87 ± 5.23%, 66.96 ± 8.48%, and 83.86 ± 15.33% at 25, 50 and 100 μg/mL), indicating that FO potently prevents PDS-induced bone resorption. Consistent with vertebral formation data, FO upregulated the expression of osteogenic genes, including z*RUNX2a*, z*RUNX2b*, z*OSX*, and z*ALP* in PDS-treated zebrafish larvae (Figure 4E). In addition, the expression of the PDS-induced osteoclastogenic genes, including z*CTSK*, *zNFACTc-1*, *zRANK*, and *zACP5b*, was

downregulated by pretreatment with FO in a dose-dependent manner. These data indicate that pretreatment with FO effectively prevents PDS-induced osteoclastogenic activity and bone resorption in zebrafish larvae.

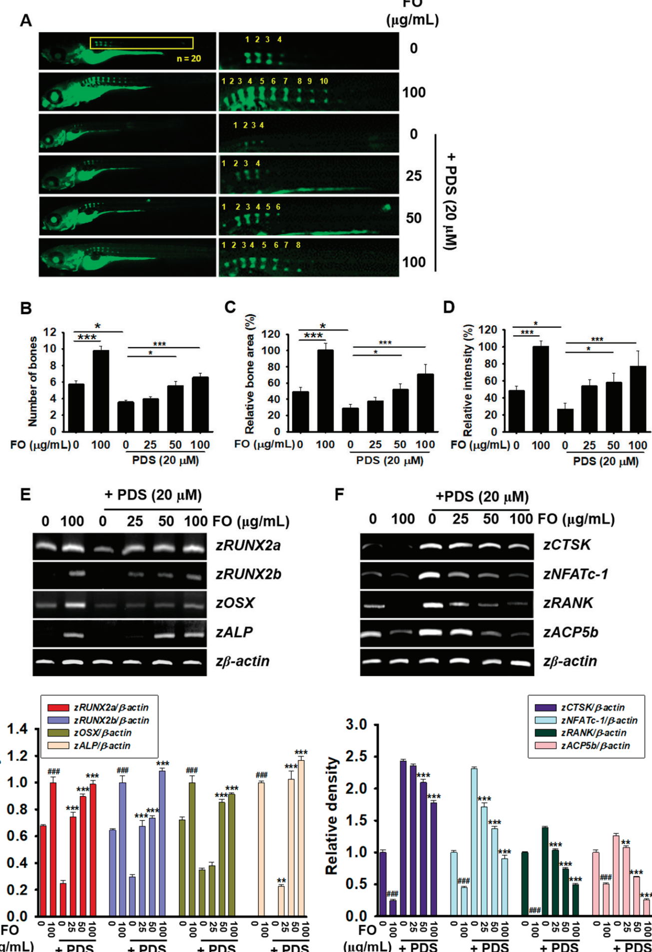

Figure 2. FO prevents PDS-induced bone resorption in zebrafish larvae. Zebrafish larvae (n = 20) at 5 days post fertilization (dpf) were pretreated with 20 μM PDS for two days prior to treatment with FO (0–100 μg/mL) for another two days. Fresh media with FO and/or PDS were replenished at 7 dpf. (**A**) At 9 dpf, zebrafish larvae were stained with 0.03% calcine and observed under fluorescence microscopy. Numbers show vertebrae (right panels). (**B**) Each vertebral number was manually counted and indicated. (**C**) Relative bone area and (**D**) bone intensity were calculated using imageJ software and expressed. All data are presented as means ± standard error of the mean (* $p < 0.05$ and *** $p < 0.001$). In a parallel experiment, total mRNA was extracted, and RT-PCR was performed to evaluate the gene expression of (**E**) osteoblast-related marker genes such as *zRUNX2a*, *zRUNX2b*, *zOSX*, and *zALP* and (**F**) osteoclast-related marker genes such as *zCTSK*, *zNFATc-1*, *zRANK*, and *zACP5b*. *zβ-Actin* was used as the internal control. All data are presented as means ± standard error of the mean (### $p < 0.001$ vs. untreated zebrafish larvae; ** $p < 0.01$, and *** $p < 0.001$ vs. PDS-treated zebrafish larvae). z: zebrafish; *RUNX2a/b*: runt-related transcription factor 2a/b; *OSX*: osterix; *ALP*: alkaline phosphatase; CTSK: cathepsin K; NFATc-1: nuclear factor of activated T-cells; cytoplasmic 1; RANK: receptor activator of nuclear factor κB; and ACP5b: Acid phosphatase 5b.

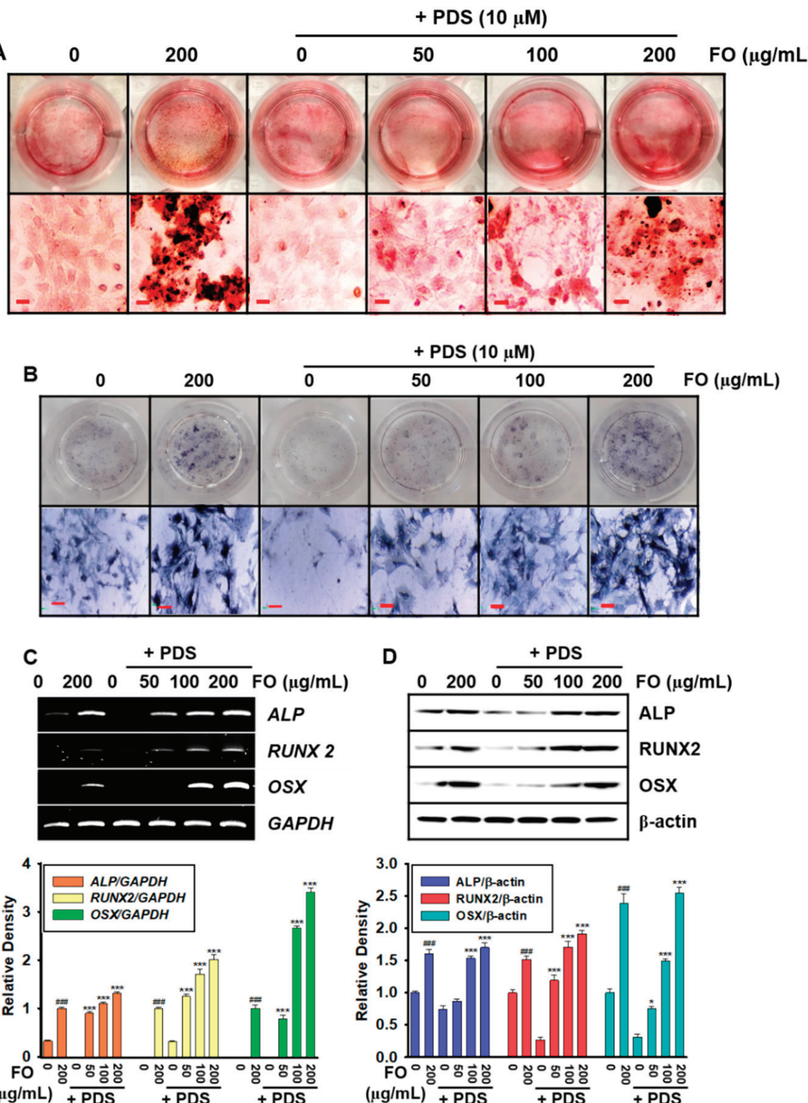

Figure 3. Prednisolone (PDS)-induced anti-osteogenic activity was inhibited by pretreatment with FO in MC3T3-E1 cells. MC3T3-E1 cells (1×10^4 cells/mL) were pretreated with FO (0–200 µg/mL) for 2 h prior to treatment with 10 µM PDS for seven days. Fresh media with FO and/or PDS were replenished every two days. At day 7, (**A**) bone mineralization and (**B**) *ALP* activity were evaluated using alizarin red staining and a TRACP & *ALP* Double-Staining Kit, respectively. (**C**) Total mRNA were extracted, and RT-PCR was performed to evaluate the gene expressions of *ALP*, *RUNX2*, and *OSX*. *GAPDH* was used as the internal control. (**D**) Total proteins were extracted, and Western blotting was performed to evaluate the expression of *ALP*, *RUNX2*, and *OSX*. β-Actin was used as the internal control. All data are presented as means ± standard error of the mean ($^{###}$ $p < 0.001$ vs. untreated MC3T3-E1 cells; * $p < 0.05$, and *** $p < 0.001$ vs. PDS-treated MC3T3-E1 cells). *ALP*: alkaline phosphatase; *RUNX2*: runt-related transcription factor 2; and *OSX*: osterix.

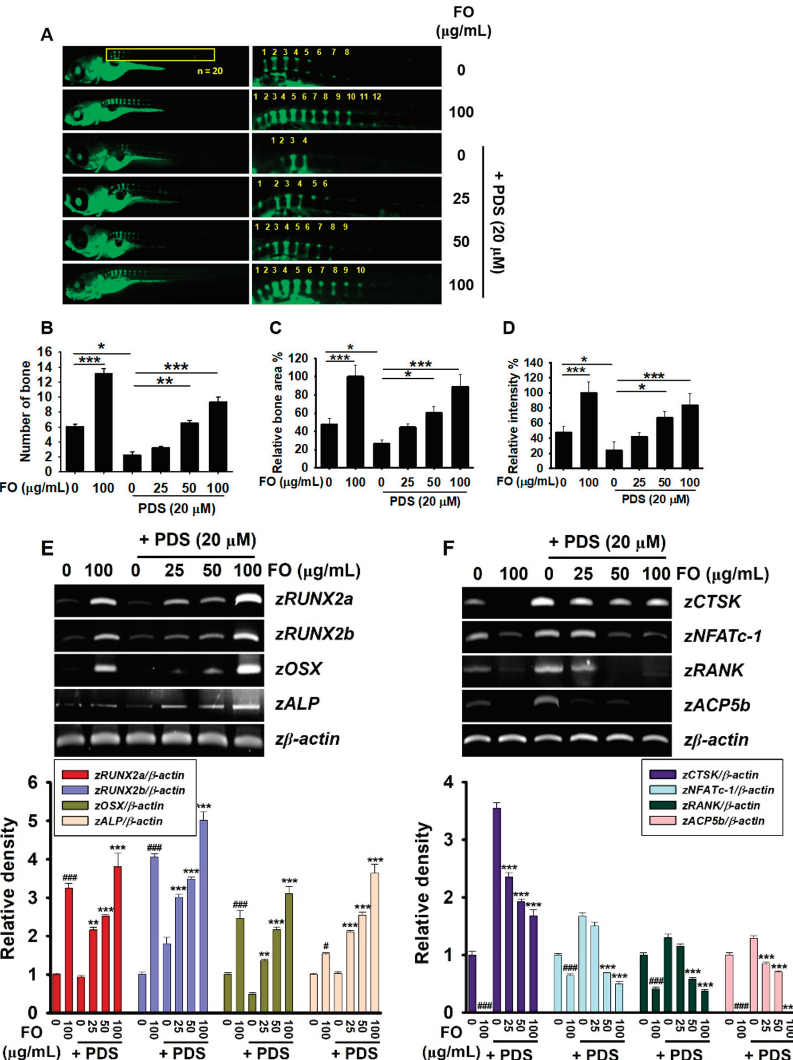

Figure 4. Pretreatment with FO restores vertebral formation in prednisolone (PDS)-post-treated zebrafish larvae. Zebrafish larvae (n = 20) at 5 days post fertilization (dpf) were treated with FO (0–100 μg/mL) for two days, and 20 μM PDS was post-treated for another two days. (**A**) At 9 dpf, zebrafish larvae were stained with 0.03% calcine and observed under fluorescence microscopy. Numbers show vertebrae (right panels) (**B**) Vertebral number was manually counted and indicated. (**C**) Relative bone area and (**D**) bone intensity were calculated using imageJ software and expressed. All data are presented as means ± standard error of the mean (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). Total mRNA was extracted, and RT-PCR was performed to evaluate the gene expression of (**E**) osteogenic genes, including *zRUNX2a*, *zRUNX2b*, *zOSX*, and *ALP* and (**F**) osteoclastogenic genes such as *zCTSK*, *zNFATc-1*, *zRANK*, and *zACP5b*. *β-Actin* was used as the internal control. All data are presented as means ± standard error of the mean ($^{\#}$ $p < 0.05$ and $^{\#\#\#}$ $p < 0.001$ vs. untreated zebrafish larvae; ** $p < 0.01$, and *** $p < 0.001$ vs. PDS-treated zebrafish larvae). z: zebrafish; *RUNX2a/b*: runt-related transcription factor 2a/b; *OSX*: osterix; *ALP*: alkaline phosphatase; CTSK: cathepsin K; NFATc-1: nuclear factor of activated T-cells; cytoplasmic 1; RANK: receptor activator of nuclear factor κB; and ACP5b: acid phosphatase 5b.

3.5. Estrogenic Activity Is Not Associated with FO

In a previous study, we confirmed that FO prevented OVX-induced bone resorption [24]. Thus, to estimate the safety of FO on the potential estrogenic activity, relative uterine weight/body weight was measured in OVX mice after administering FO for four weeks. A significant decrease in uterine weight/body weight was observed in the OVX mice (Figure 5A). Intraperitoneal injection of E2 significantly increased the relative uterine weight/body weight in OVX mice. However, oral administration of FO did not influence relative uterine weight. According to the ERE-luciferase assay in ER-responsive breast cancer MCF-7 cells, no significant luciferase activity was observed at the concentrations of FO from 2×10^{-4} to 2×10^{2} µg/mL. However, E2 slightly increased ERE-luciferase activity from 10^{-11} M and to the maximum activity at 10^{-8} M (Figure 5B), which indicates that FO stimulates bone formation in an estrogen-independent manner. Genistein, a well-known phytoestrogen, also caused significant ERE-luciferase activation at 10^{-6} and 10^{-5} M. As expected, corticosterone (10^{-11} to 10^{-4} M) used as a negative control did not affect ERE-luciferase activity. Furthermore, to evaluate whether FO possesses an ER agonist effect, the ERE-luciferase reporter gene construct was transfected into MCF-7 cells, and the activity was measured. As depicted in Figure 5C, E2 significantly increased ERE-luciferase activity (4.32 ± 0.14) compared with that in the untreated MCF-7 cells (0.97 ± 0.02); however, in the presence of ICI 182,780, the activity strongly decreased to 1.70 ± 0.08. Meanwhile, no significant difference in the activity was observed in FO-treated cells (0.92 ± 0.03). Additionally, E2 significantly increased the proliferation of MCF-7 cell from 1.00 ± 0.04 to 1.44 ± 0.02 compared with that of the untreated MCF-7 cells; however, in the presence of ICI, E2-induced cell proliferation decreased to 1.14 ± 0.03 (Figure 5D). Consistent with the luciferase activity data, FO did not cause significant changes in cell proliferation either in the absence (0.85 ± 0.02) or presence (0.81 ± 0.02) of ICI. Overall, these results indicate that FO does not affect the estrogenic activity.

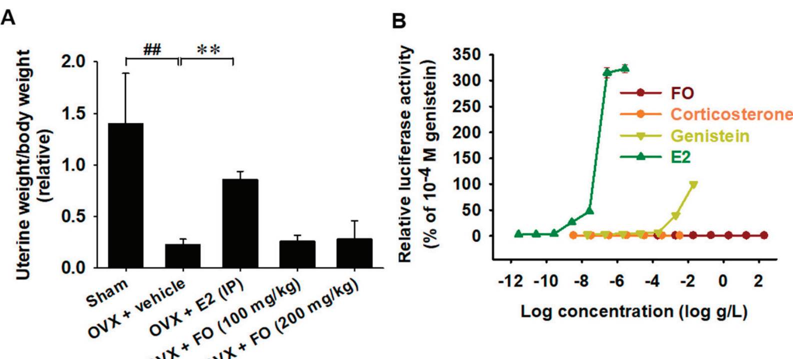

Figure 5. *Cont.*

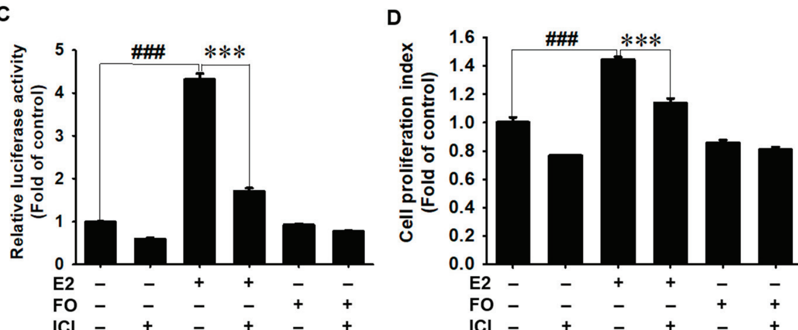

Figure 5. FO is not related to estrogenic activity in ovariectomy (OVX)-induced mice and estrogen response element (ERE)-luciferase transfected MCF-7 cells. (**A**) OVX-induced mice were orally fed with 100 mg/kg and 200 mg/kg FO and intraperitoneally (IP) injected with 10 μg/kg 17β-estradiol (E2) for 4 weeks. After termination, uterine weight was determined by calculating uterine weight in body weight. (**B**–**D**) MCF-7 breast cancer cells (1×10^4 cells/mL) transfected with human estrogen response element (ERE)-luciferase reporter plasmid for 24 h and (**B**) concentration–response curve of transcriptional activation for FO (2×10^{-4}–2×10^2 μg/mL), corticosterone (10^{-11}–10^{-5} M), genistein (10^{-10}–10^{-4} M), and E2 (10^{-14}–10^{-8} M) were measured using luciferase activity. (**C**,**D**) In a parallel experiment, FO (200 μg/mL) and E2 (10^{-9} M) were treated in the presence or absence of 10 μM ICI 182,780 (ICI), and (**C**) relative luciferase activity and (**D**) relative cell proliferation were calculated. All data are presented as means ± standard error of the mean (and $^{###}$ $p < 0.001$ and $^{##}$ $p < 0.01$ vs. untreated MCF-7 cells; *** $p < 0.001$ and ** $p < 0.01$ vs E2-treated MCF-7 cells).

4. Discussion

Synthetic adrenal glucocorticoids such as PDS are prescribed as anti-inflammatory and immunosuppressive agents; however, their long-term administration is considered the main etiology of secondary osteoporosis [12,13]. Currently, anti-bone resorptive agents such as bisphosphonates are successfully employed as treatment options. However, low drug efficacy with several off-target effects, including renal toxicity, esophageal ulcers, and acute phase reactions, have been reported [18]. Therefore, natural anabolic agents have gained extensive attention owing to their high efficacy and minimal side effects [7,19]. In our previous studies [21,22], we demonstrated that FO is a potent bone anabolic agent that promotes osteoblast differentiation, bone formation, and growth performance through the Wnt/β-catenin-mediated IGF-1 signaling pathway. In addition, we reported that FO significantly prevented ovariectomy-induced bone resorption by inhibiting osteoclast differentiation and activity [23,24]. The ultra-performance liquid chromatography (UPLC) for amino acids of FO revealed that FO contained approximately 24.5% GABA from the total amino acid content [21]. Furthermore, in our recent studies, we found that GABA directly regulated growth performances in zebrafish larvae through $GABA_A$ and $GABA_B$ receptors, indicating the potent influence of GABA in FO-mediated growth performances [27]. Nevertheless, the therapeutic and preventive effects of FO have not been elucidated in glucocorticoid-induced secondary bone resorption. In this study, we demonstrated that FO effectively cured and prevented PDS-induced anti-osteogenic activity and bone resorption in MC3T3-E1 and zebrafish larvae.

Osteoblast differentiation is tightly regulated by key transcription factors, including *RUNX2* and *OSX* [28,29]. He et al. [30] discovered that PDS downregulated the transcription of *RUNX2* and *OSX* in zebrafish larvae and induced bone resorption. In addition, the downregulation of *RUNX2* and *OSX* transcriptional activities has been found in PDS-treated mice concomitant with decreased bone area and thickness [16], confirming that PDS has a negative impact on osteoblast differentiation. In our previous study [31], direct action of PDS was also found to downregulate the gene expression of *OCN*, *ALP*, and *Col1α1* in both mouse preosteoblast MC3T3-E1 and zebrafish larvae, impairing the syn-

thesis of extracellular matrix components. Furthermore, PDS remarkably stimulates the expression of matrix metalloproteases (MMPs), including MMP-2, MMP-9, and MMP-13 in mice [32], which subsequently degrades all types of extracellular matrix (ECM) proteins, suggesting that the anti-osteoblast activities of PDS may also be linked to the degradation of ECM components. Furthermore, osteoclastogenesis was previously identified in response to PDS treatment in both zebrafish and mouse models, accompanied by specific expression of osteoclast-specific genes [16,30,31]. Interestingly, we found that FO, under both PDS-pretreated and post-treated conditions, cured and prevented PDS-mediated bone mineralization and resorption in a concentration-dependent manner. Nevertheless, whether FO directly influences the formation of ECM components remains unknown. Although FO has therapeutic and preventive effects on PDS-induced bone resorption, the efficacy of FO should be evaluated in glucocorticoid-mediated osteoporosis at the clinical level.

Endocrine-disrupting compounds (EDCs) such as bisphenol A and 4-nonylphenol have been reported in marine invertebrates and fish [33,34]. Le Curieux-Belfond et al. [35] demonstrated the short-term bioaccumulation of E2 in the pacific oyster, *C. gigas*, suggesting that EDCs have adverse effects on differentiation, development, proliferation, and reproduction through endocrine disturbance. In this regard, excessive consumption of oysters and transmission of EDCs may also disrupt the endocrine system in humans. Bateman et al. [36] demonstrated that EDCs led to decreased osteoblast differentiation by targeting *RUNX2* and other osteoblastic marker genes and increased adipogenic differentiation. Therefore, it is essential to prove the absence of EDCs in food resources prior to consumption. OECD guideline 455 (TG455) has recently imposed standard guidelines for the screening and testing of potential EDCs [37]. In our ovariectomy-induced osteoporosis model, E2 restored relative uterine weight. However, FO did not influence the weight, which indicates that FO promotes osteoblast differentiation and vertebral formation regardless of endocrine disturbances. Additionally, E2 induced ERE-luciferase activity in estrogen-responsive MCF-7 breast cancer cells and increased proliferation, but not in FO-treated MCF-7 cells. Previously, we also reported that FO did not directly bind to estrogen receptors and androgen receptors and did not show any changes in the weight of the androgen- and estrogen-dependent organs [31]. Overall, these results indicate that FO promotes osteoblast differentiation and vertebral formation regardless endocrine disturbance.

In summary, our results revealed the therapeutic and preventive effects of FO in PDS-induced osteoporosis. Our findings show that FO may be a potent pharmacological food source to downregulate bone-resorptive diseases such as osteoporosis, by activating osteoblast differentiation and bone formation.

5. Conclusions

This study demonstrated the therapeutic and preventive effects of FO on PDS-induced bone resorption. We suggest that FO may be a promising candidate for the treatment and prevention of osteoporosis. Nevertheless, further evaluation through human clinical tests is required to determine whether FO has anti-osteoporotic effects. With respect to its potential as a new natural bone anabolic agent with high efficacy and minimal side effects, it is necessary to verify the effect of FO in clinical trials. In addition, FO did not affect estrogen activity and endocrine disturbance during bone formation, so it can be used as an excellent safeguard for adolescents and women.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data used to support the findings of this study are available from the corresponding author upon request.

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

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Comparative Assessment of the Antibacterial Efficacies and Mechanisms of Different Tea Extracts

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Abstract: Tea is a popular beverage known for its unique taste and vast health benefits. The main components in tea change greatly during different processing methods, which makes teas capable of having different biological activities. We compared the antibacterial activity of four varieties of tea, including green, oolong, black, and Fuzhuan tea. All tea extracts showed antibacterial activity and Gram-positive bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*) were more susceptible to tea extracts than Gram-negative bacteria (*Escherichia coli* and *Salmonella typhimurium*). Green tea extracts inhibited bacterial pathogens much more effectively in all four varieties of tea with the minimum inhibitory concentration (MIC) values at 20 mg/mL, 10 mg/mL, 35 mg/mL, and 16 mg/mL for *E. faecalis*, *S. aureus*, *E. coli*, and *S. typhimurium*, respectively. Catechins should be considered as the main antibiotic components of the four tea extracts. Total catechins were extracted from green tea and evaluated their antibacterial activity. Additional studies showed that the catechins damaged the cell membrane and increased cell membrane permeability, leading to changes in the relative electrical conductivity and the release of certain components into the cytoplasm. Tea extracts, especially green tea extracts, should be considered as safe antibacterial food additives.

Keywords: tea extracts; pathogenic bacteria; antibacterial activity; catechins; cell membrane

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1. Introduction

Increased foodborne illness caused by foodborne spoilage and pathogenic bacteria has been a major food safety challenge and caused widespread concern [1]. Pathogenic bacteria have been reported to pose serious threats to human health, such as causing food poisoning, toxic shock syndrome, and infections [2]. In recent years, the chemical preservatives used in food have been reported causing respiratory illnesses or other health risks [3]. Therefore, there is a need to develop novel types of effective plant-derived antimicrobial compounds with safety, biodegradability, and fewer side effects [4]. Some plant extracts, including tea leaves (*Camellia sinensis* (L.) O. Kuntze), have been reported to display good inhibitory effects against pathogenic bacteria and may support the development of antimicrobial supplements [5,6].

Tea is one of the most widely consumed beverages worldwide and is considered to exert various pharmacological effects, such as hypoglycemic, anti-obesity, anti-carcinogenic, antioxidant, anti-arteriosclerotic, and antibacterial activities [7–9]. More than 300 different commercial teas have been produced and consumed world-wide. Generally, based on the manufacturing process, teas can be classified into several groups: non-fermented tea (green tea), semi-fermented tea (oolong tea), fully fermented tea (black tea), and post-fermented tea (dark tea) [10]. The active components that play key roles in most of the biological activities of tea are known to be catechins [11]. The presence of different forms of catechins and their derivatives in different teas made these compounds capable of having different biological activities [12]. Due to being steamed or panned, catechin oxidation is prevented in green tea, and polyphenols are essentially maintained in their monomeric

forms, such as (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epigallocatechin gallate (EGCG) [13]. Black tea leaves are subjected to crushing, withering, and a full fermentation process allowing enzyme-mediated oxidation where catechin derivatives are condensed to form theaflavins and thearubigins [14]. Oolong tea has a processing sequence similar to that of black tea but with a shorter oxidation duration, and contains both catechins and theaflavins [15]. Dark teas are post-fermented under controlled conditions of high humidity and temperature with fungi, which greatly decrease the contents of catechins and form tea pigments such as theabrownins [16]. Fuzhuan tea is a typical dark tea. Special fungal fermentation process of Fuzhuan tea promoted the special fungal flower aroma and mellow taste [17]. Other factors, such as the geographical location, growing conditions, variety, and preparation of the infusion, also probably influence the composition of the tea [18–20].

Tea flush extract and extracts of various tea products have shown a wide range of an-timicrobial activities against *Bacillus cereus*, *Campylobacter jejuni*, *Escherichia coli*, *Clostridium perfringens*, *Helicobacter pylori*, *Legionella pneumophila*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Vibrio cholerea*, etc. [4,21]. A comparative assessment of antimicrobial activity reported that green tea has greater antimicrobial activity than black tea [22–24]. The catechins of green tea are mainly responsible to inhibit bacterial growth. EGCG is the most abundant green tea catechin and shows strong bactericidal activity against methicillin-resistant *S. aureus* and penicillinase-producing *S. aureus* by inhibiting the biosynthesis of β-lactams [25,26]. The harvesting season also influences the antibacterial activity of tea, and oolong tea leaves prepared in the summer have been shown to exhibit the strongest activity, followed by those prepared in the spring, winter, or fall [27]. Catechin levels might be the main factor affecting the antibacterial activity of tea, which is influenced by the degree of fermentation and harvesting season. Some organic acids have shown highly antimicrobial activity against a diverse range of pathogens [28]. Kombucha, fermented from tea leaves, has antibacterial activity mainly due to the present of organic acids, such as the acetic acid [29]. Tea tree essential oil has also been reported to have highly antibacterial properties [30]. However, the precise antibacterial spectrum of tea extracts is still difficult to assess. Therefore, green tea, oolong tea, black tea, and Fuzhuan tea manufactured from the same batch of fresh leaves were used in this study to explore the efficacy and possible mechanisms of antibacterial activity, which will be helpful for developing new bacteriostatic agents.

2. Materials and Methods

2.1. Microorganisms and Materials

Bacteria stains including *E. faecalis* ATCC29212, *S. aureus* ATCC25923, *E. coli* ATCC25922, and *S. typhimurium* ATCC29213 were obtained from the College of Food Science and Engineering, Northwest Agriculture and Forestry University and maintained in slants of nutrient agar (NA) at 4 °C. All microorganisms were cultured in nutrient broth (NB) at 37 °C for 24 h. NA and NB were procured from Beijing Land Bridge Technology Company Limited (Beijing, China). Catechin standards, gallic acid, and caffeine were purchased from Shanghai Yuanye Biotechnology (Shanghai, China). High-performance liquid chromatography (HPLC) grade methanol, acetonitrile, and trifluoroacetic acid were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Bradford protein assay kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other reagents and solvents were purchased from the China National Pharmaceutical Group Corporation (Beijing, China) and were of analytical grade. Purified water (18.2 MΩ) was prepared using a Millipore Mill-Q Ultrapure Water System (Billerica, MA, USA).

2.2. Preparation of the Tea Extracts

Fresh tea leaves (*Camellia sinensis cv.* Fuding-dabaicha) were harvested from the tea station at Northwest Agriculture and Forestry University, Yangling, China. The same fresh

green shoots were washed thoroughly with water and manufactured into green tea, oolong tea, black tea, and Fuzhuan tea by different manufacturing processes (Figure 1).

Tea extracts were prepared according to the described methods [31]. Briefly, four different varieties tea cut into thin slices and extracted twice with purified boiling water (tea/water w/v = 1:20) for 7 min. The solutions were collected and concentrated by a RE-52AA vacuum rotary evaporator (Shanghai Yarong Biochemistry Instrument Factory, Shanghai, China), and then freeze-dried (Coolsafe 110-4, Labogene ScanVac, Lynge, Denmark).

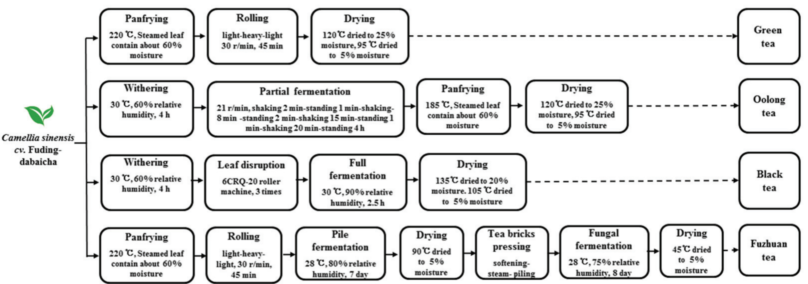

Figure 1. Main manufacture process of various teas.

2.3. *Extraction of Catechins*

Total catechins (TCs) were isolated from green tea as described previously [32]. The tea leaves were cut into thin slices and extracted twice with 85% ethanol at 35 °C for 30 min. After filtration, the solutions were collected and concentrated. Equal volume of chloroform was added to remove the caffeine, lipids, and chlorophyll. The aqueous phase was extracted with equal volume of ethyl acetate three times. The combined ethyl acetate fractions were collected and freeze-dried to obtain the TCs.

2.4. *Chemical Identification of Tea Extracts*

The total polyphenol content was determined according to the Folin-Ciocalteu method [33]. The amino acid content was assessed according to the China National Standard (B/T 8314-2013, GAQSIQ, China, 2013). The amounts of water soluble carbohydrates were measured by the anthrone-sulfuric acid method [34]. Caffeine, gallic acid, and catechins were identified and quantified by HPLC-UV (Agilent 1100VL, Agilent Technologies Inc., Santa Clara, CA, USA) as described in our previous work [35]. Separations were performed at 35 °C on an Agilent TC-C18 column (5 μm, 250 mm × 4.6 mm i.d.). A gradient mobile phase consisting of water (0.1% formic acid, A) and methanol (0.1% formic acid, B) were eluted as follows: 0–2 min, 80–75% A; 2–6 min, 75–70% A; 6–10 min, 70–75% A; 10–13 min, 75–70% A; 13–20 min, 70% A; 20–23 min, 70–75% A; 23–25 min, 75–80% A; 25–30 min, 80% A, at a flow rate of 1.0 mL/min. The injection volume was 5 μL and detection wavelength was 278 nm. The total theaflavins, thearubigins, and theabrownins were measured according to Zhong [36].

2.5. *Bacterial Susceptibility Test*

The antibacterial activities were evaluated by the disc diffusion method in agar plates according to Goni et al. [37]. Each sterile petri plate (90 mm) was prepared with 15 mL of NA. After solidifying, 100 μL of each culture of bacteria (1.0×10^7 cfu/mL) was spread on the surface of agar plates. A sterile filter paper disc (6 mm) immersed in test solution was placed on the plate followed by incubation at 37 °C for 24 h. Tea extracts were used at the concentrations ranging from 20 mg/mL to 80 mg/mL and TCs were used at concentrations ranging from 1 mg/mL to 10 mg/mL. After incubation, the diameter of the inhibition zone (DIZ) was observed and measured in millimetres (mm), and the experiments

were performed in triplicate. Sterile water was used as a control. The experiments were performed in triplicate with triplicate samples.

2.6. Determination of the Minimum Inhibitory Concentration (MIC)

The lowest concentration of tea extract to inhibit bacterial growth was determined by the broth dilution method according to a previous method [38]. In brief, 100 μL of each bacterial culture (1.0×10^7 cfu/mL) was inoculated into a test tube containing 5 mL of NB followed by the addition of serially diluted aqueous test solutions at different concentrations. All tubes were incubated at 37 °C for 24 h. The percent inhibition was calculated by measuring the absorbance at 600 nm with a UV-Visible Spectrophotometer (Shanghai, China). The MIC values were determined as lowest concentration at which no growth or turbidity was seen. Negative control contained non-inoculated medium and positive control contained inoculated without extract samples.

2.7. Membrane Permeability Assay

The membrane permeability of the bacterial cells was evaluated by measuring the change in electrical conductivity according to the method of Kong et al. [39]. The bacterial cells in their late exponential phase were harvested from NB using a centrifuge at 3000× *g* for 10 min. The cells were washed and re-suspended in 5% glucose until the electric conductivities of the cells were stable. TCs (at the MIC concentration) were added into 5% glucose and measured electrical conductivity with a conductivity meter (SevenEasy, Mettler Toledo, Switzerland).

2.8. Membrane Integrity Assay

The membrane integrity of the bacterial cells was evaluated by measuring the release of cell constituents, including nucleic acids, solute sugars, and proteins, into the cell suspension [40]. Bacterial cells in their late exponential phase were harvested from NB using a centrifuge at 3000× *g* for 10 min. The cells were washed and re-suspended in 0.1 M phosphate buffer solution (PBS, pH 7.4) followed by the addition of TCs (at the MIC concentration). After treatment, the suspensions were collected using a centrifuge at 6000× *g* for 5 min. The absorption at 260 nm was measured to determine the change in nucleic acid content by using a UV-visible spectrophotometer (Shanghai, China). The suspension was also collected to determine the concentration of solute sugars according to the anthrone-sulfuric acid method [34] and the concentration of proteins with a Bradford protein assay kit. An untreated sample was used as a control.

2.9. Statistical Analysis

All analyses were performed in triplicate using triplicate samples, and the results are expressed as the mean ± standard deviation. Significance differences for multiple comparisons were determined using one-way ANOVA with SPSS (SPSS, Chicago, IL, USA) at $p < 0.05$.

3. Results and Discussion

3.1. Comparison of the Antibacterial Efficacy of Different Tea Extracts

The antibacterial activity of different tea extracts on Gram-positive (*E. faecalis* and *S. aureus*) and Gram-negative (*E. coli* and *S. typhimurium*) bacteria were evaluated. As shown in Table 1, tea extracts showed varying degrees of antibacterial efficacy against the tested microorganisms in dose-dependent manners at concentrations ranging from 0 to 80 mg/mL. However, different tea extracts exhibited different antibacterial efficiencies. Green tea extracts showed the highest antibacterial activity against both Gram-positive and Gram-negative bacteria, with the largest the DIZ value at the same concentration of all four tea extracts, which was consistent with previous reports [22,41]. Almajano et al. [22] observed that green tea has high inhibitory effect on several microorganisms, and the magnitude of this was comparable to that of commercial infusion. Green tea kombucha has more exten-

sive inhibition on pathogenic bacteria than black tea kombucha [41]. The MIC values of the tea extracts against the test bacteria were consistent with the DIZ values (Table 2). Green tea extracts showed the highest antibacterial activity against the test microorganisms with the lowest MIC values, followed by oolong tea extracts, Fuzhuan tea extracts, and black tea extracts.

Table 1. DIZ of tea extracts against tested bacteria.

Test Solution		DIZ (mm)			
		E. coli	*S. typhimurium*	*E. faecalis*	*S. aureus*
Green tea	20 mg/mL	6.60 ± 0.19^{c}	8.80 ± 0.15^{c}	8.50 ± 0.16^{a}	21.16 ± 0.08^{c}
	40 mg/mL	7.20 ± 0.09^{b}	14.25 ± 0.19^{b}	19.80 ± 0.22^{b}	24.50 ± 0.16^{b}
	80 mg/mL	12.40 ± 0.17^{a}	22.00 ± 0.13^{a}	25.90 ± 0.13^{a}	31.33 ± 0.12^{a}
Oolong tea	20 mg/mL	6.10 ± 0.04^{c}	7.20 ± 0.10^{c}	7.50 ± 0.06^{c}	18.15 ± 0.12^{c}
	40 mg/mL	6.70 ± 0.11^{b}	12.65 ± 0.07^{b}	16.90 ± 0.11^{b}	22.60 ± 0.13^{b}
	80 mg/mL	11.40 ± 0.17^{a}	20.00 ± 0.11^{a}	21.90 ± 0.09^{a}	27.33 ± 0.17^{a}
Black tea	20 mg/mL	6.10 ± 0.08^{b}	6.10 ± 0.07^{c}	6.40 ± 0.05^{c}	12.02 ± 0.09^{c}
	40 mg/mL	6.30 ± 0.13^{b}	9.90 ± 0.03^{b}	14.80 ± 0.08^{b}	18.33 ± 0.16^{b}
	80 mg/mL	10.10 ± 0.04^{a}	15.30 ± 0.11^{a}	20.00 ± 0.13^{a}	25.50 ± 0.13^{a}
Fuzhuan tea	20 mg/mL	6.10 ± 0.18^{b}	6.30 ± 0.13^{c}	7.10 ± 0.13^{c}	14.33 ± 0.11^{c}
	40 mg/mL	6.40 ± 0.21^{b}	10.75 ± 0.12^{b}	16.50 ± 0.16^{b}	20.83 ± 0.08^{b}
	80 mg/mL	11.93 ± 0.10^{a}	17.10 ± 0.14^{a}	20.80 ± 0.08^{a}	25.17 ± 0.17^{a}

Different small letters in the same column indicate a significant difference at $p < 0.05$ level.

Table 2. MIC of tea extracts against tested bacteria.

Test Solution	MIC (mg/mL)			
	E. coli	*S. typhimurium*	*E. faecalis*	*S. aureus*
Green tea	35	16	20	10
Oolong tea	45	20	32	12
Black tea	65	36	40	16
Fuzhuan tea	45	38	32	18

Comparing the inhibitory activities of the tea extracts against Gram-negative and Gram-positive bacteria, it was found that tea extracts had stronger antimicrobial activity against Gram-positive bacteria (*E. faecalis* and *S. aureus*) than Gram-negative bacteria. *S. aureus* was the most susceptible bacterium, and *E. coli* was the most resistant bacterium to all tea extracts. The destructive response to the tea extracts of the Gram-positive bacteria compared to the Gram-negative bacteria might be due to the structural differences in the cell walls of the bacteria. The cell walls of Gram-positive bacteria is mainly composed of peptidoglycan and teichoic acid, which are permeable to solute sugars, amino acids, and most ions [42]. However, the cell walls of Gram-negative bacteria are complex and composed of peptidoglycan and lipopolysaccharide outer membranes, which are hard to damage by external factors [1,43]. In addition, the outer membrane of Gram-negative bacteria with a strong negative charge can control the flow of certain substances in and out [42].

3.2. Catechins Play the Main Role in Antibacterial Efficacy

In order to clarify the inhibitory mechanism on the antibacterial efficacy of the tea extracts, the basic active components were determined (Table 3). Green tea extracts contained the highest polyphenol (27.10%) and catechin (21.30%) contents, followed by oolong tea extracts (21.60% and 11.16%, respectively), Fuzhuan tea extracts (19.60% and 2.22%, respectively), and black tea extracts (17.26% and 1.67%, respectively). The components of catechins in different tea extracts are different (Table S1). During the fermentation process,

large amounts of catechins are reduced and oxidized to form tea pigments [12]. The highest contents of theaflavin (1.80%), thearubigins (16.13%), and theabrownins (17.80%) were detected in the black tea extracts, followed by those in oolong tea extracts (0.87%, 3.13%, and 3.19%, respectively). Due to the special pile-fermenting process and fungal-fermenting process, Fuzhuan tea had a high content of thearubigins (6.76%) and theabrownins (18.77%). The contents of gallic acid and caffeine were the highest in black tea extracts (0.78% and 5.23%, respectively) and Fuzhuan tea extracts (0.75% and 5.48%, respectively), followed by oolong tea extracts (0.55% and 4.12%, respectively), and the contents of these compounds were the lowest in green tea extracts (0.13% and 3.40%, respectively). Carbohydrates were consumed by microorganisms as a carbon source in the special pile-fermenting process and fungal-fermenting process, and the carbohydrate content was the lowest in the Fuzhuan tea extracts (8.91%). There was no significant difference in amino acid content among the four tea extracts.

Table 3. The main constituents of different tea extracts (%).

Tea Extracts	Polyphenol	Catechin	Gallic acid	Caffeine	Carbohydrate	Amino Acid	Theaflavin	Thearubigin	Theabrownin
Green tea	27.10 ± 0.05^{a}	21.30 ± 0.05^{a}	0.13 ± 0.01^{c}	3.40 ± 0.02^{c}	10.41 ± 0.10^{b}	4.88 ± 0.16^{a}	0.09 ± 0.01^{d}	2.71 ± 0.04^{d}	1.70 ± 0.02^{d}
Oolong tea	21.60 ± 0.04^{b}	11.16 ± 0.03^{b}	0.55 ± 0.02^{b}	4.12 ± 0.01^{b}	10.24 ± 0.07^{b}	4.64 ± 0.24^{a}	0.87 ± 0.01^{b}	3.13 ± 0.01^{c}	3.19 ± 0.06^{c}
Black tea	17.26 ± 0.03^{c}	1.67 ± 0.01^{d}	0.78 ± 0.04^{a}	5.23 ± 0.02^{a}	13.24 ± 0.01^{a}	4.73 ± 0.11^{a}	1.80 ± 0.01^{a}	16.13 ± 0.01^{a}	17.80 ± 0.06^{b}
Fuzhuan tea	19.60 ± 0.08^{d}	2.22 ± 0.05^{c}	0.75 ± 0.004^{a}	5.48 ± 0.05^{a}	8.91 ± 0.27^{c}	4.43 ± 0.37^{a}	0.24 ± 0.02^{c}	6.76 ± 0.06^{b}	18.77 ± 0.15^{a}

Different small letters in the same column indicate a significant difference at $p < 0.05$ level.

The catechin components are mainly responsible for several biological activities [44,45]. Line expression analysis showed that the catechin content was positively correlated with the antibacterial capacity of the tea extracts (Table 4). TCs were extracted from green tea extracts, and their antimicrobial activity was determined. As shown in Table 4, the TCs showed dose-dependent antibacterial efficacy against the tested microorganisms at concentrations ranging from 0 to 10 mg/mL. Regarding the different components of bacterial cell wall components, Gram-positive bacteria were more susceptible to TCs than Gram-negative bacteria, with larger DIZ values and lower MIC values (Table 4). Catechin groups are oxidized and polymerized to generate theaflavins, thearubigins, and theabrownins during the fermentation process, and no inhibition zone was observed in the discs containing those tea pigments (data not shown). Therefore, catechins should play the main role in the antibacterial efficacy of all four tea extracts.

Table 4. DIZ and MIC of TCs extracted from green tea against tested bacteria.

Microorganisms	DIZ (mm)			MIC (mg/mL)	Correlation
	1.0 mg/mL	5.0 mg/mL	10.0 mg/mL		
E. coli	6.30 ± 0.04^{b}	11.50 ± 0.16^{d}	12.20 ± 0.29^{d}	2.5	0.9866
S. typhimurium	6.30 ± 0.05^{b}	14.30 ± 0.19^{c}	24.60 ± 0.18^{c}	2.0	0.9946
E. faecalis	7.30 ± 0.05^{b}	17.90 ± 0.08^{b}	27.20 ± 0.09^{b}	2.0	0.9791
S. aureus	9.90 ± 0.22^{a}	22.20 ± 0.10^{a}	32.20 ± 0.32^{a}	1.5	0.9997

Different small letters in the same column indicate a significant difference at $p < 0.05$ level.

HPLC detection and quantitative analysis, as shown in Figure 2, determined the individual TC components. These components were EC (4.03%), (+)-catechin (C, 2.36%), EGC (13.16%), (-)-gallocatechin (GC, 2.87%), EGC (9.73%), EGCG (48.20%), and (+)-gallocatechin gallate (GCG, 12.79%). Comparing the green tea extracts, the TCs had an analogous composition and proportion. EGCG was the main catechin component in both the TCs extracted and the green tea extracts and has been reported to have superior antibacterial effects on *B. anthracis*, *E. coli*, and *S. aureus* [5,27]. Parvez et al. [46] observed that the MIC values of

EGCG were 1250 μg/mL against *E. coli* and 625 μg/mL against *S. aureus*. Ignasimuthu et al. [43] measured the MIC values of EGCG for *Bacillus subtilis* (130 μg/mL), *S. aureus* (200 μg/mL), *E. coli* (580 μg/mL), and *Yersinia enterocolitica* (620 μg/mL). Generally, Gram-positive bacteria were more susceptible to EGCG than Gram-negative bacteria.

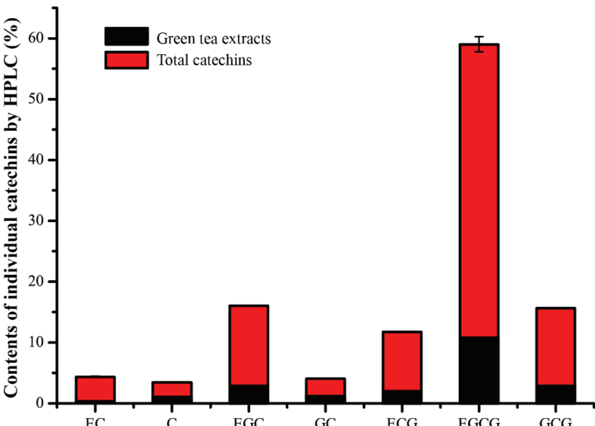

Figure 2. Individual catechin contents determined by HPLC.

3.3. Antibacterial Mechanism of Catechins

The antimicrobial mechanism of polyphenols is believed to be associated with precipitation of bacterial cell membrane proteins by the reaction of polyphenols [47,48]. Many phenolic acids and flavonoids have been found to cause cell membrane perforation and/or membrane fluidity reduction, leading to cytoplasmic membrane damage [49,50]. To further clarify the antimicrobial mechanism of catechins, the membrane permeability and integrity of TC-treated *E. coli* and *S. aureus* were determined.

The cell membrane, as a protective barrier for bacteria, will be destroyed by strong antimicrobial agents, leading to internal electrolyte leakage. Changes in relative electrical conductivity were examined as a reflection of the permeability of bacterial cell membranes. As shown in Figure 3A,B, the electrical conductivity for both *E. coli* and *S. aureus* similarly increased after treatment with TCs at their MICs (2.5 mg/mL and 1.5 mg/mL, respectively). The growth trends rapidly increased in response to TCs during the first 2 h, and then the increases slowed down. However, overall, *S. aureus* showed higher electrical conductivity after TC treatment than *E. coli*, indicating that *S. aureus* was more susceptible to catechins increasing the permeability of their cell membrane, causing more cellular leakage.

Membrane integrity was determined by measuring the release of nucleic acids, proteins, and solute sugars after treatment with TCs at their MICs (2.5 mg/mL and 1.5 mg/mL for *E. coli* and *S. aureus*, respectively). The release of intracellular nucleic acids, as an index of cell lysis, can be revealed by changes in optical density at 260 nm [51]. The optical density values at 260 nm increased to 0.214 and 0.455 after *E. coli* and *S. aureus* treatment with TCs, respectively (Figure 3C,D). Organic macromolecule proteins are the basic organics of cells and were shown to be released into the supernatants of the bacteria treated with TCs. More proteins were released into the supernatants of *S. aureus* after TC treatment than in those of treated *E. coli* (Figure 3E,F). Sugar is the primary carbon source and energy storage material for bacteria. The release of intracellular sugar was consistent with the trend of nucleic acids and proteins, which indicated that TCs could lead to sugar leakage through the cell membrane (Figure 3G,H).

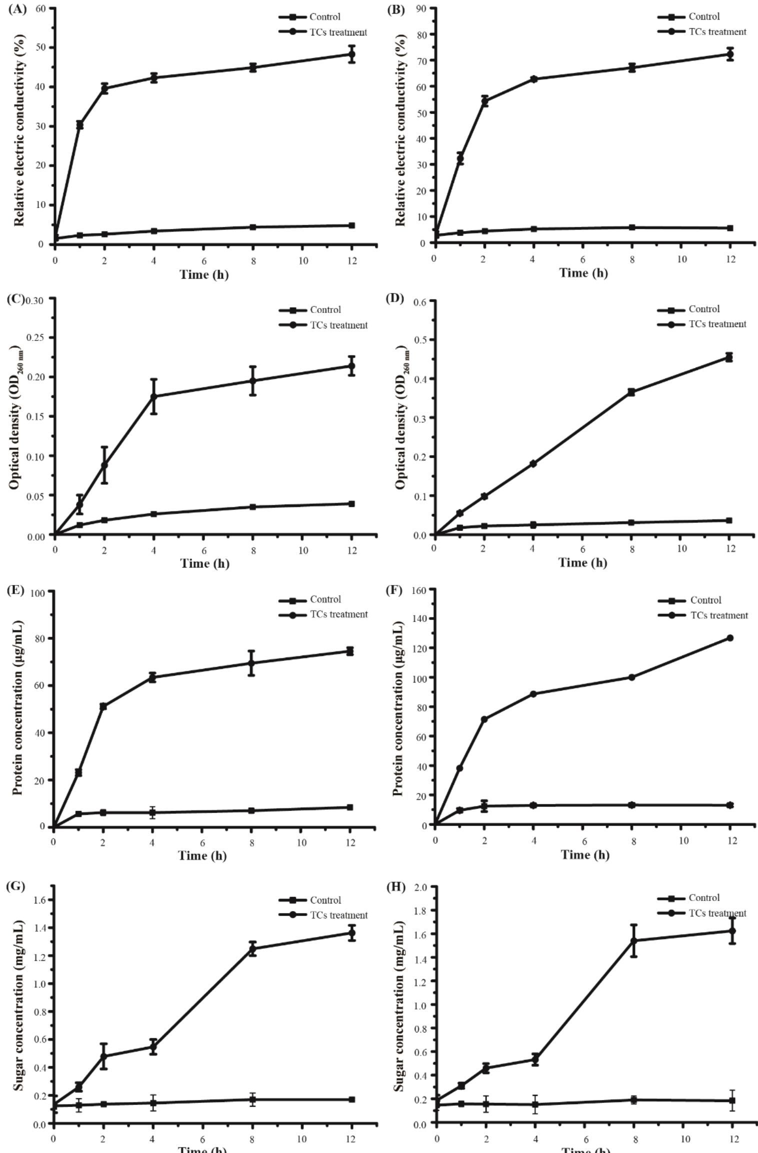

Figure 3. Effects of TCs on the membrane permeability and integrity of *E. coli* and *S. aureus*. (**A**) Changes in the relative electrical conductivity of *E. coli*, (**B**) Changes in the relative electrical conductivity of *S. aureus*, (**C**) Release of nucleic acids from *E. coli*, (**D**) Release of nucleic acids from *S. aureus*, (**E**) Release of proteins from *E. coli*, (**F**) Release of proteins from *S. aureus*, (**G**) Release of solute sugars from *E. coli*, and (**H**) Release of solute sugars from *S. aureus*.

Catechins are rich in phenolic hydroxyl groups and polycyclic structures and have a high affinity for biomacromolecules such as lipids, proteins, hydrocarbons, and nucleic acids [48]. This high affinity enables catechins to react with the bacterial cell membrane, which makes the cell structure unstable, alters cell membrane fluidity, and destroys its integrity. These phenomena have been demonstrated by Ignasimuthu et al. [43]. EGCG octaacetate has been found was two-fold higher antibacterial activity against *E. coli* than

EGCG, due to its higher lipophilic properties [43]. In our study, the changes in relative electrical conductivity and the release of nucleic acids, proteins, and solute sugars from TC-treated *E. coli* and *S. aureus* were measured. The membrane permeability and integrity changed after treatment with TCs, which indicated that the antimicrobial mechanism of catechins was associated with membrane damage and caused subsequent leakage of the intracellular constituents.

Generally, the membrane permeability and integrity of *S. aureus* were more susceptible to catechins than *E. coli*, which might be partly responsible for the stronger antibacterial activity of catechins against *S. aureus* than *E. coli*. Catechins have been reported to fail to cross the lipopolysaccharide layers of Gram-negative bacteria but exhibit antibacterial activity by binding to the peptidoglycan layer of Gram-positive bacteria [42,43]. The structural differences between Gram-positive and Gram-negative bacteria should be the main reason for the difference in the antibacterial activity of catechins.

4. Conclusions

This study compared the antibacterial activity of four kinds of tea extracts (green, oolong, black, and Fuzhuan tea) which were manufactured from the same fresh green shoots with different manufacturing processes. Green tea contained the highest the catechins and showed the best antibacterial activity against Gram-positive bacteria (*E. faecalis* and *S. aureus*) and Gram-negative bacteria (*E. coli* and *S. typhimurium*). Catechins should be considered as the main antibacterial active ingredient in tea extract, and their antibacterial activity were confirmed by test DIZ and MIC values against the test bacteria. The membrane permeability and integrity of *E. coli* and *S. aureus* changed after treatment with catechins. The results suggest that the antibacterial activity of tea extracts is due to the interaction of catechins with the bacterial cell membrane.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods11040620/s1, Table S1: The monomer components of catechin in tea extracts and GTPs (%).

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Review

The Impact of Ellagitannins and Their Metabolites through Gut Microbiome on the Gut Health and Brain Wellness within the Gut–Brain Axis

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Abstract: Ellagitannins (ETs) are a large group of bioactive compounds found in plant-source foods, such as pomegranates, berries, and nuts. The consumption of ETs has often been associated with positive effects on many pathologies, including cardiovascular diseases, neurodegenerative syndromes, and cancer. Although multiple biological activities (antioxidant, anti-inflammatory, chemopreventive) have been discussed for ETs, their limited bioavailability prevents reaching significant concentrations in systemic circulation. Instead, urolithins, ET gut microbiota-derived metabolites, are better absorbed and could be the bioactive molecules responsible for the antioxidant and anti-inflammatory activities or anti-tumor cell progression. In this review, we examined the dietary sources, metabolism, and bioavailability of ETs, and analyzed the last recent findings on ETs, ellagic acid, and urolithins, their intestinal and brain activities, the potential mechanisms of action, and the connection between the ET microbiota metabolism and the consequences detected on the gut–brain axis. The current in vitro, in vivo, and clinical studies indicate that ET-rich foods, individual gut microbiomes, or urolithin types could modulate signaling pathways and promote beneficial health effects. A better understanding of the role of these metabolites in disease pathogenesis may assist in the prevention or treatment of pathologies targeting the gut–brain axis.

Keywords: ellagitannins; ellagic acid; urolithins; ET-rich foods; gut–brain axis; antioxidant effects; anti-inflammatory effects; neuroprotective effects; anti-cancer effects

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1. Introduction

Numerous beneficial effects on human health, such as antioxidant, anti-inflammatory, anti-carcinogenic, cardioprotective, and prebiotic properties, have been reported following the consumption of many fruits, fruit juices, nuts, seeds, and beverages, these effects being due to their high content of antioxidant polyphenols, including tannins [1–8].

Tannins are secondary plant metabolites present in plants, foods, and beverages [9–11]. Among the parts of the plants that contain tannins are the bark, stems, roots, seeds, buds, leaves, and fruits [9,12]. Among foods containing tannins are fruits (grapes, blackberries, raspberries, strawberries, blueberries, cranberries, black and red currants, pomegranate, mangoes, apples, peaches, apricots, pears, guava) [9,13,14], juices [14,15], nuts (walnuts, cashews, hazelnuts, almonds, pecans, pistachios) [9,13,14], legumes (beans, lentils, cowpeas, peanuts, peas) [14,16], cereal grains (barley, rice, buckwheat, sorghum) [14], and beverages (wine [17], cognac [18], tea [19]).

Of the two main groups of tannins, namely, hydrolysable tannins and condensed tannins, ellagitannins (ETs) belong to hydrolysable tannins. They are non-flavonoid oligomeric

polyphenols, which provide very high free-radical scavenging in vitro activity. While condensed tannins are composed only of flavonoids (flavan 3-ol or flavan 3, 4-diol), without a sugar core, hydrolysable tannins are composed of ellagic acid (EA) and gallic acid with a sugar core [9]. After hydrolysis, ETs release EA and both groups are associated with important health effects [20–22].

Taking into account the poor absorption of ETs and EA, and consequently, the negligible bioavailability of ETs, respectively, the very low bioavailability of EA, the beneficial effects observed following the consumption of ET-containing foods are explained by their extensive metabolism to urolithins. The urolithins circulate in the bloodstream and reach the systemic tissues at relevant levels [20,23]. Therefore, urolithins might be responsible for the health effects [24] attributed to the consumption of ET-rich foods, herbal teas (green and black *Camellia sinensis* teas), and herbal medicinal products [20,23,25].

Although the widespread presence of ETs in nature has been reported [26–32], consumer exposure to dietary ETs is relatively low, especially in some European countries, where the daily intake of ETs has been estimated to be around 5 mg/day, with berries being the main ETs contributors [33]. This is due to the relatively low ETs content in many food sources. In contrast, a higher daily ETs intake of 12 ± 37 mg/day was reported in the Finnish diet, with the largest dietary contribution to ETs intake in the Finnish cohort being strawberries [33]. Similar results were reported for the American diet, where the estimated dietary ETs intake was 12 ± 13 mg/day, with fresh berries contributing almost half of the estimated dietary ET intake (42%), while nuts, fruit juices, and preserves accounted for the other half of dietary ET intake [34]. Therefore, the consumption of ET-rich foods, such as berries and nuts, belonging to the group of functional foods, could have a positive impact on preventing lifestyle-related diseases.

ETs are food compounds that have been rather neglected by food scientists, nutritionists, and consumers until recently [13,35]. In the past, tannins were considered antinutritional compounds and were removed by food-processing techniques; nowadays, tannins are of interest due to their numerous biological activities [35,36].

The key property underlying the prevention and/or reduction of oxidative stress-related chronic diseases and the most discussed characteristic of polyphenols is their ability to scavenge reactive oxygen species (ROS), as well as oxidatively generated free radicals derived from important cellular macromolecules, such as lipids, DNA, and proteins [5,11]. Oxidative stress is due to an imbalance between the production and accumulation of ROS in cells and tissues and the antioxidant defense capacity, being the main mechanism responsible for several human diseases [5,37]. Unlike single-target drugs that alleviate symptoms without eradicating the cause, natural polyphenols from food sources have proven multi-target abilities to counteract oxidative stress [37]. In the case of ETs, the antioxidant efficiency is explained by the presence of several hydroxy functions in the ortho position and the strong ability to donate a hydrogen atom and support the unpaired electron [33]. Thus, ETs and EA demonstrated in vitro great potential in the treatment of oxidative stress-mediated human diseases, comparable to that of urolithins, but in vivo-only urolithins proved to be responsible for the beneficial effects [37].

As is well known, oxidative stress and inflammation are closely related pathophysiological processes, each of them being able to easily induce the other, and both occur simultaneously in many pathological conditions, including diabetes and diabetic complications, hypertension and cardiovascular diseases, neurodegenerative diseases, alcoholic liver diseases, chronic kidney diseases, cancer, and aging [38,39]. In addition to their antioxidant effects, ETs and EA have shown strong anti-inflammatory activities [3,21,40,41]. Therefore, several studies have managed to confirm the effectiveness of ETs and EA in the treatment of chronic inflammatory diseases and conditions [42–44].

The scientific insights presented in this review about the in vivo metabolisms of ETs, but also about the antioxidant and anti-inflammatory, neuroprotective, and anti-cancer activities of ETs and their metabolites, tend to support the hypothesis that the potential health benefits of dietary ETs could reduce the impact of chronic and degenerative diseases

and aging-related diseases, such as cancer, diabetes, cardiovascular diseases, and central nervous system disorders [45].

In the present review, we aimed to report relevant and recent aspects of the role of ETs and their microbiota-derived metabolites in gut and brain health documented on in vitro, in vivo, and in some new clinical studies.

We conducted a narrative review of the literature using the academic databases Pubmed and ScienceDirect for the search and collection of literature. Major keywords, such as "polyphenols", "hydrolysable tannins", "ellagitannins", "ellagic acid", "urolithins", "chemical structure", "sources", "metabolism", "bioavailability", "biological activities", "antioxidant", "anti-inflammatory", "neuroprotective", "anti-cancer", "gut", "intestine", "brain", "gut–brain axis", and "in vitro", "in vivo", and "clinical studies" were used individually or in combination during the literature survey. We considered original research articles written in English and based our search on their importance and relevance to the field. As it was necessary to focus on the most impactful and relevant aspects, we included published review articles and book sections only where appropriate. We selected publications between January 2000 and October 2022 but generally focused on the most recently published articles. The non-English publications were excluded.

2. Chemistry of Ellagitannins

ETs are esters of hexahydroxydiphenic acid (HHDP) with polyols, such as glucose or quinic acid [46]. By hydrolysis of ETs, with acids or bases, HHDP is obtained, which is spontaneously lactonized to EA [22].

EA is a dilactone formed by combining two molecules of gallic acid [47]. Following glycosylation, methylation, or methoxylation of EA hydroxyl groups, numerous ETs derivatives appear in plants [33,46].

Depending on the number of HHDP groups present in the molecule, ETs can be classified into monomeric, oligomeric, and polymeric ETs [12,35]. Monomeric ETs are made up of a single HHDP group bound to a glucoside core [12]. Monomeric ETs tend to polymerize, forming dimers, oligomers, and polymers, in which the monomeric units are C-O-C bonded [12,48]. In addition, ETs are grouped into monomeric (e.g., nupharin A, geraniin, tellimagrandin II, punicalagin, eugeniin, davidiin, casuarictin, and corilagin), dimeric (e.g., sanguiin), oligomeric (e.g., agrimoniin, nupharin E, nupharin C, and hirtellin A), and C-glycosidic ellagitannins (e.g., vescalagin, castalagin, casuarinin, and stachyurin) [14,48].

There is a great structural diversity among ETs that depends on the variations in the position, frequency, and stereochemistry of the HHDP units, the galloylation degree, and/or the anomeric stereochemistry of the sugar moieties [33,35]. The high structural diversity and complexity of the ET molecules (Figure 1) influence their hydrolytic susceptibility, but also the ease with which ETs will undergo various chemical reactions, such as transformation, isomerization, and oligomerization [35,48]. The chemical structure of ETs has a great influence on their efficiency as antioxidants, the efficacy being related to the degree of hydroxylation and decreasing with the presence of a sugar moiety [33].

Hexahydroxydiphenic acid (HHDP)
Ellagic acid
alpha-Punicalagin (R=H, R′=OH)
beta-Punicalagin (R=OH, R′=H)
Corilagin

Figure 1. The chemical structures of some ellagitannins and ellagic acid.

3. Sources of Ellagitannins

Among the hydrolysable tannins, ETs are found in more plant families compared to gallotannins [14,49]. Although over 1000 natural ETs have been identified in nature to date, only a few of them are predominant in foods [35,50]. As previously mentioned, the main food sources of ETs are fruits, nuts, and seeds, but ETs may also be present in herbs, roots (used as folk medicine), and alcoholic beverages matured in wooden barrels, while EA has also been found in some types of honey [14,35,48,51].

Among fruits, abundant sources of ETs are several berries, such as raspberries, blackberries, cloudberries, blueberries, cranberries, gooseberries, currants, and strawberries [48,51]. ETs are also found in many other fruits, including pomegranates, mangos, persimmons, guavas, plums, apricots, peaches, bird cherries, and muscadine grapes [35,46]. In addition, some fruits consumed in Brazil have been reported as food sources of ETs [52,53]. Of these, the highest ET content was reported for jabuticaba, grumixama, and cambuci, while Camu-camu (Amazonian fruit) and Surinam cherry (Brazilian cherry), respectively white and red guavas, showed intermediate and low levels of ETs [52]. Moreover, rambutan (*Nephelium lappaceum* L.) peels are a potential source of ETs [54].

Regarding alcoholic beverages, EA and ETs are the most representative phenolic compounds of wine and spirits aged in barrels. ETs can represent up to 10% of the dry weight of the oak heartwood, being transferred to the wine during aging [17,55,56]. All the eight ETs identified in the traditional oak species, namely castalagin, vescalagin, granidin,

and roburins (A, B, C, D, and E), were found in wines aged in oak barrels, the first two being generally the most abundant and representing nearly 50% of the total ETs [17,18,55,57,58]. Once in the wine, the ETs undergo continuous transformations, such as reactions with the components of the wine, as well as oxidation and hydrolysis reactions, or may be involved in tannin condensation [17,55,58]. The products resulting from these reactions contribute to the feeling of bitterness and astringency and could affect the wine color [17,56,58]. In particular, ethyl derivatives, such as β-1-*O*-ethylvescalagin, complexes colored red-orange with anthocyanins that alter the wine color, and also flavonoids, such as flavano-elagitanins (e.g., acutissimin A, acutissimin B, epiacutissimin A, and epiacutissimin B) [17,55,58]. Wines aged in oak have high levels of hydroxybenzoic acid derivatives, in particular EA, which comes from the hydrolytic decomposition of oak ETs [46]. It has been reported that young cognac "eaux-de-vie" aged in barrels also contains all eight ETs mentioned above [18].

The occurrence of ETs and EA in some natural products is shown in Table 1.

Table 1. Contents of ellagitannins and ellagic acid in fruits, nuts, and beverages.

Food Product	ETs/EA	Content	Reference
Fruits			
Raspberry fruit (*Rubus idaeus* L.)	Sanguiin H-10	0.62 mg/g dw *	[26]
	Sanguiin H-6	9.56 mg/g dw	[26]
	Lambertianin C	9.79 mg/g dw	[26]
	Ellagic acid	0.31 mg/g dw	[26]
Blackberry fruit (*Rubus fruticosus* L.)	Lambertianin C	11.0 mg/100 g rw **	[30]
	Lambertianin A	39.8 mg/100 g rw	[30]
	Ellagic acid	11.8 mg/100 g rw	[30]
Strawberry (*Fragaria* × *ananassa* Duch.)—fruits of six cultivars	Pedunculagin	0.24–1.38 mg/g dw	[29]
	Potentillin	0–1.69 mg/g dw	[29]
	Casuarictin	0.12–1.30 mg/g dw	[29]
	Sanguiin H-6	0.12–1.55 mg/g dw	[29]
	Agrimoniin	0.89–13.11 mg/g dw	[29]
	Fragariin A	0.34–1.47 mg/g dw	[29]
Pomegranate (*Punica granatum* L.) —peels of seven cultivars	Pedunculagin	8.2–11.8 mg/g dw	[27]
	α-punicalagin	17.4–24.7 mg/g dw	[27]
	β-punicalagin	25.3–32.9 mg/g dw	[27]
	β-punicalin	3.1–7.1 mg/g dw	[27]
	Ellagic acid hexoside	2.9–6.3 mg/g dw	[27]
	Galloyl-HHDP-gluconic acid	1.1–1.7 mg/g dw	[27]
	Galloyl-HHDP-hexoside	1.0–2.8 mg/g dw	[27]
	HHDP-hexoside	1.7–3.2 mg/g dw	[27]
	Ellagic acid	2.1–5.3 mg/g dw	[27]
Mango peel and seed (*Mangifera indica* L.)	Valoneic acid dilactone	Not mentioned	[59]
	Ellagic acid	1.13–13.67 mg/100 g dw	[60]
Costa Rican guava peel and flesh (*Psidium friedrichsthalianum* Nied.)	Geraniin isomer 1	120.6 mg/100 g dw (peel, flesh)	[28]
	Geraniin isomer 2	9.3 mg/100 g dw (peel); 103.9 mg/100 g dw (flesh)	[28]
	Castalagin isomer 1	28.3 mg/100 g dw (peel); 39.6 mg/100 g dw (flesh)	[28]
	Castalagin isomer 2	84.6 mg/100 g dw (peel); 41.7 mg/100 g dw (flesh)	[28]
Muscadine grapes (*Vitis rotundifolia* Michx.)	Sanguiin H-5	0.03–0.91 mg/g	[48]
	Ellagic acid	32.1 mg/100 g fw # (seed); 59.1–61.5 mg/100 g fw (skin); 14.63–17.70 mg/100 g fw (fruit)	[61]

Table 1. *Cont.*

Food Product	ETs/EA	Content	Reference
Jabuticaba (*Myrciaria cauliflora* Mart.)	Castalagin	78.4 mg/100 g fruit fw	[62]
	Vescalagin	28.7 mg/100 g fruit fw	[62]
	Pedunculagin	9.8 mg/100 g fruit fw	[62]
Yellow grumixama fruit (*Eugenia brasilienses* Lam.—γ variety)	Castalagin/vescalagin, pedunculagin, strictinin, potentillin/casuarictin	92 mg EAE [##]/100 g fw	[63]
Purple grumixama fruit (*Eugenia brasilienses* Lam.—α variety)	Pedunculagin, strictinin, potentillin/casuarictin, tellimagrandin I	82−243 mg EAE/100 g fw	[63]
Camu-camu (*Myrciaria dubia* (Kunth) McVaugh)	Alnusiin	Not mentioned	[53]
Java plum/black plum fruit (*Syzygium cumini* (L.) Skeels)	Vescalagin	0–1.0 mg/g dw	[64]
	Galloyl dihexahydroxy-diphenoyl glucose	1.0–7.5 mg/g dw	[64]
	Digalloyl dihexahydroxy-diphenoyl glucose	0.97–5.6 mg/g dw	[64]
	Ellagic acid	1.1–11.9 mg/g dw	[64]
Nuts			
Walnuts (*Juglans regia* L.)—pellicle of six cultivars	Pedunculagin/casuariin (2 isomers)	3.1–13.3 mg/g fw	[31]
	Strictinin/isostrictinin (3 isomers)	1.9–9.6 mg/g fw	[31]
	Trigalloyl-HHDP-glucose (4 isomers)	2.9–27.5 mg/g fw	[31]
	Tellimagrandin 1 (3 isomers)	3.1–27.9 mg/g fw	[31]
	Pterocarinin A	0.5–2.8 mg/g fw	[31]
	Castalagin/vescalagin	9.5–35.9 mg/g fw	[31]
	bis-HHDP-glucose derivative	20.2–26.1 mg/g fw	[31]
	Casuarin/casuarictin	8.6–39.8 mg/g fw	[31]
	Ellagic acid	17.5–23.3 mg/g fw	[31]
Pecans (*Carya illinoinensis* (Wangenh.) K.Koch)	Valoneic acid dilactone	9.45 μg/g acetonic crude extract	[65]
	Ellagic acid	132.0 μg/g acetonic crude extract	[65]
Chestnut (*Castanea sativa* Mill.)—peels of cultivar Longal	Vescalagin	67.5–109.4 μg/g dw	[32]
	Castalagin	49.6–100.4 μg/g dw	[32]
	Ellagic acid	47.6–3542.6 μg/g dw	[32]
Beverages			
Red oak-aged wines	Castalagin	0.61–11.8 mg/L	[57]
		0.07–11.43 mg/L	[17]
	Vescalagin	0.194–6.4 mg/L	[57]
		0.06–1.32 mg/L	[17]
	Grandinin	0.36–3.4 mg/L	[57]
		0.02–0.63 mg/L	[17]
	Roburin A	0.01–0.24 mg/L	[17]
	Roburins B + C	0.07–1.56 mg/L	[17]
	Roburin D	0.04–0.91 mg/L	[17]
	Roburin E	0.115–2.08 mg/L	[57]
		0.02–0.79 mg/L	[17]
	Ellagic acid	7.88–11.61 mg/L	[66]

Table 1. *Cont.*

Food Product	ETs/EA	Content	Reference
White oak-aged wines	Castalagin	0.328 mg/L	[57]
	Vescalagin	0.14 mg/L	[57]
	Grandinin	0.061 mg/L	[57]
Rosé oak-aged wines	Castalagin	4.69 mg/L	[57]
	Vescalagin	2.3 mg/L	[57]
	Grandinin	3.5 mg/L	[57]
	Roburin E	1.5 mg/L	[57]
Cognac eaux-de-vie	β-1-*O*-ethylvescalagin	1.1 mg/L	[18]
	β-1-*O*-ethylvescalin	0.73–4.66 mg/L	[18]
	Brandy tannin A	0.77–10.28 mg/L	[18]
		0.4–4.2 mg/L	[67]
	Brandy tannin B	0.5–5.50 mg/L	[18]
	Castalagin	1.34–4.90 mg/L	[18]
	Vescalagin	0.06–0.64 mg/L	[18]
	Grandinin	0.11–1.50 mg/L	[18]
	Roburin A	0.01–0.07 mg/L	[18]
	Roburin B	0.01–0.17 mg/L	[18]
	Roburin C	0.03–0.61 mg/L	[18]
	Roburin D	0.26–1.48 mg/L	[18]
	Roburin E	0.03–0.61 mg/L	[18]
Commercial cognac	β-1-*O*-ethylvescalin	0.87–1.58 mg/L	[18]
	Brandy tannin A	1.7–3.38 mg/L	[18]
		0.03–7.7 mg/L	[67]
	Brandy tannin B	0.37–1.21 mg/L	[18]

* dw—dry weight; ** rw—raw material; # fw—fresh weight; ## EAE—ellagic acid equivalents (total ellagitannin content expressed as total ellagic acid); HHDP—hexahydroxydiphenic acid.

4. Metabolism and Bioavailability of ETs and EA

Unlike condensed tannins, there are fewer studies on the absorption, metabolism, and bioavailability of ETs in humans. In contrast, animal studies have provided more information.

According to bioavailability studies, it appears that ETs are not absorbed as such due to their increased size and polarity [11,35,68]. ETs that are sensitive to acidic hydrolysis in the stomach and basic hydrolysis in the duodenum release the bislactone EA, which is poorly absorbed, while hydrolysis-resistant ETs end up almost intact in the large intestine [11,35,69].

Poor intestinal absorption of EA has been observed in some human studies, in which, after oral administration of pomegranate juice, low plasma concentrations of EA were detected and no intact forms of ETs. The likely explanation for the poor absorption of EA is its low water solubility and its ability to complex calcium and magnesium ions in the gut [14,35,70].

In the lower gastrointestinal tract, released EA is converted to dimethylated ellagic acid glucuronide, which is further metabolized by the human intestinal microbiota (*Gordonibacter urolithinfaciens*, *Gordonibacter pamelaeae,* and *Ellagibacter isourolithinifaciens*) to hydroxy derivatives of dibenzopyran-6H-6-one, called urolithins [11,35,48,71,72].

Gut microbiota can convert EA into urolithins (Figure 2) via lactone ring cleavage, decarboxylation, and dehydroxylation reactions, starting with the metabolite pentahydroxy-urolithin and continuing with tetrahydroxy- (urolithin D (UD), urolithin E (UE) and urolithin M6 (UM6)), trihydroxy- (urolithin C (UC) and urolithin M7 (UM7)), dihydroxy- (urolithin A (UA) and iso urolithin A (iso-UA)), and monohydroxy- (urolithin B (UB)) dibenzopyran-6-one metabolites [25,51,73]. The urolithins are further incorporated into the enterohepatic circulation [48].

Following the consumption of ET-rich foods, the urolithins were found in human plasma and urine [14,35]. Since studies have shown a long-term persistence of urolithins in

the body after dietary intake of ETs, the urolithins are considered bioactive forms of ETs and EA, responsible for the anti-cancer effects demonstrated by food ETs in vivo [11,35,69].

Figure 2. Ellagic acid and some of its microbial metabolites, the urolithins.

However, the inter-individual variability of the colonic microbiota affects the metabolism and bioavailability of ETs, leading to inter-individual variations in urolithins production [14,35,69,74]. Thus, in studies performed on healthy volunteers, "low excreters" and "high excreters" of urolithins were identified, respectively [33,35]. Recently, a stratification of individuals according to their urinary urolithin excretion status has been proposed [23,25,35]. Thus, three gut microbiota-associated metabotypes were defined: metabotype A, which includes those producers of only UA as the final urolithin; metabotype B, which includes subjects who produce UB and iso-UA in addition to UA; and metabotype 0 (corresponding to "low excreters"), individuals that cannot produce final urolithins, in their case only the pentahydroxy-urolithin precursor is detected [21,23,25].

While metabotype 0 was reported to be approximately constant (10%) in a large age range (5-90 years), for the other two metabotypes the distribution is significantly influenced by age, as follows: metabotype A being predominant at early ages (85%) and decreasing in adulthood (up to 55%), with the parallel increase in metabotype B (from 15% to 45%). After the age of 40, the proportion of the three metabotypes (0, A, and B) remains unchanged (10%, 55%, and 45%, respectively) [25,75].

In addition to age, the main determining factor of the distribution of urolithin metabotypes in the population, other factors, including diet, physical activity, health status, sex, weight, or body mass index, could have a potential influence.

Regarding the diet, it has been observed that it can modulate to a certain extent the gut microbiota involved in the ETs-EA metabolism pathway. Thus, following a chronic consumption of pomegranate extract with a high content of ETs (1.8 g extract containing 425 mg ETs), non-producer individuals (metabotype 0) became producers (metabotype A or metabotype B) [76].

Cortés-Martin et al. [77] also reported a significant association between increased physical activity and the prevalence of metabotype B, especially between 5 and 18 years. In contrast, these authors observed no relationship between metabotype and sex.

The association between metabotype and disease risks was revealed in a study that included only adults. In their case, the prevalence of metabotype B increased in people with chronic illness (metabolic syndrome or colorectal cancer) associated with gut microbial imbalance (dysbiosis) [78]. When larger age ranges were considered, the association was no longer confirmed [77].

Another study described a potential correlation between the prevalence of metabotype B and obesity. Thus, the gut dysbiosis associated with overweight and obesity affected the metabolism of EA and ETs, the percentage of metabotype B individuals, characterized mainly by the production of iso-UA and/or UB from EA, being more abundant in overweight-obese than in normal-weight subjects [79].

These differences observed in the distribution of urolithin metabotypes may be the result of differences in the human gut microbiome associated with health status.

Despite the potential associations that could explain the differential capacity of individuals to metabolize EA derivatives into urolithins, some authors argue that the distribution of urolithin metabotypes in the population is mainly determined by aging, which indicates that the gut microbiota involved in the metabolism of ETs and EA is developmentally regulated [77,80].

Consequently, the biological activities of ET-rich foods, such as anti-inflammatory and anti-cancer effects, may differ from person to person, depending on the composition of the intestinal microbiota [74].

5. Biological Activities and Mechanisms of Action of ETs, EA, and Urolithins

The mechanisms of action underlying the biological activities of ETs are not fully elucidated even at present. The hypothesis first launched by Cerdá et al. [81] in 2005 is still supported. According to it, urolithins and/or their possible phase II conjugates formed in vivo are responsible for the biological activities of dietary ETs. Their anti-cancer, anti-inflammatory, cardio-metabolic, antioxidant, and neuroprotective effects [20,25,81] have been demonstrated following in vitro testing, but the in vivo studies are still limited [25,45].

5.1. Antioxidant Activity of ETs, EA, and Urolithins

Numerous health benefits reported for EA, including anti-inflammatory, cardioprotective, neuroprotective, hepatoprotective, gastroprotective, nephroprotective, antibacterial and antiviral properties, cancer preventive and suppressive effects, respectively inhibition of oxidative stress, were partially attributed to the antioxidant activity of EA [37,82–84]. EA is considered a very effective antioxidant, with a similar scavenging activity to other major antioxidants, such as vitamins C and E [39,82,84]. Due to its ability to regenerate and not being reduced after metabolism, EA can provide continuous protection against oxidative stress, even at micromolar concentrations [84].

Taking into account its modes of action, EA can be considered an antioxidant with multiple functions [37].

Thus, EA can act as a primary antioxidant (type I, or chain breaking), i.e., acting as a free-radical scavenger, based on its ability to transfer the phenolic H atom to a free radical, exerting its antioxidant effects mainly through three mechanisms of action, namely: single electron transfer (SET), hydrogen atom transfer (HAT) and sequential proton loss hydrogen atom transfer (SPLHAT), respectively [37,84–86].

Also, EA can act as a secondary antioxidant (type II, or preventive), exerting its effects against free radicals due to its ability to inhibit the endogenous production of oxidants and especially of the hydroxyl radical (•OH), the most reactive and electrophilic among oxygen-based radicals [37].

The secondary antioxidant activity of EA is also related to EA's ability to chelate ionic metals such as copper, iron, nickel, and cadmium, providing an additional mechanism

of protection against oxidative stress [37,82]. Thus, by chelating and subtracting metals such as Fe^{2+}, Fe^{3+}, and copper ions involved in the production of free radicals, EA prevents low-density lipoprotein (LDL) oxidation [37,84].

In addition to scavenging prooxidant agents, EA can modulate many cell signaling pathways. It has the ability to activate the nuclear factor erythroid 2-related factor 2/antioxidant response element (Nrf2/ARE) pathway that has a major role in cytoprotection [87]. Thus, EA increases the activity of some antioxidant and detoxifying enzymatic systems (superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase, and catalase (CAT)) by regulating the Nrf2 redox-sensitive transcription factor, after UV-B light-induced oxidative stress in human dermal fibroblast [88,89].

Its structure, with two lactones and four phenolic hydroxyl groups that have the ability to produce hydrogen-bonding interactions, allows it to inhibit also some enzymes involved in the radical generation, such as various cytochrome P450 isoforms, lipoxygenases, cyclooxygenase (COX) and xanthine oxidase, thereby inhibiting the excessive production of ROS and reactive nitrogen species (RNS) [39,82,90,91].

However, through some of its hydroxyl groups, EA can exert prooxidant activity under certain conditions (such as high doses, high concentrations of transition metal ions, alkaline pH, or the presence of oxygen molecules) [37,92–94]. This is specific to small polyphenols and appears little or not at all in the case of high molecular weight polyphenols, such as ETs [94].

The most used methodologies reported evaluating the chemical antioxidant capacity of the phenolic compound family, including ETs and their metabolites, are the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), the ferric reducing ability of plasma (FRAP) and oxygen radical absorbance capacity (ORAC) assays [21]. Of these, the ABTS, DPPH, and FRAP assays are based on the SET mechanism, while the ORAC assay measures antioxidant inhibition of peroxyl radical-induced oxidation, thus reflecting classical radical chain-breaking antioxidant activity by the HAT mechanism [95].

Most studies point to a strong correlation between antioxidant properties and the structure of ETs [14]. Thus, the antioxidant and free-radical scavenging capacity increases as the degree of polymerization increases, being stronger for high molecular weight ETs [14,96]. This was explained by the presence of several hydroxy functions in the ortho, which have a greater ability to donate a hydrogen atom and support the unpaired electron, in the case of high molecular weight ETs, compared to those with low molecular weight [33,97].

It is considered that among the hydroxy groups present in the structure of high molecular weight ETs, especially the multiple pyrogallol-type galloyl units have a major contribution to the particularly strong antioxidant activity [11,96]. Therefore, Pfundstein et al. [98] reported a stronger DPPH scavenging capacity and FRAP for punicalagin and punicallin, which have a higher degree of hydroxylation than EA. Similar results were obtained by Seeram et al. [97] in the Trolox equivalent antioxidant capacity (TEAC) assay, namely, a higher antioxidant capacity for punicalagin compared to EA.

On the other hand, the results obtained by Sun et al. [99] express the opposite for the DPPH scavenging test and the FRAP test, where the trends for activities decreased in the order of EA > punicalagin > punicallin and only when acting against O_2^-, punicalagin and punicallin showed much higher activities than EA, and their relative activities decreased in the order of punicalagin > punicallin > EA. Despite the observed differences, the results confirmed the strong antioxidant capacities of the three compounds, punicalagin, punicallin, and EA.

Comparing punicalagin, punicallin, and corilagin in the ORAC, FRAP, and DPPH assays, Pfundstein et al. [98] found that punicalagin, with a total of 16 gallic hydroxyl groups, was the best in the ORAC and FRAP assays, corilagin with only 9 hydroxyls was the best in the DPPH assay, while punicallin with 10 hydroxyls in the highly restrained gallagyl unit was the weakest in all 3 assays, molecular flexibility proving to be the key

factor and the decrease in the inhibitory activity taking place with the increase in the rigidity of the molecule.

Regarding the presence of free galloyl groups in the structure of ETs, it was observed that the radical-reducing power was stronger in the case of ETs with a higher number of free galloyl groups; thus, the HO· scavenging capacity of telimagrandin II, which has three galloyl groups, was better than that of telimagrandin I, with only two galloyl groups [96]. In contrast, dimeric ETs agrimoniin and gemin A demonstrated similar scavenging activities, despite the different number of galloyl groups [96]. These different results may be due to different molecular characteristics of the compounds, such as spatial structure and solubility, but also to different radical properties [96,99].

As an example, Gulcin [100] reported that low molecular weight compounds more easily access the radical site, being more active in scavenging DPPH, while high molecular weight compounds may react slowly or even be inert in the DPPH scavenging capacity.

Even though some ETs have shown strong antioxidant effects in vitro, they cannot be extrapolated in vivo due to the metabolism of ETs and EA by the intestinal microbiota to urolithins, with an antioxidant activity different from that of ETs.

This was confirmed in the study by Sun et al. [99], where oxidative stress was induced in mice with oxidized fish oil. Thus, while administration of punicallin and punicalagin resulted in significant reduction of oxidative stress (decreasing the malondialdehyde (MDA) level, increasing the activities of GPx and SOD enzymes) only in the gut, EA had the most effective protective effects against oxidized fish oil-induced oxidative damage in all tested tissues, namely intestine, plasma and liver. Therefore, punicalagin and punicallin are not absorbed into the bloodstream due to their large size and cannot exert their full antioxidant potential in other tissues than the intestine where they are hydrolyzed to EA and metabolized to urolithins by the intestinal microbiota [99,101,102].

Panchal and Brown [102] showed that an extract from European oak bark used in red wine maturation containing a mixture of ETs (vescalagin, castalagin, roburin E, grandinin, and EA) improved oxidative stress markers in high-fat diet-fed Wistar rats. Thus, reduced plasma concentrations of MDA and increased plasma GPx activity revealed a marked antioxidant response and protective effects mediated by oak-derived ETs, similar to those produced by pomegranate-derived ETs.

Considering the strong antioxidant properties demonstrated by foods rich in ETs, exploring the antioxidant capacity of urolithins has become a research topic of great interest in recent years. The urolithins most commonly found in humans and animals are UA, iso-UA, and UB [37].

Compared to ETs, urolithins demonstrated a modest in vitro antioxidant activity, with variable activity values, depending on the tested metabolite and the assay used [37,103,104].

Thus, the first study supporting these claims, conducted by Cerda et al. [103], reported an antioxidant activity of the metabolite 3,8-dihydroxy-6H-dibenzo[*b*,*d*]pyran-6-one (UA) of 42-fold and 3570-fold lower than that of punicalagin with DPPH and ABTS assays, respectively. Recent studies also reported for UA an antioxidant activity 23-fold lower than that of EA, in the DPPH assay [104,105].

The results obtained by other antioxidant tests, such as FRAP or thiobarbituric acid reactive substances (TBARS), did not confirm the antioxidant activity of UA in plasma or colon mucosa [15].

However, using the ORAC assay, Ito [106] reported that all tested urolithins exhibited strong antioxidant properties compared to ascorbic acid, with UA being the strongest among them. In addition, metabolites exhibited more potent antioxidant activities in the ORAC assay than in intact ETs, such as geraniin and corilagin. In addition, following oral administration of geraniin to rats, plasma ORAC scores increased with increasing plasma UA concentration.

In another similar study, comparing the antioxidant activities of polyphenol metabolites with those of intact functional polyphenols geraniin, chlorogenic acid, and (−)-epigallocatechin gallate (EGCG) by an ORAC assay, stronger antioxidant activity was

reported for the metabolites than for their original compounds, with UA showing the most potent antioxidant activity among all metabolites [107].

The greater antioxidant capacity of urolithins compared to EA was also confirmed in the study carried out by Kallio et al. [95], where the ORAC values for EA and urolithins A and B were 4.25, 6.67, and 5.77 Trolox equivalents, respectively, and the $ORAC_{UA}/ORAC_{EA}$ ratio was 1.57.

Thus, the antioxidant capacity of urolithins proved to be questionable following the results obtained through the DPPH, FRAP, and ABTS antioxidant assays (all based on the SET mechanism), while only the ORAC assay identified urolithins as antioxidants [37,95,104]. Unlike EA, which, due to its chemical structure, can exert its antioxidant effects both through SET and HAT reactions, unconjugated urolithins, having no other functional groups in their structure than one (UB) or at most two phenolic hydroxyl groups (UA), that act as hydrogen donors, will exert their antioxidant effects only through HAT reactions [82,84,95]. Since the electron-withdrawing carboxyl group of urolithins is part of a lactone ring, it cannot promote the SET mechanism [37,95].

Just based on the fact that they exert their antioxidant effects exclusively by the HAT mechanism, urolithins cannot be considered less potent antioxidants, especially since the HAT mechanism and oxidation induced by peroxyl radicals are considered to be more biologically relevant than the SET mechanism or oxidation induced by other oxygen radicals [95,108].

Therefore, the assumption that urolithins could have a higher antioxidant capacity than originally thought is still being investigated, aiming to prove that these metabolites can have an important contribution as antioxidants in the body after the oral administration of intact ETs.

5.2. In Vitro Studies Attesting to the Beneficial Influence of ETs and Their Metabolites on the Gut–Brain Axis

ETs, high molecular weight polyphenols, are metabolized by animal and human gut microbiota to bioavailable urolithins, low molecular weight metabolites. As urolithins rapidly undergo phase II metabolism following gut absorption, the in vitro studies should be conducted on urolithins, as well as their phase II respective glucuronides to fully evaluate the biological activity mechanisms.

The gut–brain axis is a bidirectional communication system involving many signaling molecules between the enteric and central nervous system (CNS). Scientific evidence suggests that crosstalk along the gut–brain axis regulates inflammatory responses, antioxidant activity, cell growth, and proliferation, modulating the homeostasis of functions in these systems. Alterations in gut–brain interactions have been recognized in several digestive and neurological disorders [109]. However, psychobiotics, bacterially mediated molecules including prebiotics, probiotics, and postbiotics, have the potential to affect microbiota–gut–brain axis signaling [110]. ETs and their postbiotic metabolites are part of the dietary intervention factors that can influence the gut–brain axis and be part of the prevention and therapeutic approaches [111].

5.2.1. Antioxidant and Anti-Inflammatory Effects

Oxidative phosphorylation (OXPHOS), a metabolic pathway that produces energy inside mitochondria, could generate an over-production of ROS under stressful conditions revealing a link between mitochondrial function and aging [112]. If the antioxidant homeostasis systems do not work properly, ROS could accumulate and induce oxidative stress and inflammatory processes, lipid peroxidation in cell membranes, initiation of mitochondrial dysfunctions, and cell apoptosis, all essential risk factors involved in the pathogenesis of many chronic diseases, including neurodegenerative diseases, cardiovascular disease, or cancer [113]. Antioxidant and anti-inflammatory pathways are basically intertwined, affecting similar biomarkers. Increased ROS concentration triggers proinflammatory signaling and mediators, such as nuclear factor kappa B (NF-κB) and COX-2,

that generate inflammatory cytokines, including interleukins IL-1β, IL-6, IL-8, and tumor necrosis factor α (TNF-α). Nevertheless, ETs and their metabolites could induce antioxidant and anti-inflammatory responses through scavenging free radicals and ROS [45]. Thus, these compounds were shown to silence the NF-κB and mitogen-activated protein kinase (MAPK) proinflammatory signaling pathways or upregulate heme oxygenase-1 (HO-1) expression by activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) and Nrf2/ARE pathways [114].

In SK-N-MC human neuroblastoma cell line, UA significantly attenuated intracellular ROS production and oxidative stress-induced apoptosis, increased cell viability, decreased the Bax/Bcl-2 ratio, and suppressed the p38 MAPK pathway phosphorylation [115]. Additionally, UA protected mitochondrial function and alleviated oxidative stress via the SIRT1/PGC-1α pathway [116]. A new study confirmed that UA could inhibit ROS generation, increase the levels of Nrf2, manganese superoxide dismutase (MnSOD), and total glutathione (GSH), and reduce TNF-α, NF-κB, and IL-6 levels by activating SIRT1 signaling [117].

Another UA anti-inflammatory activity mechanism was mediated through the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor implicated in numerous physiological and pathological cellular mechanisms, such as immunity, energy homeostasis, and epithelial barrier function [118]. The study of Singh et al. [119] showed that UA significantly enhanced gut barrier function and inhibited inflammation via activation of AhR-Nrf2-dependent pathways that increased epithelial tight junction proteins. It was exposed that gut-derived metabolites presented anti-inflammatory AhR ligand activities and, through targeting the gut–brain axis, could treat neuroinflammatory diseases [120]. These AhR agonists generated by the gut microbiome can cross the blood-brain barrier and diminish CNS inflammation via activating AhR ligands [121]. Recent evidence showed that urolithins, especially UA, could play key roles in multiple sclerosis (MS) pathogenesis through alteration of the intestinal epithelial barrier function [122]. These results were confirmed by Hering et al. [123], revealing that UA could reverse the proinflammatory dysregulation induced by TNF-α in colonic HT-29/B6 cells, while in ileum-like Caco-2 cells, EA strengthened barrier function, a key feature of intestinal health. Besides reinforcing the barrier function per se, Iglesias et al. [124] showed that EA could also inhibit TNF-α triggered negative effects, including IL-6 and IL-8 release, increased intercellular adhesion molecule-1 (ICAM-1) and Nod-like receptor protein 3 (NLRP3) expression, or increased mitochondrial oxidant production. Mechanistically, EA acted primarily through the inhibition of NF-κB and extracellular signal-regulated kinases (ERK) 1/2 pathways, breaking the cycle of inflammation and oxidative stress. This is in agreement with previous in vitro results in which UA significantly decreased the expression of proinflammatory cytokines IL-6 and TNF-α through modulation of miR-27 expression and the ERK/peroxisome proliferator-activated receptor gamma (PPAR-γ) signaling pathway [125].

Knowing that endothelial Akt-kinase plays an important role in the pathogenesis of cardiovascular complications in diabetic patients, the effects of different polyphenols, including UA, on Akt phosphorylation (pAkt) in endothelial cells were assayed [126]. UA, but not UB, UC, or UD, was among the strongest pAkt inhibitors, which can be linked to the two OH-groups at the C3 and C8 positions in the UA structure. Moreover, UA may even be considered a therapeutic candidate against diabetic podocytopathy, as UA treatment enhances podocyte viability and reduces ROS levels [127].

The in vitro anti-inflammatory activities of UA, iso-UA, UB as well as their respective glucuronides were performed on THP-1 human cell line-derived macrophages, RAW 264.7 murine macrophages, and human primary macrophages. UA, the most active metabolite, inhibited the human immune cell inflammatory response induced by TLR4 receptor stimulation and increased the production of anti-inflammatory cytokines, IL-10 and transforming growth factor beta 1 (TGF-β1). The anti-inflammatory activity mechanism was based on the inhibition of NF-κB translocation to the nucleus and MAPK phosphorylation contribution [128].

Further studies on RAW 264.7 cells showed that UA treatment prevented NF-κB and AP-1 activation, inhibited Akt and Jun N-terminus kinase (JNK) phosphorylation, significantly diminished the intracellular accumulation of ROS, reduced the activation of NADPH oxidase (NOX), the main source of ROS in activated macrophages [129]. These outcomes confirmed that, in lipopolysaharide (LPS)-challenged RAW 264.7 murine macrophages, UA could lower nitric oxide (NO) production through inhibition of the induced NO synthase (iNOS) protein, decreased the expression of TNF-α, IL-1β, and IL-6 mRNA, besides inhibiting NF-κB p65 nuclear translocation and p50 DNA-binding action, processes associated with anti-inflammatory activity [130,131].

In murine J774.1 macrophages, UA also inhibited the production of proinflammatory proteins, ROS, and NO, and blocked p65 NF-κB nuclear translocation [132].

A common anti-inflammatory strategy includes the decrease in biosynthesis of prostaglandins and leukotrienes via inhibition of the COX-2 and 5-lipoxygenase (5-LOX) pathways. A recent in vitro study revealed that UA, iso-UA, and UC could reduce the formation of the 5-LOX/COX-2 pathway hemiketal eicosanoids (HKE_2 and HKD_2), novel mediators of inflammation [133].

UA might be used as a natural immune-suppressant in some inflammatory diseases, as UA treatment decreased $CD4^+$ T cell proliferation and upregulated the miR-10a-5p expression, which in turn restrained store operated Ca^{2+} entry (SOCE) and suppressed the activation of murine $CD4^+$ T cells [134].

5.2.2. Neuroprotective Effects

There are many in vitro studies that assessed the neuroprotective effects of EA or urolithins an different line cells in different experimental conditions and revealed several molecular mechanisms of action.

Investigations on LPS-stimulated BV2 microglia cells confirmed that UA significantly reduced the production of proinflammatory cytokines, TNF-α and IL-6, via the activation of SIRT-1 and autophagy initiation [135]. Equally, UA treatment attenuated neuroinflammation in BV-2 microglia, decreased proinflammatory cytokine expression, reduced inducible nitric oxide synthase gene expression, and suppressed JNK/c-Jun pathway activation [136]. Moreover, UB inhibited the TNF-α, IL-6, and IL-1β concentrations and suppressed NF-κB activity and phosphorylation of JNK, ERK, and Akt. In addition, UB increased the production of anti-inflammatory cytokine IL-10 and the phosphorylation of AMP-activated protein kinase (AMPK), associated with anti-inflammatory and antioxidant activities [137].

It was suggested that the potential mechanisms of the anti-inflammatory activity of UA and UB in LPS-treated BV2 murine microglia could be via the inhibition of NF-κB p65, Erk1/2, p38 MAPK, and Akt phosphorylation and signaling pathways. The two urolithins suppressed mRNA levels of proinflammatory TNF-α, IL-6, IL-1β, iNOS, and COX-2 genes [138].

Interestingly, in an experimental autoimmune encephalomyelitis (EAE) study, the most common model for MS, the EA treatment did not affect the cause of inflammation like urolithins did but rather the consequences, such as demyelination, through the stimulation of ceramide biosynthesis [139].

The characteristic features of the Alzheimer's disease (AD) brain are the accumulation of amyloid beta (Aβ) in extracellular senile plaques and intracellular hyper-phosphorylated tau protein, as well as oxidative stress accompanied by mitochondrial dysfunction [140].

As mitophagy deficit is a key factor in AD mitochondrial dysfunction, the use of urolithins can restore mitochondrial homeostasis by inducing mitophagy and biogenesis [24]. Activation of SIRT, AMPK or PGC1-α pathways and inhibition of mammalian target of rapamycin (mTOR) modulated by UA treatment-induced mitophagy and mitochondrial biogenesis [141].

One of the main strategies for the treatment of AD is the inhibition of Aβ-induced neurotoxicity. An in vitro study revealed that EA and its metabolites, UA, UB, UM5, UM6, attenuated the Aβ-induced toxicity in PC12 cells promoting neurite outgrowth, reducing

ROS production, and inhibiting neuronal apoptosis. The results identified AKT1, Insulin-like growth factor1 receptor (IGF1R), NFKB1, epidermal growth factor receptor (EGFR), and MAPK14 genes as main targets of EA and urolithins, therefore, the possible neuroprotective action mechanisms were associated with PI3K-Akt, MAPK, and Ras signaling pathways [142].

Another approach for the prevention of AD is the regulation of mitochondrial calcium influx and mitochondrial ROS (mtROS) accumulation, which were noticed in diabetic patients. In neuroblastoma cell line SH-SY5Y, UA treatment reduced high glucose-induced amyloidogenesis, maintaining mitochondrial calcium and ROS homeostasis [143]. Additionally, UA treatment increased expression of genes of mitochondrial biogenesis and OXPHOS, suggesting hormetic effects [144].

UB presented suitable radical scavenging potential against ABTS, DPPH, OH^- and $O_2{}^-$, thus could modulate oxidative stress, a significant factor in the progression of chronic diseases, such as dementia [145]. Moreover, on H_2O_2-treated neuron-2a cells, UB significantly reduced apoptosis rate and ROS production, and increased viability.

Monoamine oxidase (MAO) enzymes (MAO-A and MAO-B) are responsible for the inactivation of monoamine neurotransmitters and could generate neurological disorders, such as depression and Parkinson's disease (PD). Treatment with UA, UB and UC significantly repressed the MAO-A activity, but none of the tested urolithins displayed strong inhibitory activity against MAO-B [146]. Similarly, UA inhibited MAO-A and tyrosinase, acted as a free-radical scavenger, and improved the physiological antioxidant defense system in Neuro-2a cells [104].

5.3. *Other Mechanisms of Action of the Urolithins*

Urolithins have been revealed to modulate cell cycle, upregulate tumor suppressor pathways, inhibit proliferation and induce apoptosis in many in vitro experiments on cancer lines including colorectal, liver, pancreas, kidney, and bladder cancer.

In a recent study, UA treatment at concentrations consistent with those found in the intestine triggered autophagy in the human colorectal cancer (CRC) cell line SW620, thereby inhibiting cell survival and metastasis [147]. UA also induced apoptosis in the CRC cell lines HT29, SW480, and SW620 by increasing the expression of proapoptotic proteins p21 and p53 and decreasing the anti-apoptotic protein expression of Bcl-2. Additionally, UA stimulates ROS production and disturbs cellular oxidation status in CRC cells, which can lead to cellular apoptosis and cell death. In contrast, UA treatment did not affect normal human fibroblast cells used as normal control [148]. Previously, UA was shown to upregulate p21 [149] and to inhibit the growth and progression of colon cancer cells and the glycosylation in a p53-dependent manner [150]. Abnormal glycosylation was noticed in major diseases, including cancer; thus, the inhibition of this process could be a potential strategy to prevent tumor cell progression. UD-related glycosylation inhibition in HCT116, SW480, and RKO colon cancer cells resulted in migration and invasion downregulation [151].

UA treatment revealed anti-proliferation in HCT-116 cells through senescence induction associated with the upregulation of p21 and p53 expressions. Moreover, UA and urolithin metabotype B reduced colony formation via the inhibition of cell cycle progression at the G_2/M phase [152].

The anti-proliferative and anti-invasive effects of UA were also demonstrated in hepatitis B virus (HBV)-positive hepatocellular carcinoma (HCC) [153]. Data showed that UA suppressed the expressions of the proteins Sp-1, widely overexpressed in neoplasms, and Lin28a, the transcriptional target of Sp-1 that could elevate the levels of certain cancer-related miRNAs. Moreover, UA elevated the expression of microRNA let-7a, which functions as a tumor suppressor and is biologically deleted in several cancers, including in HCC patients with HBV infection. UB also exposed the anti-proliferative properties of HCC cells via increasing phosphorylated β-catenin expression and inhibiting Wnt/β-catenin signaling [154].

Another mechanism for UA-induced anti-proliferative and proapoptotic effects was through downregulation of the PI3K/AKT signaling and mTOR pathways. mTOR, a serine/threonine kinase, contributes to cancer cell growth and survival, while PI3K signaling activation is associated with poor prognostic in cancer patients [155]. In pancreatic ductal adenocarcinoma (PDAC) cell lines, UA simultaneously inhibited PI3K/Akt and mTOR activation and mediated the anti-tumor activities, with minimal impact on normal pancreatic epithelial cells [156]. Knowing the extensive crosstalk between PI3K/Akt/mTOR and MAPK pathways, a dual combined reduction of both pathways should be considered for better therapeutic efficacy in human PDAC cell lines [157].

Through downregulation of the PI3K/Akt/mTOR pathway, UA inhibited the proliferation and migration of PDAC cells and increased apoptosis. Even EA, the upstream compound of UA, blocked the cell cycle and reversed epithelial to mesenchymal transition in PDAC by restraining several carcinogenic pathways activated in PDAC, such as NF-κB, COX-2, and Wnt [158].

UA could be a promising therapeutic candidate for cholangiocarcinoma (CCA), the second most common primary hepatic malignancy. In a study on the human intrahepatic CCA cell lines HuCCT-1 and SSP-25, UA treatment-induced G2/M phase cell cycle arrest, thus repressing cell proliferation, and exerted anti-tumor effects by suppressing the Akt/WNK1 signaling pathway and inducing autophagy [159].

The anti-proliferative effect of UA was also demonstrated in the UMUC3 bladder cancer cell line. UA treatment could inactivate the cyclin B1/cdc2 kinase complex and additionally lessened phosphorylation of Akt and ERK, thus downregulating PI3K/Akt signaling pathway [160].

6. In Vivo Preclinical and Clinical Evidence for Beneficial Effects of ETs, EA, and Urolithins on Gut–Brain Axis

In order to capture the most relevant results of recent research studies, in this manuscript, we have focused our attention on in vivo original articles and trials published in the last 5 years that revealed the effects of ETs, EA, and urolithins on the gut–brain axis.

6.1. In Vivo Studies

6.1.1. In Vivo Studies Performed at the Intestinal Level

Table 2 summarizes the relevant in vivo studies selected for this review highlighting the antioxidant, anti-inflammatory, and anti-tumor effects of ETs, EA, and/or urolithins in the gut.

Table 2. Preclinical studies attesting the antioxidant, anti-inflammatory, and anti-tumor effects of ellagitannins, ellagic acid, or their metabolites at the intestinal level.

Substances Tested (ST)—Doses	Preclinical Model	Main Results	Reference
EA	C57BL/6J mice were divided into 3 groups: control, standard pellet diet supplemented with 0.1% EA, and with 0.3% EA, respectively, for 21 consecutive days.	EA treatment increased jejunal villus height (0.3% EA) and enhanced the enzymatic activities for the jejunal lactase and sucrase (both 0.1% and 0.3% EA), and the alkaline phosphatase (0.3% EA). EA (0.3%) showed significant antioxidant effects by increasing the mRNA expression of Nrf2 and HO-1, the enzymatic activities of the superoxide dismutase and catalase, and reducing the malondialdehyde level in the jejunum. EA (0.3%) proved a suitable ability to regulate intestinal microbiota: increased the count of *Lactobacillus* species and decreased the count of *Escherichia coli*.	[161]
EA—10 mg/mL	Castor oil-induced diarrhea in mice. BALB/c mice were divided into 4 groups: (1) control; (2) castor oil (orally, 0.2 mL); (3) castor oil (orally, 0.2 mL) + EA (orally, 0.3 mL) after 30 min; (4) 0.1 mL GW9662 (i.p., 1 mg/mL) and castor oil (orally, 0.2 mL) + EA (orally, 0.3 mL) after 30 min.	Transcriptome, histological assay, and qRT-PCR were performed on ileum tissues. EA protected the ileum of mice against castor oil-induced diarrhea, reducing inflammation. The pretreatment with GW9662, a PPAR-specific antagonist, inhibited the anti-inflammatory effect of EA.	[162]
EA-supplemented feed (500 mg/kg)	Healthy weaned piglets (30 days old) received feed supplemented with EA twice daily for 40 days vs. a control group fed with the same feed without EA.	EA reduced the diarrhea rate, significantly increased weight gain, and diminished the serum diamine oxidase (DAO) levels of weaned piglets. Transcriptome sequencing revealed the ability of EA to down-regulate the expression of some genes involved in seven pathways related to immune response. EA modulated the microbiota composition in the cecum and rectum.	[163]
Pomegranate peel extract (PPE) containing 11% EA	A colonic inflammation model induced by dextran sodium sulfate (DSS) in mice (7-day cycles with 2% DSS in drinking water and 7 days of drinking water without DSS, for 42 days). Swiss Webster mice receiving 2% DSS were divided into 5 groups: control, PPE-dose 1 (240 mg/kg/day), PPE-dose 2 (480 mg/kg/day), ASP (43 mg/kg/day), and ASP (43 mg/kg/day) + EA (26 mg/kg/day).	PPE (dose 2) significantly increased the caspase-3 expression in mice colon tissues collected three days after the last treatment of DSS.	[164]
Pomegranate mesocarp decoction (PMD)—300 mg/kg; Polysaccharide components of PMD (PCs)—300 mg/kg; Ellagitannin components of PMD (ECs)—45 mg/kg	Colitis-induced abdominal pain in male Sprague–Dawley rats by 2,4-dinitrobenzenesulfonic acid (DNBS). ST was orally administered for 14 days.	ECs have been more effective in reducing visceral pain than the equivalent dose of PMD, both at 7 and 14 days of repeated administration. All three treatments significantly diminished the inflammation degree of the colonic mucosa and the fibrosis state. However, ECs are the responsible bioactive compounds of PMD, and PCs support and enhance their effects.	[165]

Table 2. *Cont.*

Substances Tested (ST)—Doses	Preclinical Model	Main Results	Reference
Two dietary strawberry extracts rich in monomeric ellagitannins (ME) and dimeric ellagitannins (DE), respectively	Male Wistar rats fed with high-fructose diets for 6 weeks: 3 groups received a diet based on corn starch (C): C, C + ME, C + DE, in parallel with other 3 groups fed with a diet containing fructose (F): F, F + ME, F + DE.	ME was more effective in reducing oxidative stress (lipid peroxidation in some tissues) and serum inflammatory biomarkers (TNF-α, IL-6), as well as the serum and liver triglyceride levels, than DE. Higher levels of ET metabolites were determined in the cecum and urine of rats receiving ME than of those fed with DE. Both ME and DE regulated the biochemical disturbances induced by a high-fructose diet. The efficacity of the ME extract was associated with systemic parameters, while that of the DE extract was associated with local microbial activity.	[166]
Feiyangchangweiyan capsule (FYC) and its main components: EA, gallic acid (GA), and syringin (SY)	A pathogen-induced pelvic inflammatory disease (PID) model in female SD rats (E. coli and S. aureus induced infection in the upper genital tract) with 11 groups: control, PID, FYC (1.2 g/kg), GA (210 mg/kg), EA (30 mg/kg), SY (35 mg/kg), GA (105 mg/kg) + EA (15 mg/kg), GA (105 mg/kg) + SY (18 mg/kg), EA (15 mg/kg) + SY (18 mg/kg), GA (70 mg/kg) + EA (10 mg/kg) + SY (12 mg/kg), and Fuke Qianjin capsule (FKC) (2.4 g/kg) as a positive control.	Histological analysis, ELISA assays, and Western blot analysis were applied to detect the expression of NF-κB, BAX, BCL-2, and JNK. The expressions of IL-1β, TNF-α, MPC-1, and BAX induced by infection were significantly reduced, while the IL-10 level and the expression of Bcl-2 were increased by FYC, but also by its main components. The anti-inflammatory effects in the rats of the GA + EA + SY group were more intense than those observed in the rats treated with dimers or monomers. PID was associated with an elevated expression of BAX and a dramatic suppression of BCL-2 expression. FYC, and especially EA and SY, have shown an increased efficacy in reversing these effects.	[167]
Extract of Chestnut bark (ENC®) (20 mg/kg/day) rich in ellagitannins (administered by gavage)	A high-fat diet (HFD) model in rats Male Sprague–Dawley rats were divided into 4 groups: control (regular diet, RD), HFD, RD + ENC®, and HFD + ENC®, for 21 days.	In HFD-fed rats, ENC® improved lipidic profile (significantly reduced TC, LDL-C, TG, and increased HDL-C), exerted antioxidative and anti-inflammatory activities, and normalized intestinal contractility in ileal and colonic tissues.	[168]
EA—0.3 g/kg of HFD; *Weizmannia coagulans* BC2000 and BC77—0.1 g of lyophilized powder (containing 4×10^{11} CFU/g)/kg feed	Animal model of HFD-induced insulin resistance: C57BL/6J male mice were divided into 5 groups: (1) Low-fat diet (LFD); (2) HFD (providing 60% of fat energy); (3) HFD + EA; (4) HFD + EA + BC77; (5) HFD + EA + BC2000.	EA and *W. coagulans* BC2000 had a synergistic effect in reducing the insulin resistance index and HFD-induced endotoxemia. EA co-administered with BC200 activated the autophagy pathway in the mouse liver, a urolithin-like effect, but not with BC77. *W. coagulans* BC2000 promoted a favorable intestinal environment for the proliferation of EA-transformable bacteria.	[169]
EA (80 mg/kg) and miR-125 (1.25 mg/kg) nanoparticles carrying a mitochondrion-directed peptide (K) and a tumor-targeted ligand (L)	A SAS-tumor bearing mouse model. BALB/c nude mice were divided into 5 groups: saline (control), EA, EA/LPN (lipid-polymer nanoparticles), EA/LPN-KL, and EA/LPN-KL + miR-125/SLN (solid lipid nanoparticles)-KL. The formulations were administered every 2 days for 20 days.	All EA formulations have shown hypoglycemic and hypolipidemic effects, but the combined formula, EA/LPN-KL + miR-125/SLN-KL, was the most effective. This formula also showed great tumor-suppression ability in SAS-tumor-bearing BALB/c mice.	[170]

Table 2. *Cont.*

Substances Tested (ST)—Doses	Preclinical Model	Main Results	Reference
Urolithin A (UA)—20 mg/kg	Animal model of cholangiocarcinoma (CCA) in xenograft female nude mice (BALB/c Slc-nu/nu) injected with HuCCT-1 cells (5×10^6 cells/100 mL of media). UA treatment was initiated 2 weeks after tumor transplantation for a period of 35 days (orally, 3×/week).	The tumor volume was calculated twice a week, and immunohistochemical analysis was performed at the end of the experiment. UA inhibited tumor growth and increased LC3-II levels. On the other hand, the phospho-kinase array demonstrated the downregulation of the Akt/WNK1 pathway. LC3-II expression was elevated in WNK1 knocked-down cells, indicating that WNK1 is the key signal for regulating autophagy. Thus, UA exerted anti-tumor effects by suppressing the Akt/WNK1 signaling pathway and inducing autophagy.	[159]
EA—100 mg/kg bw	Animal model of oxidative stress induced in C57BL/6J mice by diquat (25 mg/kg bw, single dose). EA was administered orally for 5 days.	EA treatment significantly reduced diquat-induced weight loss and mitigated oxidative stress in jejunum: reduced ROS production, up-regulated the mRNA expression of Nrf2 and the antioxidant enzymes (GPx1 and HO-1). ML385, a specific Nrf2 inhibitor, counteracted the EA effects on jejunum oxidative stress.	[171]
UA (synthetic)—daily dose of 0.114 mg/kg bw in drinking water	Animal inflammation model: microbiota-depleted IL-10−/− mice perorally infected with *C. jejuni* (on days 0 and 1). UA treatment was initiated on day 2 post-infection (p.i.) for 5 consecutively days vs. a placebo group (water).	Versus the placebo group, UA improved the early (<24 h) clinical status of infected mice, reduced pathogen loads in the ileum, immune cell and apoptotic epithelial cell abundance in the colon (by histopathology and immunohistochemistry), and the secretion of proinflammatory biomarkers (IFN-γ, TNF-α, MCP-1, and IL-6) both in the intestinal tract and in the extraintestinal compartments (lung, liver, kidney, serum).	[172]

Akt—protein kinase B; bw—body weight; EA—ellagic acid; GPx1—glutathione peroxidase 1; HDL-C—high-density lipoprotein-cholesterol; HFD—high-fat diet; HO-1—heme oxygenase-1; IFN-γ—interferon γ; IL—interleukin; LDL-C—low-density lipoprotein-cholesterol; MCP-1—monocyte chemotactic protein-1; Nrf2—nuclear factor erythroid 2-related factor 2; ROS—reactive oxygen species; TC—total cholesterol; TG—triglycerides; TNF-α—tumor necrosis factor α; UA—urolithin A.

Xu et al. [161] highlighted the benefits of EA treatment on the intestinal health of C57BL/6J mice. EA stimulated the increase in jejunal villus height (in a dose corresponding to 0.3% from the daily diet, after 21 days of daily treatment), promoted digestion by enhancing the enzymatic activities for the jejunal lactase and sucrase (even at 0.1% EA from daily diet), exercised significant antioxidant effects by stimulating the Nrf2 signaling pathway and positively regulated intestinal microbiota. In an animal model of diquat-induced oxidative stress in C57BL/6J mice, Zhang et al. [171] also found that EA treatment mitigated jejunum oxidative stress via the Nrf2 signaling pathway.

The protective effects of EA also extend to the ileum, as demonstrated by Chen et al. [162]. In an animal model of castor oil-induced diarrhea, EA exercised antioxidant and anti-inflammatory effects and improved mouse immunity by activating the PPAR signaling pathway. The involvement of this signaling pathway in the effects of EA was demonstrated using a PPAR antagonist, GW9662, which inhibited both the antidiarrheic and anti-inflammatory effects of EA. Moreover, RNA sequencing and qRT-PCR revealed that EA treatment increased the expressions of PPAR-gamma and decreased the expressions of inflammatory biomarkers (IL-1β, IL-6, TNF-α, and NF-κB), confirming the outcomes observed in vitro.

The ability of EA to modulate different signaling pathways was noticed by Lu et al. [163] by transcriptome sequencing in healthy weaned piglets (30 days old). After 40 days of repeated treatment (500 mg/kg), the antidiarrheic and intestinal barrier-stabilizing effects of EA were correlated with the expressing modulation of 401 genes in the jejunal mucosa tissue. Among the downregulated genes (238), genes belonging to seven signaling pathways involved in the immune response were highlighted, underlining the anti-inflammatory potential of EA through multiple biological pathways. In addition, EA promoted the proliferation of IPEC-J2 jejunal enterocytes, increased the expression of Zonula occludens-1 (ZO-1) and Occludin proteins implicated in the tight junction structure of enterocytes and intestinal permeability and promoted a favorable gut-intestinal microbiota in the cecum and rectum. Thus, EA has an important role in protecting the intestinal mucosal barrier function.

Parisio et al. [165] compared the effects of an extract of pomegranate mesocarp (300 mg/kg) with the equivalent amount of the component ellagitannins (45 mg/kg), respectively with an equal amount of lipopolysaccharide components (300 mg/kg), on colitis-induced abdominal pain in rats. All three preparations tested showed antinociceptive effects after 14 days of daily administration, reduced the total amount of mast cells in the colon, decreased the number of degranulated mast cells, and decreased the density of collagen fibers in the colonic mucosal stroma. Through such anti-inflammatory effects, ETs and their metabolites (EA, urolithins) are positively involved in regulating intestinal permeability, controlling the function of the intestinal barrier mucosa, and maintaining tight junctions between epithelial cells. Moreover, through their anti-inflammatory activity, ETs showed significant efficacy in reducing chronic visceral pain related to irritable bowel syndrome or inflammatory bowel diseases.

Fotschki et al. [166] demonstrated that the effects of ETs may be different depending on their type. Thus, monomeric ellagitannins (ME) generate higher levels of bioactive metabolites in the cecum and urine than dimeric ellagitannins (DE). Both ME and DE reduced serum total cholesterol (TC), LDL, and triglycerides (TG) levels and liver oxidized glutathione (GSSG) concentration in rats fed with high-fructose diets and increased the GSH:GSSG ratio, decreased lipid peroxidation in some tissues and revealed blood serum anti-inflammatory activity. These effects were more significant for ME, while DE significantly reduced local microbial activity.

Infections of the digestive tract or pelvic region are usually associated with dysbacteriosis and inflammatory status. Li et al. [167] studied the effects of three polyphenols: EA, gallic acid, and syringin, both individually and in binary associations and then as a tertiary mixture, in a pathogen-induced pelvic inflammatory disease (PID) model in female rats. Acting synergistically, the tertiary mixture exerted the most intense anti-

inflammatory effect. The anti-inflammatory action was boosted through controlling apoptosis, activation, and downregulation of the anti-apoptotic BCL-2 and proapoptotic BAX proteins, respectively, and by inhibiting the stimulation of JNK and p38 MAPK, thus regulating the NF-κB signaling pathway. Similarly, UA treatment reduced the abundance of the apoptotic epithelial cells in the colon of mice infected with *Campylobacter jejuni* and attenuated the proinflammatory immune responses in the intestinal tract as well as in the extraintestinal compartments [172].

A pomegranate peel extract containing 11% EA-activated caspase-3, an apoptosis executor, in a colonic inflammation model induced by dextran sodium sulfate (DSS) in mice, in a dose corresponding to 26 mg EA/kg bw, which is considered to be the effective anti-inflammatory dose of EA, according to the literature [164]. Nevertheless, EA from pomegranate peel extract was shown to increase the expression of the Bax/Bcl-2 ratio and induce cancer cell apoptosis [173]. Moreover, the pomegranate peel extract could enhance the expression of caspase-9 and induce apoptosis via the mitochondrial pathway [174]. Additionally, UA treatment exerted anti-tumor effects in a rodent model by inhibiting the Akt/WNK1 signaling pathway and inducing autophagy in cholangiocarcinoma cells [159]. These data are particularly important because they make pomegranate a possible and promising candidate in the targeted treatment of cancer. Thus, Lo et al. [170] have already tested the efficiency of a combination of EA and miR-125 nanoparticles to reduce tumor growth through the modulation of the mitochondrial dysfunctions and energetic metabolism of cancer cells.

In a high-fat diet (HFD) model in rats, rich ET chestnut bark extracts normalized intestinal contractility and exerted antioxidative and antiadipose activities suggesting a potential approach to overweight-related diseases [168]. Insulin resistance, one of the consequences of HFD-induced dysbiosis, could be prevented by the synergistic action of EA and certain probiotics such as *Weizmannia coagulans*. In addition, the combination of EA and *W. coagulans* BC2000 inhibited HFD-induced endotoxemia and activated the hepatic autophagy pathway in a manner similar to that of urolithin [169].

6.1.2. In Vivo Studies Performed at the Cerebral Level

Several recent studies have shown a strong correlation between gut microbiota dysbiosis and inflammatory processes both in the gastrointestinal tract and extraintestinal compartments [163,172]. The prebiotic role of polyphenols, which have the ability to modulate gut microbiota, is well known. As already stated, their metabolites formed in the gut under the action of the microbiota, considered as metabolites of the microbiota, are bioactive compounds with important biological actions for the health of the host organism and the maintenance of its homeostasis [71]. ETs and EA, as well as urolithins, are the focus of many researchers due to their antioxidant, anti-inflammatory, and anti-cancer effects, as well as the anti-atherogenic, neuroprotective, pro-mitophagy, etc., effects, and they are currently the molecules of pharmaceutical interest.

Table 3 summarizes recent studies selected from the literature highlighting the impact of different ETs, EA, urolithins, or natural sources (extracts or juices) rich in ETs, on the gut–brain axis, with important benefits for brain health and neuronal processes.

Table 3. Preclinical studies attesting the antioxidant, anti-inflammatory, and neuroprotective effects of ellagitannins, ellagic acid, or their metabolites in the brain.

Substances Tested (ST)—Doses	Preclinical Model	Main Results	Reference
EA—p.o. administered in 3 doses: high (H), medium (M), and low (L) doses	Aging model obtained with D-gal (100 mg/kg/day, s.c., 8 weeks) in male Sprague–Dawley (SD) rats divided into 6 groups: (1) control, (2) D-gal, (3) positive control (vitamin E, 150 mg/kg, by gavage), (4) H-EA (D-gal + 150 mg EA/kg/day), (5) M-EA (D-gal + 100 mg EA/kg/day), (6) L-EA (D-gal + 50 mg EA/kg/day).	EA restored the antioxidant defense system (evaluated by SOD, CAT, GSH-Px, and T-AOC activities, and MDA levels, respectively) in the liver and brain of D-gal-induced aging rats, especially in H-dose. The treatment with M and H doses of EA for 8 weeks has significantly mitigated the D-gal-induced inflammation (TNF-α, IL-6, and IL-1β levels in serum) and the liver function decline (ALT and AST levels). Histopathological analysis showed that the H-dose of EA was more protective and kept the morphological structure in both the liver and brain. EA treatment significantly downregulated the expression of Bcl-2 and Bax proteins and showed anti-apoptotic effects in a concentration-dependent manner.	[175]
UA—p.o. administered in 3 doses: high (H), medium (M), and low (L) doses	I. Aging model obtained with D-gal (150 mg/kg/day, s.c., 8 weeks) in male Institute of Cancer Research (ICR) mice were divided into 5 groups: (1) control (Ctrl), (2) D-gal, (3) H-UA (D-gal + 150 mg UA/kg/day), (4) M-UA (D-gal + 100 mg UA/kg/day), (5) L-UA (D-gal + 50 mg UA/kg/day). II. Additional experiment with 4 groups: - 2 groups of 2-month-old mice: control and UA (150 mg UA/kg/day for 2 months); - 2 groups of 12-month-old mice: control and UA (150 mg UA/kg/day for 2 months).	UA treatment significantly ameliorated D-gal-induced behavioral impairments (in Open field, Morris Water Maze, and Object–Place Recognition tests). UA significantly lowered the AChE and MAO levels and the oxidative stress (the activities of SOD, CAT, GSH-Px, T-AOC, and MDA levels, respectively) in the brain of D-gal-induced aging mice. UA showed neuroprotection against D-gal-induced aging downregulating miR-34a in the hippocampal tissue and activated autophagy by upregulating SIRT1 and downregulating the protein expression of p53/p21 and the mTOR signaling pathway.	[176]
Urolithin B (UB)—i.g. administered in 3 doses: high (H), medium (M), and low (L) doses	I. Aging model obtained with D-gal (150 mg/kg/day, s.c., 8 weeks) in male C57BL/6 mice were divided into 5 groups: (1) control, (2) D-gal, (3) H-UB (D-gal + 150 mg UB/kg/day), (4) M-UB (D-gal + 100 mg UB/kg/day), (5) L-UB (D-gal + 50 mg UB/kg/day). II. Additional experiment with 4 groups: - 2 groups of 2-month-old mice: control and UB (150 mg UB/kg/day for 2 months); - 2 groups of 12-month-old mice: control and UB (150 mg UB/kg/day for 2 months).	Long-term UB treatment significantly ameliorated the behavioral features, learning, and memory function (in Open field, Morris Water Maze, and Y-maze tests) in D-gal-induced aging in mice. These outcomes were correlated with a significant reduction of AGE levels and elevation of Cu, Zn-SOD, and CAT expressions and activities in the brain. UB inhibited the apoptosis of hippocampal neurons induced by D-gal, downregulated the JNK signaling pathway, prevented the cytochrome c release from isolated mitochondria, increased the activation of Akt and p44/42 MAPK, and promoted the neuronal survival via the PI3K/Akt pathways.	[145]

Table 3. *Cont.*

Substances Tested (ST)—Doses	Preclinical Model	Main Results	Reference
EA—50 mg/kg/day, intragastric (i.g.)	Mouse model of AD: Male APP/PS1 double-transgenic and wild-type (WT) C57BL/6 mice were divided into 4 groups: (1) WT, (2) WT+EA, (3) APP/PS1, (4) APP/PS1 + EA, received EA or the same volume of 10% DMSO for 60 days.	EA treatment improved learning and memory abilities and ameliorated cognitive deficits in APP/PS1 mice, reduced neuronal cell apoptosis, the expression of caspase-3 level and the amyloid aggregates in hippocampus. EA also significantly inhibited tau hyperphosphorylation and decreased the expression of pSer199-tau and pSer396-tau in the hippocampus of APP/PS1 mice. Moreover, EA treatment significantly increased the expression of pSer473-AKT and decreased the pTyr216-GSK3β levels in APP/PS1 mice.	[177]
EA—50 mg/kg, p.o.	AD animal model induced with $AlCl_3$ in male Wistar rats divided into 4 groups: (1) control, (2) EA (50 mg/kg, p.o., for 4 weeks), (3) AD model (50 mg $AlCl_3$/kg, p.o., for 4 weeks), (4) AD + EA (50 mg $AlCl_3$/kg, p.o., for 4 weeks, followed by 50 mg EA/kg/day, p.o., for 2 weeks).	The discrimination index for the novel object recognition test (NORT) was significantly increased by EA therapy in AD rats. EA treatment significantly increased SOD, GSH, and TAC levels and decreased MDA levels in the serum of AD rats. The neurofibrillary tangles and neuritic plaques in the entorhinal cortex (ERC) sections were reduced in the AD+EA group. Antioxidant activity of EA treatment (increased SOD mRNA expression and modulated the amyloid precursor protein toxicity and caspase-3-mediated apoptosis) was correlated with the restoration of ERC thickness in AD+EA rats vs. AD rats.	[178]
EA—50 mg/kg bw/day (EA50) and 100 mg/kg bw/day (EA100), i.p., for 21 days	Animal model of memory impairment and anxiety induced by sleep deprivation (SD). C57BL/6J mice were divided into 4 groups: (1) control, (2) SD, (3) SD + EA50, (4) SD + EA100.	EA ameliorated learning and memory deficits and alleviated anxiety-like behaviors in SD mice. EA treatment improved neuron survival, reversed dendritic spine density, and reduced shrinkage and loss of neurons in the hippocampus of SD mice. EA restored the SOD and GPx activities, decreased MDA levels, and activated the Nrf2/HO-1 pathway in the hippocampus of SD mice. EA also reduced the IL-1β, IL-6, and TNF-α hippocampal levels, normalized the expression levels of TLR4, MyD88, NF-κB p65, and p-IκBα, and inhibited the TLR4-mediated innate immune responses. Moreover, EA showed neuroprotective effects on glutamate-induced toxicity via both the Nrf2 and TLR4 signaling pathways.	[179]

Table 3. *Cont.*

Substances Tested (ST)—Doses	Preclinical Model	Main Results	Reference
EA—50, 75, and 100 EA mg/kg, by gavages, 3 times daily for one week	Animal model of brain inflammation induced by cerebral ischemia/reperfusion (I/R). Male Wistar rats were divided into 6 groups: (1) control (surgery without any I/R) + vehicle (Veh); (2) I/R + Veh; (3–5) I/R + EA; (6) positive control (intact rats received 100 mg EA/kg).	Only the higher dose of EA (100 mg/kg) improved post-ischemic complications: it significantly increased the neurological signs scores, significantly reversed all tested behaviors, restored the BBB permeability, and decreased brain edema and the brain tissue cytokine levels (TNF-α and IL-1β) vs. I/R + Veh rats.	[180]
UA—300 mg, p.o.	Mouse model of Alzheimer's disease (AD): APPswe/PS1ΔE9 (APP/PS1) mice received 300 mg of UA dissolved in 0.5% carboxymethylcellulose each day for 14 days vs. 2 control groups: APP/PS1 transgenic mice and wild-type mice that received only the vehicle (0.5% carboxymethylcellulose).	UA ameliorated spatial learning and memory impairment, prevented neuronal apoptosis in the cortex and hippocampus, and enhanced hippocampal neurogenesis in APP/PS1 mice. UA also decreased Aβ plaque deposit levels in the cortex and hippocampus, attenuated reactive gliosis, and significantly reduced microglia and astrocyte activation in APP/PS1 mice. UA treatment increased the expression of p-AMPK and decreased the activation of P65NF-κB and P38MAPK.	[181]
UA—2.5 mg/kg/day, i.p., for 8 weeks	A streptozotocin (STZ)- induced diabetic mouse model. Male CrljOri:CD1(ICR) mice were divided into 2 groups: (1) STZ-control and (2) STZ-UA.	UA treatment improved cognitive impairment, APP and BACE1 expressions, Tau phosphorylation, and Aβ deposition in STZ-induced diabetic mice. UA decreased blood glucose levels, but only in control mice and not in the STZ-injected mice.	[143]
UA—2.5 mg/kg bw; egpigallocatechin gallate (EGCG)—25 mg/kg bw	Animal model of late-onset AD using humanized homozygous amyloid beta knockin (hAbKI) mice were divided into 3 groups: (1) control, (2) UA (i.p., 3×/week for 4 months), (3) UA+EGCG (i.p., 3×/week for 4 months).	Both UA and UA + EGCG have been effective in counteracting the onset of pathological features of AD in hAbKI mice: enhanced phenotypic behavior, significantly increased mitochondrial biogenesis proteins (Nrf2, TFAM), both mitophagy (PINK1, Parkin) and synaptic proteins (synaptophysin, PSD95), as well as autophagy proteins (Beclin, ATG5, LC3B1, LC3B2, BCL2). Neuroinflammatory biomarkers (microglial marker Iba1 and astrocytic marker GFAP) were reduced, and the neuronal marker NeuN was significantly increased by both UA and UA + EGCG treatments. Dendritic spines and lengths, mitochondrial length, and mitophagosomal formations were also increased by both treatments. However, combined treatment UA+EGCG was stronger and more effective than only UA for most of the determinations performed.	[182]

Table 3. *Cont.*

Substances Tested (ST)—Doses	Preclinical Model	Main Results	Reference
Juglans regia (Gimcheon 1ho cultivar, GC)—lyophilized ethanolic extracts (obtained with 80% ethanol at 40 °C for 2 h), containing EA and ellagitannins (pedunculagin/casuariin isomer, strictinin, tellimagrandin I, EA-O-pentoside, and EA)—20 mg/kg bw (GC20) and 50 mg/kg bw (GC50), p.o.	A diabetic animal model with cognitive dysfunction in mice induced by a high-fat diet (HFD) for 12 weeks. Male C57BL/6 mice were divided into 4 groups: (1) control, (2) HFD, (3) HFD+GC20, (4) HFD + GC50. The lyophilized extracts were administered for 4 weeks.	GC significantly restored the behavioral and memory dysfunction in HFD-induced diabetic mice for both doses (GC 20 and GC50). GC also improved serum lipid profile and reduced white adipose tissue (WAT) and liver fat mass and showed antioxidant effects (reduced levels of MDA—in hepatic and cerebral tissues—and serum AGEs, and increased serum level of FRAP). GC inhibited AChE activity in cerebral tissue, suppressed AChE expression, and upregulated choline acetyltransferase expression vs. HFD group. GC restored mitochondrial membrane potential, regulated the mitochondrial ROS production and the protein expressions associated with synaptic disorders and neuronal apoptosis: decreased p-JNK, p-tau, and Aβ levels, upregulated p-Akt (Ser 473) and insulin-degrading enzyme (IDE), and downregulated BAX and caspase-3 expression levels vs. HFD group. Moreover, GC ameliorated TNF-α, IL-1β, p-NFκB, and caspase-1 expression levels and upregulated heme oxygenase-1 (HO-1) expression levels vs. LHFD group.	[183]
Pomegranate juice (PJ) rich in ellagitannins (galloyl-hexoside, EA-hexoside, pedunculagin, casuarinin) and EA, containing 18.90 ± 0.96 g/L (EA equivalents per L of juice)	Rat model of Parkinson's disease (PD) induced with rotenone (1.3 mg/kg bw/day, s.c., for 35 days). Male albino Wistar rats were divided into 4 groups: (1) control (vehicle only), (2) PJ (500 mg/kg bw/day, i.g.), (3) rotenone only (1.3 mg/kg bw/day, s.c.) from the 11th day (ROT), (4) PJ (500 mg/kg bw/day, i.g.) + rotenone from the 11th day (PJ + ROT). Experiment included 10 days pretreatment with PJ and 35 days with PJ + ROT combined treatment.	PJ improved postural stability in rats affected by rotenone, enhanced neuronal survival, protected against oxidative damage (reduced MDA levels and increased CAT, GPx, GST activities in midbrain) and α-synuclein aggregation, increased the activity of mitochondrial aldehyde dehydrogenase, and normalized the expression of anti-apoptotic Bcl-xL protein.	[184]
PJ	Rat model of Parkinson's disease (PD) induced with rotenone (1.3 mg/kg bw/day, s.c., for 35 days). Male albino Wistar rats were divided into 4 groups: (1) control (vehicle only), (2) PJ (500 mg/kg bw/day, i.g.), (3) rotenone only (1.3 mg/kg bw/day, s.c.) from the 11th day (ROT), (4) PJ (500 mg/kg bw/day, i.g.) + rotenone from the 11th day (PJ + ROT). Experiment included 10 days pretreatment with PJ and 35 days with PJ + ROT combined treatment.	PJ treatment proved neuroprotection against PD: improved vertical activity in rotenone-injected rats mitigated the reduction of the olfactory discrimination index to the level observed in control animals, attenuated the depletion of the dopamine (DA) and 3,4-dihydroxy-phenylacetic acid (DOPAC) in the midbrain.	[185]

Table 3. *Cont.*

Substances Tested (ST)—Doses	Preclinical Model	Main Results	Reference
EA, α-lipoic acid (LA), myrtenal (Myrt)—50 mg/kg (i.p.)	Rat model of Parkinson's disease (PD) induced with 6-hydroxydopamine (6-OHDA) (intrastriatal injection). Male Wistar rats divided into 5 groups: (1) control, (2) striatal 6-OHDA-lesioned control, (3–5) striatal 6-OHDA-lesioned rats pre-treated for 5 days with EA, LA, and Myrt.	All three compounds (EA, LA, and Myrt) improved learning and memory performance as well as neuromuscular coordination in rats with 6-OHDA-induced PD. Moreover, all these compounds significantly decreased lipid peroxidation (LPO) levels and restored catalase (CAT) activity and DA levels that were impaired by the challenge with 6-OHDA.	[186]
EA—10 mg/kg and 50 mg/kg, p.o.	Animal model of dopamine (DA) neuronal damage induced by lipopolysaccharides (LPS). Male Wistar rats were divided into 5 groups: (1) control, (2) EA (50 mg/kg), (3) LPS, (4) LPS+EA (10 mg/kg), and (5) LPS +EA (50 mg/kg).	EA (50 mg/kg) attenuated LPS-induced DA neuronal loss and ameliorated the decrease in tyrosine hydroxylase expression in the substantia nigra neurons. EA also attenuated the activation of the NLRP3 inflammasome in microglia and inhibited the NLRP3 inflammasome signaling pathway activated by LPS. The protein expressions of Iba-1 and proinflammatory cytokines IL-1β, TNF-α, and IL-18 were significantly suppressed by EA treatment.	[187]
EA—100 mg/kg and 200 mg/kg, i.p. and p.o.	Male albino Swiss mice—i.v. pentylenetetrazole (PTZ) seizure threshold test, maximal electroshock seizure test (MEST), grip-strength test, and chimney test, vs. valproic acid as reference drug.	EA (100 mg/kg) significantly increased the threshold of mice injected with PTZ for the first myoclonic twitch and generalized clonic seizures associated with the loss of the righting reflex, but not the threshold for forelimb tonus. In the MEST test, EA affected the threshold for the tonic hindlimb extension only at the high dose (200 mg/kg). EA did not have any effects on neuromuscular strength and motor coordination in mice at any of the doses tested.	[188]
Corilagin—10 mg/kg and 20 mg/kg, i.p.	Male Wistar rats—i.v. PTZ seizure threshold test.	Corilagin significantly reduced the epileptic events and improved cognitive function (in the Morris water maze (MWM) navigation test), reduced TNF-α and increased IL-10 levels, reduced ROS production, mitochondrial swelling, and carbonic anhydrase activity in the brain tissues in a dose-dependent manner. Corilagin treatment prevented structural damage of neurons and maintained the number of surviving cells vs. control group.	[189]
UA—10, 25, or 50 mg/kg/day, p.o.	Animal model of an experimental autoimmune encephalomyelitis (EAE) in C57BL/6 female mice immunized with MOG_{35-55}.	The dose of 25 mg/kg significantly suppressed the progress of EAE. UA treatment decreased demyelination, significantly inhibited inflammatory cell infiltration, reduced neuroinflammation (lowered numbers of M1-type microglia and inhibited the activation of dendritic cells).	[190]

Table 3. *Cont.*

Substances Tested (ST)—Doses	Preclinical Model	Main Results	Reference
Pomegranate peel extract (PEm) rich in EA and punicalagin, vs. EA—50 mg/kg/day of EA	Animal model of EAE in C57BL/6 female mice immunized with $MOG_{35–55}$.	Both EA and PEm showed comparable efficiencies to reduce the progression of the EAE and to ameliorate the clinical symptoms in mice. Spinal microgliosis and astrocytosis were significantly reduced by the PEm treatment as well as a clear reduction of the CD45 staining in the spinal cord was observed.	[191]
UA—2.5 mg/kg, i.p.	Mouse model of traumatic brain injury (TBI). Male C57BL/6J mice were divided into 3 groups: (1) control, (2) TBI + vehicle, (3) TBI + UA.	UA attenuated neuronal apoptosis following TBI (significantly reduced the cleaved caspase-3 expression and increased the Bcl-2 expression vs. TBI+vehicle), reinforced neuronal autophagy (increased the immunopositivity of LC3 and p62, two neuronal autophagy markers), and downregulated (PI3K)/Akt/mTOR (decreased Akt and mTOR expression levels) and Akt/IKK/NFκB signaling pathways (decreased the phosphorylation levels of IκB, IKKα, and NFκB).	[192]
Oenothein B—100 or 500 mg/kg/day, p.o.	Male ddY mice received oenothein B once a day for 3 days (from days 1 to 3) or 7 days (from days 1 to 7).	Oenothein B (only 100 mg/kg/day) activated extracellular signal-regulated kinase 2 (ERK2) in the hippocampal region of healthy mice, the ratio pERK2/ERK2 being significantly increased after 7 days of daily treatment. The activation of cAMP response element-binding protein (CREB), reflected by pCREB/CREB ratio, was slightly increased, but without statistical significance ($p = 0.0866$).	[193]
UA (30 µg UA/100 µL PBS, i.p.)	BALB/cJInv female mice were chronically infected with *Toxoplasma gondii* (*T. gondii*). UA treatment started 2 days post-infection and continued daily for 5 weeks vs. a control group (injected with vehicle).	All UA-injected mice survived throughout the entire experiment vs. 60% in the control group (40% of the infected control mice died 10-days post-infection). UA treatment inhibited cyst formation in the brain and altered the response of infected mice toward cat odor.	[194]

AChE—acetylcholinesterase; AD—Alzheimer's disease; D-gal—D-galactose; EA—ellagic acid; EAE—experimental autoimmune encephalomyelitis; EGCG—egpigallocatechin gallate; GC—*Juglans regia*, Gimcheon 1ho cultivar; HFD—high-fat diet; Iba-1—ionized calcium-binding adapter molecule-1; i.g.—intragastric; IL—interleukin; i.p.—intraperiotoneal; i.v.—intravenous; MEST—maximal electroshock seizure test; $MOG_{35–55}$—myelin oligodendrocyte protein 35–55 peptide; NLRP3—Nod-like receptor protein 3; 6-OHDA—6-hydroxydopamine; PBS—phosphate-buffered saline; PD—Parkinson's disease; PEm—pomegranate peel extract; PJ—pomegranate juice; p.o.—per os; PTZ—pentylenetetrazole; ROT—rotenone; SD—sleep deprivation; TNF-α—tumor necrosis factor-α; UA—urolithin A; UB—urolithin B; WT—wild-type.

Aging is a complex process involving multiple pathophysiological events, such as oxidative stress, chronic low-intensity inflammation called "inflamm-aging", mitochondrial dysfunctions, impaired protein homeostasis, and epigenetic mechanisms [88]. D-galactose (D-gal) is used experimentally in murine models of aging, because in high doses it induces free radicals and ROS, and oxidative stress, which accelerates senescence, including the brain [195]. Thus, Chen et al. tested the anti-aging potential of EA [175], UA [176], and UB [145] in murine models of aging obtained by i.p. injection of D-gal (100–150 mg/kg/day) for 8 weeks. EA (150 mg/kg/day, per os (p.o.)) clearly showed anti-aging potential, antioxidant and anti-inflammatory effects as well as hepatoprotective and neuroprotective properties against the toxicity of chronic exposure to high doses of D-gal. EA could prevent or attenuate the progression of aging-induced structural changes in the brain and liver by modulating the expression of some aging-related proteins [175]. UA (150 mg/kg/day, p.o.) showed evident neuroprotection, kept the morphological structure of neurons in the CA3 region of the hippocampal tissue, improved the cognitive functions impaired by D-gal, exercised antioxidant effects in the brain, and modulated the neuromediator levels by decreasing acetylcholinesterase (AChE) and MAO levels. The neuroprotective and anti-aging effects of UA are based on the regulation of autophagy through activation of the miR-34a-mediated SIRT1/mTOR signaling pathway [176]. UB (150 mg/kg/day, p.o.) also promoted neuronal survival, ameliorated neurological deficits and cognitive functions in D-gal-induced aging mice, and protected brain against oxidative stress. UB can be considered, together with UA, as brain health care products for the prevention of age-related neurodegenerative diseases such as AD or PD [145].

The development of AD is related to genetic factors but also depends on oxidative stress conditions and neuroinflammation [178]. EA has been tested in various animal models of AD or cognitive impairment at doses of 50–100 mg/kg body weight (bw)/day administered orally or parenterally. Thus, Zhong et al. [177] investigated the neuroprotective potential of EA (50 mg/kg/day) in APP/PS1 double-transgenic mice vs. wild-type (WT) C57BL/6 mice after repeated oral administration for 60 days. EA suppressed Aβ production and reduced Aβ deposition in the brain of APP/PS1 mice, and ameliorated the cognitive deficits of these animals in Morris water maze (MWM) test. In the same time, EA treatment activated threonine-protein kinase (Akt) and downregulated the activity of glycogen synthase kinase (GSK)3β, thereby reducing the tau protein hyperphosphorylation in the hippocampus by the RAC-α serine/Akt/GSK3β signaling pathway. UA (300 mg, p.o., 14 days) also decreased the Aβ plaque accumulation in the cortex and hippocampus of APP/PS1 mice, mitigated the neuroinflammation via reducing reactive gliosis and downregulating the AMPK/NFκB and AMPK/p38MAPK inflammatory signaling pathways, activated the hippocampal neurogenesis and reduced the neuronal apoptosis [181]. In a streptozotocin (STZ)-induced diabetic mouse model, parenteral UA treatment (UA —2.5 mg/kg/day, i.p., 8 weeks) was also efficient to suppress high glycemia-induced Aβ neuronal amyloidogenesis and tau hyperphosphorylation. The molecular mechanisms involved were the decreasing of mitochondrial calcium influx and mitochondrial ROS accumulation, and downregulation of amyloid precursor protein (APP) and β-secretase-1 (BACE1) expressions. UA also reversed the high glucose-activated AhR signaling and suppressed the transglutaminase type 2 (TGM2) expression [143]. Moreover, UA (2.5 mg/kg/day, i.p., 3×/week, 4 months), showed protective effects against human Aβ peptide-induced toxicities in an animal model of late-onset AD using humanized homozygous amyloid beta knockin (hAbKI) mice, both alone and in combination with EGCG (25 mg/kg/day). The phenotypic behavior (including motor coordination, locomotor abilities, working memory, and spatial learning and memory) was significantly enhanced, mitochondrial biogenesis improved, synaptic, mitophagy, and autophagy genes upregulated, and mitochondrial dysfunctions significantly reduced. The combined therapy UA + EGCG was more effective and stronger than UA alone [182].

Ramadan et al. [178] tested EA (50 mg/kg) to assess its effects on the episodic memory (by the novel object recognition test (NORT)) and the changes in the entorhinal cortex

(ERC) in an AD Wistar rat model induced with $AlCl_3$. The ERC is the most affected cortical area in AD patients and its progressive deterioration is involved in early memory loss. By the antioxidant activity, EA treatment restored the discrimination index in NORT test and reduced the structural changes in the ERC sections in AD rats, thus restoring episodic memory.

The sleep disorder (SD) could affect neurobehaviors, including cognition and memory. EA (50 mg/kg and 100 mg/kg) mitigated the neurobehavioral abnormalities associated with memory impairment and anxiety (in the MWM, NORT, object location, open field (OF), and elevated plus maze (EPM) tests) in SD mice after i.p. administration for 21 days. The EA treatment ensured neuron survival and the high dendritic spine density in the hippocampus of SD mice by reducing oxidative stress via the Nrf2/ARE pathway activation and by inhibiting the neuroinflammation via Toll-like receptor 4 (TLR4) downregulation [179].

In an animal model of brain inflammation induced by cerebral ischemia/reperfusion (I/R) performed in Wistar rats, Falahieh et al. [180] found that EA (100 mg/kg/day, p.o.) restored the blood-brain barrier (BBB) permeability, significantly reduced the neuroinflammation and brain edema, and improved all neurological signs scores and neurobehavioral abnormalities (in EPM, OF, and the forced swimming tests). In a mouse model of traumatic brain injury, UA (2.5 mg/kg, i.p.) treatment significantly reduced brain edema and protected the tight junction proteins and the BBB function in the injured cortex, thus improving the mouse neurological function. The neuroprotective effect of UA is mediated by its anti-inflammatory activity and autophagy enhancement via the inhibition of the PI3K/Akt/mTOR and Akt/inhibitor of NFκB (IκB) kinase (IKK)/NFκB signaling pathways [192].

These outcomes are very important because they demonstrate the ability of EA or its microbial metabolites, the urolithins, to improve/restore not only the permeability of the intestinal barrier but also the permeability of the BBB affected by pathophysiological processes as cerebral ischemia/reperfusion or traumatic brain injury, and encourage further research to explain the mechanisms of action on the gut–brain axis in more detail.

Moon et al. [183] prepared ethanolic extracts of *Juglans regia* (Gimcheon 1ho cultivar, GC) rich in EA and ETs and tested them in a diabetic mouse model with cognitive dysfunction. GC extracts had the ability to restore the neurobehavioral and memory dysfunction induced by high glycemia (in Y-maze, passive avoidance, and MWM tests). Moreover, GC extracts showed antioxidant effects, anti-inflammatory activity via the JNK/NFκB signaling pathway, suppressed synaptic disorders by regulating the cerebral cholinergic system, regulated mitochondrial activity and neuronal apoptosis pathway.

PD is another neurodegenerative disease that develops progressively under conditions of oxidative stress and neuroinflammation. It is characterized by loss of dopaminergic neurons and dopamine depletion in the midbrain substantia nigra pars compacta (SNpc), motor and olfactory dysfunctions, and the accumulation of the misfolded α-synuclein that forms Lewy bodies [184]. The beneficial effects of EA and ETs in PD have been shown in preclinical studies in various animal models. Thus, Kujawska et al. [184,185] tested pomegranate juice (PJ) on rats with rotenone-induced PD and found that the pretreatment with PJ followed by simultaneous exposure to rotenone and PJ provides neuronal protection from oxidative stress, enhanced the activity of mitochondrial aldehyde dehydrogenase, normalized the expression of anti-apoptotic Bcl-xL protein, prevents accumulation of α-synuclein and dopamine (DA) depletion in the midbrain, and improved olfactory function. They also highlighted the presence of UA in the brain and its possible involvement in the neuroprotective action of PJ.

Tancheva et al. [186] tested three antioxidant compounds: EA (50 mg/kg), α-lipoic acid (LA), and myrtenal (Myrt), on rats with PD induced with 6-hydroxydopamine (6-OHDA) intrastriatal injected. All compounds showed a significant antiparkinsonian effect, improved neuromuscular coordination, learning, and memory performance (in apomorphine-induced rotation, rotarod, and passive avoidance tests), and restored dopamine levels impaired by 6-OHDA injection. DA neuronal loss experimentally induced with LPS in rats was signifi-

cantly mitigated by EA (50 mg/kg). The mechanism involved was a significant reduction of neuroinflammation via the suppression of microglial NLRP3 inflammasome signaling activation. The neuroprotective properties of EA were also reflected by the reduction of tyrosine hydroxylase levels in substantia nigra neurons [187].

MS is characterized by inflammatory cell infiltration and demyelination in the CNS [190]. Two studies performed on EAE in mice immunized with $MOG_{35–55}$ showed the ability of EA (50 mg/kg/day), a pomegranate peel extract (PEm) rich in EA and punicalagin [191], and of UA (25 mg/kg/day) [190], respectively, to suppress the progress of EAE and to ameliorate the clinical symptoms. A significant reduction of microglia activation and astrocytosis, particularly in the gray matter of the spinal cord, as well as a reduction of the CD45 staining at this level, suggest the potential therapeutic benefit of EA or PEm treatment in MS [191]. UA significantly reduced infiltrating mononuclear cells in CNS, but its effect on the immune response was of mild intensity at the periphery [190]. These studies highlighted the real potential of ETs, EA, and/or UA in the treatment of autoimmune diseases such as MS.

EA (100 mg/kg) [188] and corilagin (10 mg/kg and 20 mg/kg), an ET found in pomegranate leaves, but also in other medicinal plants [189], modulated seizure susceptibility in pentylenetetrazole (PTZ)-induced animal models of seizures.

Oenothein B, a dimeric ET widely distributed in various medicinal plants, has a hydrophilic structure and may not have been able to pass through the BBB. Okuyama et al. [193] found that oenothein B activated neurotrophic factors in the hippocampal region of healthy mice: ERK2 and cAMP response element-binding protein (CREB), known as an important regulator of the expression of brain-derived neurotrophic factor (BDNF). Thus, its neuroprotective effects are mediated through not only its anti-inflammatory activity but also by the activation of some neurotrophic factors in the brain.

Neurotoxoplasmosis, a chronic infection with *Toxoplasma gondii* (*T. gondii*) characterized by the persistence of parasite cysts in the brain, is associated with high mortality in immunocompromised patients and with poor tolerability of the current therapy, which is not effective against parasite cysts. UA was tested and has been shown to be effective in the treatment of mice chronically infected with *T. gondii*, reducing the parasite cyst formation and significantly prolonging survival. Thus, UA is a promising natural bioactive compound that can be used in the treatment of neurotoxoplasmosis [194].

6.2. Clinical Studies

Oxidative stress and inflammatory conditions play a major role in the pathogenesis of irritable bowel syndrome (IBS). In a double-blind, placebo, parallel randomized clinical trial (RCT) in patients with IBS (22 EA vs. 22 placebo), the intake of 180 mg of EA per day for 8 weeks reduced abdominal pain and distention, flatulence, and rumbling [196]. Moreover, EA consumption significantly ameliorated the quality of life (QOL), increased total antioxidant capacity (TAC), and lowered MDA, C-reactive protein (CRP), and IL-6 levels, corresponding to an attenuated overall score of IBS-QOL [197]. These changes were not detected in the placebo group.

In a double-blind, randomized, placebo-controlled trial conducted in middle-aged and older adults (98 PJ vs. 102 placebo), daily consumption of PJ (236.5 mL) for 1 year kept stable the ability to learn visual information vs. the significant decline observed in the placebo group [198].

In another double-blind, parallel, placebo-controlled trial (11 PJ vs. 9 placebo), stool and plasma were collected at baseline and after 1 year of PJ consumption and analyzed to investigate the tryptophan (Trp) metabolism. Plasma level of indole propionate (IPA), a microbial metabolite of Trp, was significantly decreased in the placebo group and it was kept stable in the PJ-treated group at the end of the study vs. baseline. Other major metabolites of Trp (serotonin, kynurenine, and indole acetate), as well as the Trp levels, did not change significantly in either group. PJ consumption was associated with a reduction of the abundance of two genera, *Shigella* and *Catenibacterium*, negatively correlated with

plasma IPA levels. These outcomes were also confirmed in vivo. Thus, the microbial Trp metabolism could contribute to the health benefits of ETs [199].

Aging is associated with a decline in mitochondrial function. Several recent clinical trials have looked at the effects of long-term high-dose UA supplementation on mitochondrial and cellular health in middle-aged and older adults [200–202]. Healthy mitochondria play a critical role in cell function and homeostasis, and impaired mitochondrial function is implicated in the onset and development of several neurodegenerative diseases [202].

Andreux et al. [202] conducted the first placebo-controlled, double-blind RCT with different doses of UA (250, 500, 1000, or 2000 mg) administered for 28 days in healthy, sedentary elderly. The results demonstrated that UA was an activator of mitophagy and induced mitochondrial gene expression in skeletal muscle.

In an RCT by Liu et al. [201], 66 adults aged 65–90 years received 1000 mg UA/day. After 4 months, plasma levels of some biomarkers of mitochondrial health, such as several acylcarnitines, ceramides, as well as CRP levels, were decreased compared to placebo. These effects were clinically correlated with a significant improvement in muscle endurance vs. placebo. Similar results were obtained by Singh et al. [200] in a placebo-controlled, double-blind RCT with 88 middle-aged adults.

Therefore, these studies certify that UA supplementation is safe and it generates increased circulating levels of UA. UA is a promising therapeutic agent with real anti-aging potential and could be used in the prevention of age-related muscle decline. Furthermore, oral administration of chemically synthesized UA is safe in humans, and 1000 mg/serving could be used as a functional food ingredient [202]. Moreover, a recent in silico analysis estimated that EA was not mutagenic or carcinogenic, had low toxicity, and showed anti-inflammatory and antimicrobial activities [203].

7. Conclusions

ETs and EA are bioactive polyphenols that are found in several plant-source foods, including walnuts, berries, and pomegranates. Urolithins, their gut microbiota-derived metabolites, are better absorbed and might be responsible for the beneficial effects ascribed to ETs and EA.

In this review, we analyzed the last recent-published findings on ETs, EA, urolithins and the intestinal and brain effects, the potential mechanisms of action and the connection between the ET microbiota metabolism and the consequences detected on the gut–brain axis.

Our review highlights much preclinical and clinical evidence indicate that attests that ET-rich foods associated with individual gut microbiome or certain urolithins, especially UA and UB, could promote beneficial health impact. Their biological actions are complex, including the modulation of many important signaling pathways involved, particularly in inflammation and aging, as well as the function stabilization of the intestinal barrier and BBB. A better understanding of the role of these metabolites in the disease pathogenesis may assist in the prevention or treatment of pathologies targeting the gut–brain axis. Based on our knowledge, this review is the first one to focus on the impact ETs, EA, and urolithins could have on the gut–brain axis.

However, future preclinical detailed toxicological evaluations and clinical investigations of individual gut microbiota composition and health status in different age groups should be conducted before the therapeutic application of EA-enriched foods in human population.

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Abbreviations

ABTS—2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid; AchE—acetylcholinesterase; AD—Alzheimer's disease; AhR—aryl hydrocarbon receptor; Akt—protein kinase B; AMPK—AMP-activated protein kinase; APP—amyloid precursor protein; ARE—antioxidant response elements; BACE1—β-secretase-1; BBB—blood-brain barrier; bw—body weight; BDNF—brain-derived neurotrophic factor; CAT—catalase; CCA—cholangiocarcinoma; CNS—central nervous system; COX—cyclooxygenase; CRC—colorectal cancer; CREB—cAMP response element-binding protein; CRP—C-reactive protein; DA—dopamine; DE—dimeric ellagitannins; D-gal—D-galactose; DPPH—2,2-diphenyl-1-picrylhydrazyl; EA—ellagic acid; EAE—experimental autoimmune encephalomyelitis; EGCG—epigallocatechin gallate; EPM—elevated plus maze; ERC—entorhinal cortex; ERK—extracellular signal-regulated kinases; ETs—Ellagitannins; FRAP—ferric reducing ability of plasma; GC—*Juglans regia*, Gimcheon 1ho cultivar; GPx—glutathione peroxidase; GSH—total glutathione; (GSK)3β—glycogen synthase kinase; GSSG—oxidized glutathione; HAT—hydrogen atom transfer; HBV—hepatitis B virus; HCC—hepatocellular carcinoma; HDL—high-density lipoprotein; HFD—high-fat diet; HHDP—hexahydroxydiphenic acid; HO-1—heme oxygenase-1; Iba-1—ionized calcium-binding adapter molecule-1; IBS—irritable bowel syndrome; i.g.—intragastric; IκB—inhibitor of kappa B; IL—Interleukin; iNOS—induced NO synthase; i.p.—intraperitoneal; IPA—indole propionate; i.v.—intravenous; JNK—Jun N-terminus kinase; LDL—low-density lipoprotein; LOX—lipoxygenase; LPS—lipopolysaccharides; MAO—monoamine oxidase; MAPK—mitogen-activated protein kinase; MDA—malondialdehyde; ME—monomeric ellagitannins; MEST—maximal electroshock seizure test; MMP—matrix metalloproteinase; MS—multiple sclerosis; mTOR—mammalian target of rapamycin; mtROS—mitochondrial ROS; MWM—Morris water maze; NF-κB—nuclear factor kappa B; NLRP3—Nod-like receptor protein 3; NORT—novel object recognition test; Nrf2—nuclear factor erythroid 2-related factor 2; OF—open field; 6-OHDA—6-hydroxydopamine; ORAC—oxygen radical absorbance capacity; OXPHOS—oxidative phosphorylation; PD—Parkinson's disease; PDAC—pancreatic ductal adenocarcinoma; PEm—pomegranate peel extract; PGC-1α—peroxisome proliferator-activated receptor gamma coactivator; PI3K—phosphatidylinositol 3-kinase; PID—pelvic inflammatory disease; PJ—pomegranate juice; p.o.—per os; PPAR—peroxisome proliferator-activated receptor; PTZ—pentylenetetrazole; QOL—quality of life; RCT—randomized clinical trial; ROS—reactive oxygen species; ROT—rotenone; SD—sleep disorder; SET—single electron transfer; SIRT—sirtuins—silent information regulators; SNpc—substantia nigra pars compacta; SOD—superoxide dismutase; STZ—streptozotocin; TC—total cholesterol; TEAC—trolox equivalent antioxidant capacity; TG—triglycerides; TGM2—transglutaminase type 2; TLR4—Toll-like receptor 4; TNF—tumor necrosis factor; Trp—tryptophan; U—urolithin; WT—wild-type.

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Review

Dandelion (*Taraxacum officinale* L.) as a Source of Biologically Active Compounds Supporting the Therapy of Co-Existing Diseases in Metabolic Syndrome

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Abstract: Nowadays, many people are struggling with obesity, type 2 diabetes, and atherosclerosis, which are called the scourge of the 21st century. These illnesses coexist in metabolic syndrome, which is not a separate disease entity because it includes several clinical conditions such as central (abdominal) obesity, elevated blood pressure, and disorders of carbohydrate and fat metabolism. Lifestyle is considered to have an impact on the development of metabolic syndrome. An unbalanced diet, the lack of sufficient physical activity, and genetic factors result in the development of type 2 diabetes and atherosclerosis, which significantly increase the risk of cardiovascular complications. The treatment of metabolic syndrome is aimed primarily at reducing the risk of the development of coexisting diseases, and the appropriate diet is the key factor in the treatment. Plant raw materials containing compounds that regulate lipid and carbohydrate metabolism in the human body are investigated. Dandelion (*Taraxacum officinale* F.H. Wigg.) is a plant, the consumption of which affects the regulation of lipid and sugar metabolism. The growth of this plant is widely spread in Eurasia, both Americas, Africa, New Zealand, and Australia. The use and potential of this plant that is easily accessible in the world in contributing to the treatment of type 2 diabetes and atherosclerosis have been proved by many studies.

Keywords: dandelion; metabolic syndrome; antioxidant activity; hypolipidemic effect; anti-diabetes; antiplatelet activity

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1. Introduction

Metabolic syndrome is affecting an increasing number of people in almost all well-developed countries. The number of people, including children and young adults, with type 2 diabetes, obesity, and atherosclerosis is growing every year. These people often do not receive comprehensive treatment, and each diagnosed component disorder of the metabolic syndrome is treated separately. At the same time, there is a growing awareness in society of the importance of proper diet and physical activity in the prevention and treatment of many diseases. People with coexisting diseases classified as metabolic syndrome, according to the definition, in addition to traditional treatment, are increasingly reaching for other methods, such as the use of herbal medicine. Herbs have accompanied man for years in medicine, cosmetology, as well as in the kitchen. There are a number of plant raw materials that affect lipid and carbohydrate metabolism and improve digestion. One such plant is the dandelion. Dandelion is used both as a medicinal agent and as food. The root is a substitute for cereal coffee, the leaves are eaten raw in salads, and syrups are made from the flowers. Dandelion has many chemical compounds that affect lipid metabolism, protect the liver, regulate blood sugar, and affect digestion and, indirectly, obesity. In addition, some compounds in dandelion regulate platelet aggregation and affect blood pressure regulation. It seems that all these properties recommend this plant for use in complementary therapy in the treatment of coexisting diseases in metabolic syndrome.

2. Definition and Etiology of Metabolic Syndrome (MetS)

Metabolic syndrome is the co-occurrence of factors such as central (abdominal) obesity, elevated blood pressure, and disorders of sugar and fat metabolism in the human body, which eventually leads to the development of cardiovascular disease and type 2 diabetes. Lifestyle has an impact on the occurrence of metabolic syndrome. The treatment of metabolic syndrome is primarily aimed at reducing the risk of developing diabetes, hypertension, and cardiovascular disease.

The first definition of metabolic syndrome was proposed by the World Health Organization (WHO) in 1998 [1]. The components of metabolic syndrome include type 2 diabetes, insulin resistance, abnormal glucose tolerance or abnormal fasting glucose, and at least two of the other criteria: microalbuminuria, reduced HDL (high-density lipoproteins cholesterol) or elevated triglycerides, or European Group for the Study of Insulin Resistance (EGIR); microalbuminuria was not considered as a component of the metabolic syndrome. Insulin resistance/hyperinsulinemia occurring together with a fasting blood glucose above or equal to 110 mg/dL (IFG), or impaired glucose tolerance (IGT), the presence of hypertension; elevated triglycerides and/or reduced HDL levels; and abdominal obesity were considered the main criteria for the diagnosis of MetS. Abdominal obesity was assessed by waist measurement rather than waist/hip ratio (WHR) or body mass index (BMI), as per the WHO definition [2]. Subsequent modifications of the MetS definition reduced the emphasis on the relevance of a specific criterion occurrence and focused on the simultaneous occurrence of at least three of the above-mentioned criteria. The focus was on simplifying the diagnosis of MetS in clinical practice by concentrating on identifying people who have an increased risk of cardiovascular disease and treating lipid and non-lipid risk factors, with particular attention paid to insulin resistance. As a result of this approach, there have been changes introduced in the diagnosis of MetS included in the report National Cholesterol Education Program, Adult Treatment Panel III (NCEP-ATP III). It was found that the MetS diagnosis criteria did not require the determination of insulin resistance, making it simple in clinical practice. Although central obesity was recognized as a risk factor underlying the development of metabolic syndrome, all components of MetS were treated equally [3]. The criteria developed by the International Diabetes Federation (IDF-International Diabetes Federation) focus on the co-occurrence of abdominal obesity (waist circumference) together with at least two factors, such as elevated triglycerides, reduced HDL-cholesterol fraction, elevated blood pressure, elevated fasting glucose, or diagnosed type 2 diabetes. As with the NCEP-ATPIII criteria, the authors concluded that determining insulin resistance, which is not easy to measure, is not a requirement for diagnosing MetS. In the report, the authors noted that abdominal obesity is strongly associated with insulin resistance, and measuring abdominal circumference is easy and quick [4–6]. According to the guidelines of the Polish Forum for the Prevention of Cardiovascular Diseases (PFPChUK), updated in 2015, metabolic syndrome is a clinical condition characterized by the co-occurrence of multiple interrelated metabolic factors that increase the risk of developing atherosclerotic cardiovascular disease and type 2 diabetes [7]. According to Polish studies, metabolic syndrome includes abdominal obesity and impaired glucose tolerance (insulin resistance and/or hyperinsulinemia), dyslipidemia (high triglycerides and/or low HDL fraction cholesterol), as well as hypertension, and the activation of pro-inflammatory and pro-thrombotic processes. The criteria for the diagnosis of metabolic syndrome according to the PFPChUK are as follows: an increased waist circumference equal to or greater than 80 cm in women and equal to or greater than 94 cm in men, a triglyceride level equal to or greater than 150 mg/dL (1.7 mmol/L) or the use of medications to reduce it, a fasting glucose level equal to or greater than 100 mg/dL (5, 6 mmol/L) or the use of hypoglycemic drugs, and reduced HDL cholesterol less than 50 mg/dL (1.3 mmol/L) in women and less than 40 mg/dL (1.0 mmol/L) in men or the use of drugs to increase its concentration, elevated systolic blood pressure equal to or higher than 130 mm Hg and/or diastolic blood pressure equal to or higher than 85 mm Hg or the use of hypotensive drugs in patients with a positive history of hypertension. According to the team's study of the PFPChUK, if

a patient meets at least three of the above criteria, metabolic syndrome can be diagnosed. The reasons for the development of MetS are linked to several complex mechanisms that have yet to be fully elucidated. There is some debate as to whether the individual elements of the MetS form separate pathological states or whether they are subject to a common broader pathogenetic process. In addition to genetic and epigenetic factors, some lifestyle and environmental factors such as overeating and physical inactivity have been identified as major contributors to the development of MetS. Disorders such as atherosclerosis, type 2 diabetes, and obesity are classified as diet-related diseases. A poor diet (consuming too many calories) is believed to increase the risk of visceral adipose tissue accumulation. One hypothesis for the development of MetS considers visceral obesity as an activating factor in insulin resistance, chronic inflammation, and neurohormonal activation [7]. Metabolic syndrome factors are presented in Figure 1.

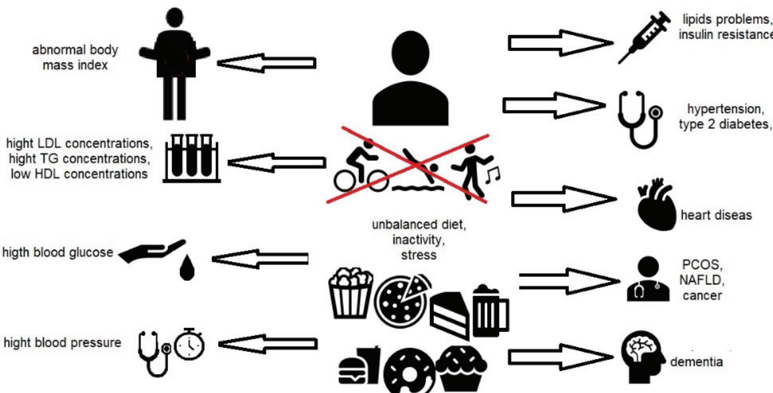

Figure 1. Metabolic syndrome factors/PCOS—polycystic ovary syndrome; NAFLD—non-alcoholic fatty liver disease/.

3. Lifestyle vs. Metabolic Syndrome

Lifestyle demonstrates a huge impact on the development of metabolic syndrome. Its change can significantly reduce the risk of MetS' possible occurrence. The results from the Diabetes Prevention Program Research Group indicated that lifestyle modification by reducing body weight by 7% and increasing physical activity to 150 min per week reduced the incidence of diabetes more effectively than metformin treatment in people without diabetes but with prediabetes [8–12]. Therefore, one of the first steps to be taken in treating metabolic syndrome is to change the lifestyle by reducing the caloric content of the meals, increasing physical activity, and reducing body weight. Health problems and conditions such as diabetes, obesity, and hypertension are treated with approved drugs providing clinically proven effects and safety of use. However, it also appears that many natural plant materials can be helpful in maintaining normal blood glucose and cholesterol levels. Among medicinal plants that may be efficient in the prevention of type 2 diabetes mellitus, the most popular are: galega (*Galega officinalis* L.), common bean (*Phaseolus vulgaris* L.), fenugreek (*Trigonella foenum-graecum* L.), alfalfa (*Medicago sativa* L.), white mulberry (*Morus alba* L.), ginger (*Zingiber officinale* Rosc.), maize (*Zea mays* L) [13]. Among the plants with the ability to affect lipid metabolism are: garlic (*Allium sativum* L.), turmeric (*Curcuma longa* L.), milk thistle (*Silybum marianum* L.), cardoon (*Cynara cardunculus* L.), Panax ginseng (*Panax ginseng* C.A. Meyer). One of these herbal plants with beneficial pharmacological effects on the set of disease factors included in the metabolic syndrome is dandelion (*Taraxacum officinale* F.H. Wigg.).

4. Dandelion—Plant Characteristics

Dandelion (*Taraxacum officinale* L. syn. *Taraxacum vulgare* L.), belonging to the Asteraceae family, is a pharmacopeial, edible plant. It probably originated from Europe; it also gradually spread to Asia, then North America, and later to some South American countries. In many European countries, it is a common weed growing in fallow fields, roadsides, meadows, and lawns. Dandelion is a perennial weed with sturdy taproot, long green leaves organized in a rose-like manner, single yellow flowers, and characteristic cotton-like fruits with many seeds that are scattered by the wind [14]. The pharmacopeial raw materials are the roots of the dandelion (Taraxaci radix), herba, and also flowers. The traditional uses of dandelion that are mentioned in the literature concern its use as a remedy in kidney diseases, diabetes, bacterial infections, diuretic, liver, kidney, and spleen disorders, and as an anti-inflammatory factor [15]. On the other hand, dandelion parts are used as food, mainly as a salad ingredient, young leaves are placed in many dishes, and the inulin-rich roots are used as substitutes for coffee or tea [15]. It has been detected that approximately 100 g of fresh leaves contain 88.5 g of water, 19.1 g of crude protein, 6.03 g of crude fat, 10.8 g of crude fiber, and 0.67 g/100 g dry matter of calcium, 6.51 g/100 g dry matter of potassium, 3.99 g/100 g dry matter of zinc, 12.6 mg/100 g dry matter of tocopherols, 156.6 mg/100 g dry matter of L -ascorbic acid and 93.9 mg/100 g dry matter of carotenoids [16]. Dandelion flower extracts can be used as flavor additives in many food products, such as desserts, candies, baked cakes, puddings, and other similar food products [17]. The main active compounds of dandelion are presented in Figure 2.

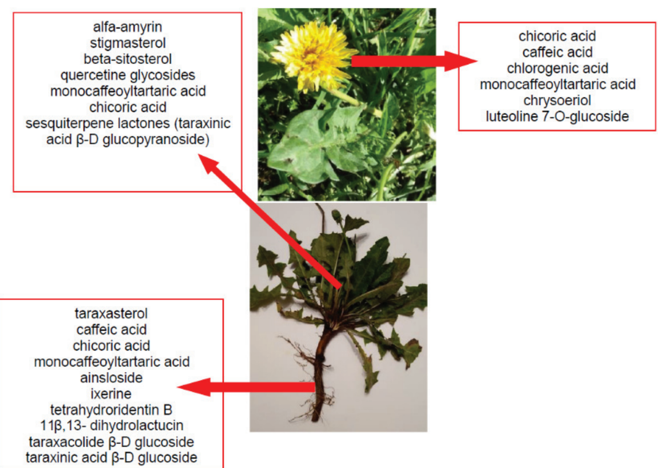

Figure 2. The main active compounds of dandelion.

Dandelion roots contain mainly sesquiterpene lactones and triterpenes and sterols (taraxasterol, taraxerol, cycloartenol, beta-sitosterol, stigmasterol) [18]. Lactones have a bitter taste and are often an ingredient in products that stimulate digestion. The literature evidence suggests that phenolic acids and sesquiterpene lactones are the main components of the dandelion root responsible for its antidiabetic potential [19]. Dandelion leaves and flowers contain polyphenols, mainly hydroxycinnamic acid derivatives (HCAs) and flavonoids (apigenin and luteolin derivatives) [20–22]. They are characterized by strong antioxidant and hypocholesterolemic properties. HCAs induce antiradical and protective effects against oxidative processes [22], while flavonoids inhibit the formation of reactive oxygen species and nitrogen by inhibiting NO synthase and COX-2 protein expression [23,24]. Chicoric acid is effective in preventing the formation and worsening of the atherosclerosis process [24]. Dandelion roots also contain significant amounts of inulin [14,20]. Inulin is a naturally occurring polysaccharide belonging to a class of dietary

fibers known as fructans. This plant is also an important source of vitamins (A, C, E, K, and B) and minerals (for example, iron and silicium), sodium, copper, zinc, magnesium, and manganese) [14,25]. Dandelion leaves are also a rich source of potassium, which may be related to the plant's diuretic activity [26]. Selected phytochemicals of dandelion are presented in Table 1.

Table 1. Selected phytochemicals of dandelion and their effects.

Name of the Phyto-Component and Parts of the Plant	Structure	Actions	References
taraxasterol (phytosterol) root	$C_{30}H_{50}O$	antihyperglycemic and anti-inflammatory properties	[27]
		anti-inflammatory activity	[28,29]
		decreases protein expression levels of PTBP1 and SIRT1, and may inhibit HBV and be a potential anti-HBV drug,	[30]
stigma sterol (phytosterols) leaf and steam	$C_{29}H_{48}O$	anti-inflammatory, anti-hyperglycemic, antimicrobial properties	[31]
chicoric acid all parts of the plant	$C_{22}H_{18}O_{12}$	antidiabetic agent with both insulin-sensitizing and insulin-secreting properties, preventing the formation and/or progression of atherosclerosis, antiradical and protective actions against oxidation processes, meanwhile, flavonoids inhibit the formation of reactive oxygen and/or nitrogen species by suppressing NO synthase and COX-2 protein expression	[32,33]
tetrahydroridentin B sesquiterpen lactone root	$C_{15}H_{24}O_4$	activated the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) in human hepatocytes, induced the Nrf2 target gene heme oxygenase	[34]
chlorogenic acid flower	$C_{16}H_{18}O_9$	antioxidant properties	[35]
		anti-inflammatory, antibacterial, antiviral, hypoglycemic, lipid-lowering, anticardiovascular, antimutagenic, anticancer, immunomodulatory	[36]
caffeic acid flower and root	$C_9H_8O_4$	anti-oxidative and immunostimulatory properties	[35]
hydroxycinnamic acids fruit	R_2-OH p-coumaric acid R_3-OCH_3; R_2-OH ferulic acid R_1-OCH_3; R_2-OH; R_3-OCH_3 sinapic acid	in experiments on plasma and platelets, using several different parameters (lipid peroxidation, protein carbonylation, oxidation of thiols, and platelet adhesion), the highest antioxidant and antiplatelet potential was demonstrated	[37]

Table 1. *Cont.*

Name of the Phyto-Component and Parts of the Plant	Structure	Actions	References
luteolin aboveground plant parts	$C_{15}H_{10}O_6$	important role in the amelioration of LPS-induced oxidative stress and inflammation.	[38]
inulin root	$C_{6n}H_{10n+2}O_{5n+1}$	influences the development of normal intestinal microflora	[39]

5. Pharmacological Activity of Dandelion for Potential Use in the Treatment of Metabolic Syndrome (MetS)

5.1. Antidiabetic Effect

According to the data provided in the scientific journals, plant products and plant-derived compounds exhibit antidiabetic effects through mechanisms such as reducing the activity of enzymes (α-amylase with β-galactosidase and α-glucosidase) that break down sugars, including polysaccharides, inhibiting renal glucose reabsorption and flow through potassium channels [19]. Different extracts (methanolic, chloroform, aqueous, petroleum ester) of dandelion root were tested for antidiabetic activity in mice with normal glycemia and alloxan-induced diabetes. In addition, the authors carried out in vitro glucose uptake assays using HepG2 and 2-NDBG. The results from the in vivo study showed that an aqueous extract of Taraxacum officinale root (400 mg/kg) caused a significant decrease in blood glucose levels (62.33%, $p \leq 0.05$), while other extracts ($p > 0.05$) showed a statistically insignificant activity in mice with alloxan-induced diabetes. No effect of the extracts on glycemia was noted in non-diabetic mice. The extracts lowered glucose levels ($p > 0.05$) in the subcutaneous glucose tolerance test. The aqueous extract showed significantly higher glucose uptake (149.6724%, $p \leq 0.05$). A phytochemical examination of the aqueous extract confirmed a higher total phenolic content than flavonoids, and chlorogenic acid, protocatechuic acid, and luteolin-7-glucoside were identified [40]. Dandelion extract also inhibited the formation of advanced glycation end products (AGEs) (IC50 = 69.4 mg/L) more effectively than the drug commonly used in diabetes, named aminoguanidine (IC50 = 138 mg/L) [41]. Dandelion leaf and root extracts and taraxinic acid β-d-glucopyranosyl ester activated nuclear erythroid-associated transcription factor 2 (Nrf2) in human hepatocytes. The leaves of *Taraxacum officinale* induced the Nrf2 target gene Hmox1. Taraxinic acid β-d-glucopyranosyl ester isolated from the leaves was found to increase Nrf2 transactivation in a dose-dependent manner. The results obtained by Esatbeyoglu et al. (2017) [34] suggest that the antioxidative activity of dandelion leaf extract is responsible for the taraxinic acid β-d-glucopyranosyl ester [34]. Other studies have tested the effect of dandelion extract on insulin secretagogue activity. Dry ethanolic extracts of *Taraxacum officinale* at concentrations ranging from 1 to 40 μg/mL were tested in vitro for insulin release from INS-1 cells in the presence of 5.5 mM glucose, with glibenclamide as a control. Insulin secretagogue activity could be observed for dandelion extracts at a concentration of 40 μg/mL [42]. Alpha-glucosidase was also inhibited by aqueous extracts of *Taraxacum officinale* depending on the origin (alpha-glucosidase from baker's yeast, rabbit liver, and rabbit small intestine) [43,44] demonstrated the antihyperglycemic effect of a

herbal preparation containing 9.7% Taraxaci radix (*Taraxacum officinale* F.H. Wigg.) in an experiment on mice with alloxan-induced non-obesity diabetes (NOD). It was noted that the extract statistically significantly reduced glucose and fructosamine levels. [44]. A follow-up study revealed the effect of the dandelion extract on the catalytic activity of glutathione S-transferases (GSTs) and the formation of malondialdehyde (MDA) in the liver of mice as an indicator of oxidative stress in early diabetes. After a 7-day administration of the dandelion extract (at a dose of 20 mg/kg body weight) to NOD diabetic mice, a significant increase in catalytic GST concentration and a statistically insignificant decrease in MDA concentration were observed [44]. Similar results were obtained by Cho et al. 2002 [45] after administering an aqueous extract of dandelion leaves to rats with streptozotocin-induced diabetes. They observed a decrease in MDA levels in the rats' liver and a significant reduction in serum glucose levels [45].

α-Glucosidase is an enzyme responsible for breaking down complex carbohydrates: di-, oligo-, and polysaccharides into simple sugars, including glucose. By inhibiting the decomposition of the alpha bonds of carbohydrates, inhibitors of α-glucosidase reduce the absorption of glucose into the blood from the gastrointestinal tract, resulting in lower postprandial glycemia. It was found that the dandelion extracts showed the ability to inhibit alpha-glucosidase [46]. In vitro studies conducted by Mir et al. (2015) [47] on methanolic and aqueous extracts of dandelion leaves, roots, and flowers confirmed their potential to inhibit α-amylase and α-glucosidase activity. It was noted that aqueous extracts inhibited enzymes more strongly than methanolic extracts, with leaf extracts showing the highest activity, followed by root extracts and the weakest activity from dandelion flowers [47]. Li and his team, based on the obtained experimental results, concluded that the aqueous extract of dandelion root, with a composition of polysaccharides (63.92 $\pm$ 1.82 mg/g), total flavonoids (2.57 $\pm$ 0.06 mg/g), total phenolic compounds (8.93 $\pm$ 0.34 mg/g), and saponins (0.54 $\pm$ 0.05 mg/g) statistically showed a significant ability to inhibit α-glucosidase and α-amylase activities [48]. Synergism was also observed between the effects of dandelion root extract and Astragalus (*Astragalus* L.) extract. In addition, it was found that mixing the extracts from these plants could alleviate insulin resistance in IR-HepG2 cells.

Choi and his team in 2018 [49] isolated from *Taraxacum officinale*, in addition to the 22 previously known compounds listed below (no. 1–22), three new butyrolactones (1–3) and three butanates (4–6), or taraxioside A-F (Figure 3) (1) 1,2,5-tri-O-p-hydroxyphenylacetyl-L-chiro-inositol, (2) chrysoeriol, (3) 5,7,30-hydroxy-40,50-dimethoxy flavone, (4) methyl 3,4-dihydroxycinnamate, (5) 5,7,40-hydroxy-30,50-dimethoxy flavone, (6) luteolin, (7) 3-glycerindole, (8) calquiqueignan D, (9) calquiquelignan E, (10) tricin 40-O-[threo-b-guaiacyl-(700-Omethyl)-glyceryl] ether, (11) tricin 40-O-[erythro-b-guaiacyl-(700-O-methyl)-glyceryl] ether, (12) loliolide, (13) epiloliolide, (14) annuionone, (15) 11b,13-dihydrotaraxinic acid b-O-glucopyranoside, (16) 3,4-dihydroxy-5,7-megastigmadien-9-one, (17) komaroveside A, (18) 6S,9R-roseoside, (19) 6S,9S-roseoside, (20) adenosine, (21) aesculetin-7-O-b-D-glucopyranoside, and (22) syringin. Their chemical structures were determined by interpreting the spectroscopic data and comparing them with data from the literature. The authors evaluated all isolates for their α-glucosidase inhibitory activity. New compounds I through VI (IC50 145.3–181.3 μM) showed inhibitory activity similar to acarbose (IC50 179.9 μM). Compounds 1 and 6 were the strongest inhibitors, with IC50 values of 61.2 and 39.8 μM, respectively. Compounds II and 6 showed mixed-type inhibition, while compound 1 and acarbose showed competitive inhibition [49].

Perumal et al. 2022 [50] studied the antidiabetic potential of a combination of dandelion and *Momordica charantia* extracts. They determined antidiabetic properties in vitro; the inhibition of α-amylase, α-glucosidase, and dipeptidyl peptidase-4 (DPP-4), and glucose-uptake in L6 muscle cells.

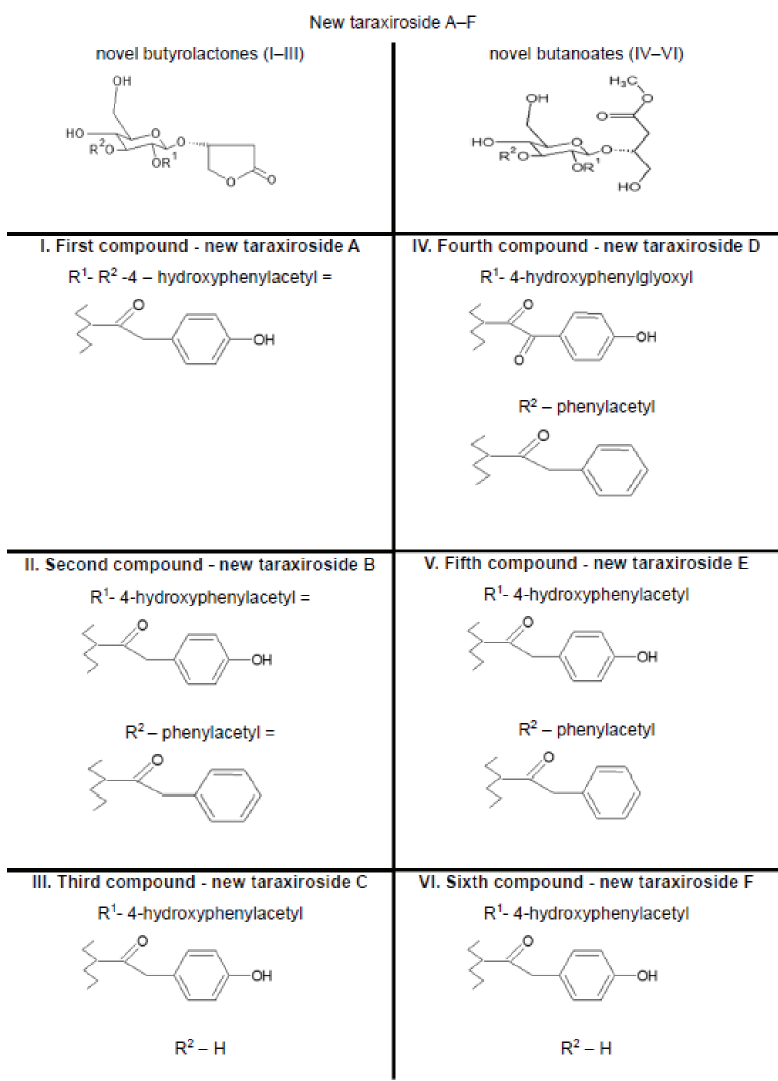

Figure 3. New compounds isolated from *Taraxacum officinale* Choi et al. 2018 [49].

The authors of the study concluded that the antidiabetic efficacy of the combination of the tested herbs was better than the aforementioned herbs used alone. A glucose tolerance test in a study involving rats with streptozotocin–nicotinamide (STZ-NA)-induced diabetes proved that the combination of herbs tested lowered blood glucose levels comparable to the effects of glibenclamide and metformin. The combination of herb extracts showed better antidiabetic properties; it increased the activity of DPP-4, α-amylase, and α-glucosidase [50]. Therefore, the authors suggest that combinations of herbs have a better phytotherapeutic potential for treating type 2 diabetes. In another study conducted by Cho et al. 2002 [45] on a rat model of streptozocin-induced diabetes, the administration of 2.4 g of dandelion aqueous extract/kg of diet reduced postprandial blood glucose levels [45]. In another study conducted in 2012 by Nnamdi and his team [51], the effects of dandelion leaves and roots on streptozotocin (STZ)-induced diabetes in rats were tested. The results provided evidence of a hypoglycemic effect after twice-daily administration of an aqueous or alcoholic extract of *Taraxacum officinale* leaves and roots in amounts of

300 and 500 mg extract/kg b.w. [51]. It was found that the ethanol extract can increase carbohydrate metabolism. It was also suggested that the ethanol extract is more effective than the aqueous extract and that the roots are more therapeutically effective than the leaves in the treatment of diabetes. The experimental results also showed that the effects of the *Taraxacum officinale* extracts were dose-dependent. However, the authors did not analyze the composition of the extracts, so it is not possible to conclude which components of the dandelion are responsible for such activity [51]. In vivo studies in rat models of non-alcoholic steatohepatitis (NAFLD) treated with dandelion extracts showed significant reductions in hepatic lipid accumulation, liver tissue and body weight, and serum cholesterol levels. After the administration of dandelion leaf extracts, insulin resistance was found to be reduced through activation of the AMPK (5′ adenosine monophosphate-activated protein kinase) pathway. Dandelion products, due to the presence of polyphenols and flavonoids in their composition, can regulate the expression of several genes whose dysfunctions contribute to lipid deposition, oxidative stress, and insulin resistance [52]. With the increasing evidence that non-alcoholic fatty liver disease increases the risk of developing type 2 diabetes, it is assumed that non-alcoholic fatty liver disease and non-alcoholic steatohepatitis are specific clinical manifestations of type 2 diabetes through the coexisting process of lipid deposition, chronic inflammation, and liver fibrosis [53]. Other studies have confirmed that polyphenols in dandelion leaf and stem extracts are useful in the treatment of type 2 diabetes and obesity. An ethanolic extract of dandelion was proved to inhibit the formation of advanced glycation end products at IC50 = 69.4 mg/L compared to the antiglycation drug-aminoguanidine (IC50 = 138 mg/L) [54]. Dandelion components also show activity in regulating the pathways responsible for insulin release, most likely by inhibiting certain enzymes involved directly and indirectly in carbohydrate breakdown in the Krebs cycle and glycolytic cycle. The mechanism of insulin release in β cells is a complex process. The ethanolic extract of dandelion at a concentration of 40 μg/mL significantly increased insulin secretion in in vitro studies on the rat INS-1 cell line [42]. In a study performed by Tousch et al. (2008) [32], it was noted that chlorogenic acid CGA is an inhibitor of glucose-6-phosphatase (G6P) in the rat liver and may contribute to intensifying glucose transport, thereby increasing ATP production and stimulating insulin secretion [32]. It is thought that CGA may also regulate β-cell function [55]. An in vivo experiment showed that CGA significantly increases hepatic mRNA expression by interacting with peroxisome proliferator-activated receptor alpha (PPAR-α). Thus, it is investigated that CGA may contribute, through the activation of peroxisome proliferator-activated receptor alpha (PPAR-α) and the stimulation of glucagon-like peptide GLP-1 production, to restore β-cell function, and thus aid in the treatment of type 2 diabetes [56].

5.2. Impact on Lipid Profile

To determine the possible use of dandelion preparations as a natural anti-obesity agent, Zhang et al. (2008) [57] examined its inhibitory activity against pancreatic lipase in vitro and in vivo. The inhibitory activity of a 95% ethanol extract of *T. officinale* and Orlistat was measured using 4-methylumbelliferyl oleate (4-MU oleate) as a substrate at concentrations of 250, 125, 100, 25, 12.5, and 4 μg/mL. To determine pancreatic lipase inhibitory activity in vivo, mice (n = 16) were orally administered corn oil emulsion (5 mL/kg) alone or with 95% ethanolic extract of *T. officinale* (400 mg/kg). The plasma triglyceride levels were measured at 0, 90, 180, and 240 min after administration. It was found that the 95% ethanol extract of *T. officinale* and Orlistat inhibited porcine pancreatic lipase activity by 86.3% and 95.7% at a concentration of 250 μg/mL. The *T. officinale* extract showed a dose-dependent inhibition of IC(50) = 78.2 μg/mL. In addition, it was discovered that a single oral dose of the extract inhibited the increase in plasma triglycerides at 90 and 180 min ($p < 0.05$) [57]. Other in vitro studies have confirmed that flavonoids related to quercetin and luteolin from dandelion inhibit porcine pancreatic lipase [58]. Mice (C57BL/6) that were fed dandelion leaf extract and a high-fat diet had lower serum triglycerides and total cholesterol compared to the control group [59]. Similar findings were reported for rabbits

fed a high-cholesterol diet (1% cholesterol) with dandelion root or leaves for four weeks. In these animals, it was also observed that the level of serum triglycerides was significantly lower compared to the control group [60]. Similar issues were the subject of another research paper. The investigation proved that dandelion extracts had an inhibitory effect on adipocyte differentiation and lipogenesis activity in 3T3-L1 pre-adipocytes. The HPLC analysis of the three plant extracts obtained from leaves and roots, which were used in this study, as well as a commercial root powder, showed the presence of caffeic acid and chlorogenic acid as the main phenolic component. It was found that there was no cytotoxicity effect in the concentrations of the extracts used in the experiments—MTT test. The authors inferred that dandelion extracts could affect adipogenesis and lipid metabolism [61]. Other researchers have searched for other biologically active compounds from a number of plants that would show properties that reduce triglyceride accumulation and increase lipolysis and induce investigated apoptosis. The work demonstrated that 18–22% v/v aqueous-ethanol extract of dandelion root effectively induced the apoptosis of human primary visceral pre-adipocytes during their differentiation while enhancing lipolysis [62]. An interesting study was conducted to reveal how the addition of dandelion extract at a rate of 0.8% to the carp's daily feed ration would affect the hydrochemical parameters (pH, dissolved oxygen, and electrical conductivity). It was found that carp fed a diet supplemented with dandelion extract did not show improved fish production characteristics compared to those found for carp from the control group. Instead, carp from the experimental groups had higher survival rates, final weights, average individual weight gain, and specific growth rates (SGR), but the differences were not statistically significant. Feed supplementation with dandelion extract significantly reduced plasma cholesterol (by 4.76%) and triglycerides (by 61.2%), which may be an interesting finding for breeders ($p \leq 0.05$) [63].

5.3. Impact on Blood Pressure

Oxidative stress is one of the factors co-responsible for the development of hypertension. In subsequent in vitro and in vivo studies, it was verified whether dandelion leaf and root extracts have sufficient antioxidant potential that is able to influence the reduction in hypertension-inducing factors. In this study, the malondialdehyde (MDA) levels were determined in lipid peroxidation assays and in rats with hypertension stimulated by free radical production. Oxidative stress was induced by N ω-nitro- L-arginine methyl ester. Aremu et al. (2019) [64] noted that the extract increased antioxidant activity and reduced lipid peroxidation in the heart, liver, kidney, and brain of the tested rats. The authors suggest that the phenolic compounds present in the extracts may also regulate nitric oxide synthase (NOS) levels and activity by affecting kinase signaling pathways and intracellular Ca^{2+} associated with NOS phosphorylation and NO production. In addition, phenolic compounds can also affect the inhibition of endothelin-1 (vasoconstrictor) and endothelial NADPH oxidase, but more research is required to confirm these theses [64].

5.4. Effects on Blood Coagulation

The inhibitory effect on platelet aggregation in humans by ethanolic extracts of dandelion root (*Taraxacum officinale* F.H. Wigg.) was examined. The extracts showed dose-dependent inhibition of platelet aggregation, where the maximum inhibition was 85% at a concentration equivalent to 0.04 g dried root/mL human platelet-rich plasma (PRP). The effect was obtained regardless of whether platelet aggregation was induced by arachidonic acid or collagen. The extracts were fractionated into two groups of compounds with masses above (Mr > 10,000) and below (Mr < 10,000). The fraction containing low-molecular-weight polysaccharides (Mr< 10,000) resulted in 91% inhibition, while the other fraction enriched in triterpenes and steroids (Mr > 10,000) showed 80% inhibition of platelet aggregation. Both at a concentration equivalent to 0.04 g raw material/mL PRP [65]. In another in vitro study, Lis and the team (2018) [66] determined the antiplatelet and antioxidant properties of four standardized phenolic fractions of dandelion. The following fractions were ana-

lyzed: two leaf fractions of 50% and 85% methanol and two petal fractions of 50% and 85% methanol. The hemostatic activity in the plasma was determined: activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT). It was found that none of the dandelion fractions tested caused damage to human platelets over the entire range tested. The results of the study show that dandelion, especially its aboveground parts containing hydroxycinnamic acid, which has antioxidant and antithrombotic effects of the hemostatic system; that is, they may be promising preparations in the prevention of cardiovascular diseases, especially those related to changes in hemostasis and oxidative stress [66]. Studies concerning the antioxidant potential of dandelion preparations have confirmed that a diet rich in dandelion preparations can be helpful in treating diseases related to oxidative stress and hemostatic disorders. Although a great number of chemical compounds present in dandelion, such as hydroxycinnamic acid and sesquiterpene lactones, were previously detected, new compounds are still being discovered and analyzed. Recently, inositol 4-hydroxyphenylacetate (PIE) esters have been characterized. In work performed by Jedrejek et al. 2019 [67], five fractions of dandelion extract were analyzed, where each was characterized by different contents of active compounds. Detailed LC-MS and chemical tests of the dandelion fractions identified about 100 phytochemical compounds, including new ones. In all concentration ranges tested (0.5–50 μg/mL), the dandelion root preparations did not cause platelet hemolysis. The results indicate that dandelion roots constitute a safe and readily available source of different classes of natural compounds with antioxidant, anticoagulant, and antiplatelet effects [67].

The aim of another in vitro study was to evaluate the activity of dandelion extracts, which were standardized for chicoric acid content. Four phenolic fractions extracted from leaves (fractions A and B) and flower petals (fractions C and D) were characterized by different concentrations of chicoric acid. The biomarkers of oxidative stress, coagulation, and platelet activation parameters were determined. The results suggest that chicoric acid has antioxidant and anti-adhesive potential. The authors noted that the fraction richest in chicoric acid (leaf fraction A) possesses anti-adhesive and anti-aggregation properties stronger than chicoric acid alone. These findings strongly suggest the possibility of a synergistic effect between the compounds in fraction A and also the presence of compounds such as phenolic acid derivatives and flavonoids, which may exhibit stronger properties than chicoric acid [68]. Relying on previous in vitro studies, another research group decided to test the effects of the same extracts in in vivo studies, conducting tests on rats. The animals were given a diet enriched in phenolic fractions obtained from dandelion leaves and petals (694 mg/kg diet/day) for 4 weeks. The phenolic fractions obtained from the dandelion leaves and petals contained, respectively, 4.10 ± 0.05 and 1.41 ± 0.07 mg of l-chicoric acid in the daily dose. It was found that supplementation with the petal fraction increased plasma thiols. The leaf fraction reduced the level of protein carbonylation and affected the lipid profile—triglycerides, total cholesterol, lipoprotein pooling index, and the plasma atherogenicity index were reduced. The authors concluded that the phenolic fractions from *T. officinale* rich in hydroxycinnamic acids should be considered as potential components of functional foods with beneficial effects on human health [33].

The phytochemical analysis of dandelion fruits is also an issue worthy of interest. It should be noted that the root, leaf, and flowers are relatively well studied. Research on dandelion fruits is rarely undertaken. However, fruits are also used in medicine and food. Lis et al. 2020 [37] obtained a methanolic extract of dandelion fruit (E1). Analysis of an extract revealed the presence of hydroxycinnamic acid (HCA) derivatives and flavone derivatives. Several new metabolites were also detected, such as biflavones and some flavonolignans. Multistage fractionation of the methanolic extract of dandelion fruit was carried out. Two fractions were prepared: phenolic acid extract (E2) and flavonoid extract (E3). The E3 extract was divided into four flavonoid fractions: A (luteolin fraction; 880 mg GAE/g), B (philonotisflavone fraction; 516 mg GAE/g), C (flavonolignans fraction; 384 mg GAE/g), and D (flavone aglycones fraction; 632 mg GAE/g). The highest antiradical activity of DPPH was exhibited by fractions A > B > Trolox, medium Trolox > E3 > E2 > E1, and the

lowest by C and D. No cytotoxic effect on platelets was noted for any of the dandelion preparations tested. Several different parameters, as well as lipid peroxidation, protein carbonylation, thiol oxidation, and platelet adhesion, were analyzed. The hydroxycinnamic acid extract (E2), flavonoid extract (E3), and luteolin fraction (A) showed the highest antioxidant and antiplatelet potential [37].

The antiplatelet potential of four fractions obtained from different parts of the dandelion (fractions A and B from roots; fraction C from leaves; fraction D from petals) on platelet activation and thrombus formation in whole blood were analyzed as well as the effect of the tested fractions on the platelet proteome were also evaluated. The authors found that fraction C from dandelion leaves reduced thrombus formation and platelet activation after collagen stimulation. None of the fractions tested caused changes in the platelet proteome. The preparations obtained from different parts of the dandelion can have a beneficial effect in the prevention and treatment of cardiovascular diseases caused by hyperactivation of platelets [69].

5.5. Dandelion vs. Obesity

To determine the possible use of the dandelion preparations as a natural anti-obesity agent, Zhang et al. 2008 [57] measured its inhibitory activity against pancreatic lipase in vitro and in vivo. The inhibitory activity of a 95% ethanol extract of *T. officinale* and Orlistat was measured using 4-methylumbelliferyl oleate (4-MU oleate) as substrate at concentrations of 250, 125, 100, 25, 12.5, and 4 μg/mL. To determine the pancreatic lipase inhibitory activity in vivo, mice (n = 16) were orally administered corn oil emulsion (5 mL/kg) alone or with 95% ethanolic extract of T. officinale (400 mg/kg). The plasma triglyceride levels were measured at 0, 90, 180, and 240 min after administration. It was found that 95% ethanol extract of *T. officinale* and Orlistat inhibited porcine pancreatic lipase activity by 86.3% and 95.7% at a concentration of 250 μg/mL. *T. officinale* extract showed a dose-dependent inhibition of IC(50) = 78.2 μg/mL. In addition, it was noted that a single oral dose of the extract inhibited the increase in plasma triglycerides at 90 and 180 min ($p < 0.05$) [57]. The mice were administered dandelion extract, which showed anti-obesity potential through a dose-dependent inhibitory effect on pancreatic lipase activity and an increase in plasma triglyceride levels. The results indicate that *T. officinale* may be an alternative to Orlistat, a drug which often causes adverse effects [57]. In another study, the anti-obesity effects of the dandelion ethanol extract were examined. The ethanolic extract of *Taraxacum officinale* was administered orally at a dose (150 and 300 mg/kg), and Orlistat was used as a reference drug. The experimental rats were fed a high-fat diet. The high-fat diet caused significant increases in body weight, fat mass, serum glucose concentration, as well as cholesterol and triglycerides levels. The authors noted that *Taraxacum officinale* extract significantly reduced body weight, lipid parameters, organ weights, and fat pad mass. The value of the study, however, is diminished by the fact that the content of phytoactive compounds in the extract was not determined. Unfortunately, it is not possible to determine which compounds are responsible for such effects [70]. The possibility of using dandelion extracts to compose formulations and functional foods affecting obesity reduction was also investigated. In the Aabideen et al. 2020 [71] experiment, various aqueous-ethanol extracts were tested for their antioxidant potential. Next, 60% of the extracts with the strongest antioxidant activity were subjected to in vivo testing. BALB/c mice weight gain, fecal fat content, and food intake were compared after an administration of an 8-week fat-rich diet. An increase in the body weight of 44.94% of mice on the high-fat diet (HFD), compared to the NDG control group of 22.21% after eight weeks was observed. After eight weeks on the HFD diet, the obese mice were divided into groups to evaluate the effects of plant extracts on obesity parameters. Treatment with plant extracts and comparative Orlistat was continued for another eight weeks. Mice given the plant extract at 300 mg/kg b.w. reduced their weight to 32.22 ± 1.86 g, and those consuming the drug Orlistat reduced their weight to 30.09 ± 1.61 g compared to the untreated group (HFD) (52.66 ± 2.03 g). The Orlistat-treated mice had a fecal fat content of 11.65%, the 300 mg/kg b.w. extract

group had a fecal fat content of 9.92% compared to the HFD mice at 5.67%. Food intake in mice in the HFD + 300 extract groups was 3.65 g/mouse/day, and HFD + Orlistat was 3.8 g/mouse/day compared to mice on the HFD diet of 4.12 g/mouse/day. The findings suggest that dandelion preparations may be an alternative to the use of the drug Orlistat [71]. A study conducted by Majewski and his team detected that the consumption of aqueous dandelion flower syrup (278.2 g/kg diet for four weeks) had beneficial effects on rat blood lipid regulation, which was manifested by increasing HDL fraction, increasing plasma superoxide radical (SOD) scavenging, and decreasing lipid peroxidation. In addition, the liver damage marker ALP content was lowered. The aqueous syrup of dandelion flowers contained hydroxycinnamic acids and flavonoids. Studies have discovered that syrup from *T. officinale* floral water at a dose of 278.2 g/kg of diet affects antioxidant levels and reduces smooth muscle contraction in the blood vessel wall. The authors concluded that phenolic compounds in flower syrup exhibit health-promoting properties. Its antioxidant activity is responsible for these properties [72].

The preparations (extracts, syrups, etc.) obtained from different parts of the dandelion (root, leaves, and flowers) show health-promoting effects. They have the ability to regulate glucose levels and lipid profile, affect digestive enzymes, and indirectly reduce obesity. However, this has been observed mainly in in vitro studies or in animal models. It was stated that there is a need for in-depth studies on this issue with healthy volunteers and people with various cardiovascular diseases, obesity, or type 2 diabetes who would be given dandelion preparations.

6. Reports on the Toxic Effects of Dandelion and its Preparation

Dandelion has been consumed as food and used as herbal medicine for centuries, and the side effects of its consumption are rather rare. Dandelion root and dandelion extracts have "generally recognized as safe" status approved by the FDA for use in dietary supplements. Fresh *Taraxacum officinale* leaves and other parts are consumed as food in many countries.

Many studies on animals have been conducted regarding the potential toxicity of this plant. Toxicological studies have been conducted, and LD_{50} at *per os* administration to mice was determined to be greater than 20 g/kg body weight [73]. In subchronic toxicity studies (4 months), no toxic effect was noted in rats fed with dandelion leaves (33% in the diet) [74]. No acute toxicity was observed in rabbits after the oral administration of dehydrated dandelion plant at a dose of 3–6 g/kg body weight. The LD_{50} (intraperitoneal injection) of the liquid extract of the herb and root for mice was 28.8 g/kg and 36.6 g/kg, respectively. It was only discovered that taraxacum acid esters could cause contact dermatitis [75]. In vitro studies have noted that dandelion infusions can inhibit cytochrome 3A4 (IC50 = 140.6 μg/mL), which may lead to interactions with the metabolism of, for example, immunosuppressive drugs [76]. In studies with rats, it was shown that doses up to 1000 mg/kg b.w. did not cause mortality when administered by the oral route, as did doses of 1600, 2900, and 5000 mg/kg b.w. [64].

Dandelion intake is generally considered safe and well-tolerated in adults if taken in moderation, but some side effects exist, such as diarrhea, upset stomach, or irritated skin. According to Yarnell et al. 2009 [74], it seems that due to its bitter content, dandelion should be consumed with caution by people with diagnosed acute gastroenteritis or reflux esophagitis [74], acute inflammation, or obstruction of the gastrointestinal tract. Allergies to dandelion may also occur. No information on dandelion toxicity or serious adverse effects in humans has been encountered in the scientific literature [77].

7. Final Remarks

Dandelion is an interesting herbal plant that can be successfully used in the food, pharmaceutical, and cosmetic industries. It manifests multidirectional pharmacological activity that is widely documented in the scientific literature. This paper presents the properties of dandelion, which can be successfully used in the treatment and prevention

of metabolic syndrome. A review of available in vivo and in vitro studies indicates that dandelion extracts can prevent diabetic complications, improve lipid metabolism, as well as exhibit inhibitory activity on sugar-degrading enzymes. The aforementioned activity, according to the definition describing metabolic syndrome, is part of the current recommendations for its treatment, the primary aim of which is to prevent the development of diabetes, hypertension, and other cardiovascular diseases. The multidirectional effects of the dandelion and its preparations are presented graphically in Figure 4.

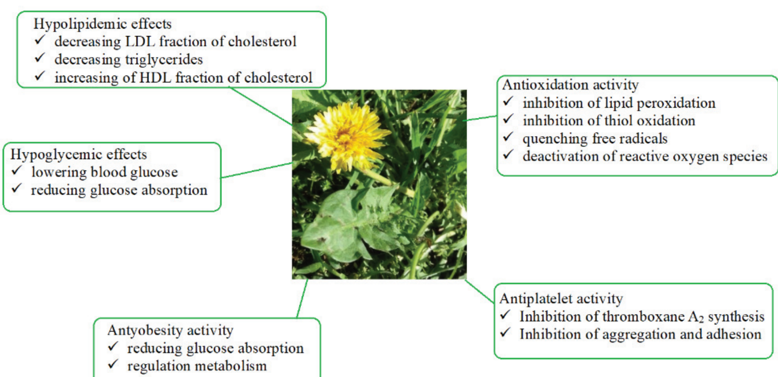

Figure 4. Multidirectional effects of dandelion and its preparations.

Many questions still remain to be clarified. Therefore, new in-depth scientific research on all biological activities of *Taraxacum officinale* in relation to human health is essential for a thorough understanding of the mechanisms of action of the preparations from this plant.

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Review

An Exploratory Critical Review on TNF-α as a Potential Inflammatory Biomarker Responsive to Dietary Intervention with Bioactive Foods and Derived Products

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Abstract: This review collects and critically examines data on the levels of tumour necrosis factor-alpha (TNF-α) in lean, overweight and obese subjects, and the effects of intervention with different foods and food products containing bioactive constituents in overweight/obese individuals. We additionally explore the influence of different single nucleotide polymorphisms (SNPs) on TNF-α levels and compare the response to food products with that to some anti-obesity drugs. Our aim was to provide an overview of the variability, consistency, and magnitude of the reported effects of dietary factors on TNF-α, and to envisage the reliability of measuring changes in the levels of this cytokine as a biomarker responsive to food intervention in association with the reduction in body weight. Regarding the circulating levels of TNF-α, we report: (i) a large intra-group variability, with most coefficients of variation (CV%) values being ≥30% and, in many cases, >100%; (ii) a large between-studies variability, with baseline TNF-α values ranging from <1.0 up to several hundred pg/mL; (iii) highly variable effects of the different dietary approaches with both statistically significant and not significant decreases or increases of the protein, and the absolute effect size varying from <0.1 pg/mL up to ≈50 pg/mL. Within this scenario of variability, it was not possible to discern clear differentiating limits in TNF-α between lean, overweight, and obese individuals or a distinct downregulatory effect on this cytokine by any of the different dietary approaches reviewed, i.e., polyunsaturated fatty acids (PUFAs), Vitamin-D (VitD), mixed (micro)nutrients, (poly)phenols or other phytochemicals. Further, there was not a clear relationship between the TNF-α responses and body weight changes. We found similarities between dietary and pharmacological treatments in terms of variability and limited evidence of the TNF-α response. Different factors that contribute to this variability are discussed and some specific recommendations are proposed to reinforce the need to improve future studies looking at this cytokine as a potential biomarker of response to dietary approaches.

Keywords: bioactive compounds; obesity; inflammation; interindividual variability; (poly)phenols; human clinical trials; genotype; genetic variants

1. The Biological Scenario of TNF-α

Tumour necrosis factor-alpha (TNF-α) is a widely investigated multifunctional cytokine that belongs to the TNF/TNFR (tumour necrosis factor receptor) superfamily. It is produced by multiple cells, primarily by circulatory and infiltrating immune cells, such as macrophages, T and B lymphocytes, natural killer cells, and monocytes, as well as by other

types of cells including adipocytes, endothelial cells, muscle cells, mast cells, fibroblasts, osteoclasts [1,2] (Figure 1). The soluble trimeric form of TNF-α is activated by binding to the receptors TNFR1 and TNFR2 and triggering multiple cell-signalling transduction pathways involved in many different processes, i.e., cell proliferation and differentiation, cell death (apoptosis) and survival, and cell communication. Maintaining normal physiological levels of TNF-α is critical for health, and TNF-α itself can contribute to this process by being involved in a negative feedback regulatory mechanism between different subpopulations of T cells (effector and regulatory T cells) [3].

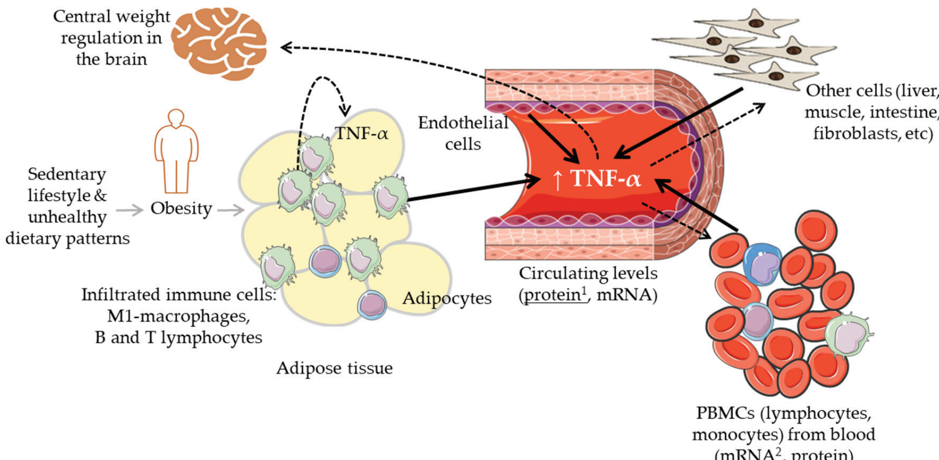

Figure 1. Graphic summary of the biological regulatory scenario of the levels of TNF-α in the context of body weight alterations. Solid black arrows indicate different cells and tissues that can contribute to the circulating levels of TNF-α; dashed arrows indicate different tissues where TNF-α can have a regulatory effect. [1]: TNF-α protein levels most commonly measured in blood (serum, plasma); [2]: *TNF-α* mRNA levels most commonly measured in peripheral blood mononuclear cells (PBMCs).

TNF-α is also a potent activator of the production of different cytokines promoting a pro-inflammatory status and thus, it has an important function in the inflammatory and immune responses. Multiple molecular and cellular mechanistic studies have pointed to the therapeutic potential of targeting the system TNF/TNFRs for the treatment of inflammatory and immune diseases [4]. In general, the long-term elevated levels of TNF-α associated with diseases such as arthritis, inflammatory bowel disease (IBD) or some specific types of cancer [5–7] can be deleterious, and the reduction in the levels of this cytokine is generally considered a beneficial anti-inflammatory effect. Thus, anti-TNF-α therapy has been successfully applied to combat some of these diseases even though it can also have some serious adverse effects [8].

It is now also clear that TNF-α has an impact on healthy metabolism as well as on metabolic diseases, particularly, on obesity-linked glucose metabolism and insulin resistance (IR) [9]. Increased levels of TNF-α have been linked with obesity and the associated low-grade chronic inflammatory status as well as with the derived cardiometabolic disorders [10,11]. Inversely, the reduction in body weight has been associated with a decrease in the levels of this and other cytokines [12]. Intriguingly, TNF-α also relates to metabolic dysfunctions such as anorexia and cachexia which share with obesity overlapping inflammatory mediators and IR but opposite relationship with fat mass [13–15]. Further, the use of anti-TNF-α antibodies (adalimumab, certolizumab, infliximab, etanercept) against some inflammatory diseases (e.g., IBD, Crohn's disease, rheumatoid arthritis, psoriatic arthritis, spondyloarthritis) has produced contrasting results with some studies reporting increases in body mass index (BMI) and other anthropometric parameters upon treatment [16–18].

Overall, the mechanisms explaining the relationship between TNF-α, body weight changes, and metabolic disorders are not yet clear. It remains thus essential to clearly understand and establish the relevance of the levels of TNF-α and of its modulation (size and direction of its fluctuation) in the context of lean physiology and increased body weight (overweight/obese) conditions. Different sets of immune cells infiltrated into metabolic organs such as the adipose or the liver tissues regulating, at least partly, the balance between metabolic homeostasis and dysfunction (Figure 1). In obesity, the accumulation of fat and enlargement of adipocytes contribute to the dysfunctionality of these cells as well as that of endothelial cells and macrophages (M1-polarized macrophages), increasing the secretion of a range of adipokines including TNF-α [2]. These elevated levels of TNF-α have been associated with impaired insulin signalling [19], and altered lipid metabolism (lipolysis/lipogenesis) [1]. It has also been indicated that TNF-α may lead to weight loss by interfering with central weight regulation in the brain through the stimulation of anorexigenic neuropeptides release as well as by influencing catabolic processes in peripheral muscle cells [17].

Overall, TNF-α is a pleiotropic molecule involved in multiple physiological and pathological mechanisms and constitutes an important target in therapies against a diversity of immunologically related disorders. In the context of obesity, a broad range of human intervention dietary studies have investigated the changes on the blood levels of TNF-α in connection with body weight changes. In this manuscript, we present an exploratory and critical revision of those studies looking at the modulation and variability in the levels of this cytokine. We examine a selection of human intervention trials with different dietary approaches including foods and food derived products containing (micro)nutrients and/or bioactive constituents. The number of studies selected and included in each section of this review is indicated in Figure 2. Also, we critically investigate the reported differences in the levels of TNF-α between lean, overweight and obese individuals, as well as the influence of specific genetic variants to this variability. Additionally, we compare the nutritional intervention results to those attained with specific anti-obesity drugs. Our main goals were to contribute to the understanding of the reliability of measuring the levels and changes in this cytokine, and to reinforce the need to improve future trials so that TNF-α may be used as a reliable biomarker of chronic inflammation associated with obesity in response to dietary intervention.

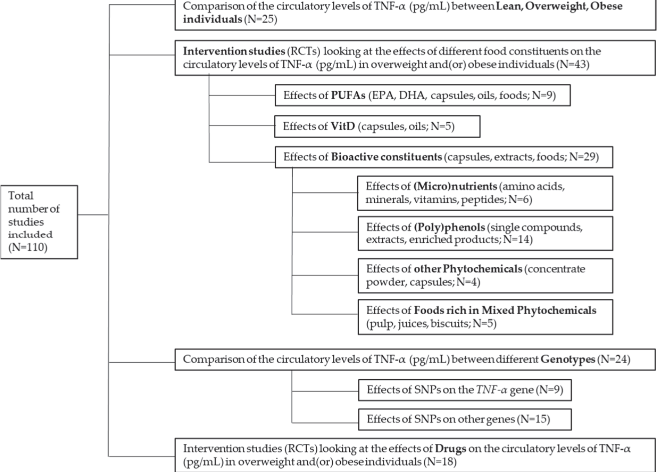

Figure 2. Diagram summarising the studies revised in this Review (N = number of studies included).

2. TNF-α Levels in Lean, Overweight and Obese Individuals

A good diagnostic biomarker has been defined as one that allows for the differentiation between the conditions under investigation (health and disease), and for identifying an individual within one of those conditions [20]. When reporting the responses of a biomarker to any treatment, i.e., dietary or drug intervention, it is thus essential to try to describe and understand the relevance of the response (both size and direction of change) within the context of the interindividual variability but also taking into consideration the differences in the values between the investigated conditions.

We reviewed a number of human studies carried out between 1995 and 2022, where the circulatory levels of TNF-α were determined in lean, overweight and obese individuals [21–45]. The results of all the studies evaluated in this section and the differences between obese and lean individuals for each study are specified in the supplementary Table S1. We examined and compared all these data in an attempt to visualize the difference in the levels of TNF-α between these metabolic conditions, and to assess whether this difference could be used with sufficient reliability to clearly differentiate between lean, overweight and obese individuals. The levels of TNF-α were most commonly reported in pg/mL and as the mean values ± standard deviation (SD) for lean, overweight, and obese individuals. We estimated the coefficient of variation (CV%) (using the standard formula as follows: (CV%) = (Standard Deviation/Mean) × 100) for each of the different subgroups in every study. A summary of the intragroup variability and levels of circulatory TNF-α in lean, overweight, obese, and very obese individuals as reported in the different human studies examined in this review is displayed in Table 1.

Table 1. Summary of the data on intragroup variability and levels of circulatory TNF-α in lean, overweight, obese, and very obese individuals as reported in the different human studies examined in this review.

Phenotype	CV% [1] (Range)	Most Commonly CV% Values (Range)	TNF-α Levels (Range)	Most Commonly Reported TNF-α Levels (Range)
Lean	(12%, ≥100%)	(≈30%, 50%)	(0.09, 82.3 pg/mL)	(≈2.0, 6.0 pg/mL)
Overweight	(37%, >100%)	(≈35%, 50%)	(0.09, 30.0 pg/mL)	(≈3.0, 6.5 pg/mL)
Obese	(11%, >100%)	(≈30%, 90%)	(0.11, 294.0 pg/mL)	(≈1.0, 10.0 pg/mL)
Very obese [2]	(21%, >100%)	(≈30%, 90%)	(1.3, 713.0 pg/mL)	(≈5.0, 10.0 pg/mL)

[1]: CV% = (Standard Deviation/Mean) × 100; [2]: This group includes participants classified as very obese, class II and III obesity, and morbidly obese.

The results show that the CV% values were typically >30% and, in some cases, well above 100%, for the lean, overweight and obese subpopulations, indicating a rather high relative intragroup variation. The data also show that the circulating levels of TNF-α displayed a very high between-studies variability. In the lean individuals, reported values expanded from <1.0 pg/mL to ~80 pg/mL; however, in most cases, the values were found in the range 2.0–6.0 pg/mL (Table 1). In general, most of the investigated studies reported higher levels of TNF-α in the overweight, obese, and/or very obese subgroups than in the lean participants. However, the differences were (S) in some studies [23–27,30,33–38,40,42] and not significant (NS) in others [21,22,28,29,31,38,39,44,45]. Also, the magnitude of the difference was highly variable. For example, in Polish women, the levels of TNF-α varied significantly ($p < 0.001$) from 2.9 ± 2.2 pg/mL in the lean participants to 6.5 ± 3.1 pg/mL in overweight individuals, 6.8 ± 3.1 pg/mL in participants with obesity, and 7.4 ± 2.6 pg/mL in very obese participants [23]. In another study also conducted in Polish women, the differences reported between lean and obese participants were 4.5 ± 2.3 pg/mL and 8.8 ± 7.0 pg/mL, respectively ($p < 0.05$) [30]. In a sample population from Israel [24], the difference found between obese and non-obese participants was also very significant, but the levels of the cytokine were much smaller (1.0 ± 0.8 pg/mL vs. 0.3 ± 0.3, $p < 0.001$). As an example of (NS) but substantial differences between individuals, in a sample population of Spanish women, the levels of TNF-α ranged from 5.7 ± 9.8 pg/mL in lean participants to

11.6 ± 19.0 pg/mL in obese ones, and 8.6 ± 12.4 pg/mL in very obese participants [22]. In addition, a few studies also reported lower levels in the overweight/obese participants than in the lean ones [32,41,43]. Likewise, a study conducted in a sample mixed population in the USA indicated that the levels of TNF-α were 0.8 ± 7.2 pg/mL in the obese participants and 82.3 ± 89 pg/mL in the lean ones [32]. Further results can be found in Table S1.

Overall, the percentage of change from lean to overweight or obese subgroups varied from a (NS) 2% increase [41] up to a maximum increase of ~1700% in some morbidly obese individuals [37], although most increases oscillated between ~20% and ~200%. The levels of TNF-α in the overweight subgroups ranged from <1.0 pg/mL up to 30 pg/mL, and in obese participants, from <1.0 pg/mL up to 294 pg/mL. In these two groups, however, most values oscillated between ≈3.0 and 6.5 pg/mL in overweight individuals and between ≈1.0 and 10 pg/mL in the obese subjects (Table 1). Participants categorized as very obese also exhibited highly variable levels of TNF-α ranging from ~1.0 pg/mL up to values as high as 713 pg/mL (Table 1). Further, the combination of being overweight or obesity with an additional disorder such as Type 2 Diabetes mellitus (T2D), Metabolic Syndrome (MetS) or Polycystic Ovary Syndrome (PCOS) did not appear to critically modify the levels of this cytokine with values also varying between <1.0 pg/mL up to 20 pg/mL (Table S1).

The overall scenario regarding the circulatory levels of TNF-α in humans shows a large intra- and between studies variability with several hundred- and, even up to several thousand-fold differences within the values attributed to each specific group of individuals. Many of the studies included in this section reported an increase in the levels of this cytokine in the overweight/obese sample populations as compared to the lean ones, and the general message conveyed is that there is an association between increased body weight and increased levels of the cytokine. Yet, looking at all the data gathered here, it was not possible to designate clear-cut ranges of TNF-α values that differentiated between lean, overweight and obese individuals, and/or to discern any clear tendency between TNF-α levels and body weight. The majority of the reported values for circulating levels of TNF-α ranged between ~1.0 and 10.0 pg/mL, with apparent independence of the body weight category posing some reasonable doubts on the potentiality of TNF-α as a biomarker to differentiate between body weight conditions.

3. Human Intervention Studies Looking at the Effects of Foods and Derived Products on the Levels of TNF-α

The most common, accessible, and first-line options for the treatment of body weight excess are behavioural changes which consist principally of adopting a healthier lifestyle, including a change in eating habits towards a healthier diet (e.g., low-calorie diet (LCD), Mediterranean diet (MD)) [46,47]. General recommendations against obesity include the specific reduction in the consumption of saturated fats and carbohydrates as well as the increased intake of plant foods. Many epidemiological and experimental studies have shown that the consumption of colourful fruits and green vegetables, which are rich in a natural blend of bioactive constituents, i.e., vitamins, fibre, phytochemicals, can improve the metabolic profile and reduce the risk of developing chronic diseases [48]. Therefore, consumption of fruits and vegetables has become a cornerstone of most dietary recommendations aimed at healthy body weight management [49]. Additionally, supplementation of the diet with a variety of those bioactive constituents including specific (micro)nutrients such as polyunsaturated fatty acids (PUFAs), vitamins, minerals, amino acids, fibre, and (or) phytochemicals ((poly)phenols, carotenoids, terpenoids, etc.) has also been widely investigated for potential metabolic and body weight benefits. These bioactive compounds have been mostly administered as (semi)purified mixed extracts, or as the constituents of enriched foods and beverages. In the following sub-sections, we have tabulated, described, and critically examined the results of a series of intervention studies (mostly randomized clinical trials (RCTs) with parallel or crossover designs) set up to investigate the effects of different foods and derived products to modulate body weight, with a focus on the TNF-α changes observed in response to dietary treatment. For each study and where it was not

reported, we have calculated the intra-group CV% and the effect size (described as the net difference between the effect in the treatment (T) and control (C) groups).

3.1. Dietary Interventions with PUFAs

Dietary fatty acids (FAs) constitute essential nutrients and an important source of energy, having a considerable range of biological functions including a modulatory role of metabolism and immune responses and inflammation [50]. Depending on their structure and degree of saturation, the different FAs can influence various aspects of human metabolic physiology, such as affecting the feeling of satiety [51] or regulating blood lipids and inflammation [52]. While saturated FAs have been considered detrimental to health, monounsaturated (MUFAs) and PUFAs, of which fatty fish are particularly rich, appear to offer potential health benefits [53]. In particular, ω-3 PUFAs, such as the eicosapentaenoic acid (EPA) and the docosahexaenoic acid (DHA), have been reported to have cardiometabolic and anti-inflammatory properties [50,54]. These (micro)nutrients could help in the treatment and prevention of body weight excess and associated inflammatory conditions and disorders. Cell culture and animal models of obesity [55] have postulated a range of potential mechanisms by which ω-3 PUFAs may exert anti-obesogenic effects, such as the promotion of mitochondrial biogenesis and fatty acid-β oxidation [56], the inhibition of adipocyte differentiation, and (or) the induction of apoptosis in pre-adipocytes [57]. Importantly, these FAs have also been shown to reduce the secretion of inflammatory cytokines, including Monocyte Chemoattractant Protein-1 (MCP-1), Interleukin-6 (IL-6), and TNF-α, in human adipose tissue as well as in mature adipocytes challenged with pro-inflammatory molecules [58]. These results have motivated the study of the effects of the consumption of ω-3 PUFAs on human body weight and body composition as part of a weight-loss strategy [59]. Nevertheless, the current evidence in adults [60,61] and adolescents [62] remains weak and contradictory.

In this section we reviewed a series of intervention trials in which overweight/obese participants received supplements containing ω-3 PUFAs, and the changes in body weight and TNF-α levels were investigated [63–71] (Table S2). These PUFAs were administered in the form of encapsulated oil to adults of both sexes [63,66,67], except for two trials that were performed on teenagers [64,65]. The intervention period lasted from ≈30 d to 180 d. The placebo (PLA) group consisted of vegetable oils such as sunflower oil, corn oil (both rich in linoleic acid), soybean oil [63,66], medium-chain triglycerides [64] or starch [65]. No other lifestyle modifications were reported, except for one study in which the subjects followed a very LCD (in both the control and experimental groups) [67]. The administered doses of ω-3 PUFAs ranged from ≈500 to 4000 mg/d, and the circulatory levels of TNF-α were mostly determined by measuring protein concentration in serum or plasma using an ELISA assay [63–70]. All these studies displayed a considerable within-group variability with some studies reaching CV% values well above 30%. Baseline levels of circulating TNF-α varied from ≈1.0 to 26.0 pg/mL. Following exposure to the ω-3 PUFAs, the modulation of those levels (calculated as the difference between the changes observed in the T and C groups, i.e., before and after intervention) ranged from a small (NS) reduction in −0.02 pg/mL [63] to an (NS) increase of +10.9 pg/mL [66]. Two studies reported significant reductions in TNF-α. In the study conducted by Lopez-Alarcon et al. [65] this reduction was not supported by numerical data, whereas only Dangardt et al. [64] reported a very significant (p = 0.008) reduction of −0.50 pg/mL. Regarding body weight changes, none of the studies showed significant (S) effects from the supplementation with ω-3 PUFAs. In the study by de Luis et al. [67] that tested the effects of the combination of DHA with a LCD, a significant reduction in body weight was observed both in the experimental and control groups, with no significant difference from the supplementation with DHA.

The results of the supplementation with several foods or oils enriched in PUFAs [68–71] are also presented in Table S2. The nutritious seeds of chia (*Salvia hispanica* L.) have been attributed metabolic and immune-regulatory properties partly due to their high content of PUFAs [72]. An intervention trial conducted on overweight/obese participants daily supple-

mented with 50 g of chia seeds showed, however, (NS) effects on body weight and TNF-α levels (small increase of +0.11 pg/mL both in men and in women) after 84 days of intervention [68]. Similarly, daily intervention with 40 g of flaxseeds, another important vegetal source of PUFAs and, in particular, of ω-3 PUFAs [72,73], did not show any significant alteration in body weight or in the levels of the cytokine (−0.2 pg/mL) in obese participants ([69]. Black cumin (*Nigella sativa* L.) oil, also a rich source of PUFAs [74], was tested in Iranian obese women (3 g/d, 56 d) with no effects on body weight. In this study, however, the authors reported a (S) reduction in the levels of TNF-α (−6.3 pg/mL, $p = 0.03$) [70]. Baru almonds (*Dipteryx alata* Vog., legume seeds) also constitute a rich source of PUFAs [75], and were investigated for their regulatory metabolic and anti-inflammatory effects in Brazilian overweight/obese women. Following 56 days of supplementation with 20 g of nuts per day, (NS) changes in body weight were reported and TNF-α was only slightly and not significantly increased (+0.7 pg/mL) [71].

3.2. Vitamin D

Vitamin-D (VitD) occurs naturally as cholecalciferol or vitamin D3, and ergosterol or vitamin D2. In humans, the quantitatively most important source of vitamin D3 is that synthesized in the skin by the sun's ultraviolet rays. Natural dietary sources of vitamin D2 are some fungi (sun-dried mushrooms) and yeast, whereas vitamin D3 can be found in salmon, fatty fish, fish liver oil, organs (e.g., liver, kidneys), meat and egg yolks. Since only few foods contain high levels of VitD, fortification of different food products such as margarine, milk and cereals with VitD is common in many countries. Furthermore, the use of oral supplements is widely recommended in order to prevent deficiencies. The recommended intake of VitD is 10 μg/d (400 IU/d). [76]. VitD plays an essential role in calcium and phosphorus homeostasis and bone health. Since this vitamin binds the vitamin D receptor (VDR), thereby triggering an effect on gene transcription and protein synthesis in many different cells and tissues, this hormone also functions as a wide-range regulator of many cellular homeostasis-, immunity-, and metabolism-related processes. For these reasons, VitD has also been broadly investigated for its role in diseases like cancer, cardiovascular disease (CVD), diabetes and inflammatory diseases [76,77]. Overall, the deficiency of VitD has been associated with some of those diseases but supplementation of the vitamin is not yet clearly related to a beneficial outcome [76]. VitD is largely stored in the adipose tissue, where this vitamin exerts an important regulatory role in the adipocyte's physiology [78]. Consistent with this, in vitro studies in human and mouse adipocyte cell cultures have shown that the VitD regulates adipose tissue differentiation and growth through multiple mechanisms, including inhibition of pre-adipocyte differentiation, inhibition of FA synthesis, reduction in lipid accumulation in vacuoles, and induction of pre-adipocyte apoptosis [79–81]. With regard to the pro-inflammatory phenotype developed by hypertrophic and hyperplastic adipose tissue, studies on human differentiated adipocytes have shown that VitD is also able to inhibit the pro-inflammatory signalling pathways activated by TNF-α and lipopolysaccharide (LPS) [82–85].

In humans, low plasma concentrations of VitD (hypovitaminosis D) have been consistently reported in overweight/obese people [86], and thus the effects of the supplementation with this vitamin on body weight have been repeatedly investigated. A recent meta-analysis examining the benefits of VitD on various measures of adiposity in healthy overweight and obese adults indicated a lack of effect on BMI, waist circumference (WC), and waist-to-hip ratio. Regarding the pro-inflammatory phenotype associated with overweight/obese individuals, the results of the supplementation with VitD were also unclear [87]. While BMI has been causally associated with several inflammatory markers (including TNF-α), mediation analyses performed to establish causality between serum VitD and serum inflammatory markers indicated no role for this vitamin as a causal mediator between BMI and systemic inflammation. The evidence of the beneficial effects of VitD as a therapeutic agent to improve inflammation in overweight and obese subjects remains poor [88,89].

We revised the results of several intervention trials in which overweight/obese adults of both sexes were treated with VitD, and changes both in body weight and levels of TNF-α were investigated [90–94] (Table S3). The intervention period varied from 56 to 365 d and the comparison was made against a PLA group which mostly consisted of vegetable oils, e.g., soy oil or a VitD–free oil (Migliol oil) [90,92], or microcrystalline cellulose [91], with no other lifestyle changes. The dose of VitD administered was between 3000 and 4000 IU/d (0.075–0.1 mg/d). In one study [94], the VitD was first given as a unique bolus of 100,000 IU/d (2.5 mg/d) followed by 4000 IU/d. TNF-α was determined mostly by measuring protein concentration in serum using an ELISA assay with baseline average levels ranging from 1.5 to 29.6 pg/mL. The results of these studies showed high within-group variability, with most CV values in the range of >30% to >100%. Reported changes in TNF-α levels following intervention were (NS) increases or decreases with values ranging from −3.5 pg/mL to +0.8 pg/mL, and only one study declared a (S) but rather small reduction in the TNF-α levels (−0.58 pg/mL, $p < 0.05$) [90]. Consistent with previous studies [87], the trials reviewed here corroborated a lack of significant effects of VitD on body weight. Overall, the data analysed in this section show no indication of the role of VitD as an anti-inflammatory agent and in controlling body weight in overweight/obese individuals.

3.3. Mixed (Micro)nutrients

A series of trials that investigated the effects on body weight and TNF-α in overweight/obese participants following intervention with other bioactive (micro)nutrients have been examined [95–100] (Table S4). The studies were primarily conducted in mixed populations of men adult men and women, with duration periods between 28 d and 90 d. The levels of protein were measured using an ELISA assay in serum [96–100], except for one study where the levels of TNF-α were measured in plasma [95]. The CV% values ranged from <10% in some cases up to 40–50% in several groups. The data regarding the modulation of the levels of TNF-α were variable and the effect of the different treatments in comparison with the PLA yielded only (NS) results ranging from a reduction of −56 pg/mL with the mixture of leucine and vitamin B6 (NuFit) [95] to an increase of +0.61 pg/mL with the black soy peptide [97]. Intervention with a NuFit active blend (leucine and vitamin B6) [95], L-arginine [96], zinc [98], and hydrolysed cod protein [100] did not show any reducing effects on body weight. Whereas, body weight was significantly reduced following the intake of black soy peptide [97], and WC was also reduced with the intake of yeast β-glucan [99].

3.4. Phytochemicals

Phytochemicals cluster a large group of organic non-essential secondary metabolites with rich structural diversity produced principally, but not exclusively, by plants. (Poly)phenols constitute one of the largest and more heterogeneous families of these naturally occurring organic compounds and are characterized by a reactive composition of aromatic rings and one or several phenolic hydroxyl groups [101]. Depending on their chemical structure, (poly)phenols are classified as flavonoids (flavones, flavonols, flavanols, flavanones, isoflavones, anthocyanins) and non-flavonoids (hydrolysable tannins, lignans, stilbenes, phenolic acids) [102]. The main dietary sources of bioactive phytochemicals, and, in particular, of (poly)phenols, are fruits (e.g., apples, grapes, pomegranates, berries, plums, etc.), vegetables (tomatoes, olives, onions, garlic, peppers, cabbage, carrots, etc.), legumes (black beans, black soybeans, etc.), cereals (black rice, rye, black sorghum, purple barley, red sorghum, etc.), chocolate, extra virgin olive oil, and beverages like green tea, black tea, red wine, and coffee [103,104].

A variety of polyphenol-containing products (extracts, freeze-dried powders, foods) have been investigated for a wide range of potential health benefits, including body weight and inflammation regulatory properties. Cell and animal models of obesity have provided extensive supportive evidence of the potential mechanisms by which many of these compounds may exert their activity against obesity, including the inhibition of adipocyte

differentiation and proliferation, reduced fat absorption, increased energy expenditure, and increased fat utilization [105]. In addition, an increasing number of intervention studies and various meta-analyses have built up the evidence of the metabolic benefits of the intake of these compounds in humans. In particular, the consumption of ellagitannins-, anthocyanins-, and flavanol-containing tea, cocoa, and apple products have been significantly associated with the reduction in BMI and WC [106,107]. In a more recent meta-analysis, it was reaffirmed that supplementation with anthocyanins (300 mg/d, 28 d) was associated with the reduction in BMI, however, body weight and WC remained unaffected [108]. Regarding the anti-inflammatory properties attributed to the (poly)phenols, there is also much pre-clinical evidence of the downregulation by many of these compounds of both the TNF-α gene expression and protein levels [109,110]. In humans, a meta-analysis examining the effects of anthocyanins on various plasma lipids and inflammatory markers highlighted the ability of these (poly)phenols to selectively modulate TNF-α levels [111]. In a more recent systematic review of human studies looking at the regulatory effects of polyphenol-containing products in post-menopausal women, it was shown that the reduction in the levels of TNF-α was, however, highly variable and inconsistent [112]. It is now well established that the large interindividual variability in response to dietary (poly)phenols poses a substantial degree of uncertainty regarding the consistency and magnitude of the metabolic and inflammatory effects that these compounds may exert in humans. The factors influencing this variability are under thorough investigation, including the complex collaboration between (poly)phenols and gut microflora. A double interaction between them, i.e., the effects of the gut bacteria on the metabolism of (poly)phenols, and the effects of (poly)phenols and resulting metabolites on the gut microbioma composition, are being explored as a major influence on the regulation of metabolic and inflammatory processes and biomarkers, including TNF-α [113].

In this section, we examined the changes in the levels of TNF-α in overweight and obese adults of both sexes following supplementation with different phytochemical-containing products from different sources [114–131] (Table S5). The first part of this table groups a series of studies where the tested products were mixed extracts or powders rich in different (poly)phenols [114–127]. The intervention period varied from ≈20 d up to 360 d, and the comparison was usually made against a PLA group with no other lifestyle modifications, with the exception of one study in which subjects in the control and experimental groups also followed a hypocaloric diet [123]. Regarding TNF-α, the circulating levels of the protein were principally analysed in serum or plasma using an ELISA test. The baseline values were highly variable and oscillated from ≈0.1 [124] to 43.01 pg/mL [127]. Once more, the results of these trials showed high intragroup variability with most CV% values above 30%, reaching values >100% in many cases. Some of the studies reported small and not statistically significant changes in the circulating levels of TNF-α (ranging between −2.6 and +2.6 pg/mL) following treatment with quercetin [114], curcuminoids [115], cocoa extract [117], grape extract (with or without resveratrol) [118,119], grape powder [120,121], and black soybean testa extract [127]. On the other hand, the levels of TNF-α were reported to be significantly decreased following intervention with curcumin (−3.5 pg/mL) or nano-curcumin (−4.8 pg/mL) [116], grape pomace extract (−1.37 pg/mL) [122], grape seed extract (−11.9 pg/mL) [123], pomegranate peel extract (−0.05 pg/mL) [124], freeze-dried strawberries (−2.7 pg/mL) [125], and frozen red raspberries (−11.0 pg/mL *post-pandrial*, −2.1 pg/mL, 28 d) [126]. Most of the studies investigated here did not show or report a (S) reduction in body weight after treatment with the exception of the study by Parandoosh et al. [123] that reported a significant higher reduction in body weight, BMI, WC, and waist-to-hip ratio in the grape seed extract group (300 mg/d containing 85% (poly)phenols) compared to the PLA group. This reduction was not confirmed in any of the other studies conducted with a polyphenol-containing grape product [118–122].

Other mixed products (extracts containing phytochemicals) have also been tested in overweight/obese individuals (Table S5), i.e., 6 capsules/d of Fruit + Vegetables concentrate powder (containing β-carotene, α-tocopherol, vitamin C, folate, (poly)phenols) [128],

6 capsules/d of Juice Plus+ Premium (2.91 mg carotene, 18.7 mg Vitamin E, 159 mg Vitamin C, 318 μg folate, 6.1 mg lutein, 1 mg lycopene and 0.15 mg astaxanthin) [129], 6 mg/d melatonin [130] and garlic tablets (1000 mg/d containing 2.5 mg of allicin) [131]. Melatonin had a (S) down-regulatory effect on the circulating levels of TNF-α protein (−0.98 pg/mL, $p = 0.02$) [130]. Also, the intake of the Fruit + Vegetable concentrate powder was reported to cause a significant but rather small reduction in TNF-α (−0.07 pg/mL, $p = 0.035$) but only in a small subgroup of participants with C-reactive protein (CRP) values ≥3.0 mg/mL [128]. None of the studies were associated with a body weight-reducing effect.

In addition to those studies conducted with phytochemical-containing capsules, extracts or powders, we have collected some examples of dietary intervention with different foods rich in mixed bioactive (poly)phenols and other phytochemicals which have also investigated for their effects on body weight and TNF-α levels [132–136] (Table S6). Interventions were carried out with the pulp from the Brazilian juçara fruit (rich in phenolics and flavonoids) [132], Queen Garnet plums (rich in anthocyanins and quercetin) [133], tart cherry juice (rich in anthocyanins, flavonoids and phenolics) [134], tomato juice (mixed carotenoids, phytosterols, phenolics, flavonoids, anthocyanins) [135], and red sorghum biscuit (mixed carotenoids, phytosterols, phenolics, flavonoids) [136]. All the studies were also conducted in obese/overweight adults of both sexes [132–136]. The intervention period varied from 20 to 84 d and the comparison was always against a PLA with no other lifestyle modifications, except for the study conducted with red sorghum biscuits where the participants from both T and C group were on a LCD [136]. TNF-α protein levels were analysed in serum or plasma [133–135] or in the supernatant of peripheral blood mononuclear cells (PBMCs) [132] using an ELISA assay. The results of these studies showed high intragroup variability, with CV% values up to 100%. The consumption of tart cherry juice or the sorghum biscuits was associated with similar and rather small (NS) reductions of circulating TNF-α (−0.21 pg/mL and −0.3 pg/mL, respectively) [134,136]. On the other hand, following intervention with the Queen Garnet plums, there was a small but (S) reduction in the levels of TNF-α protein (−2.0 pg/mL, $p < 0.05$) [133]. Supplementation with tomato juice also significantly reduced TNF-α in the entire sample population of overweight/obese participants (−8.3 pg/mL, $p < 0.05$) and in the subgroup of only overweight individuals (−5.3 pg/mL, $p < 0.05$) whereas in the obese participants a large but (NS) increase of TNF-α levels was observed (+21.3 pg/mL) [135]. None of the studies reported a (S) reduction in body weight.

4. The Influence of Genetic Variants on TNF-α Levels

As already seen in the previous sections, the circulatory levels of TNF-α show a large variability which may be partly due to a substantial genetic contribution to TNF-α regulation. Nonetheless, the consequences of the presence of different genetic variants on the transcription and final levels of TNF-α are complex and not yet understood. Various single nucleotide polymorphisms (SNPs) have been described within the promoter region of the *TNF-α* gene at different positions (e.g., −308 G/A, −1031 T/C, −863 C/A, −857 C/T, −575 G/A, −376 G/A, −244 G/A, −238 G/A) [137]. One of the most investigated polymorphisms is the −308 G/A (rs1800629) which has been associated with higher serum concentrations of TNF-α as well as with increased susceptibility for obesity, cardio-metabolic disorders (IR, T2D, MetS) and some inflammatory and immune-related disorders [138]. Nevertheless, the results are still rather inconsistent. We have reviewed a number of studies reporting the levels of TNF-α in different populations of mixed adults across the different genotypes for some of those variants, predominantly for the −308 G/A [41,42,137,139–144] (Table S7). As repeatedly indicated in the previous sections, we found a very high intra-subgroup (genotype) variability with most CV% values well above 30% and in many cases >100%. The levels of TNF-α in the homozygous reference subgroups were also very variable with values oscillating between some very low levels, e.g., 0.095 pg/mL, up to values as high as ≈60 pg/mL corroborating large differences between studies. Overall, the differences across the genotypes were rather small, highly variable and mostly (NS) (values ranging from a minimum reduction

of −6.0 pg/mL up to a maximum increase of +43.0 pg/mL although most data ranged between −0.8 and +0.5 pg/mL). We only found two studies that reported significant increased levels of TNF-α associated with the presence of the rare variant, in overweight MetS patients (p = 0.001, +38.0 pg/mL) [42], and in patients with abdominal aortic aneurism (p = 0.045, +50.1 pg/mL) [144]. Our revision suggests that the presence of the −308 G/A does not appear to have a general increasing effect on the levels of TNF-α.

We additionally reviewed a number of studies reporting the levels of TNF-α across the different genotypes for other variants associated with different genes related with obesity and metabolic disorders [137,145–158] (Table S8). We also found a very high genotype intra-subgroup variability (CV% >40% in most cases), highly variable levels (0.11–90 pg/mL) in the homozygous reference subgroups, and small and mostly (NS) differences across the genotypes except for some specific SNPs. Like so, the TNF-α levels were (S) reduced in Chinese adults with T2D with the rare variant −87 T > C of *PPARD* ($p < 0.05$, −1.7 and −3.1 pg/mL) [146], in malnourished elderly people with the rare variant of rs769214 of *CAT* ($p < 0.001$, −3.2 and −3.9 pg/mL) [153], in patients with heart failure and with the rare genotype for rs1800471 of *TGFB* (p = 0.042, −7.0 pg/mL) [154], and in healthy individuals with the rare rs2234632 variant of the gene *ZIP2* (p = 0.011, −6.1 pg/mL) [155]. On the other hand, a few other SNPs were found to have more elevated levels of TNF-α in individuals with the rare variant, e.g. FOK1/*VDR* in obese participants (p = 0.01 pg/mL, +7.5 pg/mL) [147], and rs141764639/*TMEM182* in normal and obese participants ($p < 0.05$, +4.5 pg/mL and $p < 0.001$, +13.5 pg/mL, respectively) [157]. These results illustrate the complex, multiple and variable increasing and decreasing effects of different SNPs on the circulating levels of TNF-α.

5. The Regulatory Effects of Anti-Obesity Drugs on TNF-α

Throughout the years, a considerable number of drugs has been developed and tested to try to combat obesity. Overall, in clinical trials, many of those anti-obesity drugs promote moderate body weight reductions (between ≈2.0 to 10.0% after several months of intervention) when compared with lifestyle modifications. Often, those drugs also promote considerable adverse events leading to poor adherence to the treatments [159,160]. Prospective and retrospective studies support the body weight-reducing effects of some of these drugs but the evidence still has considerable gaps [161]. Only a limited number of drugs (e.g., orlistat, naltrexone-bupropion, liraglutide, phentermine-topiramate, semaglutide) are currently approved by the Food and Drug Administration as an alternative therapy to promote weight loss in human adults [162]. We collected and analysed several human studies where the participants were treated with some of these anti-obesity drugs, and changes in body weight and in the levels of TNF-α were reported [29,163–179] (Table S9). Our main aim was to evaluate the relevance and significance of these changes and whether there was a clear association between body weight and TNF-α in response to drug treatment. The studies were primarily conducted in obese/overweight adults of both sexes (mixed) which, in some cases, were also reported to be associated with other disorders such as T2D, hypertension, dyslipidaemia, etc. Some of the investigated trials were carried out using different chemical drugs (orlistat, L-carnitine, sibutramine, diacerein, hydroxychloroquine (HCQ), troglitazone, rosiglitazone, rimonabant, lacidipine, candesartan, metformin (MET) [29,163–175], and various other studies tested the effects of the protein/peptide-like molecules etanercept, exenatide, and liraglutide [176–179]. The drug intervention period varied from ≈60 d up to more than a year, and the comparison was generally done against lifestyle modifications (i.e., LCD) and/or against a widely used reference drug (i.e., MET). Most TNF-α levels were analysed in serum or plasma using the ELISA test. The TNF-α baseline values were again highly variable and oscillated between 0.9 and 132.6 pg/mL. Like in the previous sections, the results of these drug trials also showed substantial intragroup variability with most CV% values going from 9% up to >100% (mostly ranging between 30 and 50%). Except for etanercept, most of the selected studies reported a significant reduction in the circulatory levels of TNF-α both in the T and the C groups.

Orlistat, a pancreatic lipase inhibitor [180], was used in three studies at a dose of 360 mg/d to test the effects on body weight and TNF-α levels in obese participants. Of those, one study suggested a slightly better reducing effect for orlistat + LCD (T) than for LCD alone(C) (−0.5 pg/mL) but the authors did not include sufficient data to support this statement [163]. A second study indicated similar effects in the T group as well as in the C group (−4.2 pg/mL, NS) [165]; only a third study reported significant differences for the drug [164]. In this latter case, the average difference in the levels of TNF-α was ≈−12 pg/mL, and thus, the drug seemed to potentiate the effects of the diet restriction (≈2.5 fold-difference). When orlistat was combined with a second compound, i.e., L-carnitine, the effects on body weight and on TNF-α levels were not improved [166]. The average reduction in the levels of TNF-α with the combined compounds was −0.9 pg/mL (NS).

A similar limited level of evidence was found for sibutramine, a drug that stimulates satiety [181]. One study reported a reduction in body weight and TNF-α with sibutramine + LCD but a C group was not included [167]. Another study reported that two doses of sibutramine (15 mg/d and 10 mg/d) reduced body weight and TNF-α levels. In this study, the highest dose promoted a slightly higher reduction in TNF-α levels than the lower dose (−0.5 pg/mL). Nevertheless, no information on the significance of the differences between the two groups was reported, and it was not addressed whether the reduction in TNF-α was associated with body weight reduction [168]. We found one additional study that reported a significantly better reduction in body weight with sibutramine + LCD. The levels of TNF-α were decreased both in the T and the C group with the sibutramine yielding slightly better but (NS) results (−1.0 pg/mL after 1 y treatment) [169]. Studies on the effects of the other chemical drugs included in this review did not support concomitant and differential effects on body weight and TNF-α for the tested drugs. Reported differences between T and C groups oscillated between –1.1 and +0.8 pg/mL and were (NS) [29,170,171,173]. Other studies did not include sufficient data supporting TNF-α reduction [172,174]. The study by Amoani et al. [175] supported a higher and (S) decrease of the cytokine (−65 pg/mL) with a higher dose of the drug MET but this was not a proper RCT but a cross-sectional study. Regarding the protein/peptide-like drugs, etacernept, a human recombinant TNF-α inhibitor [182], was reported to be associated with an increase in the levels of TNF-α and there were (NS) differences vs. the C group changes or association with body weight [176]. Drugs of the glucagon-like peptide 1 type such as exenatide and liraglutide [177–179] did not show (S) and/or associated reductions in body weight and TNF-α levels. For example, treatment with exenatide + MET (T) reduced the levels of TNF-α by ≈−6.0 pg/mL vs. acarbose + MET (C), however, this difference was (NS) [178].

Overall, the data included in this section show weak evidence of the reducing effects on body weight and TNF-α of the tested anti-obesity drugs. The reported changes in TNF-α levels ('effect size') were small and mostly (NS). It was not possible to infer that any drug or drug-diet combination reduced the levels of TNF-α consistently and significantly, in association with body weight loss nor that the drugs were more effective than diet restriction.

6. General Discussion

Healthy foods and/or bioactive constituents have been long attributed with many benefits and, in particular, with anti-obesity and anti-inflammatory effects, mostly supported by many cell and animal studies reporting the reduction in the levels (gene and/or protein expression) of related biomarkers such as the pro-inflammatory cytokine TNF-α [109,110]. Despite the increasing number of intervention trials performed in this research area, proving these properties in humans remains challenging. In multiple meta-analyses and reviews looking at the beneficial effects of dietary bioactive constituents in humans, the overall recurrent messages remain the same, i.e., 'the heterogeneity in the trial design including differences in the sample population, analytical procedures and data assessment leads to inconsistent results and increases the difficulty in interpreting those effects', 'interindividual variability in the responses to diet precludes the full understanding and establishment

of its efficacy and true health benefits' [106,107,112,113,183–185]. In the present review, we have explored the situation in a series of human intervention studies in which changes in the levels of TNF-α were investigated in response to different dietary approaches. Our aim was to contribute to the understanding of the current state of these studies and to highlight and reinforce the procedures that must be implemented in future intervention trials looking at the modulation of this important cytokine as a potential biomarker of the inflammatory response to diet. During the completion of the present article, another review looking at the effects of phytochemicals and herbal bio-active compounds on TNF-α in overweight and obese individuals was released [183]. The authors also collected and examined a number of clinical trials reporting changes in TNF-α in response to different foods and products containing mixed bioactive compounds. They concluded that there were some products with promising effects but they also found that the results were not uniform and that nearly 60% of the selected studies reported a lack of effect. In the present review, we have revisited some of those studies as well as others to critically examine the specific reported changes in the levels of TNF-α in response to dietary treatment in overweight and/or obese participants. A summary of the different intervention studies examined is presented in Table 2, where we recapitulate the data and assess the variability (CV%) of the TNF-α levels as well as the reported absolute effects (size, direction and significance). We also revise some of the main features of the trials (group sample size, source and doses of test product, intervention duration, analytical method) and the reported findings on body weight modulation for further discussion. Also, we explore the influence of a range of SNPs on the levels and variability of TNF-α.

Table 2. Summary of the results from the human intervention studies gathered in this review looking at the effects of different dietary products and several anti-obesity drugs on the circulatory levels of TNF-α in overweight/obese participants in relationship with body weight management.

	Study Characteristics				**TNF-α Levels, Variability, Change**			
Dietary approach	Number of RCTs (*N*: range of size per arm in the RCTs)	Doses (range) Duration (range)	Population phenotype	Samples Method	Baseline levels (range)	Intragroup variability (CV% range)	Effect description: Average difference T–C (pg/mL range) Significance (S/NS)	Effect description: Overall reported body weight message
(Micro)nutrients								
ω-3 PUFAs (EPA, DHA; oils capsules)	5 RCTs (*11*, *49*)	(500, 4000 mg/d) (30, 180 d)	Overweight/ Obese/ Obese (IR, T2D)	Serum, plasma ELISA	(1.09, 25.8 pg/mL)	(13%, 59%)	−0.5 (S) (−0.02, +10.9) (NS)	(NS) effects on body weight.
PUFAs (foods)	4 RCTs (7, *45*)	(3, 50 g/d) (56, 84 d)	Overweight/ Obese/ Obese (glucose intolerant)	Serum, plasma ELISA, Milliplex	(0.6, 24.0 pg/mL)	(5%, 86%)	−6.3 (S) (−0.2, +0.7) (NS)	(NS) effects on body weight.
Vitamins (e.g., Vitamin D)	5 RCTs (*10*, *83*)	(0.075, 0.1 mg/d) (56, 365 d)	Overweight/ Obese/ Obese (IR)	Serum, ELISA, Automated immunoassay	(1.7, 39.1 pg/mL)	(7%, >100%)	−0.6 (S) (−3.5, +5.9) (NS)	(NS) effects on body weight.
Other mixed (micro)nutrients (amino acids, peptides, minerals, complex polysaccharides, etc.)	6 RCTs (*10*, *35*)	(30 mg/d, 9 g/d) (28, 90 d)	Overweight/ Obese/ Obese (MetS)	Serum, plasma ELISA	(0.22, 411 pg/mL)	(5%, 47%)	(−56, +0.61) (NS)	(S) effects on body weight only with black soy peptide. (S) WC reduction only with yeast β-glucan

Table 2. *Cont.*

Study Characteristics					TNF-α Levels, Variability, Change			
Phytochemicals								
Mix extracts, powders rich in (poly)phenols	15 RCTs (*8*, *93*)	(15, 600 mg/d) (21, 360 d)	Overweight/ Obese/ Overweight/ obese (cardiac, CAD, T2D, MetS, hypertension, knee OA)	Serum, plasma ELISA, Chemiluminesceimmunoassay	(0.13, 43.0 pg/mL)	(4%, >100%)	(−0.05, −11.9) (S) (−2.6, +2.6) (NS)	(S) effects on body weight only with a grape seed extract (85% (poly)phenols).
Mix extracts, powders rich in other phytochemicals	4 RCTs (*16*, *92*)	Variable quantities (mg/d) (40, 112 d)	Mixed Overweight/ Obese/ Overweight/ obese (knee OA)	Serum, plasma ELISA	(1.04, 62.8 pg/mL)	(4%, >100%)	(−0.07, −0.98) (S) (−0.21, +14.0) (NS)	(NS) effects on body weight.
Foods/beverages containing mixed phytochemicals	5 RCTs (*10*, *53*)	(5, 45 g/d; 250, 330 mL/d) (20, 84 d)	Overweight/ obese Overweight/ obese (hypertension)	Serum, plasma ELISA	(2.9, 73.2 pg/mL)	(12%, 100%)	(−2.0, −8.3) (S) (−0.21, +21.3) (NS)	(NS) effects on body weight.
Anti-obesity drugs								
Chemical drugs	11 RCTs (*7*, *190*)	(2, 2000 mg/d) (56, 570 d)	Obese Obese (T2D, hypertension, dyslipidaemia) Overweight/ obese (IR, T2D, PCOS, hypertension)	Serum, plasma ELISA, Bioplex, EIA	(2.94, 132,6 pg/mL)	(9%, >100%)	(−12.1, −65.0) (S) (−4.2, +0.8) (NS)	Unclear effects on body weight and no association with TNF-α.
Protein/peptide drugs	4 RCTs (*12*, *54*)	(µg to mg/d) (s.c.i.) (84, 180 d)	Overweight/ Obese/ Overweight/ obese (CAD, T2D)	Serum, plasma ELISA	(0.9, 34.6 pg/mL)	(36%, >100%)	ND (−6.0) (NS)	Unclear effects on body weight and no association with TNF-α.

CV% = (Standard Deviation/Mean) × 100; CAD: coronary artery diseases; IR: insulin resistance; MetS: Metabolic Syndrome; PCOS: Polycystic Ovary Syndrome; T2D: Type 2 Diabetes mellitus; OA: Osteoarthritis.

Overall, the results of our analysis show that: (i) many of the estimated intra-group CV% values were well above 30% and, in some cases, reached values ≥100%; (ii) the circulating levels of TNF-α were highly variable and ranged from less than 1.0 pg/mL up to some examples of several hundred pg/mL; (iii) the reported changes in this cytokine following intervention with the different dietary approaches (effect) included both statistically (S) and (NS) decreases or increases of the protein with independence of the size effect, which ranged from −0.05 pg/mL up to +21.3 pg/mL. Within this intricate scenario of high intra-group and between-studies variability it was not possible to: (1) propose clear limits for the circulating levels of TNF-α as a biomarker to differentiate between lean, overweight, and obese individuals, (2) discern distinct down-regulatory effects of the levels of this cytokine by any of the different dietary approaches investigated (PUFAs, Vitamin-D, mixed (micro)nutrients, (poly)phenols or other phytochemicals), and (3) perceive what an effect-size (response) of this cytokine to dietary changes might be. In addition, we found that in most of the revised trials there was not a body weight response (body weight reduction) following intervention, nor a clear association between the body weight and the TNF-α responses. In comparison with the dietary approaches, the studies revised here looking at the effects of different anti-obesity chemical drugs and proteins showed similarities in terms of trials design and variability of the results. In addition, they showed a lack of clear response of the cytokine and of an association with body weight reduction, giving consistent evidence of the difficulty in understanding TNF-α as a responsive biomarker. Nutritional and pharmacological research should go hand-in-hand to further understand variability in the response to treatment, and to move towards more

personalized anti-obesity and anti-inflammatory treatments. In the next paragraphs, we discuss some of the factors that contribute to the observed variability, and propose specific recommendations to boost the quality of future intervention trials looking at TNF-α as a biomarker of anti-inflammatory response in relationship with body weight management.

A first critical issue is 'sample size'. It has been repeatedly indicated that 'given the variability of the results and the generally small size effects observed in dietary interventions with bioactive constituents, larger clinical trials are needed' [106,107,112,113,183–185]. Indeed, it is widely acknowledged that larger group sizes will improve the validity of the results. However, it is unclear how large they need to be to investigate changes in the levels of TNF-α? Taking into consideration the results of our revision and assuming a large overall intra-group variability CV% > 100% (i.e., 150%) and a medium size effect (d = 0.5), we estimated that the sample size should be ≈35 subjects per group (p-value < 0.05, 80% power) [186,187]. For a small size effect (d = 0.2), the sample size would increase up to 220 participants per arm. With a few exceptions, most of the studies investigated here were carried out in groups of less than 30 participants and, in some cases, of even less than 10 participants. Despite the well-known difficulties of recruiting and maintaining a large number of participants for this kind of intervention study, we recommend that future intervention dietary studies looking at the regulation of the levels of TNF-α in overweight/obese people must make an effort to increase the number of participants and to incorporate at least a minimum of 35 subjects in the T and C groups. Another highly variable feature regarding intervention trials looking at the beneficial effects of dietary bioactive (micro)nutrients and (or) phytochemicals is that related to the 'duration of intervention'. It has been indicated that the greatest number of trials investigating the effects of mixed (poly)phenols have been designed as 'chronic' to look at the long term effects of these products [185]. However, the duration of these 'chronic' studies is also very variable. The reasons that lead the investigators to choose different extension periods may be various (e.g., resources availability; having a limited period of time to reduce possible toxicity, side effects or the rate of participants quitting the study; the effect might be observed quite soon; or, just the opposite, the effects might be seen in the long term, etc.). We found that the time period in the studies investigated here ranged from a minimum of 20 d up to ≈1 y, making the comparison between studies difficult. Although it is not a trivial issue to decide which intervention period is appropriate, we suggest that normalizing the intervention period to, for example, an average intermediate time of ≈6 months may provide a good opportunity to detect effects on TNF-α levels as well as to facilitate a later comparative revision or meta-analysis of those studies under review.

Intervention studies looking at the health benefits of the consumption of dietary bioactive constituents have been most commonly conducted when using as the 'test products' readily available mixtures of compounds in the form of enriched extracts, oils, foods, beverages. In the particular case of (poly)phenols, the greatest numbers of studies have been performed using (poly)phenol-rich foods or (poly)phenol-rich extracts, mainly those from berries, grapes, cocoa, soy, olive oil, pomegranate, flaxseed, nuts, tea or red wine [185]. The intervention studies gathered in this review looking at the response of circulating TNF-α to intervention with bioactive products have also been mostly conducted using heterogeneous mixtures of compounds (PUFAs, vitamins, minerals, proteins, phytochemicals, (poly)phenols) in different forms and doses, all of which increase the variability between studies as well as the difficulty in ascribing a particular result (effect) to a particular compound or type of compounds. This is additionally aggravated by the need to compare the T groups to appropriate C groups. Many studies report this C group as the one taking a PLA which is not always fully characterized and, sometimes, is even a different type of product, e.g., PUFAs compared to sunflower oil [63], corn starch [65] or corn oil [66]. The comparison between a similar mixture of bioactive compounds that only differentiates in the presence or concentration of the specific test compound (s) is a better control protocol that has been reported in some cases [23,118,119], but it is not an approach that can be easily and frequently implemented. Further, in comparison with drug

testing (typically performed with chemically synthesized single pure molecules), not many individual pure natural bioactive compounds have been validated in humans for their effects against obesity and inflammatory conditions. For example, resveratrol is a natural bioactive (poly)phenol widely investigated for multiple health benefits including weight loss in obese individuals as well as for its anti-inflammatory properties. In a very recent report, a number of studies looking at the effects of resveratrol in obesity were critically assessed, with the conclusion reached that the results were still inconsistent due to the study design heterogeneity including major differences in many factors (sex, age, BMI, health conditions, doses, source, intervention period, etc.) [184], some of which are also further discussed and reinforced here. The authors already proposed the need to establish standardized guidelines to improve the depositing of relevant information in future trials and best practices for selecting studies that will be better used in future metanalyses. We herein reinforce the need to improve future intervention trials looking at the effects of the intake of bioactive foods and derived products on TNF-α and, for this purpose, we also propose further standardization of the study design. Along these lines, it may be worth focusing first on some individual pure test bioactive compounds such as the well-known and widely investigated resveratrol, curcumin, quercetin, or (epi)catechin, and try to definitively show evidence of their anti-inflammatory and anti-obesity effects in a specific subgroup of individuals, i.e., 'responders'. The next step would be to replicate those results and to prove whether the compound retains its properties within an extract or a food.

It has also been recently shown that the results of the analyses of several cytokines, including TNF-α, were different across various different assays tested (singleplex, multiplex, ELISA). The authors also showed that the enzyme-linked immunosorbent ELISA assay displayed high precision and sensitivity. However, there was not a good correlation between the serum and plasma samples analysed using this method [188]. In the studies investigated in our review, the most common analytical procedure used to detect and quantify the circulating levels of TNF-α was an ELISA immunoassay (from different commercial providers) although a few other methods were also applied (multiplex, automated immuno-assay, chemiluminescence-immunoassay). The analyses were performed indistinctly in plasma or serum samples. Since the variability in the performance across different analytical platforms or in different type of samples can also greatly contribute to a lack of agreement in the reported levels of the inflammatory biomarkers, it remains essential that further investigations in this area and, in particular, in the case of TNF-α, are carried out to provide investigators with a most suitable and unique reference analytical protocol to minimise differences across studies.

The baseline characterization of the individuals taking part in a particular trial, so that at the point of departure the features of the participants are as homogeneous as possible, is an essential issue not yet fully resolved in studies looking at the beneficial properties of dietary bioactive compounds. It is still rather common to find studies carried out in mixed sample populations in terms of sex, age, body weight and/or disease status. However, each of these features constitute a well-recognised factor contributing to variability. Detailed phenotyping of trial participants for as many as possible of those potentially modulating factors has been already recommended [189]. In our revision, we focused on dietary intervention trials that investigated the potential reduction in body weight and of the levels of TNF-α; thus, most of the studies were conducted in individuals with body weight excess. Nevertheless, we found that the participants were often a mixed group of overweight and/or obese individuals and that the health status was not always clearly established or that, in some cases, the participants were additionally characterised by other metabolic or inflammatory disorders (i.e., T2D, MetS, IR, hypertension, etc.). The presence of these and other disorders can clearly contribute to the large variability in the levels of TNF-α and in the variability of the changes in this cytokine in response to the dietary intervention. In a recent meta-analysis, the values of TNF-α have been reported to vary from ≈0.4 to ≈300 pg/mL in healthy adults and from ≈0.1 to nearly 1000 pg/mL in adults suffering

from Obstructive Sleep Apnea Syndrome (OSAS). These authors also reported an interaction between the disease and the BMI of the patients [190]. The data collected here regarding the different TNF-α levels between lean, overweight and obese individuals corroborate a very broad range of values and support the evidence of a large variability in the levels of this cytokine and of the difficulty in establishing clear differences between different phenotypes. The message of a thorough characterization and definition of the sample population to be investigated must be reinforced and pursued in future studies. This is particularly important when looking at individuals with body weight excess, where a much-improved definition and focus on a particular group or sub-group must be stated and supported by stricter ranges of BMI and of additional cardiometabolic health criteria. Additionally, and not always clearly specified in all the studies, the consumption of other chemicals and drugs must be clearly stated. This is especially important when looking at the differences in the levels of TNF-α between healthy subjects and overweigh/obese individuals with associated pathologies.

The presence of different SNPs in the regions involved in the regulation of the transcription of a gene might affect gene expression and protein levels, and eventually influence the circulating levels of such protein and constitute a potential associated risk to develop a disease [191]. In the case of TNF-α, many different SNPs, and in particular the rs1800629, have been long investigated for their association with the susceptibility to develop different diseases, including infections, cancer, inflammatory diseases, obesity and cardiometabolic disorders, with, however, varied and inconclusive outcomes [138]. On the other hand, there are not many studies investigating the circulating levels of the protein across the different genotypes for each polymorphism. In our review, we have gathered a number of those studies reporting the influence of different SNPs on the levels of circulating TNF-α. Overall, we corroborated a very broad range of values reported for this cytokine. Also, the effects of most of the investigated SNPs appear to be, in general, small, (NS) and highly variable. Only a few of the rare variants were associated with some (S) increases or decreases of the cytokine in specific subpopulations. Although this revision compiles only a limited number of studies and variants, it illustrates the complexity and difficulty of deciphering the overall effects of our genetic make-up on the levels of TNF-α as well as the variability attributable to this factor.

Even more complex and difficult is demonstrating the influence of genetic variants on the response to dietary intervention. Studies looking at the interaction between genotype and the intake of dietary bioactive (micro)nutrients on TNF-α are scarce and heterogeneous. In the study conducted by Curti et al. [192], the authors investigated the effect of lifestyle and dietary advice on the cytokine across the genotypes for two SNPs (TNF-α −308 G/A and IL6 −174 G/C). They reported downregulatory effects of the intervention on the cytokine but no differences between the reference and the rare variants. Similarly, de Luis et al. [139] found no differences in the levels of TNF-α across the TNF-α −308 G/A genotypes, following intervention with a diet rich in MUFAs or with a diet richer in PUFAs [69]. In a more recent intervention study with mixed vegetables containing two doses of folate, the authors reported a larger and more significant reduction in the levels of TNF-α (−5.12 pg/mL, $p = 0.0004$) only with the highest dose in the subgroup of overweight/obese women with the TT genotype for the *MTHFR* C677T polymorphism [145]. These results suggest some influence of the genotype on the response of TNF-α to the intake of folate and exemplifies a simple approach that tries to elucidate the interaction between one SNP and the dietary intake of a particular test product. Nevertheless, the situation is a lot more intricate, with so many SNPs potentially affecting the levels of TNF-α. A different and more complex approach is one that examines the effects of multiple SNPs. In a recent review [193], the interaction between 91 SNPs and the intake of fat, carbohydrates and protein on body weight loss concluded that the majority of those interactions were (NS) and thus the evidence was inconclusive. These results show once again the difficulty in finding and demonstrating the interaction between genetic variants and diet. Research on the combined effects of different SNPs on TNF-α levels and of the response of this

important cytokine to intervention with dietary foods or products with anti-obesity and anti-inflammatory bioactivity is still in its infancy and requires further investigation. Some of the strategies that need to be implemented to advance the understanding of the effects of genetic variants in the human responses to dietary intervention (nutrigenetics) have been already highlighted [113]. With a combination of in vitro and animal studies, as well as molecular and genomics approaches, we need to identify the proteins and genes that are involved in the mechanisms of response as well as the list of SNPs that can affect the expression and regulation of those genes. Next, the effects of all these candidate SNPs on the levels of TNF-α in response to diet need to be evaluated in well-characterised populations carrying the different variants and in sufficiently powered trials.

One final and additional point that we would like to reinforce here is the need for and importance of increasing the quality of the data presentation [112]. While reviewing all the articles included here, we came across very different ways of data presentation with, often, important missing information. In the specific case of TNF-α, we propose that reporting the results should be normalized to the same most common units (pg/mL) and the inclusion of all the results from the intervention (control, treatment, pre-, post-, effect size, mean ± SD, CV%, *p*-values for all comparison). Requesting common protocols of reporting results will importantly contribute to the improvement of future comparative studies and the understanding of the overall outcomes.

7. Conclusions

TNF-α has been widely investigated in preclinical studies looking at the metabolic and anti-inflammatory benefits of a wide range of foods and bioactive food constituents yet its validation in human trials remains unsolved. A large variability in the circulating levels of the protein as well as in the response of this important cytokine to dietary intervention is partly attributable to a range of factors related with the design of the studies but also with human intrinsic causes. One such factor is the presence of a complex mixture of genetic variants that may positively and negatively influence the production of TNF-α. Improving and normalizing the study design and incorporating the genotyping of an increasing number of genetic variants into the intervention trials will contribute to understanding the large interindividual variability of the cytokine as well as to improving the comparative analyses of multiple results from different studies, increasing the evidence and validity of TNF-α as a biomarker of response to diet.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods11162524/s1, Table S1. Reported differences in the circulatory levels of TNF-α between lean, overweight and obese individuals; Table S2. Reported changes in the circulatory levels of human TNF-α following dietary intervention with PUFAs-containing foods and products; Table S3. Reported changes in the circulatory levels of human TNF-α following dietary intervention with VitD supplements; Table S4. Reported changes in the circulatory levels of human TNF-α following dietary intervention with products containing mixed bioactive (micro)nutrients; Table S5. Reported changes in the circulatory levels of human TNF-α following dietary intervention with different products containing (poly)phenols and other phytochemicals; Table S6. Reported changes in the circulatory levels of human TNF-α following dietary intervention with foods rich in mixed bioactive phytochemicals, Table S7. Circulating levels of TNF-α across different genotypes for various SNPs located within the regulatory region of the TNFα gene; Table S8. Circulating levels of TNF-α across different genotypes for different SNPs located in various genes related to obesity, inflammatory and metabolic disorders; Table S9. Reported changes on the circulatory levels of human TNF-α following intervention with specific drugs in relation with obesity treatment.

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