International Journal of
Molecular Sciences

Special Issue Reprint

Therapeutic Potential of Antioxidants in the Prevention of Human Diseases

Edited by
Kota V. Ramana

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Guest Editor

Kota V. Ramana

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Guest Editor
Kota V. Ramana
Department of Biomedical
Sciences
Noorda College of
Osteopathic Medicine
Provo
USA

Editorial Office
MDPI AG
Grosspeteranlage 5
4052 Basel, Switzerland

This is a reprint of the Special Issue, published open access by the journal *International Journal of Molecular Sciences* (ISSN 1422-0067), freely accessible at: https://www.mdpi.com/journal/ijms/special_issues/ELF3HU7Y27.

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

Lastname, A.A.; Lastname, B.B. Article Title. *Journal Name* **Year**, *Volume Number*, Page Range.

ISBN 978-3-7258-4669-6 (Hbk)
ISBN 978-3-7258-4670-2 (PDF)
https://doi.org/10.3390/books978-3-7258-4670-2

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About the Editor

Kota V. Ramana

Kota V. Ramana is a Professor at Department of Biomedical Sciences, Noorda College of Osteopathic Medicine, Provo, UT-USA. He is also an adjunct professor at Roseman University of Health Sciences. The chief objectives of his lab investigations are to examine the involvement of cellular metabolism and oxidative stress signals in inflammation. He uses various genetic, biochemical, and cell biological approaches to analyze inflammatory responses regulated by cellular lipid metabolites leading to secondary diabetic complications and cancer. He has published more than 140 peer-reviewed articles. He is also serving as an editorial board member of several journals.

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Editorial

The Therapeutic Potential of Antioxidants in the Prevention of Human Diseases

Kota V. Ramana

Department of Biomedical Sciences, Noorda College of Osteopathic Medicine, Provo, UT 84606, USA; karamana@noordacom.org

The recent surge in various chronic infectious and non-infectious diseases worldwide has created an urgent need for safe and effective prevention and treatment options to control and manage these diseases. For example, although the recent COVID-19 pandemic is now well under control, the emergence of various new variants still requires vigorous investigations and the development of new therapeutic strategies [1]. Increased oxidative stress has been associated with the pathogenesis of a wide array of human disorders, including cardiovascular diseases, cancer, neurodegenerative conditions, diabetes, and inflammatory disorders. Oxidative stress occurs due to an imbalance between the production of reactive oxygen species (ROS) and cellular antioxidant defense mechanisms. An increased number of ROS can damage cellular components such as DNA, proteins, and lipids. ROS can also alter various cellular metabolic and inflammatory signaling pathways involved in the pathophysiology of various diseases.

Several anti-inflammatory and anti-oxidative agents have shown potential in controlling and managing human disease complications. In the past, natural medical practices such as Ayurveda, Unani, and traditional Chinese medicine have been used, employing various plant-based treatments to control human diseases without knowing their molecular mechanisms of action. However, studies from the past few decades and recent ongoing studies have shown that natural antioxidants derived from various plants have the capacity to neutralize oxidative stress and the inflammatory signaling pathways responsible for multiple pathologies. Antioxidants, both endogenous, such as glutathione, superoxide dismutase, and catalase, and exogenous, such as vitamins and plant polyphenols, could play a critical role in maintaining redox homeostasis and protecting tissues from oxidative injury.

Further, several preclinical and clinical studies have identified the therapeutic potential of antioxidants [2–5]. The dietary intake of antioxidant-rich foods has been associated with a reduced risk of chronic diseases. Moreover, targeted antioxidant therapies using curcumin [6], benfotiamine [7], quercetin [8], and other polyphenols [9] have shown promising results in improving disease outcomes in animal models, and some of these agents have undergone clinical studies for multiple disease pathologies. Some studies have also investigated the use of antioxidants as adjunct therapies to improve drug resistance and the efficacy of the treatment, particularly in various cancer chemotherapies.

Despite recent advances, the translation of antioxidant therapy to the clinical setting remains complex and needs further investigation. The efficacy of antioxidants can depend on their dosage and bioavailability and the disease context. Further, the unnecessary and or excessive intake of antioxidants or vitamins could disrupt the physiological redox balance and redox signaling. This leads to increased innate immune and inflammatory responses and unintended consequences. Therefore, a better understanding of how antioxidants regulate redox signaling is critical for their therapeutic development.

The studies published in this Special Issue include a combination of cutting-edge research articles and comprehensive review articles to explore the role of antioxidants in disease prevention and treatment. From molecular mechanisms to clinical applications, these articles offer insights into how antioxidant strategies can mitigate oxidative stress and improve human health.

The first study by Jang et al. explores the potential of herbal medicine as a treatment for postmenopausal osteoporosis using multi-scale network and random-walk-based analyses. By prioritizing herbs based on their protein target relevance to osteoporosis, they have identified promising herb candidates for modulating various protein targets relevant to osteoporosis, such as *Benincasae Semen*, *Glehniae Radix*, and *Houttuyniae Herba*. This study also identified active compounds like falcarindiol and tetrahydrocoptisine, which could regulate inflammation and bone metabolism pathways, providing new insights into herbal strategies for managing osteoporosis.

The study by Jovanovic et al. evaluated the antidiabetic potential of xanthone-rich extracts from *Gentiana dinarica* and *Gentiana utriculosa plants.* These extracts were analyzed for their secondary metabolite content and assessed for their antioxidant activity, α-glucosidase inhibition, and anti-hyperglycemic effects in glucose-loaded Wistar rats. The findings from this study indicated that extracts with higher concentrations of specific xanthones, norswertianin, and decussatin derivatives exhibited the most substantial potential to control blood glucose levels and scavenge free radicals, thus supporting their therapeutic relevance to diabetes management. Further, the study by Fonseca et al. investigated the antifungal potential of caffeine against *Trichophyton mentagrophytes*, a common cause of skin infections. Caffeine exhibited significant anti-dermatophyte activity with a minimum inhibitory concentration (MIC) of 8 mM, disrupting the fungal cell wall's components and ultrastructure and inducing autophagic-like changes. Caffeine also protects human keratinocytes during infection and inhibits fungal spore germination. These results suggest that caffeine is a potential therapeutic and preventive agent for dermatophytosis.

Another article by Hernandez et al. examined the effects of adding Coenzyme Q10 (CoQ10) to an antioxidant supplement (Nutrof Total®, Thea Pharmaceuticals, Keele, UK) on human retinal cells under oxidative stress. The combined supplement (NQ) showed synergistic benefits by controlling oxidative stress and mitochondrial dysfunction. This study thus suggests that CoQ10 could be a promising therapeutic strategy for degenerative retinal diseases such as age-related macular degeneration and diabetic retinopathy.

The randomized crossover clinical trial study by Lambadiari et al. has reported on the effects of the Mediterranean (MD) and Ketogenic (KD) diets on patients with psoriasis (PSO) and psoriatic arthritis (PSA) over 22 weeks. Both diets significantly reduced weight, body mass index, waist circumference, total fat, and visceral fat. However, only the KD showed significant improvements in disease activity and inflammatory markers, suggesting it may be more effective in managing PSO and PSA symptoms.

Another interesting study by Sandoval et al. evaluated the effects of β-carotene on the pancreas of C57BL/6 mice administered with moderate amounts of ethanol. Mice exposed to moderate-dose alcohol who were treated with β-carotene showed significantly higher amylase and lower lipase levels and notable differences in pancreatic fibrosis and islet structure across groups. These results suggest that antioxidant treatments like β-carotene may help to mitigate ethanol-induced pancreatic tissue damage.

The review article by Frost et al. explored the significance of water-soluble B vitamins in cancer progression and prevention. The authors discussed the role of B vitamins in cancer, where they can support tumor suppression and, in some cases, promote cancer progression by influencing metabolic pathways like glycolysis and mitochondrial function. The authors also emphasized the need for further studies to better understand how to

balance B vitamin intake to optimize cancer prevention and treatment outcomes. Flavonoids are known for their anti-inflammatory and antioxidant properties. Flavonoids have shown promise in improving liver health and modulating gut microbiota, a key factor in metabolic-associated fatty liver disease (MAFLD) progression. Another review by Sokal-Dembowska et al. discussed the current evidence on how specific flavonoids may help to prevent or slow MAFLD. Further, they elaborated on how flavonoids could be used in personalized dietary strategies for managing liver diseases. Finally, Jaiswal et al. discussed the various pharmacological activities of *Leopoldia comosa* (LC), a Mediterranean plant traditionally used in food and medicine. The authors briefly discussed LC's multiple biological activities, such as its antioxidative, anti-inflammatory, and anti-carcinogenic effects, and outlined the gaps in the research into LC's pharmacological effects and its therapeutic development.

Although recent growing evidence supports the therapeutic efficacy of natural antioxidants in the prevention and management of various human diseases, there are some significant gaps that need further investigation. One major gap is the inconsistency between preclinical in vitro or animal model findings when compared to human clinical outcomes. These studies sometimes show differences in bioavailability, dosage, and long-term effects. Additional clinical studies are necessary to address the variability in genetics, metabolism, and microbiota, which can influence antioxidant efficacy and safety profiles. Further, identifying standardized methodologies to evaluate antioxidant activity and limited large-scale, long-term clinical trials to confirm their preventive benefits across diverse populations could help advance antioxidant treatments to the clinical setting. Furthermore, novel targeted antioxidant delivery and personalized nutrition strategies could also help establish interventions for human diseases.

Acknowledgments: I sincerely acknowledge all authors and reviewers whose valuable contributions made this Special Issue successful. I also acknowledge the outstanding support of the journal's editorial staff.

Conflicts of Interest: The author declares no conflicts of interest.

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International Journal of
Molecular Sciences

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Article

Identifying Herbal Candidates and Active Ingredients Against Postmenopausal Osteoporosis Using Biased Random Walk on a Multiscale Network

Boyun Jang [1], Youngsoo Kim [1], Jungbin Song [2], Young-Woo Kim [3,*] and Won-Yung Lee [4,5,*]

[1] IntegroMediLab Co., Ltd., Seoul 04626, Republic of Korea
[2] Department of Herbal Pharmacology, College of Korean Medicine, Kyung Hee University, Seoul 02447, Republic of Korea
[3] School of Korean Medicine, Dongguk University, Gyeongju 38066, Republic of Korea
[4] School of Korean Medicine, Wonkwang University, Iksan 54538, Republic of Korea
[5] Research Center of Traditional Korean Medicine, Wonkwang University, Iksan 54538, Republic of Korea
* Correspondence: ywk@dongguk.ac.kr (Y.-W.K.); wonyung21@wku.ac.kr (W.-Y.L.)

Abstract: Postmenopausal osteoporosis is a major global health concern, particularly affecting aging women, and necessitates innovative treatment options. Herbal medicine, with its multi-compound, multi-target characteristics, offers a promising approach for complex diseases. In this study, we applied multiscale network and random walk-based analyses to identify candidate herbs and their active ingredients for postmenopausal osteoporosis, focusing on their underlying mechanisms. A dataset of medicinal herbs, their active ingredients, and protein targets was compiled, and diffusion profiles were calculated to assess the propagation effects. Through correlation analysis, we prioritized herbs based on their relevance to osteoporosis, identifying the top candidates like Benincasae Semen, Glehniae Radix, Corydalis Tuber, and Houttuyniae Herba. Gene Set Enrichment Analysis (GSEA) revealed that the 49 core protein targets of these herbs were significantly associated with pathways related to inflammation, osteoclast differentiation, and estrogen metabolism. Notably, compounds such as falcarindiol from Glehniae Radix and tetrahydrocoptisine from Corydalis Tuber—previously unstudied for osteoporosis—were predicted to interact with inflammation-related proteins, including IL6, IL1B, and TNF, affecting key biological processes like apoptosis and cell proliferation. This study advances the understanding of herbal therapies for osteoporosis and offers a framework for discovering novel therapeutic agents.

Keywords: postmenopausal osteoporosis; herbs; active ingredients; multiscale network

1. Introduction

Postmenopausal osteoporosis has become a critical global health concern, particularly within aging female populations, where incidence rates continue to rise. This condition, characterized by reduced bone mass and compromised bone microarchitecture, leads to diminished bone strength and an increased risk of fractures. Recent studies have indicated a prevalence of osteoporosis of approximately 23% in women and 11.7% in men, underscoring its disproportionate impact on postmenopausal women [1]. In these women, the sharp decline in estrogen levels disrupts bone remodeling processes by influencing various physiological mechanisms that sustain bone homeostasis. Estrogen deficiency stimulates osteoclast differentiation and activity, accelerating bone resorption to levels that exceed bone formation, thereby decreasing bone density, deteriorating bone structure, and heightening fracture susceptibility [2]. Although the current postmenopausal osteoporosis treatments, including antiresorptive agents like bisphosphonates and selective estrogen receptor modulators (SERMs), and anabolic agents, such as parathyroid hormone (PTH) and teriparatide, provide mechanisms to control bone loss and stimulate bone formation, they

exhibit limitations. Efficacy can vary across patient profiles, and these therapies may fail to adequately lower fracture risks in advanced osteoporosis cases. Additionally, long-term bisphosphonate use is associated with potential adverse effects, such as gastrointestinal issues, osteonecrosis of the jaw (ONJ), and atypical fractures, which can impede patient adherence [3–5]. Furthermore, the underdiagnosis and undertreatment of osteoporosis in postmenopausal women highlight a significant gap in disease management, necessitating the development of safer and more effective therapeutic strategies to improve long-term outcomes [6].

Herbal medicine offers therapeutic efficacy through its multi-compound composition at low concentrations, allowing for interactions with multiple targets across various biological pathways. This multi-target approach is particularly promising for managing postmenopausal osteoporosis, a complex disorder influenced by hormonal changes and multiple physiological factors. Studies have shown that medicinal herbs and their active compounds can improve bone mineral density and stimulate bone regeneration, highlighting their potential as therapeutic options [7,8]. For instance, clinical trials administering Cimicifugae Rhizoma to postmenopausal women demonstrated reduced bone resorption and increased bone formation without significant side effects [9–11]. Additionally, herbs with known anti-osteoporotic properties, such as Sambuci Lignum and Salviae Miltiorrhizae, along with their extracts or active compounds, have effectively prevented bone loss in ovariectomized osteoporosis animal models. These benefits appear to be mediated through mechanisms that promote osteoblast differentiation, regulate osteoclastogenesis, and inhibit collagen degradation [12–15]. A recent review article further highlighted that diospongins, isolated from Dioscorea spongiosa, exhibit promising biological activities supportive of bone health, including potential anti-osteoporotic effects [16]. Collectively, these findings underscore the therapeutic potential of medicinal herbs for postmenopausal osteoporosis, supporting their development as safe and novel treatment options.

Herbal medicine has long been widely used to manage a range of symptoms and diseases. However, the lack of understanding regarding its molecular mechanisms in the human body limits its development and broader application. Advances in systems biology tools, however, have enabled deeper insights into the mode of action of herbal medicines, particularly for herbs with multi-compound, multi-target properties [17]. Network pharmacology has been instrumental in mapping the complex interactions within biological networks, identifying rational drug targets, and providing a comprehensive overview of disease treatment through interconnected biological pathways. For example, network pharmacology has been used to elucidate the mechanisms of action of Mori Folium (dried leaves of *Morus alba* L.) in diabetes and to identify potential herbal antidepressants, facilitating the selection of an optimal herbal combination for further experimental validation [18,19]. Additionally, the recently proposed multiscale interactome framework, which integrates biological functions and physical protein–protein interactions, provides a robust platform for predicting a drug's therapeutic potential on specific disease targets and mechanisms. This approach also enables the exploration of new therapeutic applications for existing drugs and supports the identification of active ingredients and the discovery of novel drug candidates [20,21].

In this study, we applied a network-based approach to identify the candidate herbs and their active ingredients with potential benefits for postmenopausal osteoporosis and to elucidate their potential mechanisms of action (Figure 1). To achieve this, we compiled medicinal herbs, their active ingredients, and associated protein targets. We then calculated diffusion profiles using biased random walks for both herb-specific and disease-related protein targets. By comparing diffusion profiles between the herbs and postmenopausal osteoporosis, we prioritized herbs with high correlation scores, indicating their potential efficacy against the condition, and identified core protein targets and biological functions involved in the proposed treatment. We further investigated the top 10 ranked herbs, assessing the available evidence to support their effectiveness and identify the novel candidate herbs not yet reported for this condition. We further calculated the propagation

effects of the individual ingredients of the candidate herbs, prioritizing those with high correlation scores for postmenopausal osteoporosis, and explored the mechanisms of these active ingredients within the multiscale network. Our approach demonstrates the potential of multiscale network analysis for the discovery of novel therapeutic herbs and ingredients and provides a foundation for future research into their therapeutic mechanisms against postmenopausal osteoporosis.

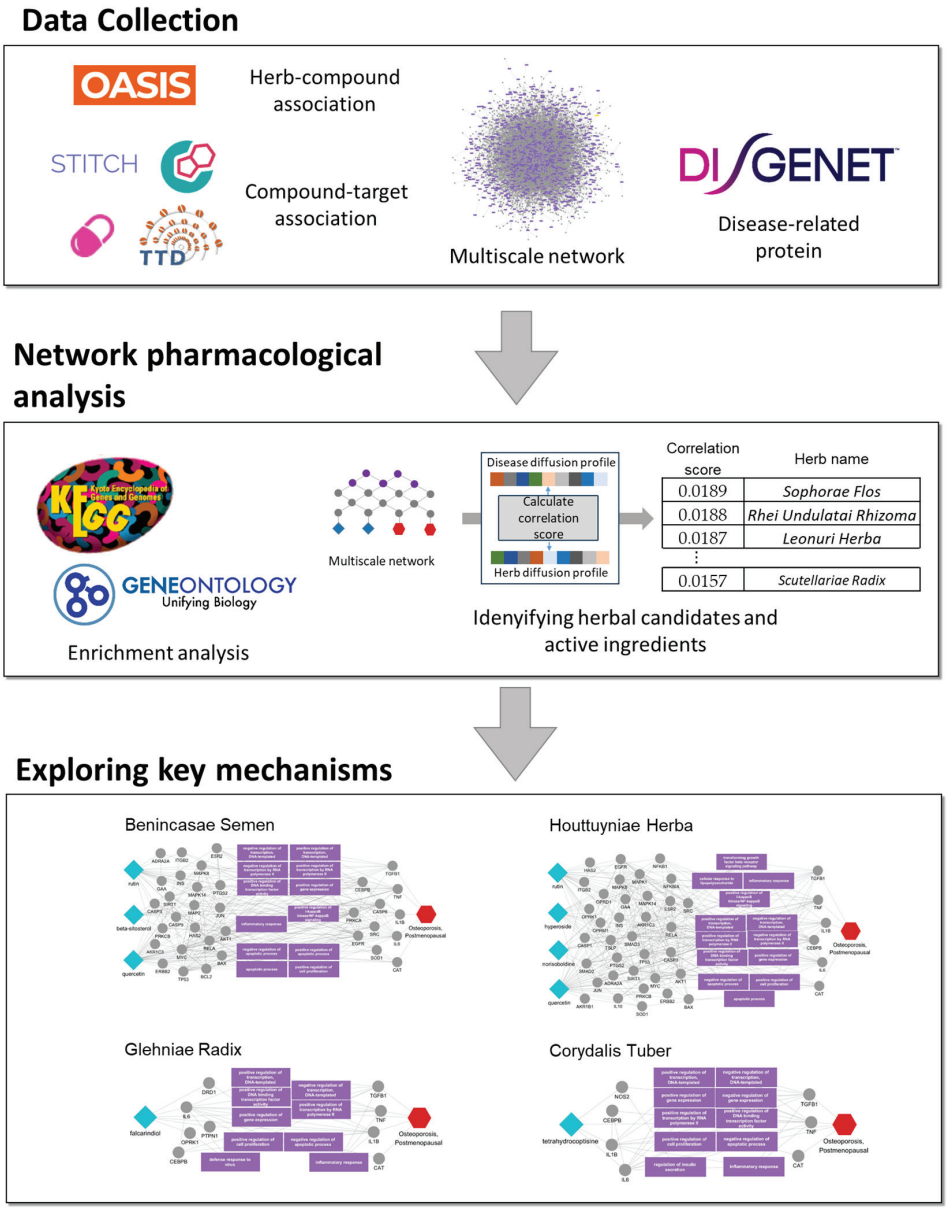

Figure 1. Schematics for identifying candidate herbs and active ingredients for postmenopausal osteoporosis. This schematic illustrates the use of multiscale network analysis to identify herbs and active ingredients with potential efficacy against postmenopausal osteoporosis. Herb–compound and compound–target associations were mapped, and disease-related proteins were identified. Diffusion profiles for herbs and disease proteins were calculated and compared, prioritizing herbs with high correlation scores. Enrichment analysis revealed key biological pathways, while individual ingredients of top herbs were further analyzed to highlight core protein targets. The bottom panel shows network diagrams for selected herbs, indicating relevant protein targets and pathways.

2. Results

2.1. Identification of Potential Candidate Herbs Against Postmenopausal Osteoporosis

To identify the herbs that are potentially effective against postmenopausal osteoporosis, we first collected herb–ingredient data from the OASIS database. Protein targets associated with these ingredients were then retrieved from validated sources, including DrugBank, TTD, and STITCH. Using this herb target data, postmenopausal osteoporosis-related targets, as well as proteins and biological functions within the multiscale network, we applied a biased random walk algorithm to calculate diffusion profiles. Correlation scores, indicating the similarity between the diffusion profiles of the herb and postmenopausal osteoporosis, were subsequently calculated. Herbs with high correlation scores were identified as promising candidates for postmenopausal osteoporosis treatment. Additionally, a hypergeometric test was conducted to assess the degree of protein overlap.

Herbs with a high correlation score and a significant association (p-value < 0.05) with disease-related proteins were prioritized. Among these, the top 10 ranked herbs were selected, specifically those with five or more active ingredients significantly associated with disease-related proteins. An enrichment value of five or higher was also confirmed between these herbs and protein targets related to postmenopausal osteoporosis, indicating that the multiscale network-based prediction model successfully identified targets closely linked to the disease. The results showed that Sophorae Flos had the highest correlation score (0.0189), followed by Rhei Undulatai Rhizoma (0.0188), Leonuri Herba (0.0187), and Benincasae Semen (0.0186), Schizonepetae Spica (0.0174) and Glehniae Radix (0.0173), each exhibiting a high correlation coefficient (Table 1).

Table 1. Top 10 ranked herbs identified strong correlation with postmenopausal osteoporosis.

Herb Name (Latin)	Correlation Score	Overlap (p-Value $^{\#}$)	Enrichment	References (PMID)
Sophorae Flos	0.0189	5/50 (3.51×10^{-11})	118.36	29058425, 39104339
Rhei Undulatai Rhizoma	0.0188	5/50 (3.51×10^{-11})	118.36	29693149
Leonuri Herba	0.0187	5/50 (3.51×10^{-11})	118.36	31524244, 33708763, 30224063, 31328430
Benincasae Semen *	0.0186	5/50 (3.51×10^{-11})	118.36	-
Schizonepetae Spica	0.0174	3/31 (1.29×10^{-6})	114.54	27550314
Glehniae Radix *	0.0173	3/30 (1.17×10^{-6})	118.36	-
Cnidi Fructus	0.0163	4/50 (2.24×10^{-8})	94.69	38507853, 31081953
Anemarrhenae Rhizoma	0.0162	4/48 (1.89×10^{-8})	98.63	16723092, 30272269
Corydalis Tuber *	0.0160	3/34 (1.72×10^{-6})	104.44	-
Houttuyniae Herba *	0.0157	4/50 (2.24×10^{-8})	94.69	-

The $^{\#}$ symbol next to the p-value indicates values obtained using the hypergeometric test, applied to evaluate the significance of overlap between datasets. An * next to the herb name marks candidate herbs strongly associated with postmenopausal osteoporosis that have not yet been investigated.

Among the top 10 ranked herbs, Sophorae Flos, Rhei Undulatai Rhizoma, Leonuri Herba, Schizonepetae Spica, Cnidi Fructus, and Anemarrhenae Rhizoma have been previously reported to benefit postmenopausal osteoporosis [22–30], indicating that our predictions effectively align with findings from the earlier studies. For Sophorae Flos, both its extract and the ingredient sophoridine have demonstrated the potential to treat osteoporosis by inhibiting osteoclast differentiation in estrogen-deficient animal models induced by ovariectomy [22,23]. Leonuri Herba, commonly used for female-related conditions, has been shown to support bone health by promoting osteoblast differentiation and inhibiting osteoclast formation [25,26]. Schizonepetae Spica exhibited protective effects in inflammation-induced bone loss models by reducing osteoclast formation and activity through the suppression of Akt and IkB phosphorylation [27]. Research on Cnidi Fructus primarily focused on its anti-osteoporotic properties, with particular emphasis on its key ingredient, osthole, which is considered the most promising compound for further

study [28,29]. In contrast, Benincasae Semen, Glehniae Radix, Corydalis Tuber, and Houttuyniae Herba have limited or no prior evidence supporting their use for postmenopausal osteoporosis treatment. In this study, however, these herbs showed high correlation scores, and significant protein overlap with the postmenopausal osteoporosis diffusion profile. These findings suggest that these herbs could serve as promising novel candidates for therapeutic strategies for postmenopausal osteoporosis.

2.2. Herb–Ingredient–Target Network Construction of the Top 10 Herbs

Subsequently, we constructed and visualized an interaction network to map the relationships among the top 10 ranked herbs, identified as having high correlation scores with postmenopausal osteoporosis, and their respective protein targets using Cytoscape 3.10.2 (Figure 2). This network comprises 443 interactions (edges) between 10 herbs and 210 targets, effectively illustrating the multi-target nature of multi-component herbs and their potential therapeutic effects through complex biological interactions. Of these targets, only 49 proteins were targeted by 3 or more of the 10 herbs, underscoring their potential role as core protein targets. Notably, IL6, IL1B, and NFKB1 were common targets across all 10 herbs, while TNF, MAPK1, RELA, and NOS2 were each targeted by 9 herbs. These findings suggest that these core protein targets have a pivotal role in the mechanisms of action of these herbs and are likely critical in the treatment of postmenopausal osteoporosis.

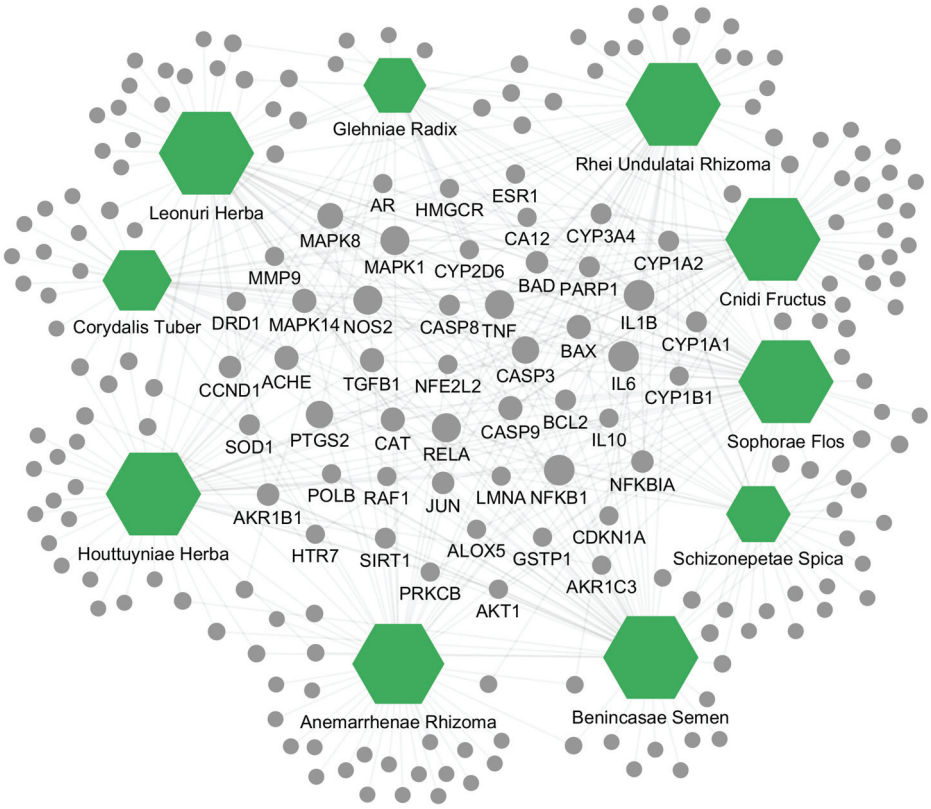

Figure 2. Herb–target interaction network of the top 10 candidate herbs with high correlation scores for postmenopausal osteoporosis. Green hexagons represent herbs, and gray circles represent protein targets. Edges indicate interactions between herbs and targets, with the size of hexagons and circles reflecting interaction frequency (ranging from 1 to 50). Names of the 49 core protein targets, targeted by three or more of the 10 herbs, are displayed.

2.3. Gene Set Enrichment Analysis (GSEA) of the Top 10 Herbs

To further investigate these core protein targets of herbs, we performed Gene Set Enrichment Analysis (GSEA) using KEGG and Gene Ontology to identify the signaling pathways and biological functions associated with the selected top 10 herbs. KEGG

analysis highlighted significant associations with several critical pathways, including the AGE–RAGE signaling pathway, apoptosis, and C-type lectin receptor signaling pathway. Additional pathways related to inflammatory cytokine activation, such as the TNF and IL-17 signaling pathways, as well as osteoclast differentiation, ranked among the top 15 pathways (Table 2). Gene Ontology analysis revealed strong associations with essential biological functions, primarily those involved in the regulation of apoptosis and response to reactive oxygen species. Notably, inflammation-related cellular responses to lipopolysaccharides and lipids also demonstrated significant associations (Figure 3, top). Further analysis of cellular component locations for these core protein targets showed a predominant localization in the mitochondria, nucleus, cytoplasmic vesicles, secretory granules, and peroxisomes, which are organelles critical for cell survival, cellular signal transduction, and bone cell homeostasis (Figure 3, middle). Notably, in the molecular function category of Gene Ontology analysis, estrogen 16-alpha-hydroxylase activity demonstrated the highest combined score, indicating a strong association. This suggests that the core protein targets are significantly involved in estrogen metabolism, a primary factor in postmenopausal osteoporosis, and thus influence osteoporosis progression (Figure 3, bottom). These findings imply that the central role of these core protein targets in regulating molecular signaling pathways and biological functions provides a strong basis for their potential application in therapeutic strategies for postmenopausal osteoporosis.

Table 2. KEGG Signaling Pathway Enrichment Analysis of Core Protein Targets.

Term	Overlap	Adjusted *p*-Value	Combined Score	Genes
AGE-RAGE signaling pathway in diabetic complications	16/100	4.48×10^{-24}	6573.87	*JUN;TGFB1;PRKCB;MAPK14;TNF;RELA;NFKB1;IL6;MAPK8;CCND1; IL1B;CASP3;BCL2;BAX;AKT1;MAPK1*
Apoptosis	17/142	1.79×10^{-23}	4700.81	*JUN;PARP1;BAD;TNF;RELA;NFKB1;NFKBIA; CASP9;MAPK8;CASP8;CASP3;LMNA;BCL2;BAX;AKT1;MAPK1;RAF1*
C-type lectin receptor signaling pathway	15/104	6.15×10^{-22}	5119.68	*IL10;JUN;MAPK14;PTGS2;TNF;RELA;NFKB1;NFKBIA; IL6;MAPK8;CASP8;IL1B;AKT1;MAPK1;RAF1*
TNF signaling pathway	15/112	1.64×10^{-21}	4589.15	*JUN;MAPK14;PTGS2;MMP9;TNF;RELA;NFKB1; NFKBIA;IL6;MAPK8;CASP8;IL1B;CASP3;AKT1;MAPK1*
IL-17 signaling pathway	14/94	8.35×10^{-21}	4863.44	*JUN;MAPK14;PTGS2;MMP9;TNF;RELA;NFKB1; NFKBIA;IL6;MAPK8;CASP8;IL1B;CASP3;MAPK1*
Pathways of neurodegeneration	20/475	3.31×10^{-19}	1325.74	*NOS2;BAD;PRKCB;MAPK14;PTGS2;TNF;RELA;NFKB1;SOD1;CASP9; IL6;MAPK8;CASP8;IL1B;CASP3;CAT;BCL2;BAX;MAPK1;RAF1*
Toll-like receptor signaling pathway	12/104	1.21×10^{-16}	2707.61	*NFKBIA;JUN;IL6;MAPK8;CASP8;IL1B;AKT1; MAPK1;MAPK14;TNF;RELA;NFKB1*
Neurotrophin signaling pathway	12/119	5.66×10^{-16}	2225.41	*NFKBIA;JUN;MAPK8;BAD;BCL2;BAX;AKT1; MAPK1;MAPK14;RAF1;RELA;NFKB1*
Relaxin signaling pathway	12/129	1.44×10^{-15}	1979.36	*NFKBIA;JUN;MAPK8;TGFB1;NOS2;AKT1; MAPK1;MAPK14;RAF1;MMP9;RELA;NFKB1*
FoxO signaling pathway	12/131	1.70×10^{-15}	1935.66	*IL10;IL6;CDKN1A;MAPK8;TGFB1;CCND1; CAT;AKT1;MAPK1;MAPK14;RAF1;SIRT1*
T cell receptor signaling pathway	11/104	6.02×10^{-15}	2130.15	*IL10;NFKBIA;JUN;MAPK8;AKT1;MAPK1; MAPK14;RAF1;TNF;RELA;NFKB1*
Sphingolipid signaling pathway	11/119	2.70×10^{-14}	1751.70	*MAPK8;PRKCB;BCL2;BAX;AKT1; MAPK1;MAPK14;RAF1;TNF;RELA;NFKB1*
Osteoclast differentiation	11/127	5.34×10^{-14}	1593.97	*NFKBIA;JUN;MAPK8;TGFB1;IL1B;AKT1; MAPK1;MAPK14;TNF;RELA;NFKB1*
NOD-like receptor signaling pathway	12/181	7.10×10^{-14}	1209.95	*NFKBIA;JUN;IL6;MAPK8;CASP8;IL1B; BCL2;MAPK1;MAPK14;TNF;RELA;NFKB1*
Prolactin signaling pathway	9/70	3.81×10^{-13}	2208.04	*MAPK8;CCND1;AKT1;MAPK1;MAPK14;RAF1;ESR1;RELA;NFKB1*

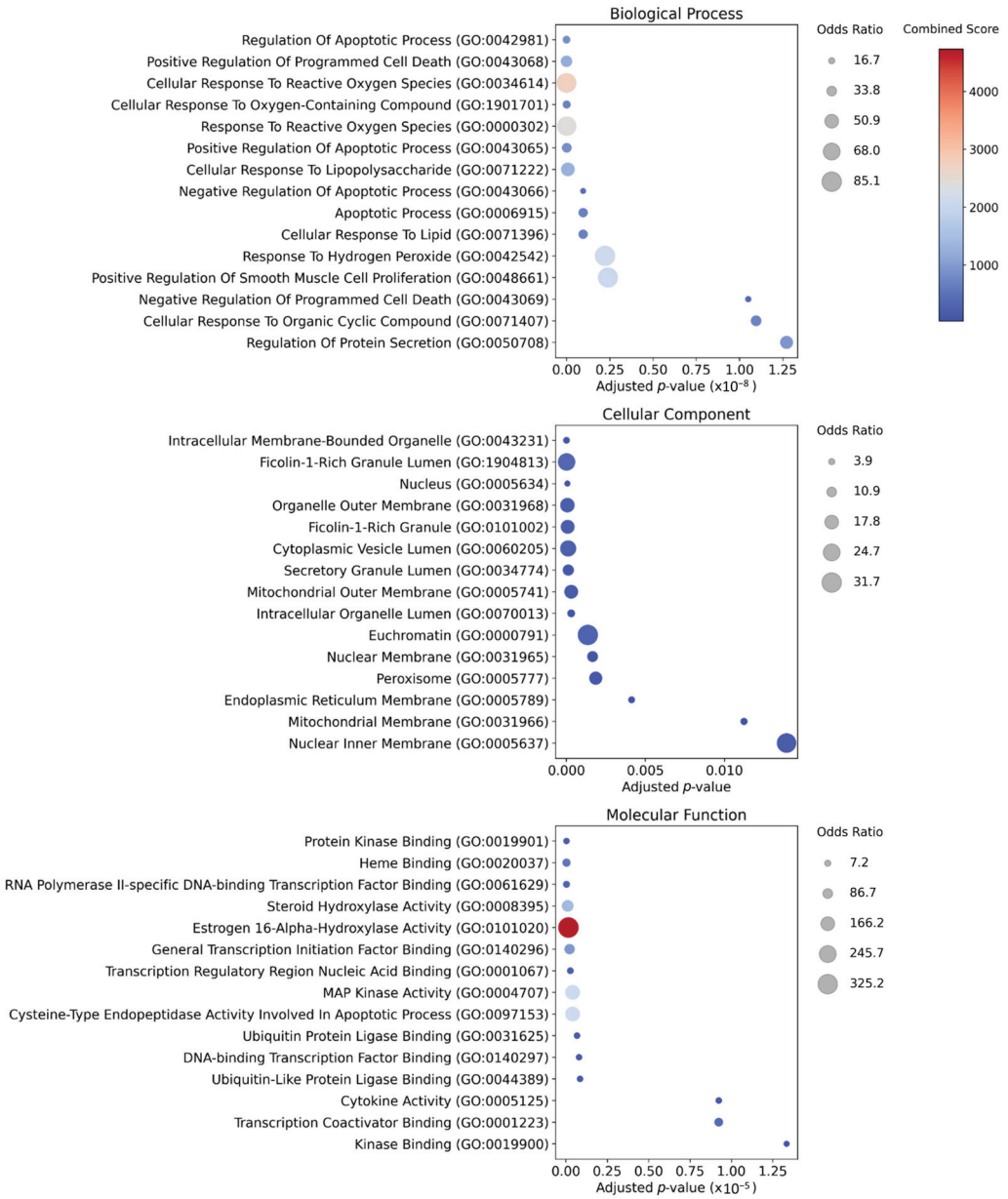

Figure 3. Gene Ontology Enrichment Analysis for Core Protein Targets. Gene Ontology enrichment analysis of the 49 core protein targets across three categories: biological processes (**top**), cellular components (**middle**), and molecular functions (**bottom**). The x-axis represents the adjusted *p*-value (indicating the association significance), bubble size corresponds to the odds ratio, and bubble color reflects the combined score, which indicates the statistical significance of each term. Visualization highlights the most significantly enriched terms across each category.

2.4. Identification of Potential Active Ingredients and Multiscale Network Mechanism Analysis of Candidate Herbs

We performed an additional analysis of the ingredients and multiscale network mechanisms of the candidate herbs, Benincasae Semen, Glehniae Radix, Corydalis Tuber, and Houttuyniae Herba, identified as potential treatments for postmenopausal osteoporosis. For each herb, our objective was to predict therapeutic mechanisms through which their ingredients might influence postmenopausal osteoporosis. Using target data for each ingredient, we applied multiscale network analysis to calculate correlation scores, evaluating each ingredient's potential impact on postmenopausal osteoporosis. Ingredients with high correlation scores and significant protein overlap were prioritized as active ingredients.

This analysis identified rutin (0.0217), beta-sitosterol (0.0161), and quercetin (0.0092) as the active ingredients in Benincasae Semen, each exhibiting high correlation scores with the postmenopausal osteoporosis diffusion profile. In Houttuyniae Herba, norisoboldine (0.0473), hyperoside (0.0261), rutin (0.0217), and quercetin (0.0092) showed significant correlations with postmenopausal osteoporosis-related targets. Glehniae Radix was characterized by falcarindiol (0.0557), and Corydalis Tuber by tetrahydrocoptisine (0.0846), each as the sole ingredient with both high correlation scores and statistically significant protein overlap within the postmenopausal osteoporosis diffusion profile (Table 3).

Table 3. Representative ingredients of candidate herbs and its association with postmenopausal osteoporosis.

Name	Pubchem CID	Correlation Score	Overlap (*p*-Value [#])	References (PMID)
Benincasae Semen				
Rutin	5280805	0.0217	5/45 (3.76×10^{-12})	31737218
β-Sitosterol	222284	0.0161	1/11 (6.62×10^{-3})	35648689
Quercetin	5280343	0.0092	5/424 (3.43×10^{-7})	38240215
Glehniae Radix				
Falcarindiol *	5281148	0.0557	3/8 (5.91×10^{-9})	-
Corydalis Tuber				
Tetrahydrocoptisine *	6770	0.0846	3/5 (1.06×10^{-6})	-
Houttuyniae Herba				
Norisoboldine	14539911	0.0473	3/10 (1.26×10^{-8})	38813717
Hyperoside	5281643	0.0261	3/20 (1.20×10^{-7})	37157916
Rutin	5280805	0.0217	5/45 (3.76×10^{-12})	28485786
Quercetin	5280343	0.0092	5/424 (3.43×10^{-7})	34592982

The [#] denotes *p*-values calculated via the hypergeometric test to assess dataset overlap significance. * indicates active ingredients with strong associations to postmenopausal osteoporosis that remain uninvestigated.

Most of the prioritized active ingredients, particularly those derived from Benincasae Semen and Houttuyniae Herba, have documented therapeutic efficacy in postmenopausal osteoporosis. In contrast, falcarindiol from Glehniae Radix and tetrahydrocoptisine from Corydalis Tuber have no prior evidence of efficacy for this condition, suggesting that these ingredients may represent novel active ingredients with potential benefits for postmenopausal osteoporosis. These findings indicate that our multiscale network approach successfully prioritized active ingredients with known or potential efficacy against postmenopausal osteoporosis.

To explore the key mechanisms underlying the activity of these active ingredients, we constructed a subnetwork comprising protein targets and biological functions significantly influenced by postmenopausal osteoporosis and these active ingredients. The constructed network for Benincasae Semen identified that rutin, beta-sitosterol, and quercetin directly interacted with disease-related proteins, including TGFB1, TNF, IL1B, IL6 and CAT. Other proteins were involved in disease-associated biological functions or were indirectly linked to disease-related proteins. The key biological functions represented included the regulation of gene expression, inflammatory response, NF-kappaB signaling, and apoptosis, all of which are associated with postmenopausal osteoporosis (Figure 4A).

Similarly, the analysis visualized the direct interactions of norisoboldine, hyperoside, rutin, and quercetin from Houttuyniae Herba with TGFB1, TNF, IL1B, IL6, and CAT. These interactions, both direct and indirect, influenced biological functions such as the TGF-beta receptor signaling pathway, cellular response to lipopolysaccharide, inflammatory response, NF-kappaB signaling, gene expression regulation, and apoptotic process (Figure 4B).

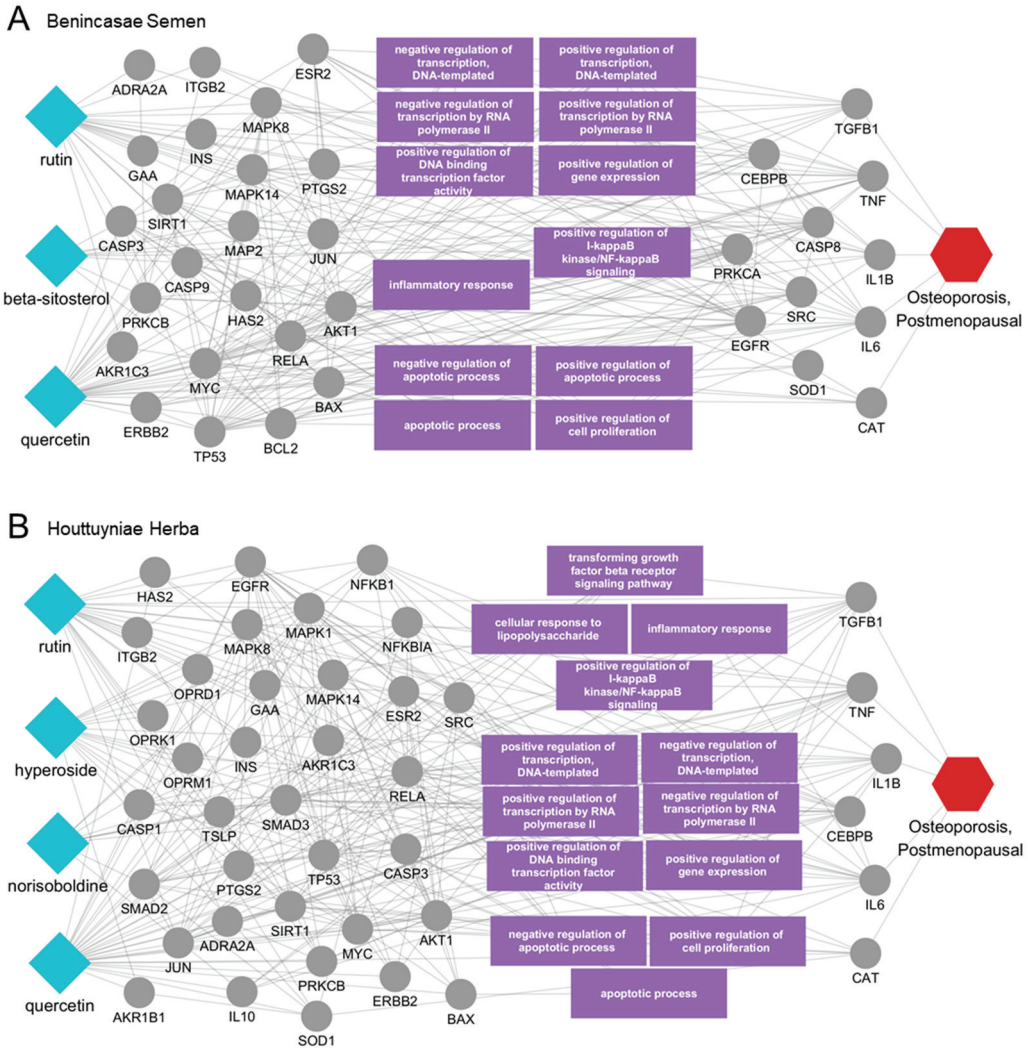

Figure 4. Predicted multiscale network mechanisms of active ingredients from candidate herbs. (**A**) Rutin, beta-sitosterol, and quercetin from Benincasae Semen, (**B**) Rutin, hyperoside, norisoboldine, and quercetin from Houttuyniae Herba. Light blue diamond shapes indicate the active ingredients, gray circles represent the protein targets, and the red hexagon denotes the disease of interest. Purple rectangles highlight distinct biological functions associated with the targets.

The multiscale network mechanisms of the novel active ingredients, previously lacking evidence, were further investigated to predict their molecular interactions that may underpin their therapeutic effects. The network indicated that falcarindiol from Glehniae Radix directly affected disease-related proteins, including IL6, IL1B, and TNF. Additionally, falcarindiol interacted with other proteins such as DRD1, PTPN1, and OPRK1, which are engaged in a network of interactions with other proteins linked to the disease. These proteins are involved in regulating gene expression, transcriptional activity, and key cellular processes, including apoptosis, cell proliferation, and inflammatory responses (Figure 5A). Next, the impact of tetrahydrocoptisine, an active ingredient in Corydalis Tuber, on disease-related targets was visualized (Figure 5B). Tetrahydrocoptisine was found to modulate postmenopausal osteoporosis by directly interacting with disease-related proteins such as IL6, IL1B, and TNF, in addition to affecting disease-associated biological functions and indirectly interacting with other disease-related proteins. The findings further suggested that the therapeutic effect of tetrahydrocoptisine was associated with the regulation of gene expression, apoptosis, cell proliferation, and inflammation. In summary, this study highlights the therapeutic potential of active ingredients from candidate herbs for post-

menopausal osteoporosis and provides valuable mechanistic insights by predicting the biological functions of the proteins involved in their therapeutic effects.

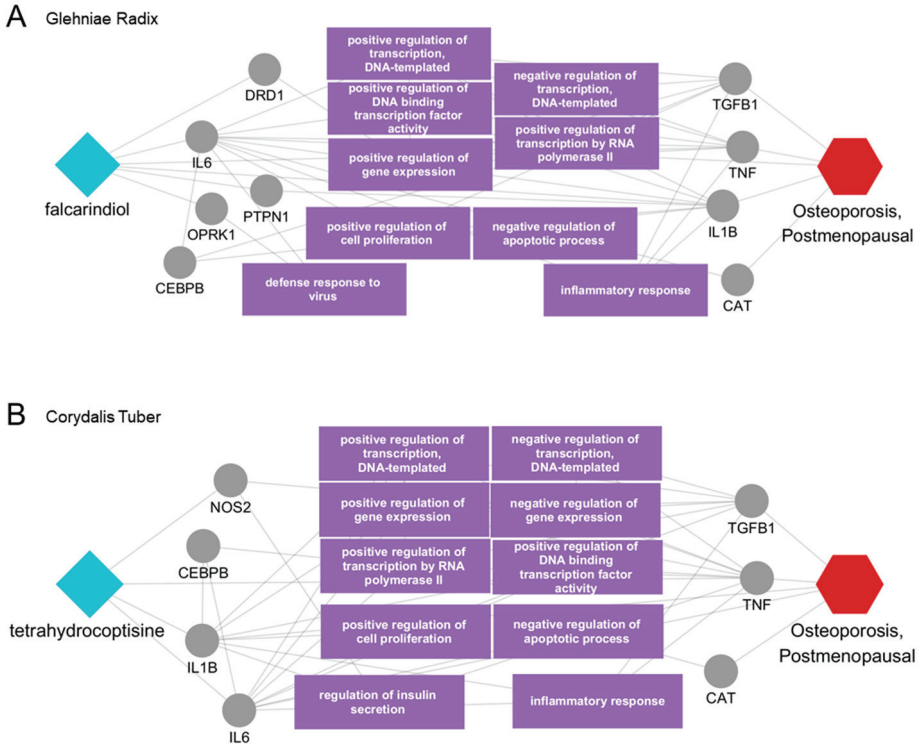

Figure 5. Predicted multiscale network mechanisms of falcarindiol and tetrahydrocoptisine. (**A**) falcarindiol from Glehniae Radix, (**B**) tetrahydrocoptisine from Corydalis Tuber. Light blue diamond shapes indicate the active ingredients, gray circles represent the protein targets, and the red hexagon denotes the disease of interest. Purple rectangles highlight distinct biological functions associated with the targets.

3. Discussion

This study employed a multiscale network and random walk-based analysis to identify the potential candidate herbs with therapeutic effects on postmenopausal osteoporosis and to elucidate their mechanisms of action. The top 10 ranked herbs, which showed high correlation scores and statistically significant associations (p-value < 0.05) within the disease-related protein network, included Sophorae Flos, Rhei Undulatai Rhizoma, Leonuri Herba, Benincasae Semen, Schizonepetae Spica, Glehniae Radix, Cnidi Fructus, Anemarrhenae Rhizoma, Corydalis Tuber, and Houttuyniae Herba. Notably, some herbs with limited or no prior evidence of efficacy for postmenopausal osteoporosis, such as Benincasae Semen, Glehniae Radix, Corydalis Tuber, and Houttuyniae Herba, were identified as novel candidate herbs. Meanwhile, herbs with previously reported efficacy, including Sophorae Flos, Rhei Undulatai Rhizoma, Leonuri Herba, Schizonepetae Spica, Cnidi Fructus and Anemarrhenae Rhizoma, further supported the robustness of the network pharmacology approach utilized in this study. Overall, these findings enhance our understanding of the effects of multi-compound traditional herbs on postmenopausal osteoporosis and provide a foundation for the discovery of new therapeutic agents.

The multiscale network analysis used in this study offered a powerful approach for assessing the broad impacts of therapeutics and diseases within the human interactome, accounting for the complex biological functions and pathways beyond protein–protein interactions [31]. Previous research has shown that this approach outperforms other network-based methods in identifying active herbal ingredients and their therapeutic effects on diseases [32]. By simulating propagation effects within the network and calculating interaction similarities using a biased random walk algorithm, prediction accuracy was

enhanced. This was achieved by adjusting the transition probabilities to favor movements toward biological functions over individual proteins. As a result, this approach provided a comprehensive view of how the protein targets of herbs or active ingredients influence the critical biological pathways and mechanisms. Previously applied to predict the therapeutic effects of polyphenols in oxidative liver damage [32], this method was extended here to postmenopausal osteoporosis. The approach successfully identified candidate herbs and their core protein targets, which impact disease-associated proteins and pathways in postmenopausal osteoporosis.

Using Gene Set Enrichment Analysis (GSEA) with KEGG and Gene Ontology, we identified the signaling pathways and biological functions linked to the core protein targets of our candidate herbs that are potentially effective against postmenopausal osteoporosis. The KEGG analysis result revealed that pathways related to inflammation and cell survival, such as the AGE–RAGE signaling pathway, apoptosis, and C-type lectin receptor signaling, play central roles in the therapeutic potential of these herbs. For example, the abnormal accumulation of advanced glycation end products (AGEs) on collagen, often linked to diabetes, activates the AGE–RAGE signaling pathway. This activation leads to the inhibition of osteoblast differentiation and the enhancement of osteoclast-mediated bone resorption, thereby contributing to bone loss [33,34], while C-type lectin receptor signaling modulates osteoporosis progression by regulating osteoclast activity and influencing the immune responses within bone tissue [35]. Moreover, the presence of signaling pathways related to inflammatory cytokines, such as TNF and IL-17, among the top ranked target-associated pathways highlighted the significant impact of inflammation on bone metabolism in postmenopausal osteoporosis [36]. The identification of osteoclast differentiation, a primary pathological mechanism of osteoporosis, further clarified the role of osteoclast activation in bone loss after menopause [2]. Pathways involved in immune response regulation, such as the Toll-like receptor and NOD-like receptor signaling pathways, were also identified. Moreover, the FoxO and Sphingolipid signaling pathways, which are associated with cell survival and oxidative stress regulation, suggested a potential role in maintaining bone cell survival and homeostasis [37,38]. Gene Ontology (GO) analysis further revealed that the targets of candidate herbs are significantly associated with biological functions related to apoptosis regulation, oxidative stress response, and inflammation which are major factors in bone cell survival and the progression of postmenopausal osteoporosis [39]. Associations were also found with cellular responses to lipopolysaccharides and lipids, which are closely linked to the inflammatory pathways highlighted the KEGG analysis. In the molecular function category, a strong association was found with estrogen 16-alpha-hydroxylase activity. This enzyme converts estrogen to 16α-hydroxyestrone (16α-(OH)E1), a metabolite known for its estrogen-like physiological effects, particularly in supporting bone formation [40]. Clinical studies on patients with postmenopausal bone loss have shown that decreased activity of estrogen 16-alpha-hydroxylase may reduce estrogen's bone-protective effects thus accelerating bone loss [41]. Collectively, these findings suggest that the therapeutic effects of candidate herbs with high correlation scores for postmenopausal osteoporosis may be mediated through core targets involved in various signaling pathways and biological functions known to influence the disease.

Our study identified novel candidate herbs, including Benincasae Semen, Glehniae Radix, Corydalis Tuber, and Houttuyniae Herba, which previously lacked clear evidence of efficacy in treating postmenopausal osteoporosis. Benincasae Semen demonstrated anti-inflammatory, nephroprotective, cytotoxic and anti-cancer effects, while Glehniae Radix possesses antioxidant, antitussive, and immune-regulating properties [42,43]. Corydalis Tuber is known for its analgesic and blood circulation promoting effects [44]. Houttuyniae Herba has a broad pharmacological profile, including antioxidant, anti-inflammatory, anti-cancer, anti-bacterial and hepatoprotective effects, with emerging interest in its skin-care applications [45,46]. These therapeutic profiles support further exploration of these herbs' bioactive compounds as candidates for expanded health applications, including postmenopausal osteoporosis. Our multiscale network analysis further demonstrated

that active ingredients in these candidate herbs exhibit high correlation scores as well as significant protein overlap with postmenopausal osteoporosis-related targets, indicating potential therapeutic relevance. The constructed subnetworks for Benincasae Semen and Houttuyniae Herba elucidated the mechanisms through which these active ingredients may act on postmenopausal osteoporosis. The direct interactions of rutin, beta-sitosterol, quercetin, norisoboldine, and hyperoside with key disease-related proteins such as TGFB1, TNF, IL1B, IL6, and CAT suggested that these compounds may modulate critical pathways involved in the pathology of postmenopausal osteoporosis. These interactions influenced essential biological functions, including the regulation of gene expression, inflammatory response, NF-kappaB signaling, and apoptosis.

Notably, falcarindiol from Glehniae Radix and tetrahydrocoptisine from Corydalis Tuber, have not been previously reported in association with postmenopausal osteoporosis. Our analysis predicted that these compounds modulate key disease-associated biological functions, including inflammatory response, apoptosis, and cell proliferation. Both falcarindiol and tetrahydrocoptisine demonstrated direct interaction with pro-inflammatory cytokines IL6, IL1B, and TNF, key drivers of osteoclastogenesis and bone resorption in postmenopausal osteoporosis by activating inflammatory pathways linked to bone loss [36]. Additionally, these compounds were found to influence gene expression and bone cell survival through indirect interactions with proteins such as DRD1, CEBPB, OPRK1, PTPN1, NOS2, TGFB1, and CAT. For example, CEBPB (C/EBPβ), a transcription factor involved in the differentiation of osteoblasts and adipocytes, modulates the balance between these cells and regulates gene expression relevant to bone formation [47]. Alterations in TGF-β1(TGFB1), as a key regulator of bone formation and resorption balance, influenced by genetic polymorphisms and estrogen deficiency are known to increase the risk of postmenopausal osteoporosis, highlighting the potential relevance of this interaction in mitigating disease progression [48–50]. Although falcarindiol, also known as (3R,8S)-falcarindiol, has been identified as a structural component of phthalides in Angelica Radix with known anti-osteoporotic activity, its role as a constituent of Glehniae Radix remains unexplored [51]. Collectively, these findings underscore the potential of these herbal compounds as novel candidates for postmenopausal osteoporosis treatment and provide valuable insights into their mechanisms of action and associated protein functions, establishing a foundation for future research and clinical applications.

Despite these findings, our study has several limitations that warrant consideration for future research. First, our method for predicting key protein targets of the herbs was based on the hypothesis of overlapping effects within the network, which might require additional optimization to improve predictive accuracy. Second, while our therapeutic effect predictions relied on a multiscale network analysis, we did not account for specific mechanisms of action, such as inhibition or activation, between the compounds and their targets. This gap suggests the need for further validation studies to elucidate these interactions. Additionally, the disease-related proteins were identified from a single database, potentially limiting the comprehensiveness of our analysis. Nevertheless, to the best of our knowledge, this study is the first to systematically identify herbal candidates and active ingredients for postmenopausal osteoporosis using a biased random walk on a multiscale network, providing a valuable foundation for future investigations.

4. Materials and Methods

4.1. Herb–Ingredient–Target Network Construction

Herbs and their ingredient data were retrieved from the OASIS traditional medicine database (https://oasis.kiom.re.kr/index.jsp, (accessed on 21 August 2024)), managed by the Korean Institute of Oriental Medicine (KIOM). The OASIS platform provides data on potential active ingredients extracted from herbs, identified through physicochemical analysis techniques such as HPLC and UPLC and validated by pharmacological and traditional medicine experts. In this study, we collected 12,871 associations between 420 herbs and 4786 ingredients, each identified through its PubChem CID. These ingredient

data served as foundation input for the subsequent network analysis. Ingredient–target interaction data, that had been experimentally validated, were compiled from reputable databases including DrugBank 5.0 [52], Therapeutic Target Database (TTD 2.0) [53], and the Search Tool for Interactions of Chemicals (STITCH 5) [54], which offer comprehensive information on established and potential targets, associated diseases, biological pathways, and drugs targeting these proteins. For precise target identification, SynGO 1.2 was used to align gene symbols and Uniprot IDs with Entrez Gene IDs [55].

A network was then constructed to represent the associations between herbs, ingredients, and protein targets. In this network, nodes represent the following three types of entities: herbs, ingredients, and protein targets. Edges represent the relationships between these entities, specifically herb–ingredient associations and ingredient–target interactions. All edges were unweighted and undirected, indicating the presence of an association or interaction without implying directionality or strength. The ingredients identified by PubChem CID were compared and integrated with the ingredient–target data. Herbs containing fewer than three target-associated ingredients were excluded to ensure sufficient data for reliable network analysis. This threshold was chosen because herbs with at least three active components are more likely to exhibit meaningful pharmacological effects and provide robust interaction data, which enhances the reliability of our predictions. The resulting network enabled the visualization and analysis of interactions among the herbs, components, and protein targets. In this network analysis, the simple pathway count for each herb was calculated, accounting for instances where multiple components influenced a single target. This process allowed for the selection of the top 50 targets, with each target's relative importance assessed accordingly.

4.2. Gene Set Enrichment Analysis (GSEA)

Biological processes and signaling pathways associated with the protein targets were identified using gene set enrichment analysis (GSEA) with the GSEApy module (version 1.1.3) in a Python 3.7 environment, facilitated through the Enrichr platform (http://amp.pharm.mssm.edu/Enrichr/, (accessed on 12 September 2024)) [56,57]. Enrichr performs enrichment analysis by drawing on various gene-set libraries, such as Gene Ontology and the Kyoto Encyclopedia of Genes and Genomes (KEGG) [58,59]. In this study, adjusted *p*-values, z-scores, and combined scores were calculated to evaluate the signaling pathways and biological functions relevant to herbal ingredient targets. The combined score, multiplying the logarithm of the *p*-value with the z-score, provided reliable results, allowing for a systematic evaluation of the effects of herbal components on specific biological pathways. All signaling pathways identified through enrichment analysis were included in the analysis, except for those specifically related to diseases.

4.3. Disease-Related Targets

In this study, we utilized postmenopausal osteoporosis-related protein data curated by Ruiz et al. to analyze the proteins associated with postmenopausal osteoporosis [20]. This dataset was sourced from DisGeNet 2019 (https://www.disgenet.org/, (accessed on 12 September 2024)), a database that rigorously maps disease–gene associations to ensure research reliability [60]. We focused solely on expert-curated disease–gene associations provided by DisGeNet for "Osteoporosis, Postmenopausal" thus ensuring relevance to our study. Data in this curated set draw from highly regarded sources, including UniProt, the Comparative Toxicogenomics Database, Orphanet, Clinical Genome Resource, Genomics England PanelApp, Cancer Genome Interpreter, and the Psychiatric Disorders Gene Association Network. We excluded disease–gene associations based on homology in animal models or derived from computational literature mining, as well as associations labeled as therapeutic. This refined disease–protein interaction network formed the basis for validating the protein information related to postmenopausal osteoporosis in our analysis.

4.4. Multiscale Network Analysis for Predicting Disease Associations

The multiscale interactome was constructed following the methodology of Ruiz et al., integrating the following three interaction types: protein–protein, protein–biological function, and biological function–function interactions [20]. Human protein–protein interaction data were sourced from the Biological General Repository for Interaction Datasets (BioGRID 3.5.178), the Database of Interacting Proteins (DIP), and the Human Reference Protein Interactome Mapping Project (HuRI), encompassing 387,626 physical interactions among 17,660 proteins. Protein–biological function interactions were derived from the human Gene Ontology database, assembling 34,777 experimentally verified associations between 7993 proteins and 6387 biological functions. Finally, biological function–function interactions were organized into a highly interconnected hierarchical structure, with 22,545 associations among 9798 functions.

Diffusion profiles were then calculated using the multiscale interactome to assess the propagation effects between the herbal ingredients and proteins associated with postmenopausal osteoporosis. This analysis utilized a biased random walk with a restart algorithm, enabling a quantitative evaluation of the influence exerted by herbal targets and ingredient targets within herbs on postmenopausal osteoporosis-related proteins. Correlation score was then calculated between herb–ingredient and disease profiles, facilitating the identification of potential candidate herbs and ingredients for treating postmenopausal osteoporosis.

The primary mechanisms of each ingredient–disease pair were identified by analyzing diffusion profiles and selecting the top k-proteins or biological functions based on their influence from either the drug or the disease. A network was then constructed from these selected entities to highlight their significance. Targets of ingredients that were not associated with disease-related proteins or biological functions were excluded. The highest-ranking entity in the diffusion profile was deemed the most essential for treatment due to its considerable influence. In our analysis, we set the value of k to 20 to ensure sufficient exploration of influential nodes. A previous study indicated that when k was set to 10, the top nodes accounted for approximately 50% of the total visitation frequency in the diffusion profile. By increasing k to 20, we were able to capture a larger portion of the visitation frequency, enhancing the comprehensiveness of our analysis. For a comprehensive explanation of the diffusion profile calculation method, including specific mathematical formulas, iterative processes, and the rationale for selecting parameter k, please refer to the prior research [20].

5. Conclusions

In conclusion, this study demonstrated a comprehensive multiscale network analysis approach to proposing novel herbs and compounds for the treatment of postmenopausal osteoporosis. This methodology enabled the identification of promising therapeutic candidates by exploring complex protein interactions and biological pathways, including previously understudied herbs. However, a limitation of this predictive approach is its inability to specify precise modes of action, such as activation or inhibition. To address this, validation of the predicted outcomes through in vitro, in vivo, and clinical studies is essential. Additionally, as database reliability and scope affect prediction accuracy, integrating the latest data and diverse sources remains a critical objective. Despite these limitations, this study presents a pioneering strategy for developing treatments for postmenopausal osteoporosis, combining multiscale network and random walk algorithms. It holds substantial academic value by introducing an innovative methodological framework for future medicinal herb research.

Author Contributions: Conceptualization, Y.-W.K. and W.-Y.L.; Data Curation, Y.K. and W.-Y.L.; Formal Analysis, B.J.; Funding Acquisition, Y.-W.K.; Investigation, B.J.; Methodology, Y.K. and W.-Y.L.; Project Administration, Y.-W.K. and W.-Y.L.; Resources, W.-Y.L.; Software, Y.K.; Supervision, W.-Y.L.; Validation, B.J., Y.K. and W.-Y.L.; Visualization, B.J.; Writing—Original Draft, B.J.; Writing—Review and Editing, J.S. and Y.-W.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by grants from the National Research Foundation of Korea (NRF) funded by the Korean government (MSIT) (grant numbers 2022R1A2C1092168, RS-2023-00243363, RS-2023-00218419, and RS-2024-00335030) and the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: RS-2020-KH093754).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting the findings of this study are included within the manuscript.

Conflicts of Interest: Authors Boyun Jang and Youngsoo Kim were employed by the company IntegroMediLab Co., Ltd. The company, which focuses on the research and development of methodologies for network pharmacology analysis of natural products, supported the data analysis for this study. However, the company does not target any specific disease or drug, and its involvement was strictly limited to providing methodological support. As such, no conflicts of interest are declared. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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International Journal of
Molecular Sciences

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Evaluation of the Antidiabetic Potential of Xanthone-Rich Extracts from *Gentiana dinarica* and *Gentiana utriculosa*

Jelena Arambašić Jovanović [1,*], Dijana Krstić-Milošević [2,*], Branka Vinterhalter [2], Svetlana Dinić [1], Nevena Grdović [1], Aleksandra Uskoković [1], Jovana Rajić [1], Marija Đorđević [1], Ana Sarić [1], Melita Vidaković [1] and Mirjana Mihailović [1]

[1] Depatment of Molecular Biology, Institute for Biological Research "Siniša Stanković", National Institute of Republic of Serbia, University of Belgrade, Bulevar Despota Stefana 142, 10060 Belgrade, Serbia; sdinic@ibiss.bg.ac.rs (S.D.); nevenag@ibiss.bg.ac.rs (N.G.); auskokovic@ibiss.bg.ac.rs (A.U.); marija.sinadinovic@ibiss.bg.ac.rs (M.Đ.); ana.saric@ibiss.bg.ac.rs (A.S.); melita@ibiss.bg.ac.rs (M.V.); mista@ibiss.bg.ac.rs (M.M.)

[2] Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", National Institute of Republic of Serbia, University of Belgrade, Bulevar Despota Stefana 142, 10060 Belgrade, Serbia; horvat@ibiss.bg.ac.rs

* Correspondence: jelena.arambasic@ibiss.bg.ac.rs (J.A.J.); dijana@ibiss.bg.ac.rs (D.K.-M.); Tel.: +381-11-2078343 (J.A.J.); +381-11-2078394 (D.K.-M.); Fax: +381-11-2761433 (J.A.J.)

Abstract: Despite the existence of various therapeutic approaches, diabetes mellitus and its complications have been an increasing burden of mortality and disability globally. Hence, it is necessary to evaluate the efficacy and safety of medicinal plants to support existing drugs in treating diabetes. Xanthones, the main secondary metabolites found in *Gentiana dinarica* and *Gentiana utriculosa*, display various biological activities. In in vitro cultured and particularly in genetically transformed *G. dinarica* and *G. utriculosa* roots, there is a higher content of xanthones. The aim of this study was to investigate and compare antidiabetic properties of secondary metabolites (extracts) prepared from these two *Gentiana* species, cultured in vitro and genetically transformed with those collected from nature. We compare HPLC secondary metabolite profiles and the content of the main extract compounds of *G. dinarica* and *G. utriculosa* methanol extracts with their ability to scavenge DPPH free radicals and inhibit intestinal α-glucosidase in vitro. Anti-hyperglycemic activity of selected extracts was tested further in vivo on glucose-loaded Wistar rats. Our findings reveal that the most prominent radical scavenging potential and potential to control the rise in glucose level, detected in xanthone-rich extracts, were in direct correlation with an accumulation of xanthones norswertianin and norswertianin-1-*O*-primeveroside in *G. dinarica* and decussatin and decussatin-1-*O*-primeveroside in *G. utriculosa*.

Keywords: *Gentiana dinarica*; *Gentiana utriculosa*; xanthones; diabetes; antidiabetic properties; norswertianin; norswertianin-1-*O*-primeveroside; decussatin; decussatin-1-*O*-primeveroside

1. Introduction

Plants, as an inexhaustible source of natural products with various medical properties, are continuously explored for the development of novel drugs [1,2]. However, it is estimated that only a small percentage of all plant species (around 15%) have been explored for the presence of biologically active compounds with pharmacological potential [3]. These compounds are products of plant secondary metabolism and are identified as secondary metabolites or phytochemicals. Plants produce a high diversity of secondary metabolites that provide protection against herbivores, different pathogens, and abiotic stress and enable the adaptation of plants to changing environmental conditions [4,5]. Unlike primary metabolites synthesized in all plant species, secondary metabolites are specific to plant species and to plant organs, including leaves, stems, roots, and flowers [6]. Secondary plant metabolites are often classified according to their chemical structures into three major classes: phenolics, terpenes, and alkaloids [7].

The most common challenge faced when using wild plants as a source for the isolation and characterization of biologically active compounds is the low availability of the plant material from nature. Secondary metabolites are accumulated by plants in small quantities. Although sufficient for initial pharmacological evaluation, those quantities are insufficient for testing on a broad range of biological activities and for mass production. Additionally, many plant species have become endangered as a result of uncontrolled harvesting and/or extreme environmental changes. In order to obtain a sufficient amount of secondary metabolites, the most commonly used approach includes biotechnology-based in vitro techniques for plant cultivation in controlled environmental conditions, such as callus culture, hairy root culture, cell suspension culture, and micropropagation [7].

The species comprising the Gentianaceae are of great importance to the pharmaceutical industry because their phytochemical constituents display vast and versatile pharmacological effects [8]. The main secondary metabolites of Gentianaceae species are phenols (xanthones and *C*-glucoflavones) and terpenoids. Aside from Gentianaceae species, the majority of xanthones are found in only one plant family—Guttiferae [9]. Xanthones are heterotricyclic compounds substituted with simple isoprene, methoxy, and hydroxyl groups at various locations on aromatic A and B rings. In the Gentianaceae, xanthones occur as simple oxygenated xanthones (mono-, tri-, tetra-, and hexaoxygenated) and xanthone glycosides (*C*- and *O*-glycosides). Xanthone-*O*-glycosides are very frequent among the Gentianaceae species while xanthone-*C*-glycosides are quite rare [10]. Bioproduction of xanthones is of great importance since these natural compounds exhibit a variety of pharmacological and health benefits [11]. Previous research was initiated with the aim to establish protocols for shoot micropropagation, excised root cultures, and hairy root cultures of *Gentiana dinarica Beck*. and *Gentiana utriculosa* L. in order to enhance the production of secondary metabolites, especially xanthone compounds [12–17]. Compared to plants grown in nature, in vitro-cultured *G. dinarica* and *G. utriculosa* had a higher content of xanthones, particularly in genetically transformed roots (hairy roots) [12,17,18]. This is highly important for *G. dinarica* because this perennial plant species is rare and endangered, limited to the Dinaric mountains of the Balkan peninsula and the Apennines mountains in Italy [19]. On the other hand, *Gentiana utriculosa* L. is an annual plant species with a wide distribution in the mountains of central Serbia [20] and in Central Europe [21]. These two Gentians are rich in tetraoxygenated xanthones with a 1, 3, 7, 8-oxygenation pattern. Norswertianin and its *O*-glycoside (norswertianin-1-*O*-primeveroside) are typical xanthones for *G. dinarica* [6,12], while decussatin (1-hydroxy-3,7,8-trimethoxyxanthone) and its *O*-glycoside (decussatin-1-*O*-primeveroside) are characteristic for *G. utriculosa* [22]. These xanthones display at least one of the various biological activities, e.g., antimicrobial [23], radioprotective [24], antitumor [25], antidepressant [26], vasodilator [27], antiulcer [28], antibacterial, antifungal, antioxidative, and hypoglycemic effects [29], as well as potential to act as a chemopreventive [30] and anticancerogenic agent [31].

The curative value of plant extracts depends on the composition and different combinations of biologically active compounds [32]. The aim of this study was to compare the antidiabetic properties of extracts derived from aerial parts and roots of *G. dinarica* and *G. utriculosa* that were collected from nature, in vitro propagated, or genetically transformed in order to find the optimal combination of active compounds for the treatment of diabetes. The importance of finding new antidiabetogenic extracts is reflected in the fact that diabetes is one of the top 10 causes of death globally and, together with cardiovascular disease, cancer, and respiratory disease, account for over 80% of all premature noncommunicable disease deaths [33]. Diabetic patients are, due to the of lack of insulin secretion and/or action, constantly exposed to high blood sugar levels, which causes oxidative stress, further leading to the development and progression of diabetes and its complications [34,35]. Because of the fact that most of the currently available antidiabetic drugs have their limitations, adverse effects, and secondary failures, the focus has been shifted towards the medical plants and their role in the therapy of diabetes.

2. Results

2.1. Qualitative and Quantitative Analysis of Secondary Metabolites in G. dinarica (Gd) and G. utriculosa (Gu) Extracts

The secondary metabolite profiles of Gd and Gu methanol extracts analyzed using the HPLC-DAD method are presented in Figure 1, and the chemical structures of compounds identified in these extracts are presented in Figure 2. It is evident that chromatograms of *G. dinarica* extracts derived from vegetative roots (Gd4) and from genetically transformed roots (Gd5 and Gd6) (Figure 1b) contain different sets of secondary metabolites from the other three examined extracts (Gd1—aerial parts of wild growing plants; Gd2—roots of wild growing plants; Gd3—shoot culture). An analysis revealed the presence of xanthones norswertianin-1-*O*-primeveroside (6), norswertianin-8-*O*-primeveroside (7), gentioside (8), and norswertianin (10) in all examined Gd methanol extracts (Figure 1a,b). Apart from these xanthones, Gd1, Gd2, and Gd3 also contained groups of bitter glycosides, including swertiamarin (1), gentiopicrin (3), sweroside (4), and amarogentin (9) (Figure 1a). Additionally, the presence of flavones isoorientin (5) and isoorientin-4-O-glucoside (2) was detected only in these extracts (Figure 1a).

On the other hand, -1-*O* primeverosides of decussatin (13) and gentiakochianin (12) along with their corresponding aglycones decussatin (16) and gentiakochianin (15) are the main xanthones identified in Gu methanol extracts (Gu1—aerial parts of wild growing plants; Gu2—shoot culture; Gu3—genetically transformed shoots; Gu4—genetically transformed roots; Figure 1c). Xanthone *C*-glucoside mangiferin (11) was detected in all examined extracts of Gu except in Gu4 (Figure 1c).

The contents of the main secondary metabolites of Gd and Gu methanol extracts are shown in Tables 1 and 2. The most abundant compounds in the aerial parts and shoots of *G. dinarica* (Gd1 and Gd3) were secoiridoids—swertiamarin, gentiopicrin, and sweroside. The amount of gentiopicrin was the most dominant. Xanthones (norswertianin, norswertianin-1-*O*-primeveroside, and gentioside) were the second group of secondary metabolites present in these extracts with much lower content (Table 1). The preponderant compound in extracts obtained from vegetative roots (Gd4) and transformed roots (Gd5 and Gd6) was norswertianin-1-*O*-primeveroside, followed by its aglycone norswertianin. However, the predominant compound in the extract of roots from wild plants was gentiopicrin, which had double the amount of norswertianin-1-O-primeveroside. The rest of the secondary metabolites (swertiamarin, sweroside, gentioside, and norswertianin) were quantified in much lower content (Table 1).

Table 1. Gd1—aerial parts of wild growing plants; Gd2—roots of wild growing plants; Gd3—shoot culture; Gd4—vegetative roots; Gd5—genetically transformed roots, clone B; Gd6—genetically transformed roots, clone 3. Values are means of three technical replicates ± S.E.M. (n = 3). For all variables with the same superscript letter, the difference between the means is not statistically significant. If two variables have different letters, they are significantly different at $p < 0.05$.

Quantitative Analysis of Secondary Metabolites in Methanol Extracts of *Gentiana dinarica*

Extract	Swertiamarin (mg/g dw)	Gentiopicrin (mg/g dw)	Sweroside (mg/g dw)	Gentioside (mg/g dw)	Norswertianin (mg/g dw)	Norswertianin -1-O- primeveroside (mg/g dw)
Gd1	39.82 ± 1.71^{b}	55.88 ± 2.13^{c}	10.52 ± 0.44^{c}	0.49 ± 0.02^{c}	0.26 ± 0.01^{e}	4.31 ± 0.15^{e}
Gd2	9.91 ± 0.34^{c}	70.15 ± 2.76^{b}	14.46 ± 0.53^{b}	11.37 ± 0.51^{a}	1.95 ± 0.081^{c}	36.65 ± 1.66^{c}
Gd3	57.47 ± 2.44^{a}	109.95 ± 4.17^{a}	21.89 ± 0.98^{a}	6.79 ± 0.29^{b}	0.63 ± 0.02^{d}	9.51 ± 0.35^{d}
Gd4				0.311 ± 0.09^{c}	19.92 ± 0.84^{b}	126.12 ± 5.31^{a}
Gd5					39.36 ± 1.53^{a}	131.08 ± 4.35^{a}
Gd6					33.78 ± 1.32^{a}	114.86 ± 4.91^{b}

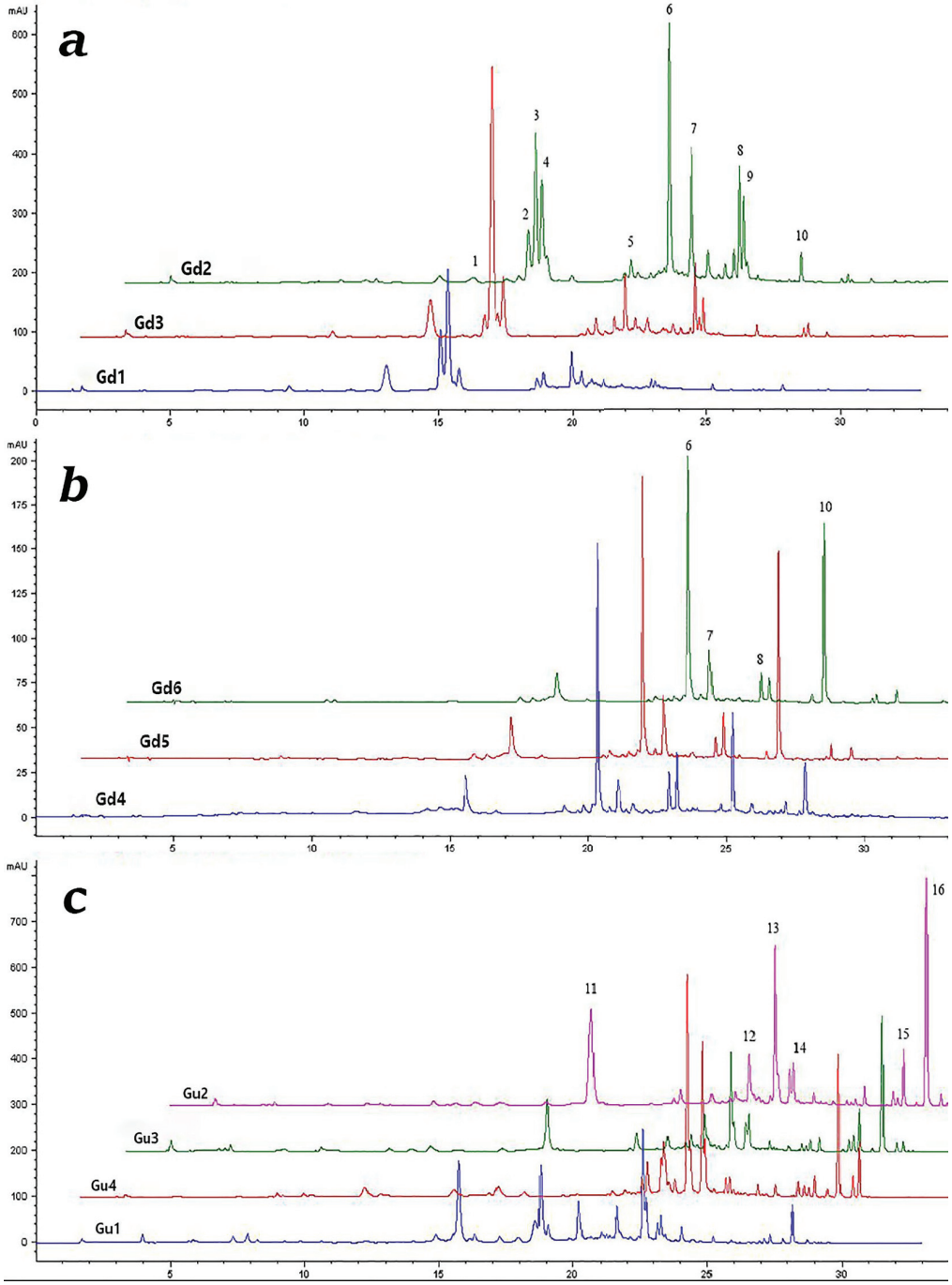

Figure 1. Comparative chromatograms of *G. dinarica* (Gd) (**a**,**b**) and *G. utriculosa* (Gu) (**c**) methanol extracts. (**a**) Gd1—aerial parts of wild growing plants; Gd2—roots of wild growing plants; Gd3—shoot culture. Peaks: 1—swertiamarin; 2—isoorinetin-4′-O-glucoside; 3—gentiopicrin; 4—sweroside; 5—isoorientin; 6—norswertianin-1-O-primeveroside; 7—norswertianin-8-O-primeveroside; 8—gentioside; 9—amarogentin; 10—norswertianin; (**b**) Gd4—vegetative roots; Gd5—genetically transformed roots, clone B; Gd6—genetically transformed roots, clone 3. Peaks: 6—norswertianin-1-O-primeveroside; 7—norswertianin-8-O-primeveroside; 8—gentioside; 10—norswertianin; (**c**) Gu1—aerial parts of wild growing plants; Gu2—shoot culture; Gu3—genetically transformed shoots; Gu4—genetically transformed roots. Peaks: 11—mangiferin; 12—gentiakochianin-1-O-primeveroside; 13—decussatin-1-O-primeveroside; 14—1,8-dihydroxy-3-methoxy-7-O-primeveroside; 15—gentiakochianin; 16—decussatin.

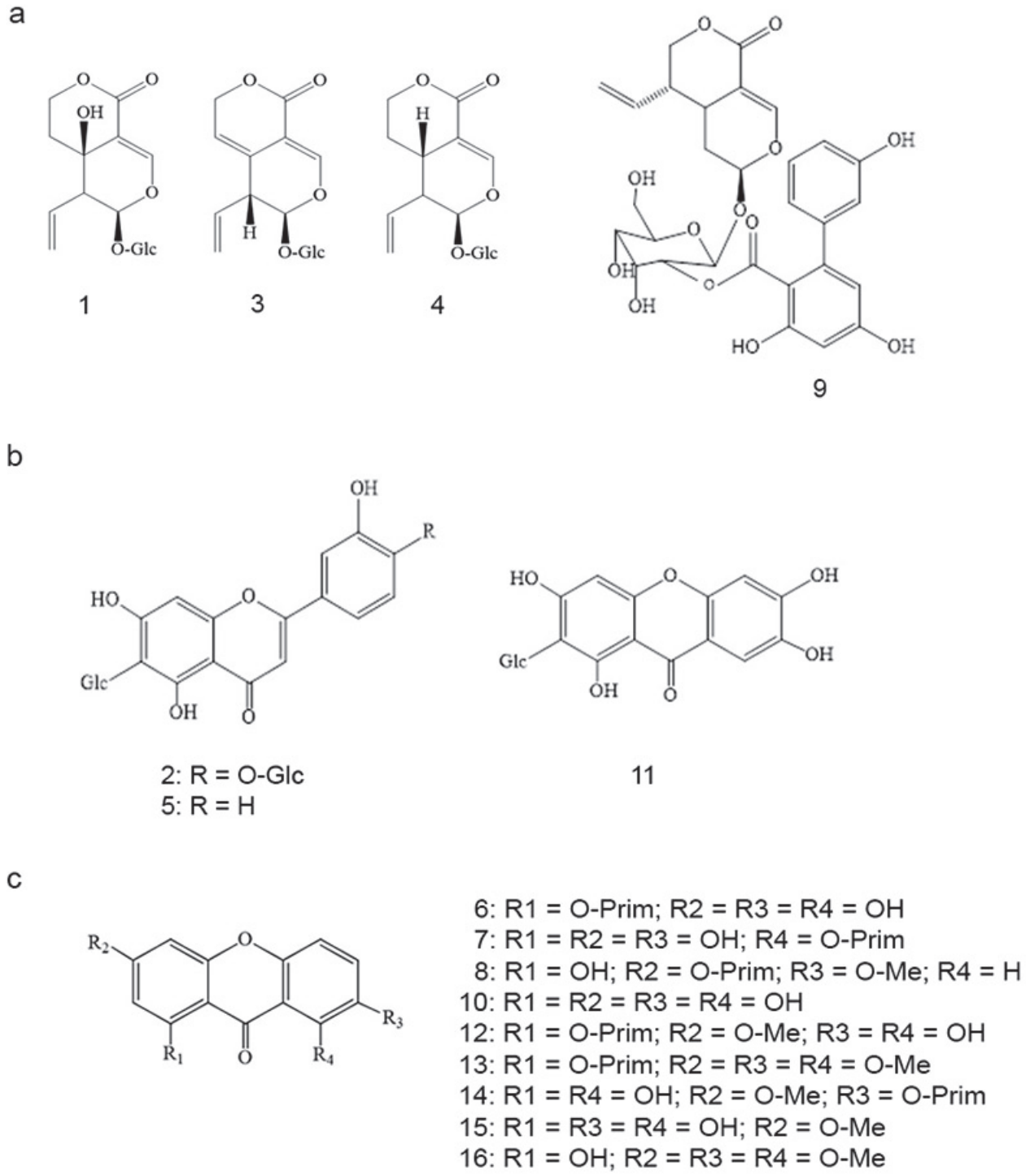

Figure 2. Chemical structures of compounds identified in methanol extracts of *G. dinarica* (Gd) and *G. utriculosa* (Gu). The order of compounds is in accordance with their increasing retention times in the chromatograms. (**a**) Bitter glycosides identified in *G. dinarica* methanol extracts: 1—swertiamarin; 3—gentiopicrin; 4—sweroside; 9—amarogentin; (**b**) C-glucoflavones and xanthone-C-glucoside identified in methanol extracts of *G. dinarica* and *G. utriculosa*: 2—isoorinetin-4′-O-glucoside; 5—isoorientin; 11—mangiferin; (**c**) xanthones identified in methanol extracts of *G. dinarica* and *G. utriculosa*: 6—norswertianin-1-O-primeveroside; 7—norswertianin-8-O-primeveroside; 8—gentioside; 10—norswertianin; 12—gentiakochianin-1-O-primeveroside; 13—decussatin-1-O-primeveroside; 14—1,8-dihydroxy-3-methoxy-7-O-primeveroside; 15—gentiakochianin; 16—decussatin.

Table 2. Gu1—aerial parts of wild growing plants; Gu2—shoot culture; Gu3—genetically transformed shoots; Gu4—genetically transformed roots. Values are means of three technical replicates ± S.E.M. (n = 3). For all variables with the same superscript letter, the difference between the means is not statistically significant. If two variables have different letters, they are significantly different at $p < 0.05$.

Quantitative Analysis of Secondary Metabolites in Methanol Extracts of *Gentiana utriculosa*			
Extract	**Mangiferin (mg/g dw)**	**Decussatin (mg/g dw)**	**Decussatin -1-O-primeveroside (mg/g dw)**
Gu1	13.12 ± 0.56 ^{b}	7.44 ± 0.31 ^{d}	26.64 ± 1.06 ^{c}
Gu2	18.73 ± 0.84 ^{a}	46.91 ± 2.15 ^{b}	38.33 ± 1.64 ^{b}
Gu3	7.43 ± 0.29 ^{c}	27.92 ± 1.22 ^{c}	23.17 ± 1.06 ^{d}
Gu4		58.91 ± 2.15 ^{a}	107.77 ± 3.15 ^{a}

Quantitative analysis of secondary metabolites in *G. utriculosa* extracts showed high amounts of xanthones decussatin-1-O-primeveroside and its corresponding aglycone decussatin in shoot culture (Gu2) and genetically transformed shoots (Gu3) and roots (Gu4) (Table 2). The highest amount of decussatin-1-O-primeveroside was recorded in the extract of genetically transformed roots (Gu4). Xanthone mangiferin was detected in higher content in Gu2 extract (Table 2).

2.2. DPPH Radical Scavenging

The in vitro antioxidant activity of the extracts was determined using a DPPH assay. As can be seen from Figure 3, extracts from *G. dinarica* vegetative and transgenic roots (Gd4, Gd5, and Gd6) displayed significantly higher scavenging activity than the other three extracts of *G. dinarica* (Gd1, Gd2, and Gd3) and all four extracts of *G. utriculosa*. Although DPPH radical scavenging activity increased with increasing concentrations of all examined extracts (Figure 3), only vegetative and transformed root extracts of *G. dinarica* displayed the DPPH radical scavenging activity at the same concentration as ascorbic acid, which served as the reference substance. These three Gd extracts reached maximal scavenging (90%) at the concentration of 1 mg/mL, while ascorbic acid reached its maximum (92%) at four times lower concentration. The remaining three extracts of *G. dinarica* and all four extracts of *G. utriculosa* showed scavenging of DPPH radicals at ten times higher concentrations than the reference substance. Among these extracts, the best activity (91%) was detected in the aerial parts of wild grown plants (Gd1) and the lowest activity (62%) in the shoot culture of *G. dinarica* (Gd3). In contrast, all examined extracts of *G. utriculosa* showed similar scavenging activity (Figure 3).

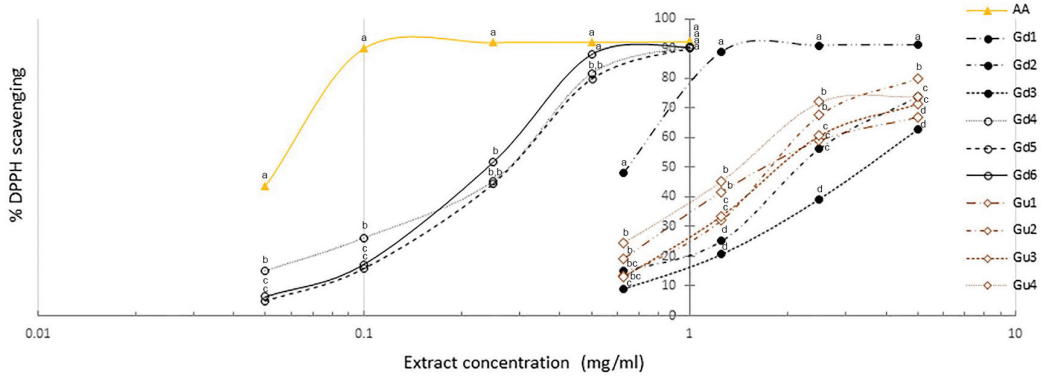

Figure 3. Comparative analysis of DPPH radical scavenging activity at different concentrations of methanol extracts isolated from *G. dinarica* and *G. utriculosa*. Gd1—aerial parts of wild growing plants; Gd2—roots of wild growing plants; Gd3—shoot culture; Gd4—vegetative roots; Gd5—genetically transformed roots, clone B; Gd6—genetically transformed roots, clone 3. Gu1—aerial parts of wild growing plants; Gu2—shoot culture; Gu3—genetically transformed shoots;

Gu4—genetically transformed roots. The results of the assays are presented as the means ± S.E.M. from three separate measurements (n = 3). For all variables with the same superscript letter, the difference between the means (at the same concentration) is not statistically significant. If two variables have different letters, they are significantly different at $p < 0.05$.

The compounds responsible for free radical scavenging activity were determined using the HPLC method with pre-chromatographic reaction of tested extracts and DPPH radicals. Figs. 4 and 5 show the comparative chromatograms of *G. dinarica* and *G. utriculosa* extracts before and after the reaction with DPPH radicals. The decrease in peak areas in the chromatogram profile of the extract revealed radical scavenging compounds. These compounds are oxidized in reaction with DPPH, which causes a decrease in their concentration. A further consequence of that reaction is a decrease in peak areas. In the *G. dinarica* extract, the largest decrease was recorded for the peak area of norswertianin-1-O-primeveroside (1) and its aglycon norswertianin (4), indicating that these xanthones had the highest antioxidant activity. The strong antioxidant activity of Gd extract is obviously correlated with the high content of these xanthones (Figure 4). However, in *G. utriculosa* extract, the peak area of xanthones was not significantly reduced, pointing out that these compounds had moderate antioxidant activity (Figure 5).

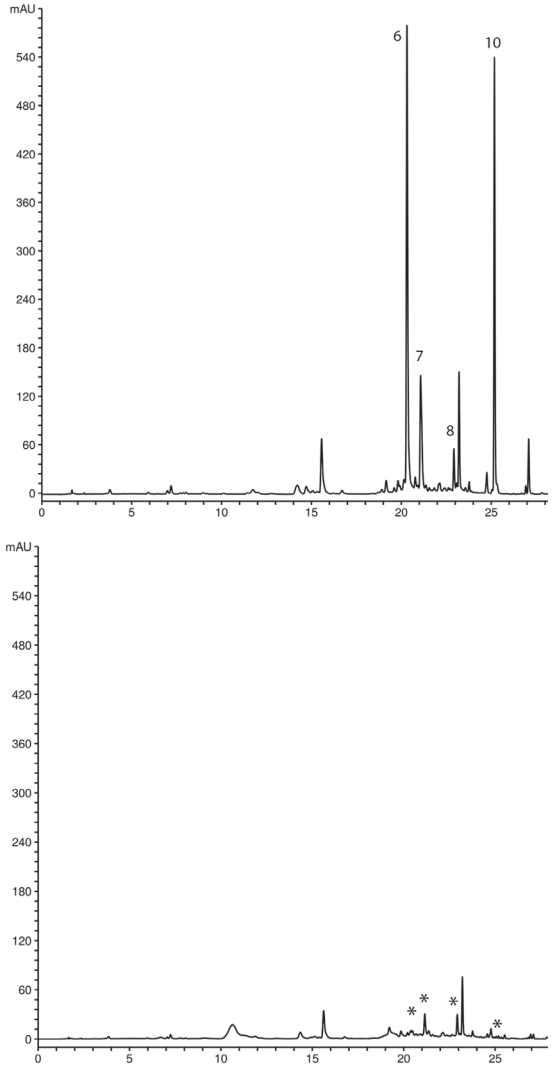

Figure 4. Chromatograms of *G. dinarica* extracts derived from transgenic roots clone B (Gd5) before ((**top**) chromatogram) and after reaction with DPPH radicals ((**bottom**) chromatogram). Peaks: 6—norswertianin-1-O-primeveroside; 7—norswertianin-8-O-primeveroside; 8—gentioside; 10—norswertianin. * The peaks of compounds that were involved in free radical scavenging activity.

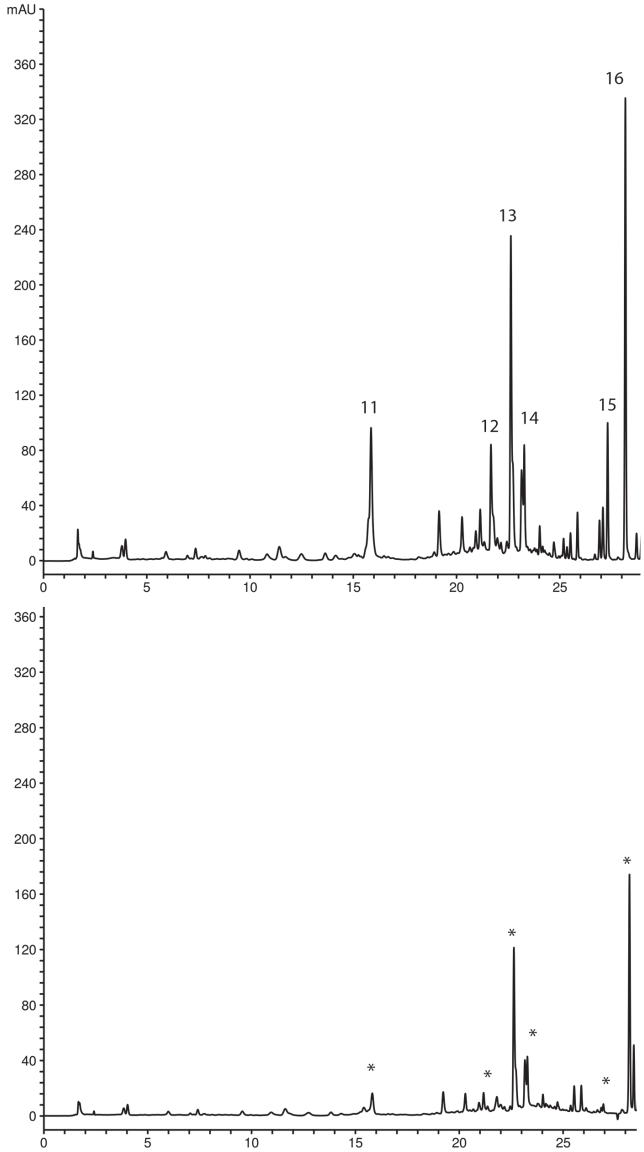

Figure 5. Chromatograms of *G. utriculosa* extracts derived from transgenic shoots (Gu3) before ((**top**) chromatogram) and after reaction with DPPH radicals ((**bottom**) chromatogram). Peaks: 11—mangiferin; 12—gentiakochianin-1-O-primeveroside; 13—decussatin-1-O-primeveroside; 14—1,8-dihydroxy-3-methoxy-7-O-primeveroside; 15—gentiakochianin; 16—decussatin. * The peaks of compounds that were involved in free radical scavenging activity.

2.3. The Inhibitory Effect of Gd and Gu Extracts on α-Glucosidase

The results in Figure 6 demonstrate the percentage inhibition of six *G. dinarica* extracts and four *G. utriculosa* extracts against the intestinal α-glucosidase (maltase). At a concentration of 1 mg/mL, examined extracts had moderate inhibitory potential towards α-glucosidase, ranging from 4.5 to 11.33% for Gd extracts and 4.56 to 28.29% for Gu extracts. On the other hand, at a concentration of 5 mg/mL, the examined extracts markedly inhibited intestinal maltase, ranging from 32.43 to 89.59% for Gd extracts and 9.36 to 64.4% for Gu extracts. The intestinal maltase inhibitory activity of Gd extracts was in the following order, from highest to lowest: Gd4, Gd5, Gd6 > Gd2 > Gd1, Gd3; for Gu extracts: Gu4 > Gu2, Gu3 > Gu1.

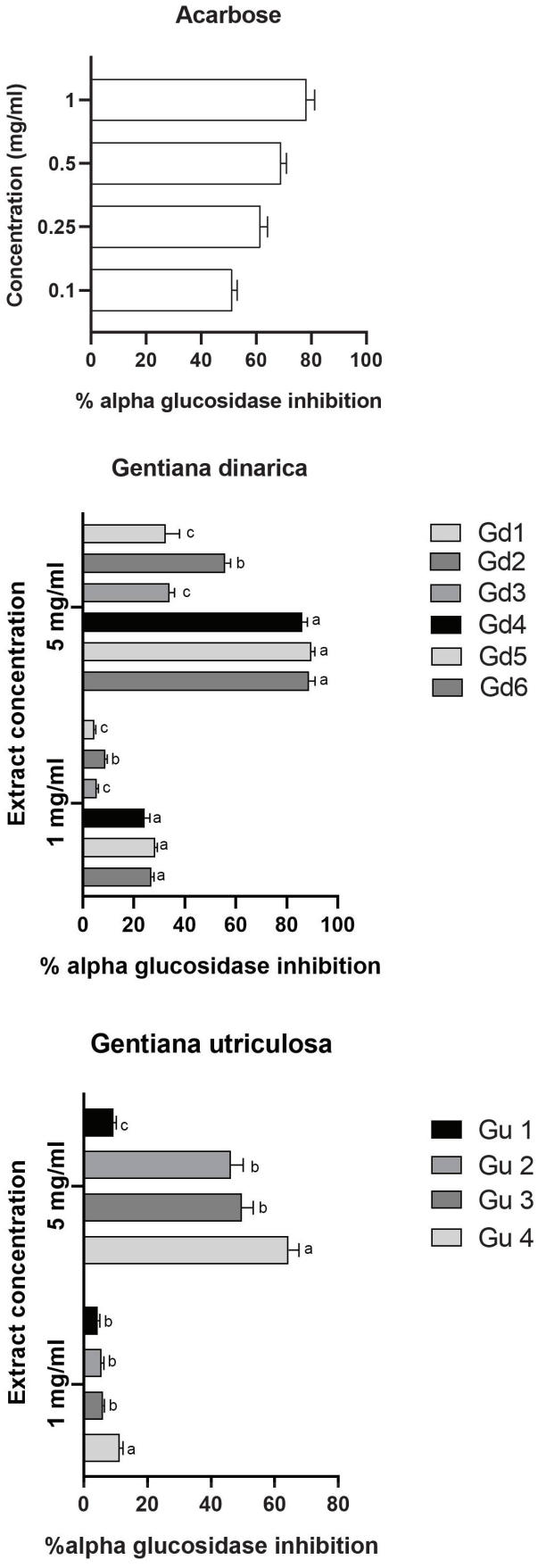

Figure 6. α-Glucosidase inhibitory activity at two different concentrations of methanol extracts obtained from *G. dinarica* and *G. utriculosa*. Acarbose was used as standard. Gd1—aerial parts of

wild growing plants; Gd2—roots of wild growing plants; Gd3—shoot culture; Gd4—vegetative roots; Gd5—genetically transformed roots, clone B; Gd6—genetically transformed roots, clone 3. Gu1—aerial parts of wild growing plants; Gu2—shoot culture; Gu3—genetically transformed shoots; Gu4—genetically transformed roots. The results of the assays are presented as the means ± S.E.M. from three separate measurements (n = 3). For all variables with the same superscript letter, the difference between the means (at the same concentration) is not statistically significant. If two variables have different letters, they are significantly different at $p < 0.05$.

2.4. Antihyperglycemic Activity of the Gd and Gu Extracts on Oral Glucose-Loaded Rats

Further analysis of the antihyperglycemic activity of the Gd and Gu extracts was conducted on selected extracts (Gu2, Gu3, Gd4, Gd5) based on their success in DPPH radical scavenging and in inhibition of alpha-glucosidase enzyme. In addition, the chosen extracts from both plants had to meet the criteria of coming from the same part of the plant but being grown in two different ways (Gu2, Gd4—in vitro propagation and Gu3, Gd—genetic transformation). The results of the oral glucose tolerance test in normal Wistar rats are shown in Figures 7 and 8. As seen in Figure 7, the initial blood glucose levels of all groups prior to drug/extract administration were equal. Following oral glucose loading (2 g/kg) to control and test groups, the blood glucose level was elevated to the maximum level after 30 min in vehicle-treated rats and decreased subsequently over time. At 15 min post-oral glucose loading, only the standard drug (glibenclamide (GLC), 5 mg/kg) treated group, and groups treated with Gu2 (400 mg/kg) and Gu3 (400 mg/kg) extracts showed a significant decline in blood glucose levels compared to the vehicle group. Likewise, the GLC 5 mg/kg-treated group and all groups treated with extracts in a dose of 400 mg/kg (Gu2, Gu3, Gd4, Gd5) showed a significant antihyperglycemic effect 30 min after glucose loading compared to the vehicle-treated group (Figure 7). In extract-treated groups, the blood glucose reached a maximum level at 60 min post-oral glucose loading. In addition, the area under the curve (AUC) in the first 60 min was significantly reduced in GLC and all extract-treated groups when compared to the vehicle group (Figure 8).

	0min	15min	30min	60min	120min	180min
Vehicle	4.94 ^{a}	7.46 ^{a}	8.26 ^{a}	7.62 ^{b}	6.18 ^{a}	5.06 ^{b}
Glc	4.7 ^{a}	6.2 ^{d}	5.7 ^{d}	4.9 ^{d}	3.7 ^{c}	3.4 ^{c}
Gu2	4.85 ^{a}	6.6 ^{c}	7.45 ^{b}	7.5 ^{b}	6.175 ^{a}	4.95 ^{b}
Gu3	5.05 ^{a}	6.425 ^{c}	7.25 ^{c}	7.35 ^{c}	6.2 ^{a}	5.4 ^{a}
Gd4	5.028 ^{a}	7.06 ^{b}	7.4 ^{b}	7.64 ^{b}	6.12 ^{a}	5.2 ^{b}
Gd5	4.96 ^{a}	7.15 ^{b}	7.375 ^{b}	7.82 ^{a}	5.94 ^{b}	5.14 ^{b}

Figure 7. In vivo antihyperglycemic effect of selected methanol extracts of *G. dinarica* and *G. utriculosa* in oral glucose-loaded rats. Glibenclamide (Glc) was used as a control drug. Gd4—vegetative roots;

Gd5—genetically transformed roots, clone B; Gu2—shoot culture; Gu3—genetically transformed shoots. Data are expressed as mean ± S.E.M.; no. of animals (N) = 5. For all variables with the same superscript letter, the difference between the means (same post-glucose overload time) is not statistically significant. If two variables have different letters, they are significantly different at $p < 0.05$.

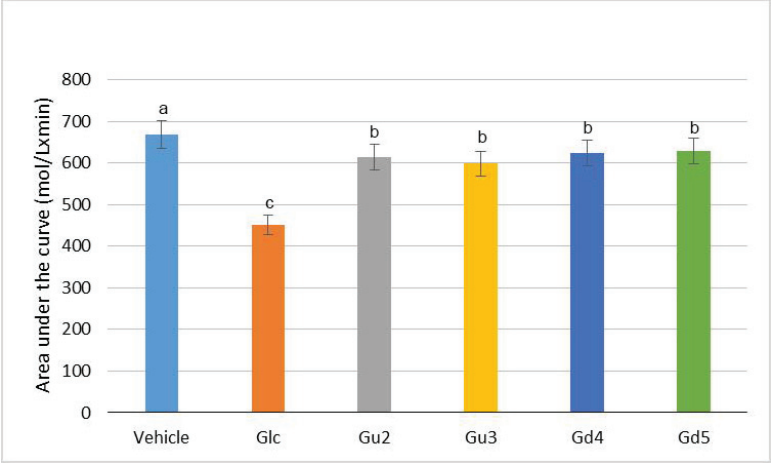

Figure 8. Area under the curve (AUC) for the first 60 min of oral glucose overload responses of Wistar rats treated with vehicle or selected methanol extracts of *G. dinarica* and *G. utriculosa* compared with standard drug. Glibenclamide (Glc)—standard drug. Gd4—vegetative roots; Gd5—genetically transformed roots, clone B; Gu2—shoot culture; Gu3—genetically transformed shoots. Data are expressed as mean ± S.E.M.; no. of animals (N) = 5. For all variables with the same superscript letter, the difference between the means (same post-glucose overload time) is not statistically significant. If two variables have different letters, they are significantly different at $p < 0.05$.

3. Discussion

Diabetes mellitus is one of the major multifactorial health problems globally and is associated with high morbidity and mortality rates. Insufficient blood sugar regulation over time leads to serious damage to the heart, blood vessels, eyes, kidneys, and nerves, causing chronic complications in diabetic patients. Conventional antidiabetic drugs improve blood glucose levels and the survival of people with diabetes but do not prevent secondary chronic complications associated with diabetes leading to an increase in the number of people living with more than one chronic condition, known as multiple long-term conditions (MLTC) [36]. Also, the side effects of conservative therapy impose restrictions on the choice of an antidiabetic drug. Therefore, despite the effectiveness of different antihyperglycemic drugs, new antidiabetogenic agents are increasingly sought among medicinal plants. In this study, the analysis of the antidiabetic potential of two *Gentiana* plant species, *G. dinarica* and *G. utriculosa*, and the association with secondary metabolite composition of their methanolic extracts revealed that xanthone-rich extracts have the most prominent antioxidant and antihyperglycemic effects.

Based on the quantitative and qualitative analysis of the extracts obtained from these two *Gentiana* species, it can be noticed that the accumulation of secondary metabolites is species-specific. Also, the quantitative and qualitative composition changes depending on the part of the plant the secondary metabolites were isolated from (aerial part or root) and the way a certain part of the plant was obtained (from nature, in vitro propagation, or genetic transformation). Differences in secondary metabolite composition between different *Gentiana* species and between underground and aerial parts of the same plant are already documented in the literature [37,38]. In accordance with previous studies [12], the dominant compounds of the extracts are derived from *G. dinarica* are secoiridoids (sweroside, swertiamarin, and gentiopicroside) and/or xanthones (norswertianin, norswertianin-1-O-primeveroside, and gentioside). Correspondingly to earlier research, in vitro culture

conditions significantly increased the accumulation of all quantified secondary metabolites, especially gentiopicrin in shoot cultures compared to samples of plants collected in nature [37,39–42]. On the other hand, as was reported earlier, in vitro propagation and genetic modification by Agrobacterium rhizogenes stimulated the production of norswertianin and norswertianin-1-O-primeveroside in transgenic roots of *G. dinarica* [43] and inhibited the production of secoiridoids and gentioside. Although genciopicrin is the main bitter constituent of Gentian roots [44], it was not detected either in vegetative or transgenic roots. In comparison to *G. dinarica*, *G. utriculosa* accumulates completely different sets of secondary metabolites, among which the most abundant were decussatin, decussatin-1-O-primeveroside, and mangiferin. This is in accordance with previous reports [17,22]. Although, according to Jensen and Schripsema [9], at least one bitter compound from the class of iridoids should be found in practically all *Gentiana* species, we did not detect any in *G. utriculosa*.

Hyperglycemia and glucose autooxidation in diabetes are responsible for the overproduction of various reactive oxygen species, causing oxidative damage to macromolecules such as lipids, proteins, and DNA. The inability of the body to effectively remove free radicals by antioxidant processes leads to oxidative stress, which affects the majority of tissues and organs in diabetic patients and promotes diabetic complications [35]. Given this, the use of natural products with antioxidant properties could have multiple beneficial effects on diabetic patients. The results of the antioxidant activity of the extracts from *G. dinarica* and *G. utriculosa*, measured through a DPPH scavenging assay, showed that extracts of both plants can scavenge the radical to a certain extent. The greatest scavenging activity, recorded in roots of *G. dinarica* obtained in vitro and by genetic transformation, was attributed to xanthones norswertianin and norswertianin-1-O-primeveroside. Uvarani et al. [45] also showed that norswertianin isolated from Swertia corymbosa (Gentianaceae) has the ability to scavenge ROS (DPPH, OH-, and NO-) and they ascribed this strong antioxidant capacity of norswertianin to the presence of the catechol moiety, which enhanced its H-donating ability. The other three *G. dinarica* extracts showed approximately ten times weaker ability to scavenge free radicals, which was in accordance with their very low level of norswertianin and norswertianin-1-O-primeveroside. Four extracts obtained from *G. utriculosa* also showed ten times weaker ability to scavenge DPPH radicals in comparison to extracts obtained from roots of *G. dinarica* (vegetative root culture and transgenic roots). Shoot culture and transgenic shoots showed the strongest scavenging activity among *G. utriculosa* extracts and this scavenging activity was attributed mainly to xanthones mangiferin, decussatin, and decussatin-1-O-primeveroside. Mangiferin is one of the most studied xanthone-C-glycosides with several beneficial properties, including antioxidant activity [46]. Unlike mangiferin, whose DPPH scavenging activity has been shown previously [47], there are no data published about radical scavenging activity and antioxidant properties of decussatin and decussatin-1-O-primeveroside.

Another effective strategy for diabetes management is the inhibition of intestinal α-glucosidase enzyme, which slows down the digestion and absorption of complex carbohydrates and in turn alleviates the increase in postprandial glycemia. Commercial drugs currently used as reversible inhibitors for α-glucosidase inhibition, such as acarbose, miglitol, and voglibose, exhibit side effects such as abdominal distension, bloating, flatulence, and possibly diarrhea [48]. Therefore, there has been a growing interest in research seeking novel new plant-derived inhibitors with improved efficacy [49]. In this study, both investigated *Gentiana* species possessed antidiabetic properties regarding the inhibition of the α-glucosidase enzyme, with different extracts having different inhibitory abilities. All extracts showed dose/concentration-dependent effects of inhibition on the activity of the α-glucosidase enzyme, suggesting a competitive type of inhibition [50]. The highest inhibitory activity recorded in vegetative and transgenic roots of *G. dinarica* was attributed to xanthones norswertianin and, particularly, norswertianin-1-*O*-primeveroside. In a previous investigation by Uvarani et al. [45], it was shown that norswertianin exhibited the most prominent inhibition of the α-glucosidase enzyme among six other xanthones

isolated from *Swertia corymbosa*. The same group of authors ascribed this inhibition to the presence of four free OH groups involved in H-bonding interactions with the α-glucosidase enzyme [45], which can be a valid explanation also for norswertianin-1-*O*-primeveroside having three free OH groups. Also, the root extract obtained from the wild growing plant showed a significant inhibitory activity that can be associated with a substantial amount of norswertianin-1-*O*-primeveroside. Among four extracts derived from *G. utriculosa*, which were less effective than the *G. dinarica* root extract, the highest inhibition of α-glucosidase enzyme was recorded in the extracts from genetically transformed roots that were rich in decussatin and decussatin-1-*O*-primeveroside. Although decussatin was found to have hypolipidemic and hypoglycemic effects in male Wistar rats fed with a high-fructose diet, the reported mechanism of action has not been through the inhibition of the α-glucosidase enzyme [51]. The other three *G. utriculosa* extracts were less effective in the inhibition of the α-glucosidase enzyme due to lower levels of decussatin and, particularly, decussatin-1-*O*-primeveroside. On the other hand, these extracts had mangiferin, a compound known for its ability to inhibit the α-glucosidase enzyme [52].

Sustained reduction in hyperglycemia is the key factor for preventing or reversing micro- and macrovascular complications, thus improving the quality of life in diabetic patients [53]. Therefore, we selected the glucose-induced hyperglycemic model to screen the antihyperglycemic activity of the Gd and Gu extracts. To determine the ability of the selected extract of these two *Gentiana* species to reduce the increased blood glucose levels, oral glucose tolerance tests were performed [54]. The criterion for the selection of the extracts to be used in further analysis was the success in DPPH radical scavenging and in the inhibition of the alpha-glucosidase enzyme. This study revealed that treatment with all four extracts at a dose level of 400 mg/kg had significantly lowered blood glucose levels in the first 60 min after glucose loading compared to the vehicle control. Treatment with extracts delayed and lowered the postprandial glucose spike, which indicates that extract-treated rats had increased glucose utilization, lower glucose absorption, or decreased glucose production from the liver compared to the control animals [55,56]. The mechanism behind this antihyperglycemic activity of *G. dinarica* and *G. utriculosa* extracts involves an insulin-like effect [51] or prevention of glucose transport at the level of some transport protein on the absorptive intestinal surface. It is known that the absence of the SGLT1 transporter prevents the absorption of glucose at the level of the small intestine in humans [57]. The main secondary metabolites in the examined extracts of these two Gentians are norswertianin and norswertianin-1-*O*-primeveroside for Gd extracts and mangiferin, decussatin, and decussatin-1-*O*-primeveroside for Gu extracts. Some of these compounds are known from the literature for their hypoglycemic effects in vivo. First of all, it has been shown that mangiferin facilitates β-cell proliferation and islet regeneration in mice with 70% partial pancreatectomy [58]. Decussatin from *Lomatogonium rotatum* (Gentianaceae) was found to produce hypolipidemic and hypoglycemic effects in male Wistar rats fed with a high fructose diet (51) and this effect has been attributed to its potential to modulate AMP-activated protein kinase (AMPK) activity in the liver. To date, no in vivo studies have been performed regarding the antihyperglicemic effects of norwertianin and norswertianin-1-*O*-primeveroside.

4. Materials and Methods

4.1. Plant Material

Plant extracts used in this study were obtained from the plant material of two *Gentiana* species collected in nature and cultivated in vitro. Extracts Gd1 (aerial parts of wild growing plants) and Gd2 (roots of wild growing plants) were derived from plants of *Gentiana dinarica* (Gd) collected in June 2015 on Mount Tara (~1300 m), western Serbia. A voucher specimen (accession number Gd072001) was deposited in the herbarium at the Faculty of Biology, University of Belgrade, Code BEOU. Extract Gd3 was obtained from *G. dinarica* shoot culture [13]. Extract Gd4 was derived from *G. dinarica* vegetative root cultures, while extracts Gd5 and Gd6 were prepared from genetically transformed roots—clone B and

clone 3, respectively. Genetic transformation of *G. dinarica* and establishment of vegetative and transgenic root cultures were previously published by Vinterhalter et al. 2015 [14].

Extract Gu1 (aerial parts of wild growing plants) was derived from *Gentiana utriculosa* collected on Divčibare Mountain (at ca. 800 m) in Serbia in June 2016. A voucher specimen (accession number Gu072002) was deposited in the herbarium at the Faculty of Biology, University of Belgrade, Code BEOU. Extract Gu2 was prepared from *G. utriculosa* shoot cultures [16], while extracts Gu3 and Gu4 were derived from genetically transformed shoots and transgenic roots, respectively. The protocol for the establishment of transgenic roots by *Agrobacterium rhizogene*-mediated transformation was reported by Vinterhalter et al. 2019 [17].

4.2. Extraction and HPLC Analysis of Secondary Metabolites

Secondary metabolite identification and content determination were performed in extracts from in vitro-derived plant material and wild growing plants. Plant material was air-dried at room temperature, ground using an electric mill, and extracted with methanol in an ultrasonic bath for 20 min. After sonication, extraction was continued by maceration in the dark at room temperature for 24 h. Then, extracts were filtered and evaporated to dryness in vacuum rotary evaporator (Buchi R-210, Flawil, Switzerland) at 50 °C. The extracts were stored at 4 °C until further analysis.

Identification and quantification of secondary metabolites of *G. dinarica* and *G. utriculosa* extracts were accomplished with chromatographic analysis (Agilent series 1100 HPLC instrument with a diode array detector, Waldbronn, Germany) on a reverse phase Zorbax SB-C18 (Agilent) analytical column (150 mm × 4.6 mm i.d., 5 µm particle size) thermostated at 25 °C. The mobile phase consisted of solvent A (0.1%, *v*/*v* solution of orthophosphoric acid in water) and solvent B (acetonitrile, J.T. Baker, Deventer, The Netherlands).

The separation of the components was performed using an elution gradient according to the earlier published method by Krstic-Milosevic et al. [59]: 98–90% A 0–5 min, 90% A 5–10 min, 90–85% A 10–13 min, 85% A 13–15 min, 85–70% A 15–20 min, 70–40% A 20–24 min, 40–0% A 24–28 min. The injection volume was 5 µL. Detection wavelengths were set at 260 and 320 nm, and the flow rate was 1 mL min^{-1}. The isolation and characterization of xanthones norswertianin-1-*O*-primeveroside and gentioside from *G. dinarica* roots were previously reported [60]. Acid hydrolysis of norswertianin-1-*O*-primeveroside with 2N HCl yielded xanthon aglycone norswertianin. Standards of xanthones decussatin-1-*O*-primeveroside and decussatin were previously isolated from the aerial parts of *G. utriculosa* (22). Xanthone mangiferin was purchased from Sigma-Aldrich, Steinheim, Germany. Commercial standards of secoiridoids swertiamarin, gentiopicrin, and sweroside were bought from Cfm Oscar Tropitzsch (Bayern, Germany). Identification of secoiridoids and xanthones was confirmed using a co-injection method using standard compounds. Quantification of secondary metabolites was performed using calibration curves in an external standard method. Standard solutions for HPLC were prepared by dissolving standard compounds in methanol. All experiments were repeated at least two times. The results are presented as mg per g of dry weight of extracts.

4.3. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Free Radical-Scavenging Assay

The free radical scavenging activity of the *G. dinarica* and *G. utriculosa* methanol extracts was determined according to a standard method of Blois [61] by measuring the decrease in the absorbance of stable free radical DPPH (2,2-Diphenyl-1-picrylhydrazyl) at 517 nm as previously performed [62]. Stock solution of extracts Gd1, Gd2, Gd3, Gu1, Gu2, Gu3, and Gu4 were diluted to a concentration of 0.625, 1.25, 2.5, and 5 mg/mL in methanol. Stock solution of extracts Gd4, Gd5, and Gd6 were diluted to a concentration of 0.05, 0.1, 0.25, and 0.5, 1 mg/mL in methanol. DPPH methanolic solution (0.5 mL, 0.25 mM) was added to a mixture of 0.1 mL of sample solutions of different concentrations and 0.4 mL Tris HCl (100 mM, pH 7.4) and allowed to react at room temperature. After 30 min, the absorbance values were measured at 517 nm. Ascorbic acid was used as a standard. All

tests were performed in triplicate. The inhibitory percentage of DPPH was calculated according to the following formula: percentage of inhibition = ((A blanc − A test)/A blanc) × 100, where A blanc is the absorbance of the methanolic DPPH solution and A test is the absorbance of DPPH in the solution with the extract or a standard (ascorbic acid).

4.4. The DPPH-HPLC Procedure

The compounds of extracts that were included in free radical scavenging activity were evaluated using the HPLC method with a pre-chromatographic reaction of tested extracts with DPPH radicals, according to the method described by Olennikov et al. [63]. The reaction mixture contained 300 µL of plant extract dissolved in methanol (10 mg/mL) and 300 µL of DPPH methanol solution (2.5 mg/mL). The control mixture consisted of 300 µL of plant extract dissolved in methanol (10 mg/mL) and 300 µL of methanol instead of DPPH solution. After incubation for 20 min at room temperature in the dark, mixtures were filtered through a 0.45 µm membrane filter and analyzed by the above-described HPLC method.

4.5. The Assessment of Inhibitory Effect of Gd and Gu Extracts on α-Glucosidase

The assessment of intestinal α-glucosidase inhibitory activity was based on the modified method previously described [64]. Briefly, 50 mg of rat intestinal acetone powder was homogenized in 1.5 mL of 0.9% NaCl solution. The solution was centrifuged at 12,000× g for 30 min and then used as the small intestinal glucosidases for maltose hydrolysis. The crude enzyme solution (20 µL) was incubated with 30 µL maltose (86 mM) and 10 µL of the extract at various concentrations, followed by the addition of 0.1 M phosphate buffer with pH 6.9 to a final volume of 200 µL. The reaction was incubated at 37 °C for 30 min. Thereafter, the mixtures were suspended in boiling water for 10 min to stop the reaction. The concentrations of glucose released from the reaction mixtures were determined with the glucose oxidase method with absorbance at a wavelength of 450 nm. Intestinal α-glucosidase inhibitory activity was expressed as the percentage inhibition using the following formula: percentage of inhibition = (A control − A sample)/A control) × 100, where A control was the absorbance without a sample and the A sample was the absorbance of the sample extract or standard (acarbose).

4.6. Anti-Hyperglycemic Activity of Selected Gu and Gd Extracts In Vivo

Experiments were performed on 2.5-month-old male Wistar albino rats weighing 220–250 g. All animals were kept under standard laboratory conditions in a climate-controlled room with a temperature of 24–26 °C, 20–60% relative humidity, and on a 12 h–12 h light–dark cycle. They were allowed free access to a standard chow diet and fresh water. All experimental procedures were approved by the Ministry of Agriculture, Forestry and Water Management—Veterinary Administration (protocol code 323-07-00055/2023-05); date of approval 9 January 2023) based on positive opinions of the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", National Institute of the Republic of Serbia, University of Belgrade, which acts in accordance with the Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes. For the realization of this in vivo experiment, 30 male Wistar rats, aged 2.5 months, were needed. Rats were randomly assigned to the appropriate experimental groups (five animals in each group). The first group served as the glucose control group to which glucose solution (2 g/kg) was administered by oral gavage. The second group served as the standard control group to which glibenclamide (GLC, 5 mg/kg, oral gavage) was administered. The third group received Gu2 extract isolated from in vitro-propagated shoots. The fourth group received Gu3 extract isolated from genetically transformed shoots. The fifth group received Gd4 extract isolated from in vitro-propagated roots. The sixth group received Gd5 extract isolated from genetically transformed roots. All extracts were applied in a dose of 400 mg/kg by oral gavage. Animals were fasted overnight prior to the experiment and in the morning the fasting blood glucose levels were measured with a blood

glucometer (Accu-Chech Active, Roche Diabetes Care, Mumbai, India). Test extracts and reference standard drugs were given to the respective groups of animals as per their body weight. After 30 min of extract/drug administration, glucose solution was administered to all groups orally by dissolving it in distilled water. Thereafter, blood glucose level was recorded at 30, 60, 90, 120, and 180 min of post-glucose overload for accessing the antihyperglycemic activity of tested extracts and standard drugs [50]. Blood samples were obtained from the tail. Less than 2 mm of tissue was cut from the tail tip, distal to the bone, with sharp scissors. Blood was obtained by direct flow or by gently massaging the tail and collecting the blood directly on a glucose test strip of blood glucometer (Accu-Chech Active, India).

4.7. Statistical Analysis

Experimental data subjected to statistical analysis were expressed as the means ± S.E.M. (standard error of mean). One-way analysis of variance (ANOVA) followed by Tukey's tests were used for multiple comparison. Statistical analysis was conducted in GraphPad Prism 8 software. Different letters indicate significant differences between extracts ($p < 0.05$).

5. Conclusions

The findings of this present study showed that all examined extracts obtained from *G. dinarica* and *G. utriculosa* showed moderate to strong antioxidant and antihyperglycemic effects correlated to the secondary metabolites composition, which differed among different parts of the plant (aerial vs. root) and was influenced by the way in which a certain part of the plant was obtained (from nature, in vitro propagation or genetic transformation). The most prominent antidiabetic properties were detected in extracts derived from vegetative and transgenic root cultures of *G. dinarica* and shoot and transgenic shoot cultures of *G. utriculosa*. The examined antidiabetic properties are in direct correlation with accumulation of xanthones norwertianin and norswertianin-1-*O*-primeveroside in *G. dinarica* and decussatin and decussatin-1-*O*-primeveroside in *G. utriculosa* extracts. In conclusion, the ability of xanthone-rich extracts to scavenge free radicals and control the rise in glucose levels might be potentially useful in the development of novel therapeutics for the treatment of diabetes.

Author Contributions: Conceptualization, J.A.J., M.M. and D.K.-M.; methodology, D.K.-M., M.M., B.V. and M.Đ.; validation, M.V., S.D., J.R. and M.Đ.; formal analysis A.U., J.R., N.G. and A.S.; investigation, J.A.J., D.K.-M., M.Đ. and S.D.; writing—original draft preparation, J.A.J., D.K.-M. and M.M.; writing—review and editing, M.V., B.V., S.D. and A.U.; visualization, S.D., A.U. and A.S.; supervision, B.V., J.A.J. and M.M.; project administration, M.V., J.R., N.G. and A.S.; funding acquisition, M.V., A.U. and N.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Science Technological Development and Innovation of the Republic of Serbia (registration No. 451-03-66/2024-03/200007).

Institutional Review Board Statement: This study was conducted in accordance with the Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes. The animal study protocol was approved by the Ministry of Agriculture, Forestry, and Water Management—Veterinary Administration (protocol code 323-07-00055/2023-05); date of approval 09/01/2023) based on positive opinions of the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", National Institute of the Republic of Serbia, University of Belgrade.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors upon request.

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

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Caffeine Protects Keratinocytes from *Trichophyton mentagrophytes* Infection and Behaves as an Antidermatophytic Agent

Diogo M. da Fonseca [1,2,3], Lisa Rodrigues [2,3], José Sousa-Baptista [1,2,3], Félix Marcos-Tejedor [4], Marta Mota [1,2,3], Rodrigo A. Cunha [1,2,3], Chantal Fernandes [2,3] and Teresa Gonçalves [1,2,3,*]

[1] FMUC—Faculty of Medicine, University of Coimbra, Rua Larga, 3004-504 Coimbra, Portugal; dmfonseca@igc.gulbenkian.pt (D.M.d.F.); josepedrobaptista1237@gmail.com (J.S.-B.); marta.mota83@gmail.com (M.M.); cunharod@gmail.com (R.A.C.)
[2] CNC-UC—Center for Neuroscience and Cell Biology of Coimbra, University of Coimbra, 3004-504 Coimbra, Portugal; lisa1cor@gmail.com (L.R.); xantal@gmail.com (C.F.)
[3] CIBB—Centre for Innovative Biomedicine and Biotechnology, University of Coimbra, 3004-504 Coimbra, Portugal
[4] Department of Medical Sciences, Faculty of Health Sciences, University of Castilla-La Mancha, 45600 Talavera de la Reina, Toledo, Spain; felix.marcostejedor@uclm.es
* Correspondence: tmfog@ci.uc.pt

Abstract: Caffeine affords several beneficial effects on human health, acting as an antioxidant, anti-inflammatory agent, and analgesic. Caffeine is widely used in cosmetics, but its antimicrobial activity has been scarcely explored, namely against skin infection agents. Dermatophytes are the most common fungal agents of human infection, mainly of skin infections. This work describes the in vitro effect of caffeine during keratinocyte infection by *Trichophyton mentagrophytes*, one of the most common dermatophytes. The results show that caffeine was endowed with antidermatophytic activity with a MIC, determined following the EUCAST standards, of 8 mM. Caffeine triggered a modification of the levels of two major components of the fungal cell wall, β-(1,3)-glucan and chitin. Caffeine also disturbed the ultrastructure of the fungal cells, particularly the cell wall surface and mitochondria, and autophagic-like structures were observed. During dermatophyte–human keratinocyte interactions, caffeine prevented the loss of viability of keratinocytes and delayed spore germination. Overall, this indicates that caffeine can act as a therapeutic and prophylactic agent for dermatophytosis.

Keywords: caffeine; antidermatophytic; dermatophytes; *Trichophyton mentagrophytes*

1. Introduction

Caffeine (1,3,7-trimethylxanthine), a purine alkaloid, is the most widely consumed psychostimulant, found in several beverages such as coffee, tea, cocoa, yerba mate, guarana, or cola nuts. Several beneficial effects for human health have been associated with caffeine intake such as increasing cognitive and memory performance and increasing healthspan upon aging. Its recognized bioactive properties include being anti-inflammatory, analgesic, and anti-tumoral [1–3]. Several studies show that the consumption of caffeine, thanks to its antioxidative properties, prevents the occurrence or progression of chronic diseases, where reactive oxygen or nitrogen species are involved [3,4]. Recently, a comprehensive review addressed the effects of caffeine on human health, as well as delivery formulations to enhance the bioavailability of caffeine [3]. Caffeine and its derivatives are also widely used in cosmetics. The cosmetic antioxidant properties of caffeine make it an important protective and preventative agent against UV damage radiation and photoaging of the skin [5,6]. This led us to hypothesize that caffeine might also be beneficial during skin infection since the antibacterial and antifungal activity of caffeine has been previously reported [7–11].

Caffeine has also been described as interfering in fungal cell growth and is widely used in in vitro assays aiming to study the mechanisms regulating cell wall synthesis [12–15] and, more recently, the mechanisms of antifungal resistance [16]. Previously, we have described the antifungal effect of spent coffee grounds against dermatophytes, a group of fungi responsible for skin infections [17]. However, the antifungal properties of caffeine on the dermatophytes involved in human skin infection have not been unraveled. Dermatophytes are filamentous fungi with a tropism for keratinized structures and are an important cause of skin, nail, and hair infections, generically designated as dermatophytosis. Although these infections are not life-threatening, they can significantly affect the patient's quality of life. The management of these infections has become an important public health issue, due to the incidence of recurrent, recalcitrant, or extensive infections [18]. The current treatment for dermatophytosis comprises topical application of antifungal agents, while administration of oral antifungals is indicated for more extensive infections, although they have limited effectiveness and toxicity [18,19].

This work reports the susceptibility of *T. mentagrophytes* to caffeine and the impact of caffeine in the progression of the in vitro infection of HaCaT human keratinocytes with this dermatophyte, together with the evaluation of its cytotoxicity, fungal spores' germination, and cell viability upon infection. The antifungal mechanism of action was approached by quantification of the main fungal cell wall components, chitin and β-1,3-glucan, and how caffeine affects the germination of fungal spores during in vitro infection.

2. Results

2.1. Antidermatophytic Activity of Caffeine

Antifungal susceptibility testing was performed to determine the minimal inhibitory concentration (MIC) of caffeine, following the EUCAST E.DEF 9.3.1 standards for filamentous fungi. The antifungal susceptibility assays showed that caffeine is an antidermatophytic with a MIC value of 8 mM for *T. mentagrophytes*.

2.2. Modulation of Fungal Cell Wall β-1,3-Glucan and Chitin in Response to Caffeine

To approach the mechanism of action of caffeine, we determined the modulation of two cell wall components of *T. mentagrophytes* as a model organism. The results showed that all the tested concentrations of caffeine caused a significant decrease in the levels of the cell wall β-(1,3)-glucan in *T. mentagrophytes* (Figure 1A).

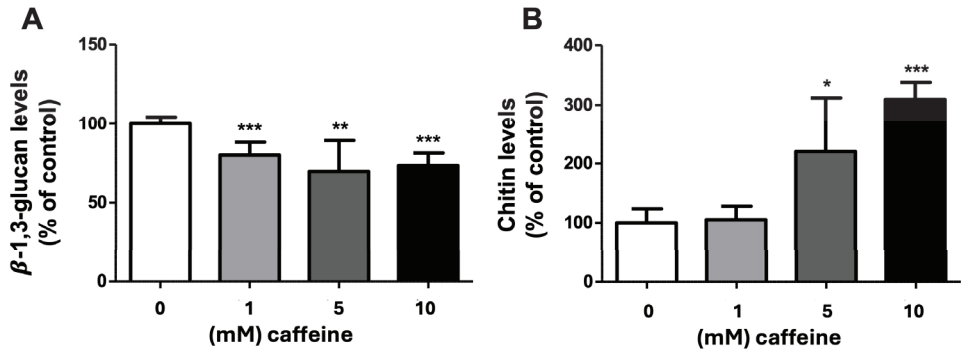

Figure 1. Effect of caffeine on β-(1,3)-glucan and chitin levels of *T. mentagrophytes* cell wall. *T. mentagrophytes* microconidia were inoculated in YME supplemented with 1, 5, or 10 mM of caffeine prior to the quantification of (**A**) β-(1,3)-glucan or (**B**) chitin levels in the fungal cell wall. Under the control condition, the fungus was grown in YME containing no caffeine. Results are presented as mean ± SEM (n = 3); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with an unpaired t-test.

The quantification of chitin in *T. mentagrophytes* grown in media supplemented with several concentrations of caffeine, showed that different concentrations of caffeine affected differently the chitin cell wall content. While 1 mM of caffeine did not change the chitin cell

wall levels, 5 mM and 10 mM concentrations of caffeine significantly increased the levels of this cell wall component of *T. mentagrophytes* (Figure 1B). At the 10 mM caffeine level, the highest concentration tested, a roughly threefold increased production of chitin occurred when compared with control conditions; this indicates that high concentrations of caffeine lead to an upregulation of chitin synthesis by this dermatophyte.

2.3. Characterization of Ultrastructural Changes

Transmission electron microscopy was used to appraise the ultrastructural changes in the hyphae of the fungi grown in caffeine-containing media. The morphology of *T. mentagrophytes* grown in the absence of caffeine (Figure 2A–D) showed fungal hyphae with a regular clear cytoplasm and a homogeneous cell wall with a smooth surface. One of the most marked features of the ultrastructure of fungal cells is a high number of mitochondria, with a normal morphology characterized by well-defined cristae and regular nuclei. Figure 2A,C,D shows the presence of large vacuoles with denser electronegative compounds inside the control fungal cells, probably representing reserve materials required for hyphal growth.

In the presence of 10 mM caffeine (Figure 2E–L), abnormal cellular morphologies and ultrastructure modifications were observed when compared with the control (Figure 2A–D). The clearest modification occurs in the cell wall surface, with a rougher surface and less homogeneous structure in fungi grown in 10 mM caffeine compared with control cells. The stability of the cell wall structure seems compromised since there were materials detaching from it (as seen in the central cell in Figure 2L). Interestingly, in Figure 2H, the chromatin seems to be migrating to opposite poles of the nucleus, which suggests that the fungal cell is undergoing mitosis and consequently dividing. Another cellular structure that exhibits modifications in the presence of caffeine is the mitochondria. Strikingly, the ultrastructure of these organelles showed an aberrant morphology with abnormal cristae (see zoomed crops in Figure 2G–J). Signs of autophagy can be observed in Figure 2F,K with the characteristic membrane whorls. Another aspect worth noting is that the extracellular medium of fungi incubated with caffeine is full of debris, not observed in the extracellular milieu of fungi grown in control conditions. Measurement of the thickness of *T. mentagrophytes* cell walls did not reveal significant alterations when grown in the presence and absence of caffeine (results not shown).

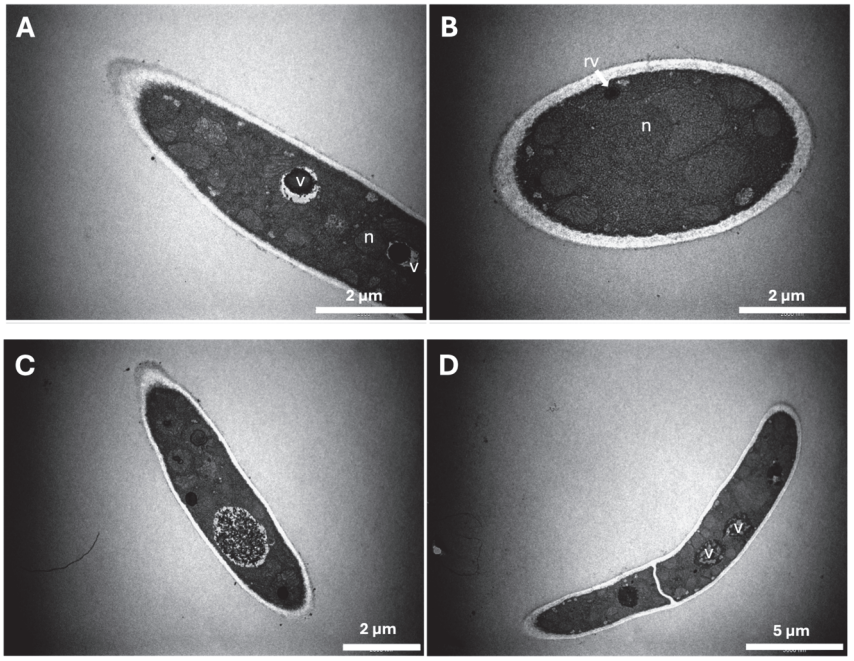

Figure 2. *Cont.*

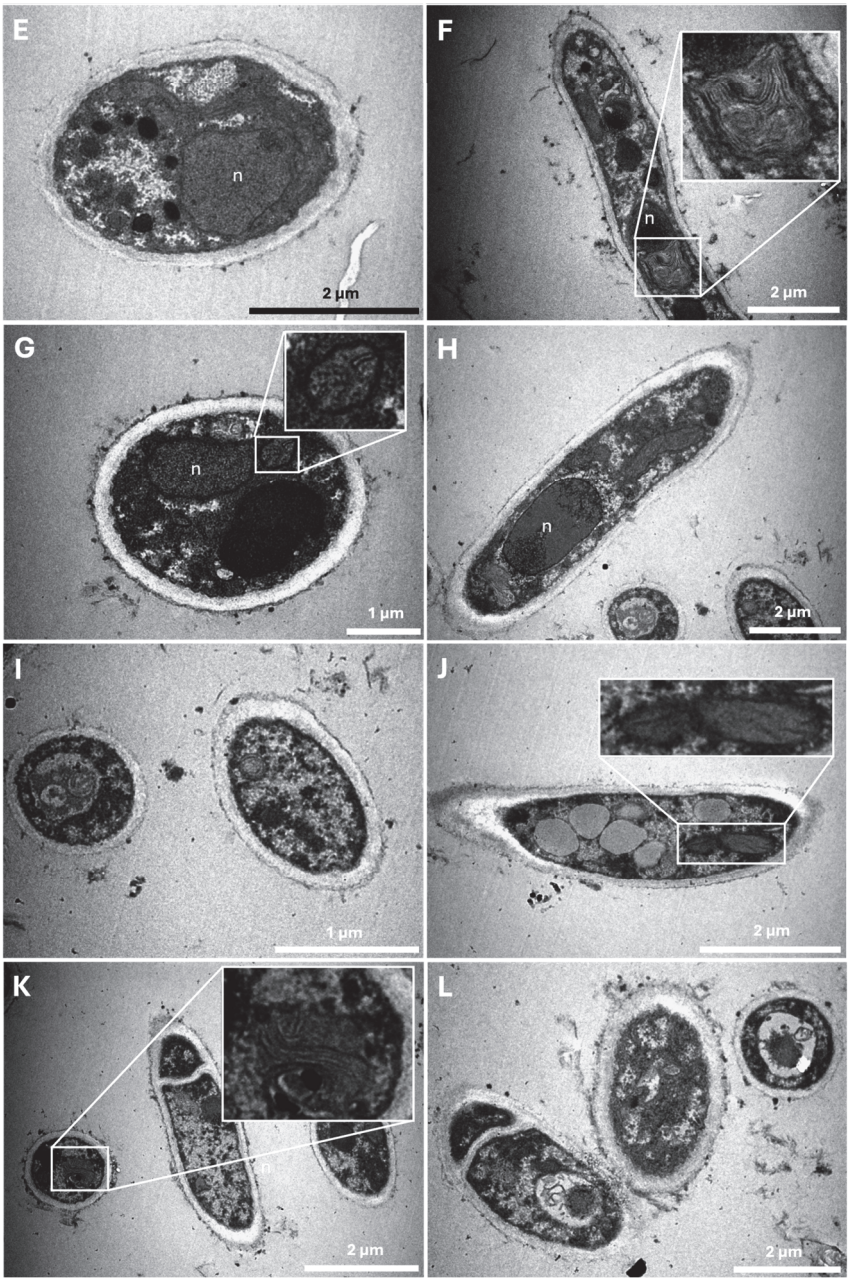

Figure 2. Impact of caffeine on *T. mentagrophytes* ultrastructure. Fungal microconidia were inoculated in YME in the absence or presence of 10 mM caffeine. Acquired images from (**A**–**D**) represent fungi grown in the absence of caffeine, and images from (**E**–**L**) represent fungi grown in media supplemented with 10 mM caffeine. "v" identifies vacuoles, "n" nuclei.

2.4. Antifungal Properties of Caffeine during Keratinocyte Infections by T. mentagrophytes Spores

2.4.1. Viability of Keratinocytes upon Infection with *T. mentagrophytes* Spores

To understand how caffeine affects the viability of HaCaT cells, control assays were performed by incubating keratinocytes with the selected caffeine concentrations, without the addition of fungal microconidia (Figure 3A).

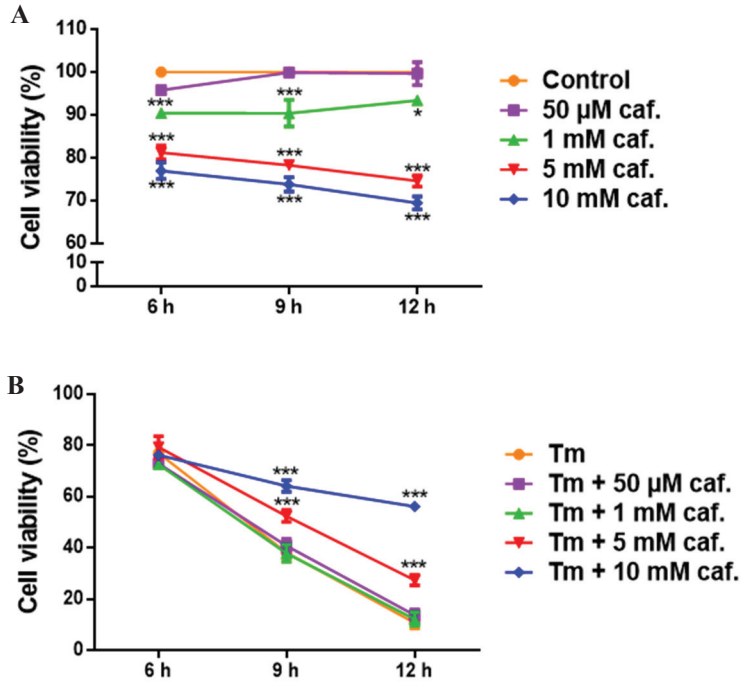

Figure 3. Effect of caffeine on HaCaT cell viability and impact during an in vitro infection with *T. mentagrophytes* (Tm) microconidia. Keratinocytes were exposed to caffeine (caf.) at different concentrations for 6, 9, and 12 h. Cell viability was quantified using the MTT assay. Under control condition, no caffeine was added to cells. (**A**) The viability of HaCaT cell was determined in the absence of fungal microconidia and (**B**) in the presence of fungal *T. mentagrophytes* spores. Results are presented as mean ± SEM ($n = 3$); * $p < 0.05$, *** $p < 0.001$ (vs. control in (**A**) and vs. Tm in (**B**)) using two-way ANOVA.

During the interaction assay period (12 h), there were no differences in the viability of keratinocytes when they were incubated in the presence of 50 µM caffeine; however, viability decayed in the presence of 1 mM, 5 mM, and 10 mM caffeine (Figure 3A). Although these results show that increasing concentrations of caffeine have a negative effect on the viability of HaCaT cells, the viability of the cells under all the conditions was above 70% (Figure 3A). A completely different scenario was observed when HaCat cells were infected with *T. mentagrophytes* (Figure 3B). In these conditions, both without or in the presence of 50 µM and 1 mM of caffeine, the viability of HaCaT cells dramatically decreased to 11% during the 12 h infection period. However, this decline in cell viability was attenuated by the presence of 5 mM and 10 mM caffeine concentrations to values of 27% and 56%, respectively (Figure 3B). This indicates that caffeine prevents the loss of viability of keratinocytes during the course of *T. mentagrophytes* infection.

2.4.2. Ungermination of Microconidia during HaCaT Infection

These assays were performed to understand how caffeine impacts dermatophyte microconidia germination during the course of an in vitro keratinocyte infection. The results obtained show that 10 mM caffeine reduced *T. mentagrophytes* spore germination during infection of human keratinocytes (Figure 4).

The presence of caffeine led to a higher number of ungerminated conidia. This effect was more evident at 9 h of infection, with a 460% increase in the number of ungerminated microconidia (Figure 4).

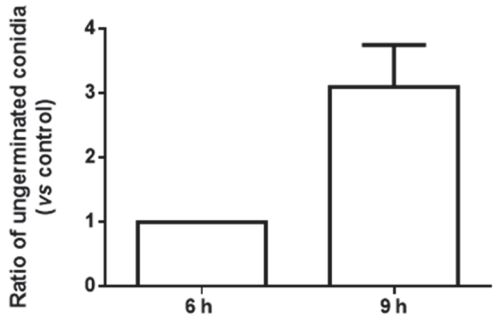

Figure 4. Effect of caffeine on *T. mentagrophytes* microconidia germination during the course of an in vitro infection. HaCaT cells were infected with *T. mentagrophytes* microconidia in the absence (control condition) or in the presence of 10 mM caffeine. After each incubation period, the number of ungerminated microconidia was counted. The number of ungerminated microconidia in control conditions (no caffeine) was normalized to 1. Results are presented relative to control as mean ± SEM (n = 3).

3. Discussion

Caffeine is a secondary metabolite present in over 100 plant species [8]. Although only a few papers report the antifungal properties of caffeine, its antidermatophytic activity was not known. We previously described that spent coffee ground extracts were endowed with antifungal properties, including antidermatophytic activity [17]. This was observed for both caffeinated and decaffeinated spent ground coffee extracts: the differences between the two extracts were minimal, but both had antidermatophytic effect. However, in that previous study, the components of the extracts were not isolated, so the effect was ascribed to a complex mixture of phytochemicals [17]. The main goal of the present work was to unveil the particular antidermatophytosis properties of caffeine.

The in vitro antidermatophytic effect of caffeine was determined using a microdilution broth, following EUCAST standards, on *T. mentagrophytes* with a MIC of 8 mM. These results are in good agreement with previous studies on the yeast *Candida albicans* that reported a MIC of caffeine of 12.5 mM [20]. Other studies also in *C. albicans* showed a different MIC of caffeine of 0.1 mM (25 mg/L) [21] using caffeine extracted from tea and 150 mM (30 mg/mL) [22] using pure caffeine. Comparison with filamentous fungi is limited because data are scarce and the few available data were obtained with different methodologies [23].

As an attempt to unravel the therapeutic target of caffeine, we quantified the modulation of β-1,3-glucan and chitin contents of *T. mentagrophytes* grown in the absence and presence of caffeine. The β-(1,3)-glucan and chitin were selected because, apart from being two structurally important polysaccharides present in this cellular structure and strongly accounting for its integrity, they are also important pathogen-associated molecular patterns (PAMPs) to consider when studying infection and inflammation phenomena triggered by these fungi [24–26]. It was observed that there was a decrease in β-(1,3)-glucan levels at all tested concentrations of caffeine together with an increase in chitin cell wall contents upon exposure to 5- and 10-mM caffeine in relation to the control. Some fungi also increase chitin levels in response to echinocandins as a salvage mechanism to compensate for the decreased contents of β-glucan [27–30]. Previously, we also reported some fluctuations of the chitin and/or β-glucan cell wall content in dermatophytes by the action of natural extracts, but we never observed such a clear paradoxal effect associated with the decreased β-glucan levels [31,32]. This may indicate that the decreased levels of β-glucan lead to a marked cell wall destabilization or that caffeine activates the chitin cell wall synthesis. Although few studies report the antifungal properties of caffeine, caffeine has been described as interfering with the growth of fungal cells and it is widely used in in vitro assays studying the mechanisms of the regulation of cell wall synthesis [13,14]. Recently, it was proposed that caffeine might be an "epigenetic drug" reducing antifungal resistance [16]. Caffeine is an inhibitor of cAMP phosphodiesterase and stimulates a dual phosphorylation of ScSlt2, the MAP kinase component of the PKC cell wall integrity signal transduction

pathway [33]. For example, the growth of *C. albicans* in a medium containing 12 mM of caffeine resulted in a significantly elevated expression from all CHS promoters [14]. Therefore, we can speculate that the two-to-three-fold increase in chitin cell wall levels now observed upon exposure to 5 mM and 10 mM of caffeine might also be due to the activation of chitin synthesis.

The impact of caffeine on the ultrastructure of *T. mentagrophytes* was evaluated by TEM and showed profound changes in response to caffeine. The surface of the cell wall was modified in the presence of caffeine and presented a rougher aspect with loosening materials to the extracellular milieu. This ultrastructural modification might be due to changes in the cell wall components as observed with other fungi treated with inhibitors of glucan synthesis, of chitin synthase, or of melanin synthesis [34]. It was found that mitochondria were scarce in caffeine-grown fungi when compared with the control and displayed an irregular morphology and abnormal cristae. In contrast, in humans, it is described that caffeine promotes mitochondrial biogenesis and improves mitochondrial functionality, playing an important protective role against oxidative stress [35–37]. The TEM study also showed that *T. mentagrophytes* grown in the presence of 10 mM caffeine had irregular structures, which appeared rolled in whorls. These abnormal structures were reported before as signs of autophagy [38]. In fact, we previously observed similar modifications with extracts of spent ground coffee and with other natural extracts [17,31,32]. Vacuoles play an important role in homeostasis and in the maintenance of a balanced chemical composition of the cytoplasm in the face of fluctuating external conditions; one of their mechanisms is the excreting of water into the cell to compensate for an unbalanced turgor pressure [39]. Since these structures appeared in higher numbers and some of them were abnormally large, it might be a mechanism of *T. mentagrophytes* to compensate for changes in the osmotic equilibrium due to a more fragile cell wall.

One of the most important objectives of this work was to study the impact of caffeine, a drug used in cosmetics, during the course of a (skin) fungal infection. For this, we studied the interaction of *T. mentagrophytes* spores with human keratinocytes (HaCaT cell line). Keratinocytes constitute most of the epithelial cells in human skin [40]. These cells are responsible for the formation of a natural barrier against physical, chemical, and microbial aggressions. Keratinocytes detect and respond to stimuli producing immune-inflammatory mediators that in turn trigger a more specific response by cells of the immune system, some of which are recruited to the site where the aggression was detected [41]. Keratinocytes also play an important role in wound healing [42]. Here, it was explored whether caffeine could interfere with the damage of human keratinocytes during the infection of *T. mentagrophytes*. It was observed that the infection of a HaCaT cell culture with spores of *T. mentagrophytes* during 12 h led to a decrease in cell viability to 11% of the control non-infected cells. The exposure to 10 mM caffeine during this 12 h interaction period prevented the loss of viability of keratinocytes. Surprisingly, caffeine alone led to a decrease in keratinocyte viability, although remaining above 70% with all the tested concentrations of caffeine. In other stressful conditions, it was described that 5 mM caffeine reduces HaCaT cell viability and promotes apoptosis upon exposure to ultraviolet B radiation [2]. It was also reported that this concentration of caffeine prevents human epidermal keratinocyte (HEK cell line) proliferation and migration, therefore suggesting that it may have an inhibitory effect in wound closure and epithelization during in vitro wound assays. The authors hypothesized that this delay, caused by caffeine in cell migration and epithelization, might likely be due to alterations of the cytoskeleton caused by caffeine, but the underlying mechanism remains to be elucidated [43]. We now describe for the first time that, during the course of an infection (in this case by *T. mentagrophytes*), the presence of caffeine-protected keratinocytes, preserving their viability. This might be explained by the antioxidant effect of caffeine since under conditions of infection, oxidative stress increases dramatically and this is usually also deleterious for the host cell, although contributing to killing the pathogen. Moreover, upon the infection of keratinocytes with a dermatophyte in the presence of caffeine, there was a decay in the germination of fungal spores. Undoubtfully, these two aspects together

indicate an alleviation of the infection process since the ability of a fungal infection such as tinea to proceed and succeed depends on the germination of spores.

The most important conclusions that were revealed by the present study are that caffeine, especially at a concentration of 10 mM, a fifteen times lower concentration than the one found in most commercially available topical solutions (3%; [6]), inhibited *T. mentagrophytes* growth. Caffeine also altered both β-(1,3)-glucan and chitin, two important structural components of the fungal cell wall required for the robustness of the fungus, for an efficient recognition of this pathogenic microorganism by target host cells and for triggering an efficient immune response to promote its clearance during an infection. It was also shown that caffeine preserved the viability of keratinocytes during an in vitro infection insult and delayed microconidia germination. Considering the unmet medical need for more effective antifungal chemotherapeutic agents or approaches for treating dermatophytosis, natural products have been a successful source for the discovery of new drugs [44–47]. Therefore, the present work is an opportunity for the use of topical formulations containing caffeine, opening novel perspectives for the implementation of a clinical trial to test the topical use of caffeine as a novel therapeutic approach to dermatophytosis.

4. Materials and Methods

4.1. Fungal Strains and Culture Conditions

T. mentagrophytes was the model dermatophyte selected. This fungus was kindly provided by Professor Carmen Lisboa of the Laboratory of Microbiology, Faculty of Medicine, University of Porto. Fungi were cultured at 30 °C in Potato Dextrose Agar medium (PDA, BD Biosciences®, San Jose, CA, USA).

Fungal spores were harvested from solid cultures, submerging the mycelial mat with 0.1% Tween 80 solution (*v*/*v*, Sigma-Aldrich®, St. Louis, MO, USA). Then, the suspensions of spores were filtered through sterile handmade sacred linen filtration systems to remove hyphae fragments. The filtered suspension was washed twice with Phosphate Buffered Saline (PBS) (10 mM Na_2PO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl (*w*/*v*), pH 7.3, by centrifugation at 16,060× *g* for 10 min, at 4 °C, and the pellets were resuspended in PBS. After another washed step under the same conditions, the pellet was resuspended either in PBS and diluted in RPMI R1383 (Sigma-Aldrich®, St. Louis, MO, USA) for posterior use for susceptibility assays or in YME (0.4% yeast extract (*w*/*v*), 1% glucose (*w*/*v*), 1% malt extract (*w*/*v*)) for liquid cultures or in DMEM D5648 (Sigma-Aldrich®, St. Louis, MO, USA) supplemented with 10% non-inactivated Fetal Bovine Serum, 10 mM HEPES, 12 mM $NaHCO_3$, and 2 mM L-glutamine for later use in infection assays. Concentration of spores in the purified suspensions was estimated in microconidia/mL using a hemocytometer.

For the quantification assays of fungal cell wall components and for transmission electron microscopy (TEM) analysis, 2×10^5 fungal microconidia were inoculated in 100 mL of liquid YME supplemented with caffeine (Sigma-Aldrich®, St. Louis, MO, USA) at concentrations of 1, 5, or 10 mM. Caffeine was added to the sterile media by aseptically adding 10 mL of each caffeine sterile stock solution (*w*/*v*). Cultures were incubated at 30 °C with constant orbital shaking at 120 rpm from 3 up to 25 days upon inoculation (control cultures without caffeine and cultures supplemented with 1 mM caffeine were grown for 3 days, 5 mM caffeine cultures for 9 days, and 10 mM cultures for 25 days to enable fungal growth).

4.2. Antidermatophytic Activity of Caffeine

The minimum inhibitory concentration (MIC) causing the inhibition of the growth of the selected dermatophytes was determined by microdilution, as previously described [17,32], following the E.DEF 9.3.1 EUCAST standards. The MIC was defined as the lowest concentration for which caffeine inhibited fungal growth.

4.3. Fungal Cell Wall β-1,3-Glucan and Chitin Quantifications

The quantification of β-1,3-glucan levels in fungal cell walls was performed using the aniline blue assay, as described before [47]. Mycelia were sonicated in 1 M NaOH, and the β-1,3-glucan concentration was determined by aniline blue fluorescence at 405 nm excitation and 460 nm emission in a fluorimeter (Spectra Max® ID3, Molecular Devices, San José, CA, USA) [47].

The quantification of chitin in the cell wall was performed by measuring the glucosamine released by acid hydrolysis of purified cell walls, as described before [31]. The absorbance was read at 520 nm on a plate reader using a SpectraMax® Plus 384 spectrophotometer (Molecular Devices, San Jose, CA, USA).

4.4. Characterization of Morphological and Ultrastructural Changes

The ultrastructural changes induced by caffeine in *T. mentagrophytes* were analyzed by transmission electron microscopy (TEM). To perform TEM analysis, the inoculum was cultured in liquid medium with 10 mM caffeine or with no caffeine (control cultures). Samples were washed and then fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h. Fixation was performed as described previously [31]. Observations were carried out on a FEI-Tecnai® G2 Spirit Bio TwinTM transmission electron microscope at 100 kV.

4.5. Infection Assays

The HaCaT cell line of immortalized human keratinocytes was obtained from the German Cancer Research Centre (Heidelberg, Germany). Cells were maintained in Dulbecco's Modified Eagle Medium D5645 (Sigma-Aldrich®, St. Louis, MO, USA) supplemented with 10% non-inactivated Fetal Bovine Serum (FBS), 10 mM HEPES, 12 mM sodium bicarbonate, and 2 mM L-glutamine (DMEM), at 37 °C in 5% CO_2 atmosphere, until reaching 70% confluency. All infection assays were performed using HaCaT cells from passages #35 to #55. For the in vitro infection assays, HaCaT cells were trypsinized (trypsin-EDTA solution; Sigma-Aldrich®, St. Louis, MO, USA) and, depending on the assay type, 1 to 2×10^5 keratinocytes/well were seeded in 12-multiwell plates. HaCaT cells were allowed to sediment and adhere overnight at 37 °C in a 5% CO_2 atmosphere and then incubated with *T. mentagrophytes* microconidia at a multiplicity of infection (MOI) of 1:1. To assess the impact of caffeine in keratinocyte–microconidia interaction, caffeine solutions were also added at different concentrations (50 μM, 1 mM, 5 mM, or 10 mM).

4.6. Keratinocyte Viability Assay

HaCaT cell viability was assessed using an MTT assay, according to manufacturer's instructions. Briefly, 12-well keratinocyte plates were incubated with *T. mentagrophytes* microconidia and different caffeine concentrations for 6, 9, and 12 h. After each incubation period, media was discarded and replaced by DMEM with MTT solution (Sigma-Aldrich®, St. Louis, MO, USA), at a final concentration of 500 μg/mL per well. Plates were incubated again for 2 h to allow keratinocytes to metabolize the compound, and dimethyl sulfoxide (DMSO; Sigma-Aldrich®, St. Louis, MO, USA) was then added to each well. The contents of each well were transferred to 96-well microtiter plates in 100 μL triplicates and absorbance at 570 nm was measured in a SpectraMax® PLUS 384 spectrophotometer.

4.7. Ungerminated Microconidia Assays

HaCaT cells and *T. mentagrophytes* microconidia were co-incubated, as described above, and, after each incubation period, the plates were transferred to ice. The wells were immediately scraped and centrifuged at 16,060× *g* for 10 min at 4 °C. After centrifugation, supernatants were rejected, and the pellets were resuspended and homogenized in ice-cold PBS. The ungerminated microconidia were counted using a hemocytometer.

4.8. Statistical Analysis

Statistical analysis was performed using GraphPad® Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as mean ± standard error of the mean (SEM), and significance values are presented as $p < 0.05$, $p < 0.01$, or $p < 0.001$ (*, **, and ***, respectively).

5. Conclusions

The main conclusions are the following: (1) caffeine, especially at 10 mM, a concentration fifteen times lower than that found in commercially available topical solutions, inhibits the growth of the dermatophyte *T. mentagrophytes*; (2) caffeine modifies both β-(1,3)-glucan and chitin, reducing the robustness of the cell wall of the dermatophyte; and (3) caffeine prevents keratinocyte cell death during an in vitro infection by *T. mentagrophytes* and delays microconidia germination.

The present work opens novel perspectives for the implementation of a clinical trial to test the topical use of caffeine as a novel therapeutic approach to dermatophytosis.

Author Contributions: Conceptualization, T.G. and R.A.C.; methodology, D.M.d.F., C.F., M.M., J.S.-B., F.M.-T. and L.R.; software, D.M.d.F., M.M. and L.R.; data curation, D.M.d.F., M.M. and L.R.; writing—original draft preparation, D.M.d.F.; writing—review and editing, C.F., L.R., R.A.C. and T.G.; supervision, R.A.C. and T.G.; funding acquisition, T.G. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financed by the European Regional Development Fund (ERDF) through the Centro 2020 Regional Operational Programme, project CENTRO-01-0145-FEDER-000012-Healthy-Aging 2020 and CENTRO-01-0145-FEDER-022095: ViraVector; the COMPETE 2020—Operational Programme for Competitiveness and Internationalisation; and the Portuguese national funds via FCT—Fundação para a Ciência e a Tecnologia, I.P., UIDB/04539/2020, UIDP/04539/2020, and LA/P/0058/2020. Chantal Fernandes funding DOI: 10.54499/DL57/2016/CP1448/CT0025 and Lisa Rodrigues funding: CEECIND/03530/2017. F.M.T. was funded through Regional Government of Extremadura and the European Regional Development Fund (ERDF) through a grant to strengthening the mobility of postdoctoral researchers in 2017 (code P017018).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be available upon request.

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

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International Journal of
Molecular Sciences

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The Scavenging Activity of Coenzyme Q_{10} Plus a Nutritional Complex on Human Retinal Pigment Epithelial Cells

Maria Hernandez [1,†], Sergio Recalde [1,†], Jaione Bezunartea [1], Maite Moreno-Orduña [2], Idoia Belza [2], Ainara Chas-Prat [1], Elena Perugini [2], Alfredo Garcia-Layana [1,‡] and Patricia Fernández-Robredo [1,*,‡]

[1] Retinal Pathologies and New Therapies Group, Experimental Ophthalmology Laboratory, Department of Ophthalmology, Clinica Universidad de Navarra, Navarra Institute for Health Research, IdiSNA, (RICORS-TERAV), 31008 Pamplona, Spain; mahersan@unav.es (M.H.); srecalde@unav.es (S.R.); jbezunartea@unav.es (J.B.); achas@external.unav.es (A.C.-P.); aglayana@unav.es (A.G.-L.)
[2] Retinal Pathologies and New Therapies Group, Experimental Ophthalmology Laboratory, Department of Ophthalmology, Clinica Universidad de Navarra, 31008 Pamplona, Spain; maimoreno@unav.es (M.M.-O.); idoiabelza@unav.es (I.B.); eperugini@alumni.unav.es (E.P.)
* Correspondence: pfrobredo@unav.es
† These authors contributed equally to this work.
‡ These authors contributed equally to this work.

Abstract: Age-related macular degeneration (AMD) and diabetic retinopathy (DR) are common retinal diseases responsible for most blindness in working-age and elderly populations. Oxidative stress and mitochondrial dysfunction play roles in these pathogenesis, and new therapies counteracting these contributors could be of great interest. Some molecules, like coenzyme Q_{10} (CoQ_{10}), are considered beneficial to maintain mitochondrial homeostasis and contribute to the prevention of cellular apoptosis. We investigated the impact of adding CoQ_{10} (Q) to a nutritional antioxidant complex (Nutrof Total®; N) on the mitochondrial status and apoptosis in an in vitro hydrogen peroxide (H_2O_2)-induced oxidative stress model in human retinal pigment epithelium (RPE) cells. H_2O_2 significantly increased 8-OHdG levels ($p < 0.05$), caspase-3 ($p < 0.0001$) and TUNEL intensity ($p < 0.01$), and RANTES ($p < 0.05$), caspase-1 ($p < 0.05$), superoxide ($p < 0.05$), and DRP-1 ($p < 0.05$) levels, and also decreased *IL1β*, *SOD2*, and *CAT* gene expression ($p < 0.05$) vs. control. Remarkably, Q showed a significant recovery in *IL1β* gene expression, TUNEL, TNFα, caspase-1, and JC-1 ($p < 0.05$) vs. H_2O_2, and NQ showed a synergist effect in caspase-3 ($p < 0.01$), TUNEL ($p < 0.0001$), mtDNA, and DRP-1 ($p < 0.05$). Our results showed that CoQ_{10} supplementation is effective in restoring/preventing apoptosis and mitochondrial stress-related damage, suggesting that it could be a valid strategy in degenerative processes such as AMD or DR.

Keywords: age-related macular degeneration (AMD); diabetic retinopathy (DR); coenzyme Q_{10}; oxidative stress; mitochondrial stress; ARPE-19; DRP-1; caspase-3

1. Introduction

Oxidative stress and mitochondrial dysfunction are involved in the pathogenesis of age-related macular degeneration (AMD) and diabetic retinopathy (DR) [1–4]. Both are complex eye disorders with multifactorial etiologies and many factors have been implicated in their pathogenesis and progression, including oxidative damage, inflammation, aging, genetic predisposition, and environmental influences. AMD is characterized by retinal pigmented epithelium (RPE) dysfunction and damage to Bruch's membrane and the choriocapillaris complex [5], and DR is a microvascular disease characterized by blood flow alterations, pericyte loss, the downregulation of endothelial cells, tight junctions, and the thickening of the basement membrane [6,7].

Mitochondria dynamics are affected by several stressors, like oxidative stress, provoking an imbalance in its fission/fusion processes [8]. Mitochondrial fission creates new

mitochondria during cell division and facilitates the segregation of damaged mitochondria, whereas mitochondrial fusion enables the exchange of intramitochondrial material between mitochondria. The balance between fission/fusion processes determines the mitochondrial morphology and adapts it to the cellular metabolic requirements [9]. Exorbitant mitochondrial fission, resulting in mitochondrial disintegration or fragmentation, may be a consequence of oxidative stress in neurodegenerative disorders [9].

Coenzyme Q_{10} ($CoQ_{10,}$) is a fat-soluble quinone involved in the mitochondrial respiratory chain, synthetized mainly in the inner membrane of the mitochondria and secondarily in the endoplasmic reticulum Golgi apparatus [10], and exerts protective roles in various metabolic, antioxidant, and inflammatory [11] and ferroptosis processes [12]. CoQ_{10} plays an essential role in the normal function of the electron transport chain and has been reported to exhibit neuroprotective activity in a range of disorders, including cerebral ischemia [13] instead of Parkinson's disease and Huntington's disease [14]. Usually, its expression decreases with age and is therefore correlated with degenerative diseases such as AMD [15]. Lower plasma levels than in the controls were observed in AMD and DR patients [15–17]. The lack of protection provided by CoQ_{10} could affect the development of AMD and DR. Therefore, CoQ_{10} has been extensively utilized for food supplements and as a dietary supplement that is very important for maintaining human health.

This study aimed to elucidate the effect of adding CoQ_{10} to a nutritional antioxidant complex, Nutrof total®, in an adult RPE cell line (ARPE-19) subjected to oxidative stress. We focused on its effect on apoptosis, cytokines release, and DNA oxidative damage, especially that related to the mitochondria. Therefore, we evaluated the mitochondrial function under oxidative stress conditions. We analyzed specifically the dynamin-related protein (DRP1), a protein that physiologically serves to eliminate damaged mitochondria during fission [18], mitochondrial DNA quantification, mitochondrial superoxide concentrations and mitochondrial membrane potential (mtΔψ) in live cells.

2. Results

2.1. CoQ_{10} Plus N Restored Oxidative Stress-Related DNA Damage

Under basal conditions, a similar response in 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels was observed in treated groups with different antioxidants (n = 3). Although a slight increase is observed in N and NQ groups, this did not reach statistical significance (Figure 1A). Oxidative stress induced by H_2O_2 revealed a statistically significant increase in DNA damage ($p < 0.05$, Figure 1B). Under an oxidant environment, all treatments were able to reduce 8-OHdG levels, although the reduction was only nearly significant in the NQ group ($p = 0.055$, Figure 1B).

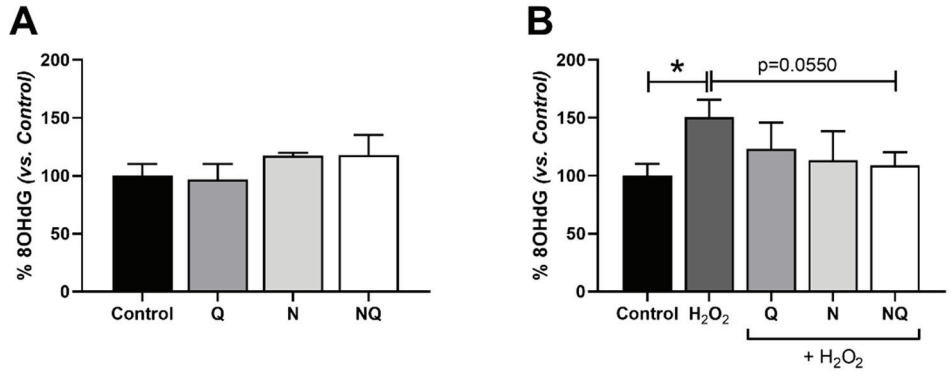

Figure 1. DNA oxidative damage analyzed as 8-OHdG levels in ARPE-19 cells' supernatants by ELISA in basal conditions (**A**) and after the addition of H_2O_2 (600 μM, 1 h) and antioxidant treatments in concomitance for 30 min (**B**) (* $p < 0.05$ vs. control) (n = 3). The application of NQ showed a tendency to significantly reduce 8-OHdG levels vs. H_2O_2 control group ($p = 0.0550$).

2.2. CoQ_{10} Plus N Protects from Early and Late Apoptosis Induced by Oxidative Stress

Early apoptosis was analyzed and quantified by active caspase-3 immunofluorescence on ARPE-19 cells after several conditions of H_2O_2 (Figure S1A) to select the appropriate concentration and incubation time ($n = 3$). Basal conditions (Figure 2A) and antioxidant treatments with induced oxidative stress (600 µM H_2O_2 for 3 h) (Figure 2B) were analyzed. Under basal standard conditions, caspase-3 immunofluorescence revealed that there is a similar fluorescence signal intensity in treated groups with antioxidants, except for the Q group which showed a statistically significant increase when compared to the control ($p < 0.05$, Figure 2A). The oxidative environment induced by H_2O_2 revealed a statistically significant increase in caspase-3 expression ($p < 0.001$, Figure 2B). N and NQ treatments in concomitance with H_2O_2 were able to significantly reduce early apoptosis induction when compared to the H_2O_2 control ($p < 0.05$ and $p < 0.01$, respectively, Figure 2B). The Q group did not show any effect on early apoptosis under the conditions used.

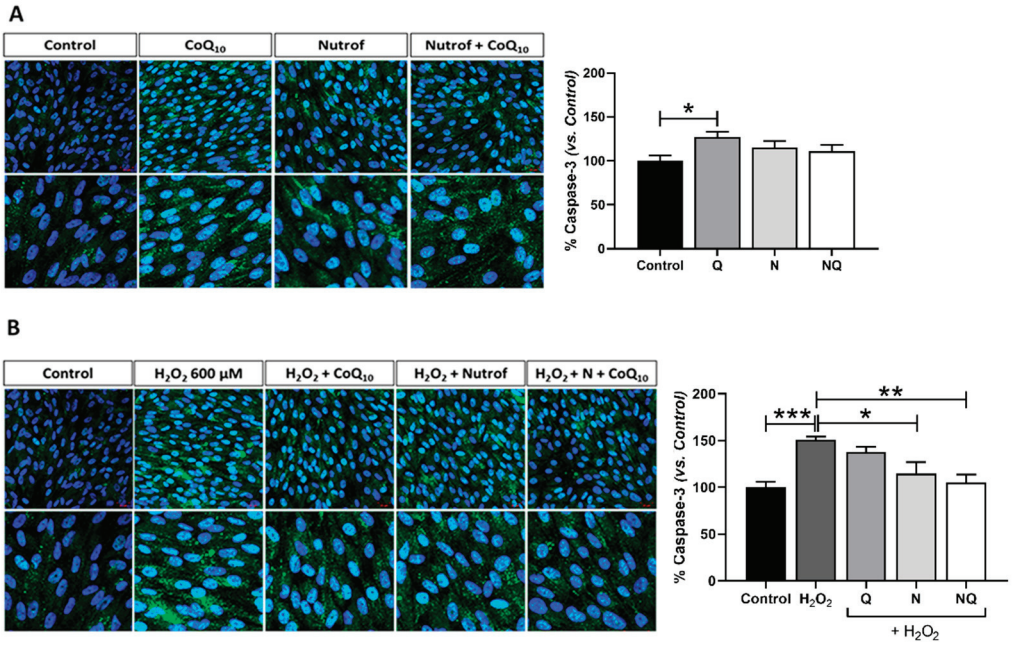

Figure 2. Percentage of the fluorescence intensity of caspase-3 (green) immunolabeling in basal conditions after Q, N, and NQ showed statistical differences between control and Q ($p < 0.05$) (**A**). Oxidative environment induced by H_2O_2 increased caspase-3 immunofluorescence vs. control group ($p < 0.001$) (**B**) ($n = 3$). After N and NQ with oxidative stress, significant differences were observed vs. H_2O_2 group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI, blue). Scale bar: 20 µm.

Furthermore, we analyzed DNA fragmentation by TUNEL in order to study the late stage of apoptosis. Under basal conditions (Figure 3A), similarly to the early apoptosis results, TUNEL revealed no changes in the fluorescence signal intensity in treated groups with antioxidants when compared to the control, except for the Q group which showed a statistically significant increase ($p < 0.05$, Figure 3A) ($n = 3$). Oxidative stress induction demonstrated an increase in the late apoptosis signal according to the experimental design showed in Table S1 ($p < 0.001$, Figure 3B). Concomitant treatment with either N, Q, or NQ were able to restore the oxidative damage (Figure 3B). Q and NQ treatment additions were able to induce a statistically significant reduction in the TUNEL signal when compared to H_2O_2 ($p < 0.05$, $p < 0.001$, Figure 3B). In contrast, although N was able to reduce the TUNEL signal, this difference was not statistically significant when compared to the H_2O_2 group (Figure 3B).

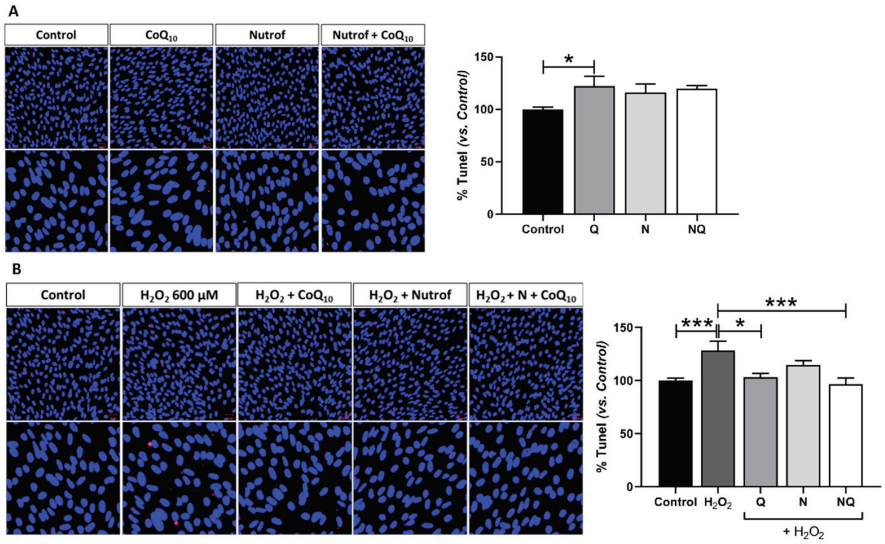

Figure 3. Percentage of TUNEL fluorescence intensity (red) in basal conditions after Q, N, and NQ showed statistical differences between control and Q (* $p < 0.05$) (**A**). H_2O_2 group showed a significant increase vs. control group (*** $p < 0.001$). After Q, N, and NQ treatments in concomitance with oxidative stress, a significant reduction was observed in Q and NQ vs. H_2O_2 group (* $p < 0.05$ and *** $p < 0.001$) (**B**) ($n = 3$). Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI, blue). Scale bar: 20 µm.

2.3. CoQ_{10} Reduces Caspase-1 Levels Increased by Oxidative Stress

ARPE-19 cells' supernatants and lysates of caspase-1, IL12-p70, IL17A, IL18, IL1β, IL6, RANTES, and TNFα were analyzed to determine intracellular levels ($n = 4$). Under standard conditions, the addition of treatments did not modify the levels of caspase-1, IL12-p70, IL17A, IL18, IL1β, IL6, TNFα, and RANTES (Figure S2) in ARPE-19 lysates. Released cytokines were also similar in the treatment groups when compared to the control (Figure S2), except for IL17A and RANTES, which showed an increase in the Q group when compared to the control ($p < 0.01$ and $p < 0.05$, respectively; Figure 4A,C). IL6 released levels were significantly reduced in the Q, N, and NQ treatments ($p < 0.01$, $p < 0.01$ and $p < 0.001$, respectively; Figure 4B).

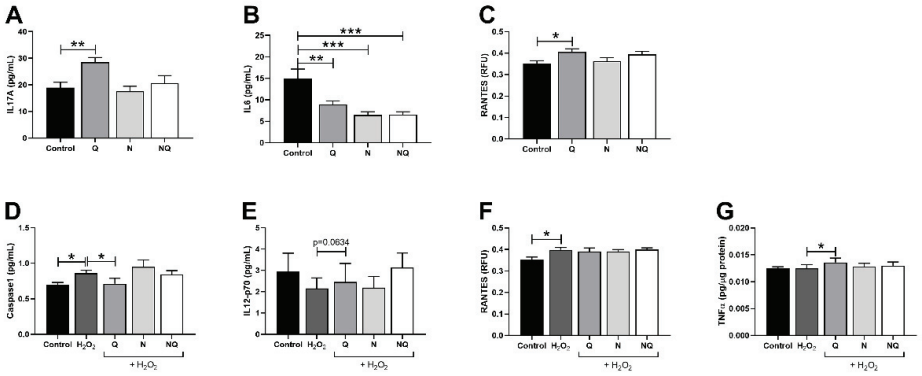

Figure 4. Quantification of cytokine levels in which changes have been observed in standard conditions and under oxidative stress with treatments Q, N, and NQ ($n = 4$). Levels of IL17A, IL6, and RANTES in ARPE-19 cells supernatant (**A**–**C**) in standard conditions. Levels of caspase-1, IL12-p70, and RANTES in ARPE-19 cells supernatant after oxidative stress conditions (**D**–**F**) and TNFα levels in lysates after oxidative stress conditions (**G**). Lysates' data are presented as pg/µg protein and supernatants' data are presented as pg/mL. RANTES data are presented as RFU. For all data mean ± SEM are presented. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. H_2O_2. Q—coenzyme Q_{10}, N—Nutrof total, NQ—Nutrof total + CoQ_{10}.

Oxidative stress induction significantly increased the caspase-1 and RANTES levels vs. the control group (Figure 4D and Figure 4F, respectively, $p < 0.05$). Only caspase-1 levels were significantly reduced after the Q addition when compared to the H_2O_2 group (Figure 4D, $p < 0.05$). However, N and NQ were not able to modify the cytokines levels (Figure 4D–G).

2.4. Interleukin (IL) 1β, Superoxide Dismutase 2 (SOD2) and Catalase (CAT) Gene Expression

Oxidative stress induction with H_2O_2 for 2 h produced a decrease in *SOD2* expression in both timepoints when compared to the control group, although it was significant only at 2 h ($p < 0.05$, Figure S4A) ($n = 4$). Under basal conditions, all antioxidant treatments (Q, N, and NQ) showed a significant reduction in *SOD2* expression with respect to the control ($p < 0.05$, Figure 5A). Antioxidant treatment (30 min) concomitance with H_2O_2 (1 h induction) provoked a significant decrease in *SOD2* expression when compared to the control ($p < 0.05$, Figure S4B,C). After 2 h of oxidative damage with H_2O_2, a significant reduction in *SOD2* gene expression was observed when compared to control group (Figure 5B, $p < 0.05$); however, the Q, N, and NQ treatments did not restore the effect, although there is a tendency for this to increase under oxidative conditions (Figure 5B).

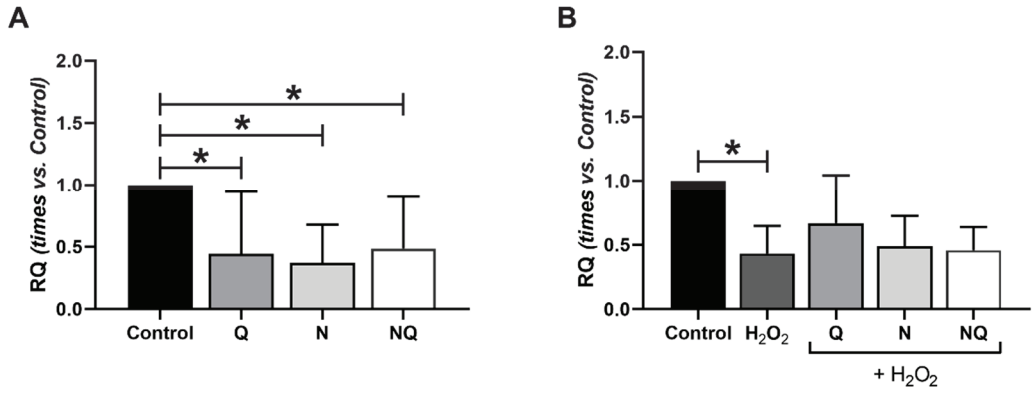

Figure 5. Quantification of *SOD2* gene expression of cultured ARPE-19 cells in standard conditions and under oxidative stress with Q, N, and NQ treatments ($n = 4$). *SOD2* expression in standard conditions showed a significant reduction with all antioxidant treatments (**A**). H_2O_2 group showed a significant decrease vs. control group (* $p < 0.05$). *SOD2* expression in ARPE-19 cells with 2 h of H_2O_2 in concomitance showed no significant reduction with treatments (**B**). For all data, mean ± SEM are presented. * $p < 0.05$ vs H_2O_2 group. Q—coenzyme Q_{10}, N—Nutrof total, NQ—Nutrof total + CoQ_{10}.

Figure 6 shows the results obtained in the comparative quantification of *ILβ1* expression ($n = 4$). After 2 h of damage with H_2O_2, a very significant decrease in *ILβ1* expression was observed ($p < 0.01$, Figure S4C), and a non-significant increase was observed after 1 h of damage (Figure S4C). Under the basal conditions, treatments showed an effect of decreasing *ILβ1* expression which was only significant for the N group vs. the control ($p < 0.05$, Figure 6A). After the administration of the antioxidant treatments in concomitance with H_2O_2 (1 h), the Q and N groups were able to significantly decrease *ILβ1* expression vs. the H_2O_2 group ($p < 0.05$, Figure 6B). After 2 h of oxidative damage, no changes were observed for all groups ($p < 0.01$, Figure S4D).

After 1 h of damage with H_2O_2, a statistically significant decrease in *CAT* expression and a non-significant increase was observed after 2 h of damage ($p < 0.05$, Figure S4E). Figure 7 shows the results obtained for *CAT* gene expression ($n = 4$). Under basal conditions, treatments did not show a statistically significant modification (Figure 7A). When used in concomitance with H_2O_2, all treatments showed a stabilizing effect against the alterations observed with oxidative stress, maintaining similar *CAT* gene expression values as the control group for both timepoints (Figure S4F and Figure 7B).

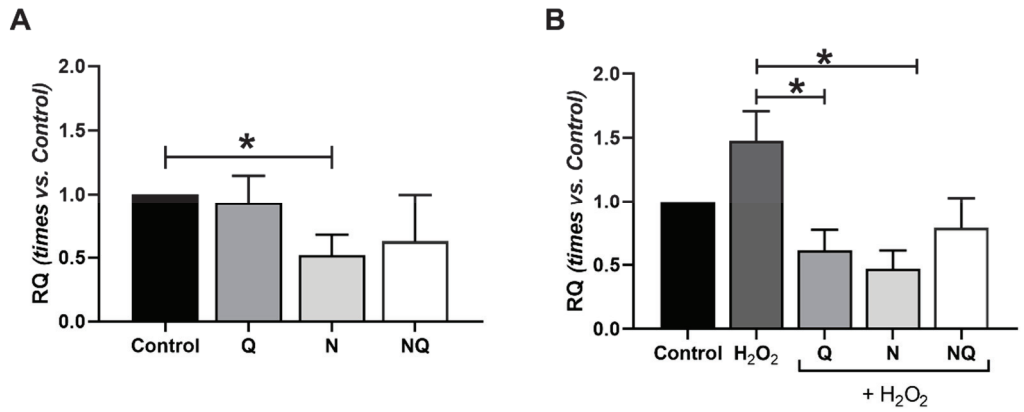

Figure 6. Quantification of *ILβ1* expression of cultured ARPE-19 cells in standard conditions and under oxidative stress with treatments Q, N and NQ ($n = 4$). *ILβ1* expression significantly decreased with N antioxidant treatment * $p < 0.05$ vs. control (**A**). *ILβ1* expression in ARPE-19 cells with 1 h of H_2O_2 in concomitance decreased after Q and N treatment (**B**) (* $p < 0.05$) vs. H_2O_2 group. Q—coenzyme Q_{10}, N—Nutrof total, NQ—Nutrof total + CoQ_{10}.

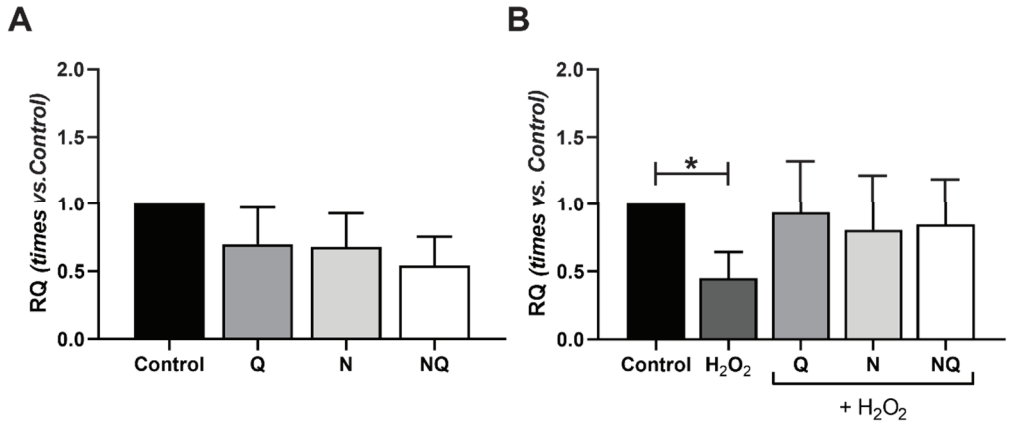

Figure 7. Quantification of *CAT* expression of cultured ARPE-19 cells in standard conditions and under oxidative stress with treatments Q, N, and NQ ($n = 4$). No changes were observed in *CAT* expression in basal conditions with antioxidant treatments (**A**). *CAT* expression in ARPE-19 cells with 1 h of H_2O_2 concomitance showed a decrease only in H_2O_2 group vs. control (* $p < 0.05$) (**B**). Q—coenzyme Q_{10}, N—Nutrof total, NQ—Nutrof total + CoQ_{10}.

2.5. Mitochondrial Dysfunctionality and Damaged Mitochondrial DNA (mtDNA)

2.5.1. Mitochondrial Superoxide Production

A mitochondrial superoxide indicator was detected using the fluorescent assay MitoSOX in live ARPE-19 cells. The dose selected to be used in the subsequent analysis was 600 µM after 2 h (Figure S5) ($n = 3$). In basal conditions, a decrease in superoxide levels was observed in N and NQ groups; however, it did not reach statistical significance (Figure 8A). The oxidative environment induced by H_2O_2 showed a statistically significant increase in superoxide quantification when compared to the control group ($p < 0.05$, Figure 8B), and only the NQ treatment was able to reduce its levels, although the reduction was not statistically significant ($p = 0.053$; Figure 8B).

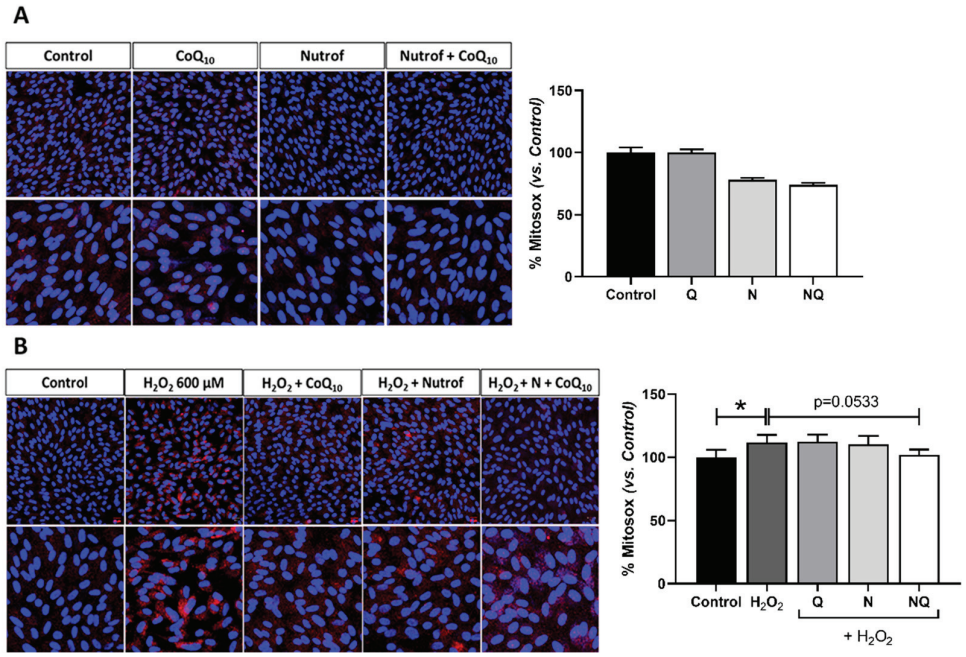

Figure 8. Percentage of mitochondrial superoxide indicator in live ARPE-19 cells measured by MitoSOX (red) in standard conditions and under oxidative stress with treatments Q, N, and NQ (n = 3). No changes in basal conditions were observed (**A**). H_2O_2 group showed a significant increase vs. control group (* $p < 0.05$), (**B**) and after H_2O_2 in concomitance, only the NQ treatment decreased MitoSOX ($p = 0.0533$) (B). Q—coenzyme Q_{10}, N—Nutrof total, NQ—Nutrof total + CoQ_{10}. * $p < 0.05$. Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Scale bar: 20 µm.

2.5.2. Mitochondrial DNA (mtDNA) Amount

Mitochondrial DNA was measured under basal conditions with antioxidant treatments (Q, N, and NQ), and no significant differences were found when compared with the control group (Figure 9A) (n = 4). Under oxidative stress induction with H_2O_2, an increase in the amount of mtDNA in the group treated only with H_2O_2 was observed, with differences close to significance ($p = 0.069$) vs. the control group (Figure 9B) (n = 4). Q and NQ treatments were able to reduce the amount of mtDNA generated by oxidative stress conditions to values similar to the control group, being statistically significant in the case of the NQ group when compared to the H_2O_2 group ($p < 0.05$, Figure 9B).

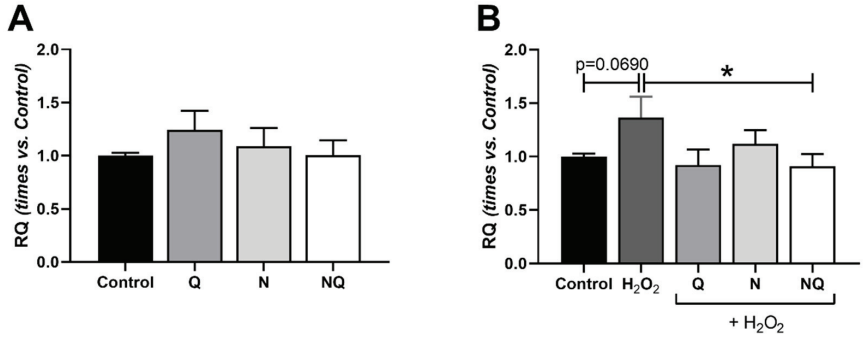

Figure 9. Mitochondrial DNA amount of cultured ARPE-19 cells measured by *12S* RT-PCR under standard conditions and under oxidative stress with treatments Q, N, and NQ (n = 4). No changes were observed in the mitochondrial DNA amount in cells treated with different treatments under basal conditions (**A**). H_2O_2 group showed an almost significant increase vs. the control group ($p = 0.0690$) (**B**) and the NQ group in concomitance with H_2O_2 significantly decreased mtDNA vs. the H_2O_2 group * $p < 0.05$ (**B**). Q—coenzyme Q_{10}, N—Nutrof total, NQ—Nutrof total + CoQ_{10}.

2.5.3. CoQ_{10} Decreases Mitochondrial Membrane Potential (mtΔψ) under Oxidative Stress Conditions

Under basal conditions, the JC-1 ratio was slightly increased in the Q group vs. the control. N and NQ groups showed a similar value when compared to the control (Figure 10A,B) ($n = 3$). After oxidative stress induction, an increase in the JC-1 ratio was observed when compared to the control group, which was not statistically significant. A statistically significant reduction in JC-1 was observed in the Q group compared to H_2O_2 (* $p < 0.05$). The N and NQ groups showed a similar value when compared to control (Figure 10C,D).

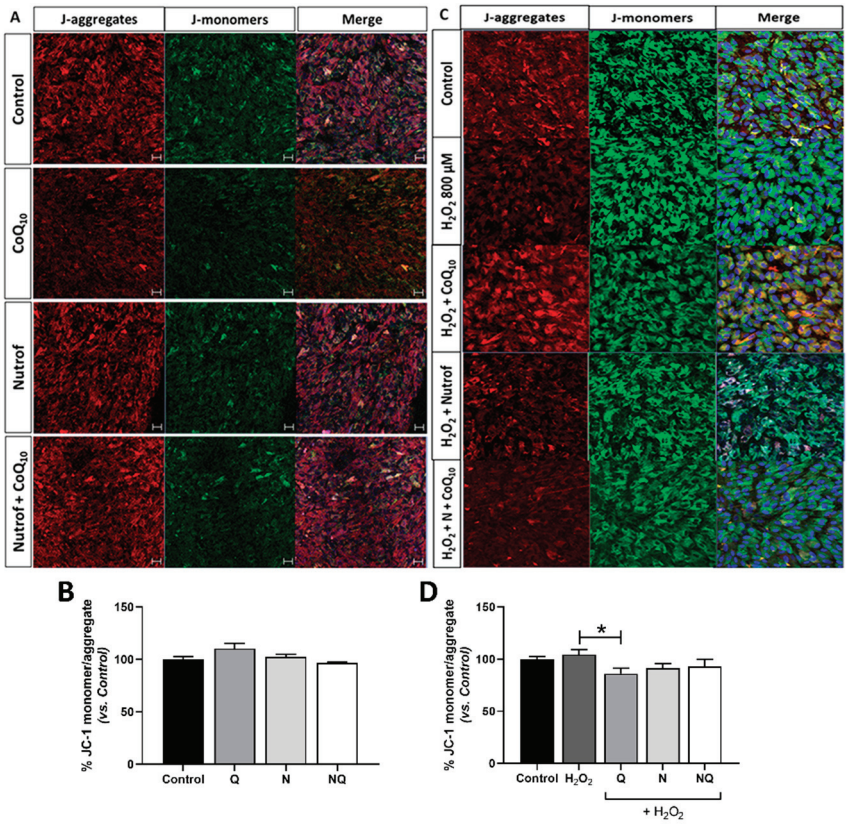

Figure 10. Mitochondrial membrane potential (mtΔψ) determined by live JC-1 measurement in ARPE-19 cells under basal conditions (**A**,**B**) and in concomitance with oxidative stress conditions with antioxidants treatments (**C**,**D**) ($n = 3$). J-monomers, green; J-aggregates, red. No changes were observed in JC-1 under basal conditions (**A**); however, in concomitance with H_2O_2 only, the Q treatment significantly decreased the mtΔψ vs. H_2O_2 group (**B**) (* $p < 0.05$). Q—coenzyme Q_{10}, N—Nutrof total, NQ—Nutrof total + CoQ_{10}. Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Scale bar: 20 µm.

2.5.4. Mitochondrial Dysfunction Determined by DRP-1 Immunofluorescence

Under basal conditions DRP-1 showed a similar fluorescence signal intensity in treated groups compared to the control group, except for the Q group which exhibited a statistically significant increase when compared to the control group ($n = 4$) ($p < 0.01$, Figure 11A,B). The oxidative environment induced by H_2O_2 revealed a statistically significant increase in DRP-1 fluorescence intensity quantification when compared to the control ($p < 0.05$, Figure 11C,D). Under the oxidative environment, treatments were able to reduce DRP-1 levels, which was statistically significant only for the NQ group when compared to H_2O_2 ($p < 0.05$, Figure 11D).

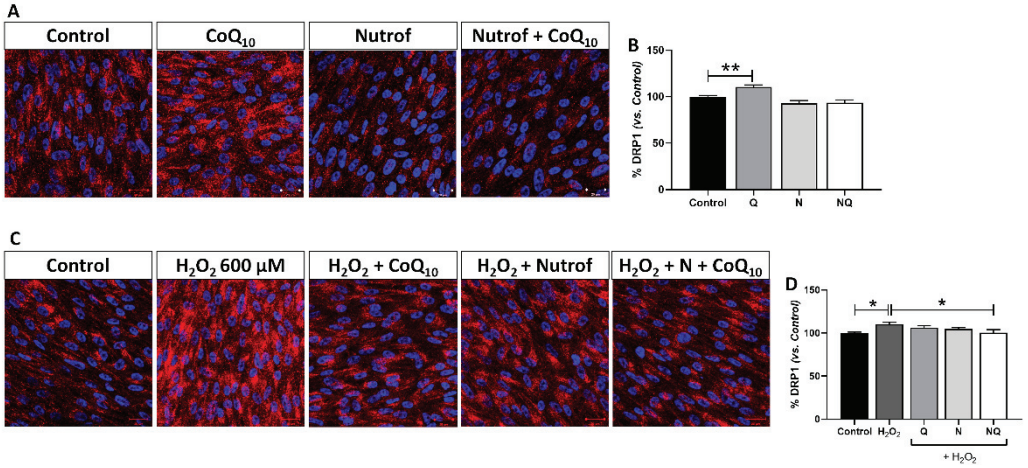

Figure 11. Percentage of mitochondrial DRP-1 (red) measurement in ARPE-19 cells under basal conditions (**A**,**B**) and under oxidative stress conditions with treatments Q, N, and NQ (**C**,**D**) ($n = 4$). Q treatment significant increased DRP-1 under basal conditions (**B**) (** $p < 0.01$). H_2O_2 group showed a significant increase vs. control group (* $p < 0.05$) (**B**). After concomitance with H_2O_2, only NQ treatment showed a significant decrease vs. H_2O_2 group (* $p < 0.05$). Q—coenzyme Q_{10}, N—Nutrof total, NQ—Nutrof total + CoQ_{10}. Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Scale bar: 20 µm.

3. Discussion

This study demonstrates that adding either CoQ_{10} or a nutritional complex (Nutrof total®), or the complex in combination with CoQ_{10}, can reverse the cellular damage induced by oxidative stress in human RPE cells in vitro. The main mechanisms through which the combined supplementation exerts RPE protection seem to relate to its antioxidant activity, its ability to reduce apoptosis, and its ability to stabilize mitochondrial parameters.

In previous studies from our group, a synergistic antioxidant and anti-inflammatory effect of Nutrof total along with vitamin D in ARPE-19 cells was also described [19]. Hundreds of papers on antioxidant synergism have been published so far, but the majority of them do not elucidate the mechanism of the synergistic activity [20,21].

CoQ_{10} is a molecule that possesses antioxidant, anti-inflammatory, and neuroprotective properties in some retinal neurodegenerative and ocular diseases [22]. In AMD and RP pathology, RPE cells and retinal endothelial cells undergo several subcellular accumulated damages, such as an increase in lesions in DNA [23–25], mitochondrial DNA degradation [26,27], cellular apoptosis [28,29], inflammation [30,31], and mitochondrial dysfunction [2,32] which contribute to the onset of the disease. All these events are strongly correlated with oxidative stress, which plays a significant role in the development of AMD and DR [33,34].

One of the most widely used biomarkers in many studies is 8-OHdG, produced by the oxidative damage to DNA by reactive oxygen and nitrogen species, which serves as an established marker of oxidative stress. High levels of mitochondrial 8-OHdG have been correlated with increased mutation, deletion, and the loss of mtDNA, as well as apoptosis in H9C2 cardiac cells [35] and astrocytes [36]. 8-OHdG is increased in ARPE-19 cells under hydrogen peroxide exposure [19,37,38], in AMD [39–41] and RD serum patients [42], and in aged RPE-choroid mice [43]. This is consistent with our results, where we observed that H_2O_2 resulted in an 8-OHdG increase in ARPE-19 cells. We have shown the capacity of CoQ_{10} to restore DNA damage as similar results in our previous studies with vitamin D [38]. Similar results were obtained in a rat model of metabolic syndrome [44], where CoQ_{10} administration dose-dependently decreased the serum 8-OHdG levels in the control group and in healthy adult subjects supplemented with CoQ_{10}, where a delay of the formation of 8-OHdG in lymphocyte DNA was observed [45]. Fluorescence studies have demonstrated that ubiquinone homologues, CoQ_{10} included, possess a strong ordering effect on the lipid

bilayer [46], and Tomasetti et al. hypothesized that the enrichment of human lymphocyte cells with ubiquinone-10 yielded an ordering and condensing effect on cell membranes, likely restricting the number of hydroxyl radicals which are capable of reaching cells' DNA [47].

The potent protective and synergistic effects of CoQ_{10} and Nutrof were also corroborated by their efficacy in inhibiting the apoptosis of RPE cells reducing the levels of caspase-3 and TUNEL, since it has been demonstrated that the exposure of ARPE-19 cells to concentrations of H_2O_2 promotes apoptosis [37]. In this sense, CoQ_{10} with Nutrof enhances oxidative stability more efficiently than the sum of the individual antioxidant effects. Similar results were obtained in the literature by adding the CoQ_{10} complex in vitro and in vivo experiments in RPE under oxidative stress and other types of retinal cells such as RGCs [48].

The activity of antioxidant enzymes, among SOD2, which occurs in the mitochondrial matrix, and CAT [49] represents an important sign of the defense mechanism against ROS-induced oxidative stress [50]. A significant decrease in the mRNA expression of *SOD2* and *CAT* was observed in the H_2O_2 group when compared to the controls. In our study, neither CoQ_{10} nor N were able to restore this effect. In contrast, CoQ_{10} has been found to reduce the *SOD2* expression after an increase in the amount of enzymes following H_2O_2 application in astrocytes [51], RGCs [52,53] and retinal layers of porcine explants [54]. Moreover, in cancer progression, SOD2 has a dichotomous role [55]. These authors observed a reduction in Sod2-to-Gpx1 and Sod2-to-catalase ratios in DRP-TpoKO mice (follicular thyroid cancer model), indicating an inability to scavenge ROS. Furthermore, a stressful situation in age-related human granulosa cells in ovaries causes a decrease in *SOD2* and *CAT* mRNA and any relative proteins [56]. CoQ_{10} is well known to be a powerful nutritional supplement with antioxidant properties; however, it also exerts a protective role during inflammatory processes [11]. The anti-inflammatory effects of CoQ_{10} have already been corroborated through various clinical studies associated with chronic diseases, in particular, cardiovascular diseases, kidney disease, chronic obstructive pulmonary disease, non-alcoholic fatty liver disease, and neurodegenerative diseases [57]. For this reason, CoQ_{10} has been proposed as a possible adjuvant treatment in viral infections that causes a systemic inflammatory response [58]. In this sense, we investigated the potential role of CoQ_{10} and the nutritional complex in the downregulation of several inflammatory cytokines. Oxidative and inflammatory mediators, such as caspase 1, IL12p70, IL17A, IL18, IL1β, IL6, RANTES, and TNFα, play a vital role in the development of AMD [59–65] and DR diseases [66–68]. Hydrogen peroxide only induced a significant upregulation of both caspase-1 and RANTES; however, CoQ_{10} restored the caspase-1, TNFα, and IL-1β levels. It seems that, for some treatments, some cytokines are released earlier than for other treatments that are kept in the intracellular area longer when compared to the control group. The combination of both treatments used had no restorative effects. These results agree with the recent meta-analyses [69] that explain the role of the declining production of pro-inflammatory cytokines by inhibiting NF-κB gene expression, which is involved in the expression of pro-inflammatory cytokines, such as TNF-α [70,71]. In addition, inflammatory cytokines such as IL-1β were markedly decreased, and the expression of antioxidant genes (e.g., *SOD1*) was notably increased in ARPE-19 cells co-exposed to CoQ_{10} and H_2O_2 when compared to cells treated with H_2O_2 alone [15]. Interestingly, a study in human peripheral blood mononuclear cells cultured and pretreated with CoQ_{10} demonstrated that TNFα secretion was significantly decreased [72], but no changes in IL-1β were observed.

Mitochondrial dysfunction in RPE is one of the most important events observed in neovascular AMD patients [2,23,73,74], and it is often associated with a decrease in the mtDNA content in many disease with the overproduction of ROS in human RPE cells [75]. The oxidation of ARPE-19 cells induced the depletion of mtDNA as demonstrated by the decrease in the mtDNA on RPE cells. Our data show that CoQ_{10} combined with Nutrof prevents mtDNA release from the mitochondria to the cytosol and the circulation. Other studies in the skeletal muscle of mice described this effect [76]. However, there were no

differences in the mtDNA content among the control or CoQ_{10}-treated groups in ischemic retinas in a murine model [52]. Anion superoxide, as an estimation of ROS production, increased after hydrogen peroxide, and this was only improved by using both treatments together in our study. Cells with hydrogen peroxide were almost statistically significant when compared to the control, probably due to the sample size. A similar effect was found with idebenone, a quinone with similarities to the naturally occurring CoQ_{10}. The treatment with idebenone significantly decreased the intracellular ROS formation [77] and ameliorated the cytotoxic effects of oxidative stress on RPE cells. In vivo investigations in age-related mice oocyte CoQ_{10} restored oocyte mitochondrial gene expression, improved mitochondrial activity [78]. Moreover, oxidative injury in rat pancreatic beta cells revealed the role of CoQ_{10} in reducing ROS levels [79].

Other mitochondrial components such as mt$\Delta\psi$ and mitochondrial membrane permeability (mPT) could be affected after oxidative stress and could initiate the degradative processes [80]. CoQ_{10} participates in the electron transport chain that takes place during aerobic cellular respiration in the mitochondria, meaning it is essential for the production of energy in cells [81,82]. In this sense, we found a beneficial effect of CoQ_{10} decreasing mt$\Delta\psi$ using a JC-1 marker. The effect was also observed but with less evidence in groups containing Nutrof. Consistent with our observations, studies have reported the same effect in ARPE-19 cells after chemical hypoxia. CoQ_{10} counteracted this phenomenon, significantly preventing mitochondrial membrane depolarization in more than 50% of ARPE-19 cells examined [48]. These authors described that CoQ_{10} is significantly more effective than other antioxidants (vitamin A, C, E) [83,84], and confer this effect to the participation of CoQ_{10} in complexes I and III of the respiratory chain with the mitochondrial permeability transition pore (mPTP); the association of ubiquinone Q_{10} with both complexes was in favor of this possibility, suggesting that CoQ_{10} could be part of the mPTP complex. In this sense, Zhong et al. proposed that the protective effect of CoQ_{10} might be associated with its role as a mobile electron transporter [85]. CoQ_{10} can correct the disorder of the electron transfer and improve the Q cycle, thus attenuating Ca^{2+} overload and cytrochome *c* release [47].

Mitochondrial dynamics is an essential process, and, in this study, we focused our attention on DRP-1 expression, known to be involved in the processes of fusion/fission and the energy regulation of the mitochondria. An abnormal activation of DRP-1 serves to eliminate damaged mitochondria during fission [18]. DRP-1 was altered after oxidative stress in ARPE-19 cells [86], in a murine model of long-term exposure to blue light, especially the ONL and RPE cells [87], in streptozotocin (STZ)-induced diabetic mice [88], and recently in a choroidal neovascularization (CNV) murine model, suggesting that mitochondrial fission in RPE contributes to angiogenesis development [89]. Our results indicated that CoQ_{10} in combination with Nutrof significantly decreased DRP-1, whereas H_2O_2 induced DRP-1 activation. In vitro studies have shown that CoQ_{10} prevented mitochondrial dynamic imbalance by reducing DRP-1 in murine neuronal HT22 cells [90] and other compounds, such as chrysoeriol, a flavonoid molecule, which protects ARPE-19 cells from oxidative stress through a decrease in DRP1 [84]. Interestingly, in vitro experiments with $Drp1^{-/-}$ cells reveal that they are protected against apoptosis [91] and DRP-1 inhibition reduced the cleavage of caspase-3 and PARP in hepatocytes [8], suggesting that targeting DRP-1 may be protective against apoptosis.

In most markers studied, CoQ_{10} has slight antioxidant activity in human RPE cells exposed to oxidative stress by treatment with hydrogen peroxide; however, CoQ_{10} increases its beneficial activity with the nutritional complex, Nutrof (Table 1), providing a strong and synergistic effect in some cases. A possible explanation in this regard could be that CoQ_{10} is capable of regenerating other sources of antioxidants, such as high levels of NADPH quinone reductase, which has been postulated to produce the reduced form of CoQ_{10} in the epidermis, and it is necessary to reduce this from ubiquinone to ubiquinol in order for it to act as an antioxidant [92]. For all these functions, CoQ_{10} must be distributed among

cell membranes, and that distribution seems to be regulated by specific proteins such as members of the UbiB family of atypical kinases/ATPases [46].

Table 1. Graphical summary showing the protective effects of CoQ_{10} on human RPE damaged by H_2O_2.

Processes	Markers	H_2O_2-RPE Cells	Antioxidant Treatment + H_2O_2		
			CoQ10	Nutrof	N + CoQ10
Oxidative and DNA stress	8-OHdG	↑ *	-	-	$p = 0.055$
	SOD2	↓ *2 h	-	-	-
	CAT	↓ *1 h	-	-	-
Apoptosis	Caspase-3	↑ ***	-	*	**
	TUNEL	↑ ***	*	-	***
Inflammation	TNF-α	Unchanged supernatants	*	-	-
		↓ Lysate	*	-	-
	Caspase-1	Unchanged supernatants	-	-	-
		↑ Lysate	*	-	-
	ILβ1	↓ **2 h	*	*	-
	RANTES	↑ Lysate	-	-	-
	IL6, IL17A, IL18	-	-	-	-
	IL12p70	↓ Lysate	$p = 0.0634$	-	-
Mitochondrial dysfunction	MitoSOX	↑ *	-	-	$p = 0.053$
	mtDNA	↑ $p = 0.069$	-	-	*
	JC-1	↑	*	-	-
	DRP-1	↑ *	-	-	*

Oxidative damage was induced by H_2O_2 and treated with CoQ_{10} (Q), Nutrof (N), and CoQ_{10} plus Nutrof (NQ). The damage caused changes in DNA, gene expression, apoptosis, increases in several inflammation markers (ILs), and alterations to mtDNA and mitochondrial functions. Furthermore, the antioxidants together with NQ were able to protect RPE cells from oxidative stress by decreasing the apoptosis and recovering mtDNA and DRP-1 levels. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

In particular, its effectiveness in reversing cellular damage and the consequent apoptosis is revealed when acting at a mitochondrial level. The CoQ_{10} levels decrease with age and in AMD and DR patients; therefore, the possibility of increasing the CoQ_{10} levels in different organs or tissues through dietary supplementation is necessary to standardize the indications for its use, composition, and dose. Two investigations have been conducted in AMD patients using CoQ_{10} as a dietary supplement [93,94]. The results have shown a slight improvement in visual function after treatment and a decrease in the area covered by drusen.

In conclusion, our results suggest that adding CoQ_{10} to the Nutrof Total formula shows a synergistic effect when compared to the individual supplementation in scavenging, restoring, and/or preventing apoptosis and mitochondrial stress-related damage in RPE cells. These results suggest that adding CoQ_{10} could be a valid strategy for ameliorating early mitochondrial changes in degenerative processes such as AMD or DR. However, although the addition of CoQ_{10} to a nutritional complex seems to be promising to improve and prevent the progression of early and intermediate stages of AMD, additional research, mainly related to bioavailability, distribution, and interactions between antioxidant molecules, is necessary.

4. Materials and Methods

4.1. Cell Culture

Human retinal pigment epithelial cells (ARPE-19) were obtained from the American Type Culture Collection (ATCC) (CRL-2302, Manassas, VA, USA) and were grown to 70% confluency in Dulbecco's modified Eagle's medium (DMEM; D6429, Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; 10270106 Gibco ThermoFisher, Paisley, UK), 1% fungizone (Gibco, Carlsbad, CA, USA), and penicillin–streptomycin (Gibco, Carlsbad, CA, USA) in a 37 °C incubator with 5% CO_2. The culture medium was replaced three times per week and split into the proper culture plate according to the subsequent experiments. After plating and reaching confluency, cells were maintained up to 2 months at 1% FBS, replacing the medium three times per week to reach the RPE phenotype until needed for the experiments as explained below.

4.2. Phenotypic Characterization by Flow Cytometry (FC) and Immunofluorescence

After reaching confluence in 24-well plates, the medium was changed to 1% FBS and replaced three times per week up to 2 months. To verify that ARPE-19 cells preserved their phenotype, RPE65 (ab231782, Abcam, Cambridge, MA, USA) and ZO1-Alexa Fluor-594 (339194, Invitrogen-Life Technologies, Carlsbad, CA, USA) were performed by FC (Figure S9A,B) and cytokeratin-18 (CK-18, M7010, DAKO, Santa Clara, CA, USA) antibodies were performed by immunofluorescence using CytoFLEX S (Beckman Coulter, Brea, CA, USA) (Figure S9C). Briefly, ARPE-19 cells were fixed with 4% of paraformaldehyde (PFA) for 10 min at 4 °C followed by three washes with FACS-Buffer (PBS 1X + 2% BSA + 5 mM EDTA). The cells were incubated in the dark for 30 min at RT with ZO1-Alexa Fluor-594 and RPE65 antibody prelabelled with FlexAble CoraLite® Plus 555 Antibody Labeling Kit for Rabbit IgG (KFA002, Proteintech, Manchester, UK) according to the manufacturer's instructions. After the incubation, the cells were washed 3 times with FACS-Buffer and were resuspended in 500 µL of FACS Buffer to measure the fluorescence. Data were analyzed with CytExpert software (Beckman Coulter, Brea, CA, USA). ARPE-19 cells (175,000 cells) were seeded on coverslips, and, after an experimental period of time, they were fixed with 4% of PFA in PBS for 10 min, washed with PBS, and labeled with active CK18 antibody diluted in blocking buffer containing 1% bovine serum albumin (BSA), 0.5% Triton X-100, 0.2% sodium azide, and 1% FBS overnight at 4 °C. Cells were incubated with the secondary fluorescent antibodies goat anti-mouse 488 (1:250, A11029, Life technologies, Gaithersburg, MD, USA) in blocking buffer for 1 h in the dark. Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA). The morphology of cells was observed under a confocal microscope (LSM800, Zeiss, Oberkochen, Germany).

4.3. Cell Viability Determination after Oxidative Stress Induction and Treatments Application

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay was used to determine cell viability using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) following the manufacturer's instructions. Experiments were carried out on 96-well plates seeded with 32,000 ARPE-19 cells per well. Once cells were confluent, a culture medium was changed to 1% FBS and maintained for 2 months. In order to select the appropriate and safe doses for the efficacy experiments, we evaluated ten doses of CoQ_{10} (0.01, 0.05, 0.1, 0.25, 0.5, 1, 2, 10, 50, 100 µM) and H_2O_2 (100, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800, 2400 µM) at different timepoints (CoQ_{10}: 1, 2, and 4 h; H_2O_2: 2, 6, and 24 h). Moreover, five doses of the N (0.01, 0.04, 0.07, 0.14, 0.70 mg/mL) and N + CoQ_{10} treatments (NQ; 0.1, 0.5, 1, 2, 10 µM CoQ_{10}) were tested at three different timepoints (1, 2, and 4 h) on ARPE-19 cells (passages p8-p14). Then, samples were subjected to the cell viability test according to the manufacturer's instructions. Results were obtained by reading the 492 nm absorbance using a Sunrise-basic Microplate reader (Tecan Austria GmbH, Grödig, Austria) and are shown in Figures S6–S8. The control group consisted of the solution used to dissolve the treatments, namely acetone 0.002% in cell culture media.

4.4. Selection of the Oxidative Stress Conditions and Treatment Concentrations

According to the results obtained (Figures S6 and S7), H_2O_2 (Panreac, Barcelona, Spain) at 600–800 μM were selected as the safe pro-oxidant stimulus to induce RPE oxidative stress. CoQ_{10} (synthetic origin, provided by Thea Laboratoires, Clermont-Ferrand, France) at 0.1 μM, Nutrof Total® (N; 0.01 mg/mL, see Table S2 for composition; Thea Laboratoires) or Nutrof Total® plus CoQ_{10} (NQ) at a total equivalent concentration of 62.34 μg/mL (Figure S8) was selected. This concentration was used for both the Q and NQ treatments in our experiments in order to have consistency in our comparisons between the Q and NQ treatments, and these concentrations were found to be non-toxic for ARPE-19 cells. Treatments were added in concomitance with the oxidative damage, as shown in Table S1.

4.5. Cell Apoptosis Evaluation by TUNEL and Caspase-3 Immunofluorescence

The apoptotic stage of the cells was also evaluated, using caspase-3 as a marker of early stage apoptosis and TUNEL as a marker of late stage apoptosis. For the TUNEL assay, an in situ cell death detection kit with TMR Red was used following the manufacturer's instructions (12156792910, Roche, West Sussex, UK). ARPE-19 cells (175,000 cells) were seeded on coverslips, and after experimental procedures, they were fixed with 4% of PFA in PBS for 10 min, washed with PBS, and labeled with active caspase-3 antibodies (1:100, G7481; Promega, Madison, WI, USA) diluted in blocking buffer containing 1% bovine serum albumin (BSA), 0.5% Triton X-100, 0.2% sodium azide, and 1% FBS overnight at 4 °C. Cells were incubated with the secondary fluorescent antibodies goat anti-mouse 488 (1:250, A11029, Life technologies, Gaithersburg, MD, USA) in blocking buffer for 1 h in the dark. Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA). The morphology of cells was observed under a confocal microscope (LSM800, Zeiss, Oberkochen, Germany).

4.6. Analysis of Mitochondrial Function

4.6.1. Analysis for Membrane Mitochondrial Potential (mtΔψ)

The MtΔψ status was performed in live ARPE-19 cells using the membrane-permeant JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) dye, which is widely used in apoptosis studies to monitor mitochondrial health. JC-1 is a ratiometric dye that forms aggregates in highly polarized/energized mitochondria and emits an orange-red fluorescence at 595 nm (red/phycoerythrin). In depolarized mitochondria, JC-1 remains as monomers and emits green fluorescence at 530 nm (green/fluorescein isothiocyanate). After culturing 175,000 cells on a 10 mm dish (Menzel-Glaser, Waltham, MA, USA), they were incubated with JC-1 (2.5 μM) for 15 min in the dark at 37 °C according to the manufacturer's instructions (T3168, Invitrogen, Molecular Probes, Inc, Eugene, OR, USA). Three passages were analyzed and images were taken under a confocal fluorescence microscope (LSM800, Zeiss, Oberkochen, Germany) at ×40 magnification. Relative levels of the intensities of the monomers/aggregates of JC-1 fluorescence were quantified using Fiji/ImageJ, an open-source Java-based image analysis software (NIH, Bethesda, MD, USA).

4.6.2. Detection of Mitochondrial Superoxide Production Using MitoSOX

ARPE-19 cells seeded on a 10 mm dish (175,000 cells per dish) (Menzel-Glaser, Waltham, MA, USA) were stained with MitoSOX Red mitochondrial superoxide indicator for live-cell imaging (M36008, Molecular Probes Inc, Eugene, OR, USA) (n = 3). Briefly, the MitoSOX component was dissolved in dimethyl sulfoxide (DMSO) in a medium without FBS to produce the mitoSOX reagent working solution in which the cells were incubated for 10 min at 37 °C and protected from light. Then, they were gently washed three times with PBS for 10 min. Finally, images were taken under a confocal fluorescence microscope (LSM800, Zeiss, Oberkochen, Germany) at ×40 magnification.

4.6.3. DRP-1 Immunofluorescence

ARPE-19 cells were plated in 96-well plates for 2 months as explained above, and after the experimental procedures, they were fixed with 4% of PFA in PBS for 10 min, washed with PBS three times, and permeabilized with blocking buffer for 10 min at 4 °C. Then, cells were incubated with the rabbit polyclonal anti-DRP-1 (1:250 dilution, ab184247, Abcam, Cambridge, MA, USA) antibody and subsequently with goat anti-rabbit Alexa fluor 594 (1:250, A-11012, Thermo Fisher Scientific, Paisley, UK). Nuclei were stained with DAPI (Sigma-Aldrich, St. Louis, MO, USA). Cells were analyzed under a confocal microscope (LSM800, Zeiss, Oberkochen, Germany) at ×40 magnification, and the intensity of fluorescence was measured using a home-made plugin tool developed for Fiji/ImageJ, an open-source Java-based image analysis software. The plugin was developed by the Imaging Platform of the CIMA Universidad de Navarra.

4.6.4. Mitochondrial DNA Amount (mtDNA)

DNA extraction was performed using the DNeasy Blood & tissue extraction kit (Qiagen, Hilden, Germany). All possible RNA was digested by a RNAse reaction. The extracted DNA was measured by spectrophotometry with the ND-1000 Spectrophotometer (Nanodrop, Thermo Fisher Scientific, Waltham, MA, USA) to check both the final concentration and DNA quality. The expression of *12S* was measured using a 7300 Real Time PCR System (Applied Biosystems; Life Technologies, Carlsbad, CA, USA) and the Taqman Assays 12S Hs02596859_g1 (Applied Biosystems; Life Technologies, Carlsbad, CA, USA). For relative calculations, we compared the Ct results of treated samples vs. the control samples in a straight pattern of decreasing concentrations of mtDNA.

4.7. Measurement of 8-Hydroxidioxiguanosine (8-OHdG) under Oxidative Stress Conditions

Oxidative damage was measured in the DNA of ARPE-19 subjected to H_2O_2 for 1 h, and antioxidant treatments were added in concomitance for 30 min. To evaluate the effect of antioxidant treatments, we added 0.1 μM of CoQ_{10} and/or 0.01 mg/mL of Nutrof to the media. Three hundred ng of DNA was evaluated using the EpiQuik TM 8-OHdG DNA Damage Quantification Direct kit #P-60003 (Epigentek, Farmingdale, NY, USA). Data are presented in % 8-OHdG vs. control.

4.8. DNA Multiplex Cytokine Analysis

Samples were subjected to H_2O_2 for 2 h (Table S1), and treatments were added in concomitance for 1 h. Then, ARPE-19 lysates and supernatants were collected, and the following cytokines levels were measured using the ELLA multiplex platform (Biotechne, Minnesota, MN, USA): Caspase 1, IL12-p70, IL17A, IL18, IL1β, IL6, RANTES, and TNFα. Cell lysates were obtained by collecting cells using tripsin and adding a lysis buffer. Then, samples were centrifuged at 13,000 rpm for 20 min, pellets were discarded, and supernatants were used to determine the intracellular cytokines' levels.

4.9. RNA Analysis: Expression of IL-1β, SOD2, and CAT

Samples were subjected to H_2O_2 for 1 or 2 h (Table S1) and antioxidant treatments were added in concomitance for 30 min or 1 h, respectively. Then, ARPE-19 lysates were collected, and the subsequent methods were performed. RNA extraction was performed using the Illustra^{TM} RNAspin extraction kit (GE Healthcare, Chicago, IL, USA). All possible DNA was digested by the DNAse reaction. The extracted RNA was measured by spectrophotometry with the ND-1000 Spectrophotometer (Nanodrop, Thermo Fisher Scientific, Waltham, MA, USA) to check both the final concentration and RNA quality. The reverse transcription of 500 ng of RNA from each sample was performed in a total of 20 μL of reaction with PCR Retrotrancription System (Quantabio, Beverly, MA, USA) under the following conditions: 25 °C—5 min; 42 °C—30 min; 85 °C—5 min. The expression of genes was measured using a 7300 Real Time PCR System (Applied Biosystems; Life Technologies, Carlsbad, CA, USA) and the Taqman Assays *SOD2* Hs00167309_m1, *IL1β* Hs01555410_m1, and *CAT*

Hs00156308_m1. *18S* and *GAPDH* genes (Hs99999901_s1 and Hs99999905_m1, respectively, Applied Biosystems; Life Technologies, Carlsbad, CA, USA) were used for normalization purposes. For relative calculation, we compared the Ct results of the *SOD2*, *IL1β*, and *CAT* expression of the control samples vs. antioxidants.

4.10. Statistical Analysis

For quantitative variables, the Shapiro–Wilk normality test was applied and all parameters were subjected to the one-way analysis of variance (ANOVA) or Kruskal–Wallis followed by the Bonferroni post hoc test. All groups were normalized by each pass and compared against a control group. Data are expressed as mean ± SEM. A difference of $p < 0.05$ was considered statistically significant. GraphPad Prism 8.0 (GraphPad Prism Software Inc., San Diego, CA, USA) was used for statistical analysis.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/ijms25158070/s1.

Author Contributions: Conceptualization, P.F.-R., M.H., S.R., and A.G.-L.; methodology, M.H., S.R., J.B., M.M.-O., I.B., E.P. and A.C.-P.; software, J.B. and A.C.-P.; validation, P.F.-R. and A.G.-L.; formal analysis, J.B., M.M.-O. and I.B.; investigation, M.H. and S.R.; resources, P.F.-R. and A.G.-L.; data curation, M.H.; writing—original draft preparation, M.H. and E.P.; writing—review and editing, P.F.-R. and A.G.-L.; visualization, S.R. and P.F.-R.; supervision, P.F.-R. and A.G.-L.; project administration, P.F.-R.; funding acquisition, A.G.-L. All authors have read and agreed to the published version of the manuscript.

Funding: This study has been funded by Thea Laboratories (Clermont-Ferrand, France) and they had no role in the interpretation of data or the writing of the manuscript. Supported by the Inflammatory Disease Network (RICORS-REI; RD21/0002/0010), Advanced Therapies Network (RICORS-TERAV; RD21/0017/0009), (Carlos III Health Institute) and the European Union "NextGenerationEU"/PRTR.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data are available within the manuscript and upon request to corresponding authors.

Acknowledgments: We greatly appreciate the expertise and assistance of Creative Pharma Services S.M.S.A. for their help in editing, writing, and revising the manuscript.

Conflicts of Interest: A.G.-L. is consultant for Bayer, Novartis, Allergan, Thea, and Roche. The rest of the authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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International Journal of
Molecular Sciences

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The Effect of a Ketogenic Diet versus Mediterranean Diet on Clinical and Biochemical Markers of Inflammation in Patients with Obesity and Psoriatic Arthritis: A Randomized Crossover Trial

Vaia Lambadiari [1,*], Pelagia Katsimbri [2], Aikaterini Kountouri [1], Emmanouil Korakas [1], Argyro Papathanasi [3], Eirini Maratou [4], George Pavlidis [5], Loukia Pliouta [1], Ignatios Ikonomidis [5], Sofia Malisova [6], Dionysios Vlachos [7] and Evangelia Papadavid [3]

[1] Second Department of Internal Medicine, Attikon University Hospital, Medical School, National and Kapodistrian University of Athens, 12462 Athens, Greece; akoun@med.uoa.gr (A.K.); mankor@med.uoa.gr (E.K.); plioutaloukia@gmail.com (L.P.)
[2] Rheumatology and Clinical Immunology Unit, Fourth Department of Internal Medicine, Attikon Hospital, Medical School, National and Kapodistrian University of Athens, 12462 Athens, Greece; pelkats@gmail.com
[3] Second Department of Dermatology and Venereology, University of Athens Medical School, 12462 Athens, Greece; argyp92@gmail.com (A.P.)
[4] Department of Clinical Biochemistry, Medical School, National and Kapodistrian University of Athens, 15772 Athens, Greece; maratueirini@gmail.com
[5] Second Cardiology Department, Attikon University Hospital, Medical School, National and Kapodistrian University of Athens, 12462 Athens, Greece; geopavi80@gmail.com (G.P.); ignoik@gmail.com (I.I.)
[6] Independent Researcher, 11142 Athens, Greece; smalisova@eviviosmed.gr
[7] Independent Researcher, 16451 Athens, Greece; dvlachos@eviviosmed.gr
* Correspondence: vlambad@otenet.gr; Tel.: +30-210-5831148; Fax: +30-210-5326454

Abstract: The effect of different diet patterns on psoriasis (PSO) and psoriatic arthritis (PSA) is unknown. The aim of our study was to evaluate the effectiveness of a Mediterranean diet (MD) and Ketogenic diet (KD), in patients with PSO and PSA. Twenty-six patients were randomly assigned to start either with MD or KD for a period of 8 weeks. After a 6-week washout interval, the two groups were crossed over to the other type of diet for 8 weeks. At the end of this study, MD and KD resulted in significant reduction in weight ($p = 0.002$, $p < 0.001$, respectively), in BMI ($p = 0.006$, $p < 0.001$, respectively), in waist circumference (WC) ($p = 0.001$, $p < 0.001$, respectively), in total fat mass ($p = 0.007$, $p < 0.001$, respectively), and in visceral fat ($p = 0.01$, $p < 0.001$, respectively), in comparison with baseline. After KD, patients displayed a significant reduction in the Psoriasis Area and Severity Index (PASI) ($p = 0.04$), Disease Activity Index of Psoriatic Arthritis (DAPSA) ($p = 0.004$), interleukin (IL)-6 ($p = 0.047$), IL-17 ($p = 0.042$), and IL-23 ($p = 0.037$), whereas no significant differences were observed in these markers after MD ($p > 0.05$), compared to baseline. The 22-week MD–KD diet program in patients with PSO and PSA led to beneficial results in markers of inflammation and disease activity, which were mainly attributed to KD.

Keywords: psoriasis; psoriatic arthritis; obesity; diet intervention; Mediterranean diet; Ketogenic diet; PASI score; DAPSA score; inflammation; interleukins

1. Introduction

Psoriasis is one of the most prevalent auto-inflammatory diseases worldwide, with an incidence of 1.9 to 3.4% in western Europe [1]. Its etiology is considered multifactorial, and it is characterized by the dysregulation of the innate and adaptive immune systems, with the activation of T helper (Th)-1 and Th-17 T cells, leading to an increased production of inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-23, IL-22, IL-17, and IL-33; tumor necrosis factor alpha (TNF-α); and interferon-gamma (IFN-γ) [2,3]. In this cascade,

inflammation plays a prominent role by promoting hyper-proliferation and angiogenesis, leading to the typical skin lesions and the articular involvement of psoriatic arthritis [4].

A growing number of studies have highlighted the association between obesity and psoriasis [5–9]. The prevalence and incidence of psoriasis is higher among patients with obesity, while obesity is an important predisposing factor for psoriasis onset, progression, and severity. Moreover, obesity exerts a negative impact on the treatment of psoriasis and increases the adverse effect of anti-psoriatic drugs [10–14]. As obesity and psoriasis represent chronic inflammatory states, many recent studies have focused on the complicated role of visceral fat, which releases a number of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-8 [15]. Adipocytokines such as leptin and resistin, which are secreted not only by adipocytes but also by macrophages in adipose tissue, contribute equally to the inflammation process [16].

According to research data, lifestyle interventions, including diet, weight loss, and physical activity, not only improve the symptoms of pre-existing psoriasis, but also constitute preventable factors for the disease in individuals who are overweight and obese [17–20]. Multiple dietary patterns such as a low-calorie diet, gluten-free diet, very-low-calorie KD (VLCKD), and Mediterranean diet (MD) have been proposed for weight loss management in patients with psoriasis [21]. MD is characterized by a high consumption of fruits, vegetables, and cereals; moderate consumption of fish, olive oil, nuts, and legumes; and low intake of poultry, eggs, and red meat [22]. Recent studies have highlighted the positive effect of this eating pattern in many cardiometabolic and auto-inflammatory disorders possibly due to the reduction in oxidative stress and inflammation [23]. However, interventional randomized clinical trials to confirm these results are still lacking and the existing data rely on observational reports.

Apart from its established benefits on metabolism, KD has been considered as an alternative treatment choice for autoimmune disorders [24–26]. The classicKD, very-low-carbohydrate KD, Atkins diet, high-fat KD, and VLCKDare different forms of KD. The main characteristic of KD is the low content of carbohydrates (less than 30–50 g/day) along with an increase in protein and fat (75–80% kcal from fat, 5–10% kcal from carbohydrates, and 15–25% kcal from protein of the total daily energy consumption) [24]. This results in a metabolism switch to fat consumption as a main source of energy, leading to an increase in fatty acids and ketone bodies, which have anti-inflammatory properties by reducing IL-β and TNF-α plasma levels [27,28]. Based on this pathophysiological background, an increased interest regarding the effect of VLCKD on psoriasis exists, yet the evidence is still scarce. According to case reports and interventional studies, VLCKDs have been associated with a substantial improvement in the course of the disease [29–31]. However, randomized studies are needed to confirm the results.

The aim of our study is to compare the effectiveness of classic MD with the isocaloric KD, each applied for 8 weeks, in patients with obesity and psoriasis along with psoriatic arthritis. Our objective is to investigate the hypothesis that beyond the weight loss, the overall management of inflammation, achieved with KD for a short period of time, may lead to faster beneficial results for patients with obesity and psoriasis.

2. Results

2.1. Study Participants

In total, twenty-six patients met the inclusion/exclusion criteria of this study and were randomly assigned to KD (n = 13) or to MD (n = 13). Overall, one patient dropped out during the KD intervention, four patients during MD, and five patients during the washout period. In total, sixteen patients completed this study and were included in the final analysis (Figure 1).

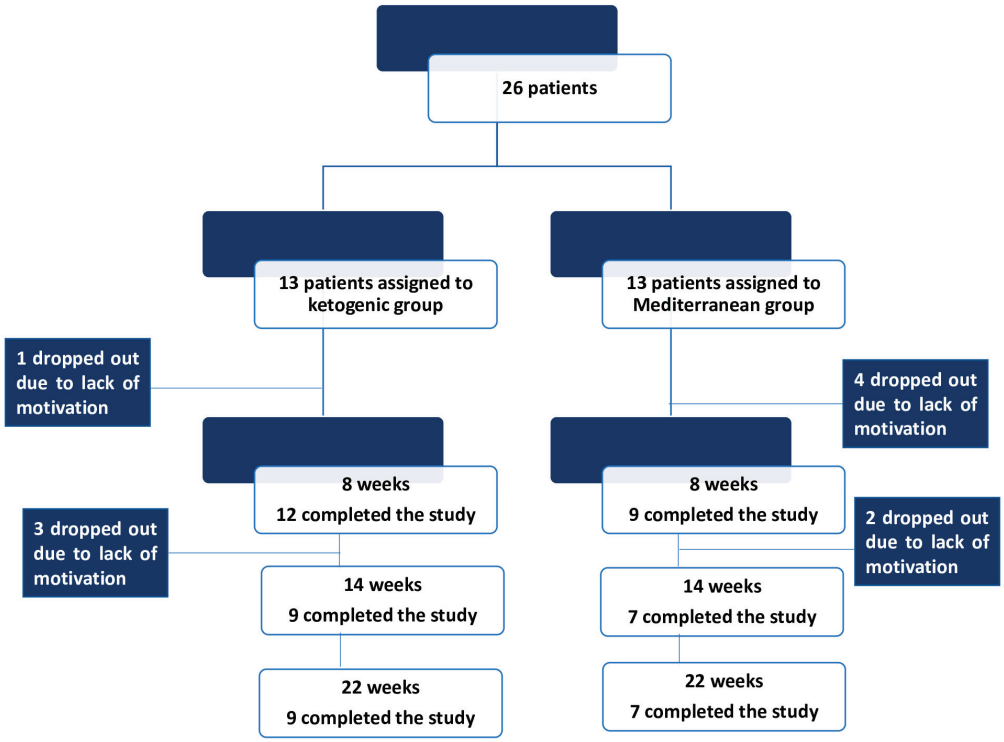

Figure 1. CONSORT flow diagram showing the progress through the phases of the trial.

The mean ± SD age of patients included in this study was 52.93 ± 7.33 years and 12 patients were female (75%). Patients included in this study displayed obesity grade II with a mean weight of 108.44 ± 19.01 and a mean body mass index (BMI) of 39.90 ± 7.60 along with increased waist circumference (WC) (122.96 ± 17.87). At baseline, patients presented moderate psoriasis with a mean Psoriasis Area and Severity Index (PASI) of 5.09 ± 5.73 along with severe PSA with a mean Disease Activity Index for Psoriatic Arthritis (DAPSA) of 46.28 ± 34.89.

2.2. Dietary Effects on Weight and Body Composition

At the end of this study, MD and KD resulted in significant reduction in weight ($p = 0.002$, $p < 0.001$, respectively) and BMI ($p = 0.006$, $p < 0.001$, respectively). Weight and BMI reductions were greater after the KD intervention (−10.63 kg) compared to MD (−7.48 kg), but not at a statistically significant level ($p = 0.168$, $p = 0.200$, respectively). MD and KD resulted in a significant decrease in WC ($p = 0.001$, $p < 0.001$, respectively). Patients after KD displayed a 2-fold higher reduction in WC compared to MD (−6.32% vs. −3.65%, $p = 0.04$). MD and KD resulted in significant reduction in total fat mass ($p = 0.007$, $p < 0.001$, respectively) and visceral fat ($p = 0.01$, $p < 0.001$, respectively), in comparison with baseline (Table 1).

Table 1. The effect of the diet intervention on anthropometric measurements and biochemical parameters.

	Baseline	MD	Δ%	KD	Δ%	*p*-Value †	*p*-Value *	*p*-Value ‖
Weight (kg)	108.44 ± 19.01	101.21 ± 17.95	−7.48	98.17 ± 17.46	−10.63	0.002	<0.001	0.168
BMI (kg/m^2)	39.90 ± 7.60	37.40 ± 7.75	−7.28	36.30 ± 7.33	−10.18	0.006	<0.001	0.200
WC (cm)	122.96 ± 17.87	118.53 ± 15.71	−3.65	115.56 ± 15.52	−6.32	0.001	<0.001	0.040
Fat Mass (kg)	46.83 ± 12.76	42.30 ± 12.84	−12.45	40.51 ± 12.80	−17.47	0.007	<0.001	0.320
Visceral Fat (%)	15.43 ± 4.38	14.18 ± 4.38	−10.79	13.50 ± 3.81	−15.20	0.01	<0.001	0.326
Glu (mg/dL)	97.28 ± 21.70	98.21 ± 32.30	−2.90	103.62 ± 23.56	4.82	0.927	0.403	0.436
SGOT (IU/L)	19.14 ± 8.82	16.76 ± 5.67	−18.79	20.52 ± 13.45	−9.57	0.285	0.759	0.574
SGPT (IU/L)	18.57 ± 11.21	15.56 ± 11.46	−23.70	14.01 ± 7.84	−25.94	0.283	0.032	0.905

Table 1. *Cont.*

	Baseline	MD	Δ%	KD	Δ%	*p*-Value †	*p*-Value *	*p*-Value ⁋
γ-GT (mg/dL)	24.40 ± 17.65	24.20 ± 20.00	−13.01	18.60 ± 12.96	−32.84	0.958	0.022	0.142
ALP (IU/L)	67.5 ± 25.72	59.83 ± 14.25	−17.99	57.75 ± 15.35	−19.76	0.371	0.127	0.935
TChol (mg/dL)	181.84 ± 45.19	185.74 ± 36.04	2.5	174.95 ± 36.24	−5.3	0.557	0.496	0.292
HDL (mg/dL)	44.57 ± 11.25	41.55 ± 7.97	−8.21	43.96 ± 7.05	−1.04	0.387	0.800	0.439
LDL (mg/dL)	110.99 ± 53.11	96.95 ± 23.02	−17.5	87.60 ± 21.23	−32.1	0.256	0.164	0.547
TGs (mg/dL)	133.63 ± 40.61	127.82 ± 47.95	−11.26	106.90 ± 29.24	−29.47	0.632	0.022	0.146

Data are presented as mean ± SD. Δ% indicates percentage change from baseline. MD: Mediterranean diet; KD: Ketogenic diet; BMI: Body mass index; WC: Waist circumference; Glu: Glucose; TGs: Triglycerides; TChol: Total cholesterol; HDL: High-density lipoprotein cholesterol; LDL: Low-density lipoprotein cholesterol; SGOT: Serum glutamic-oxaloacetic transaminase; SGPT: Serum glutamic pyruvic transaminase; γ-GT: Gamma-glutamyl transferase; ALP: Alkaline phosphatase. † Comparisons between baseline and after MD, * comparisons between baseline and after KD, ⁋ comparisons between MD and KD.

2.3. Biochemical Parameters

Patients did not display any significant alteration in aminotransferases, gamma-glutamyl transferase (γ-GT), and alkaline phosphatase (ALP) ($p > 0.05$ for all parameters). KD resulted in significant reduction in serum glutamic pyruvic transaminase (SGPT) ($p = 0.032$) compared to baseline, while no significant change in SGPT was observed after MD ($p > 0.05$), in comparison with baseline. Any of the examined diet patterns resulted in significant reduction in total cholesterol (TChol), low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL) ($p > 0.05$ for all parameters). However, patients after KD displayed a substantial reduction in triglycerides (TGs) ($p = 0.022$) in comparison with baseline, showing an almost 3-fold higher reduction compared to MD (−11.26% versus −29.47%). No significant change in TGs was observed after MD ($p > 0.05$) (Table 1).

2.4. Clinical Markers of Disease Activity

After KD, patients displayed a significant reduction in PASI ($p = 0.04$) compared to baseline, showing a 2-fold higher reduction compared to an MD diet (−61.58% vs. −31.24%, $p = 0.038$). No significant change in PASI was observed after MD ($p = 0.278$). KD resulted in significant reduction in DAPSA ($p = 0.004$) compared to baseline, showing a 3-fold higher reduction compared to an MD diet (−98.62% vs. −32.64%, $p = 0.034$). No significant change in DAPSA was observed after MD ($p = 0.06$) (Table 2 and Figure 2).

Table 2. The effect of the diet intervention on clinical markers of disease activity.

	Baseline	MD	Δ%	KD	Δ%	*p*-Value †	*p*-Value *	*p*-Value ⁋
PASI	5.09 ± 5.73	3.82 ± 3.93	−33.24	3.15 ± 4.88	−61.58	0.278	0.040	0.038
DAPSA	46.28 ± 34.89	34.89 ± 30.17	−32.64	23.30 ± 16.75	−98.62	0.060	0.004	0.034

Data are presented as mean ± SD. Δ% indicates percentage change from baseline. MD: Mediterranean diet; KD: Ketogenic diet; PASI: Psoriasis Area and Severity Index; DAPSA: Disease Activity Index for Psoriatic Arthritis. † Comparisons between baseline and after MD, * comparisons between baseline and after KD, ⁋ comparisons between MD and KD.

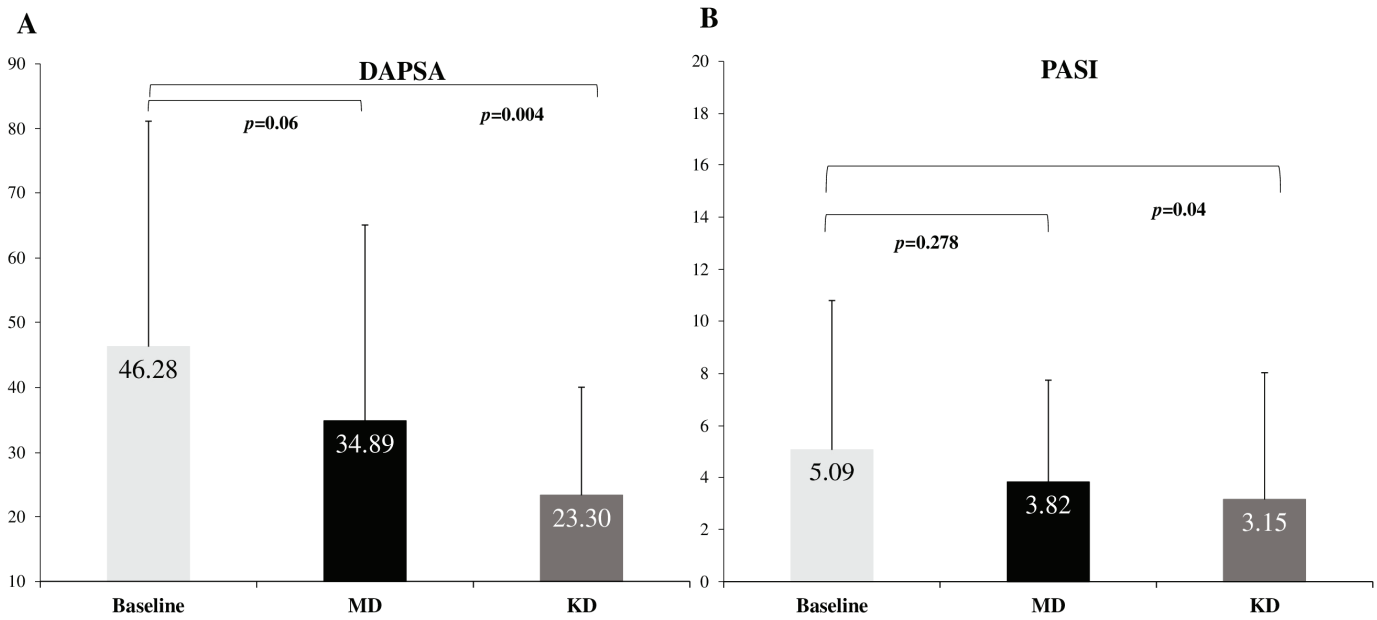

Figure 2. The effect of diet intervention on (**A**) DAPSA and (**B**) PASI. MD: Mediterranean Diet; KD: Ketogenic Diet; PASI: Psoriasis Area and Severity Index; DAPSA: Disease Activity Index for Psoriatic Arthritis.

2.5. Biochemical Markers of Inflammation

After KD, patients displayed a significant reduction in IL-6 (p = 0.047) compared to baseline, showing a 2-fold higher reduction compared to MD (−55.6% vs. −20.56%, p = 0.041). No significant change in IL-6 was observed after MD (p = 0.666), compared to baseline. KD resulted in substantial reduction in IL-17 (p = 0.042) and IL-23 (p = 0.037), whereas no significant differences were observed in the above markers in MD ($p > 0.05$; Table 3) (Figure 3), compared to baseline. No change in IL-22 was observed in all patients ($p > 0.05$) (Table 3).

Table 3. The effect of the diet intervention on biochemical markers of inflammation.

	Baseline	MD	Δ%	KD	Δ%	*p*-Value †	*p*-Value *	*p*-Value ⁋
IL-6	9.85 ± 17.94	8.17 ± 12.85	−20.56	6.33 ± 12.47	−55.6	0.666	0.047	0.041
IL-17	11.44 ± 20.10	5.29 ± 6.74	−116.25	4.66 ± 8.72	−145.49	0.243	0.042	0.687
IL-23	23.59 ± 11.04	19.15 ± 9.70	−23.18	17.86 ± 9.97	−32.08	0.151	0.037	0.540
IL-22	190.24 ± 166.10	213.43 ± 211.86	10.79	240.87 ± 256.35	20.83	0.584	0.328	0.368

Data are presented as mean ± SD. Δ% indicates percentage change from baseline. MD: Mediterranean diet; KD: Ketogenic diet. † Comparisons between baseline and after MD, * comparisons between baseline and after KD, ⁋ comparisons between MD and KD.

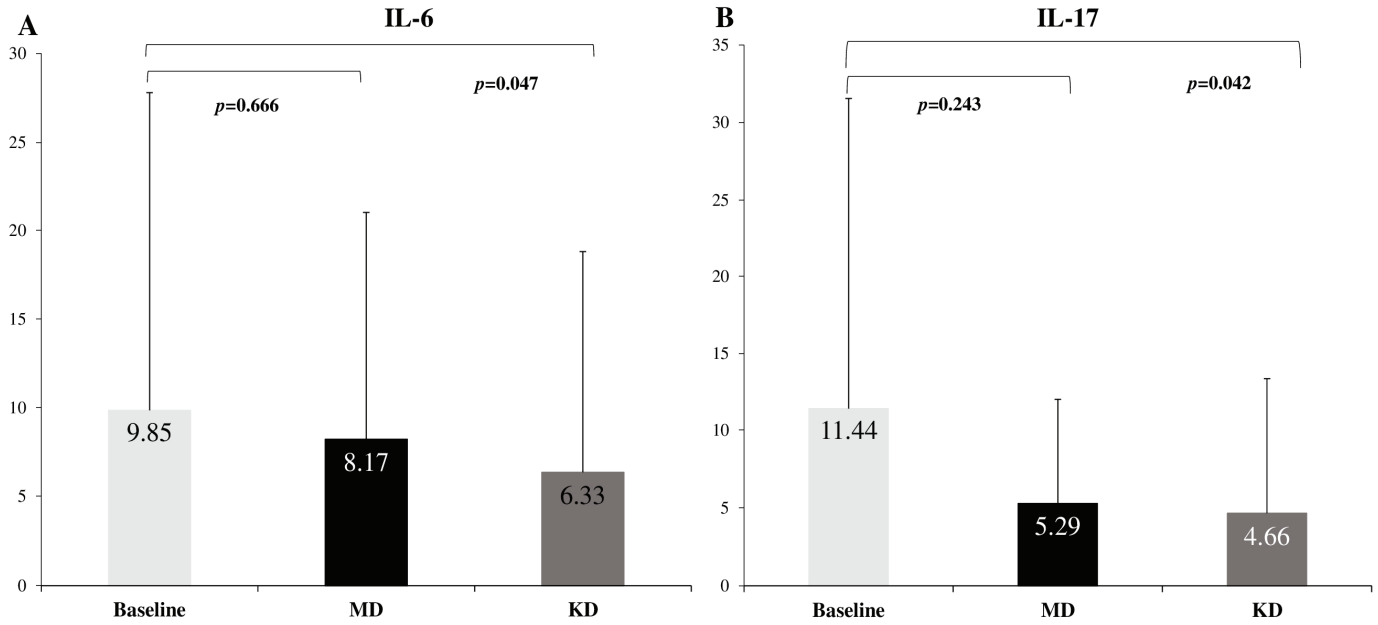

Figure 3. The effect of diet intervention on (**A**) IL-6 and (**B**) IL-17. MD: Mediterranean diet; KD: Ketogenic diet.

3. Discussion

According to our results, the combined 22-week MD–KD program for patients with PSO and PSA on stable anti-psoriatic pharmacological treatment led to weight loss and enhancements in clinical and biochemical markers of inflammation. However, these favorable effects were mainly attributed to KD, as statistically significant reductions in clinical scores of disease activity and inflammatory markers were observed only in this dietary regimen. This study supports the correlation between weight loss and reduced disease activity and the beneficial effect of KD on the inflammatory burden of psoriasis. To our knowledge, this is the first randomized study that examined the effects of MD and KD on disease parameters and markers of inflammation in patients with PSO and PSA.

Regarding the effect of KD on patients with psoriasis, literature data are still scarce. The first relevant publication was a case report by Castaldo et al. about a 40-year-old female patient with recurrent moderate-to-severe plaque psoriasis, psoriatic arthritis, and metabolic syndrome, who received conventional treatment with adalimumab. After a disease flare, VLCKD was introduced, and led to a significant reduction in a PASI score > 80% along with the resolution of psoriatic arthralgia [29]. These favorable results were reproduced in a study that included drug-naive patients, where the intervention consisted of a 4-week and a 6-week hypocaloric, low-glycemic-index, Mediterranean-like diet. Similar to our results, a body weight reduction of 12% and a significant reduction in the PASI score (mean change of −10.6) [31] were indicated. Regarding inflammatory markers, a low-calorie KD in 30 patients with psoriasis led to decreased IL-1β and IL-2 levels, along with 10% weight loss and 50% reduction in the PASI score [30]. The pathophysiological pathways through which KD ameliorates the pro-inflammatory milieu in psoriasis have not yet been fully elucidated. The KD improves oxidative stress via the activation of nuclear factor erythroid-derived 2 (NF-E2)—related factor 2 (Nrf2), which in turn promotes the activation of the peroxisome proliferator-activated receptor-gamma (PPAR-γ) and which may elicit anti-inflammatory effects through the inhibition of nuclear factor kappa B (NF-Kb) activation [32,33]. Furthermore, β-hydroxybutyrate inhibits the NLRP3 inflammasome in lipopolysaccharide (LPS)-stimulated human monocytes, resulting in the reduction in IL-1β and IL-18 [34]. A further mechanism explaining the protective activity of the KD against oxidative stress is the intracellular modulation of the NAD^+/NADH ratio. An increased

NAD^+/NADH ratio protects against reactive oxygen species (ROS) and plays an important role in cellular respiration, mitochondrial biogenesis, and redox reactions [35].

The implementation of a Mediterranean dietary pattern has also exhibited beneficial results in terms of autoimmunity. Skoldstam et al. [36] reported that patients with rheumatoid arthritis after MD displayed a significant reduction in the disease activity score (DAS28), and comparable results were shown in a recent study by Vadel et al. (ADIRA trial), which demonstrated the positive effect of a Mediterranean-like, anti-inflammatory diet on disease activity in patients with rheumatoid arthritis [37]. Regarding the association with psoriasis and MD, a number of studies are also available [38–40]. What needs to be pointed out, however, is that these studies retrospectively evaluated the adherence to MD through the use of validated questionnaires, which inevitably lack the robustness of data compared to interventional studies. For instance, a case–control study by Barrea et al. was the first that showed that patients with psoriasis had a lower adherence to MD (assessed by PREDIMED questionnaire) compared to the control group [39]. Even more importantly, psoriasis severity (assessed by PASI score and C-reactive protein (CRP)) was negatively associated with the intake of extra-virgin olive oil, fruits, nuts, fish or seafood, vegetables, and legumes, and it was positively correlated with red meat intake [39]. In the same notion, a cross-sectional observational study with a larger sample (n = 35.735 patients) by Phan et al. showed an inverse association between adherence to a Mediterranean diet (assessed by MED-LITE) and psoriasis severity [39]. These beneficial effects of MD on disease activity and inflammation markers were also demonstrated in our study, despite not achieving statistical significance. The reason for this discrepancy could be the small size of the sample, along with the fact that the patients already suffered from psoriatic arthritis, which implies the significantly more severe inflammatory burden of the disease compared to the aforementioned trials. Based on these data, the Medical Board of the National Psoriasis Foundation recommends a trial of MD in patients with psoriasis, but the need for large-scale, interventional trials is still underlined [41].

The positive effects of MD cannot be attributed specifically to its every single component separately, but it is rather the combination of all the different macro- and micronutrients, which exert the favorable anti-inflammatory effects. However, extra-virgin olive oil seems to play the most important role [42]. Oleic acid, a monounsaturated fatty acid (MUFA), is its major constituent, and is highly effective in decreasing the oxidization of LDL [43]. In a study by Loued et al., the consumption of extra-virgin olive oil for 12 weeks significantly increased the anti-inflammatory activities of both HDL and paraoxonase 1 (PON1) and decreased serum levels of inter cellular adhesion molecule (ICAM-1), which is one of the most prominent adhesion molecules with pro-inflammatory properties. Olive oil down-regulates pro-inflammatory cytokines such as IL-6 and TNF-α [42].

The present study has several strengths. First, we employed a crossover design to reduce the effects of inter-individual variation and maximize the statistical power from the available sample size. We also implemented a 6-week washout period, which we believe to be sufficient to normalize effects from the prior dietary period. In addition, it is the first study that is interventional in nature and performs a direct comparison between these two dietary patterns.

One limitation of our study is its small sample. Furthermore, our results could not easily be extrapolated to a real-world setting as the patients were evaluated at bi-weekly intervals, which ensured a high degree of adherence but it is not easily feasible in everyday clinical practice. Nevertheless, from a clinical point of view, the beneficial effect of diet interventions as an adjunct treatment to conventional pharmacological therapy was confirmed, and these two dietary regimens could be implemented alternately in patients with psoriasis.

The present study demonstrated that the combined 22-week Mediterranean–Ketogenic diet program for patients with PSO and PSA led to beneficial results in indices of disease activity and pro-inflammatory markers. These favorable effects were mainly attributed to KD, but MD also showed a beneficial tendency. These findings further establish the

association between dietary interventions and auto-inflammatory disorders and emphasize the need for more, large-scale interventional trials to compare different dietary patterns.

4. Materials and Methods

4.1. Study Population

Sixteen patients who were admitted to the dermatology unit with the diagnosis of psoriasis and psoriatic arthritis were enrolled in this study. Inclusion criteria were the following: (1) age above 18 years, (2) BMI $\geq$ 30 kg/m^2, (3) diagnosis of psoriasis and psoriatic arthritis, (4) constant systematic treatment with biologic agents and/or synthetic-disease-modifying anti-rheumatic drugs (DMARDs) for at least 3 months, (5) PASI score improvement < 75%, with moderate or severe activity in joints (>3 swollen and >3 tender joints or DAPSA < 14). Exclusion criteria were the following: participation in another study, eGFR < 60 mL/min/1.73 m^2, malignancy, severe hepatic disorder, HbA1C > 10%, and use of glucagon-like-petide-1 analogues.

4.2. Study Design

This is a randomized, open-label, controlled crossover study conducted from May 2020 to January 2022 at the Unit of Diabetes, Second Department of Internal Medicine, in collaboration with the Dermatology Department in Attikon University Hospital. Clinical and medical measurements, as well as lifestyle intervention sessions, were performed at the Attikon University Hospital. The patients were randomly assigned to two groups, starting either with MD or KD for a period of 8 weeks. After a 6-week washout interval, the two groups were crossed over to the other type of diet for the same 8-week period (Figure 4).

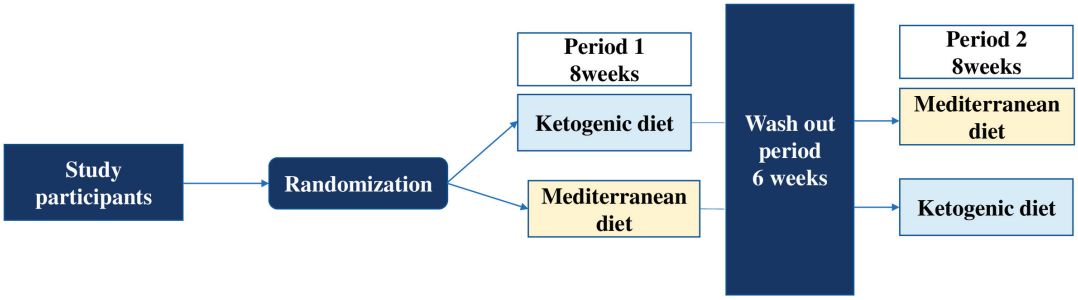

Figure 4. Design and analysis of crossover trial.

Basic characteristics, including age, gender, smoking status, alcohol consumption, physical activity level, medical history, concomitant medication, and dietary habits, were obtained at baseline. All participants underwent a physical examination during the first visit. Before and after each intervention, the study team performed anthropometric measurements, assessment of the PASI and DAPSA score, and blood sampling for biochemical markers of inflammation and hormones.

The study protocol was approved by the ethics committee of Attikon University Hospital (171/09-04-2020) before the beginning of the enrollment and any other procedure. All participants provided written informed consent before study initiation. This study was carried out in accordance with the Declaration of Helsinki (Trial Registration: Clinicaltrials.gov, accessed on 13 January 2024; Identifier: NCT06164860).

4.3. Anthropometric Measurements

Anthropometric parameters were obtained at the beginning and at the end of each intervention. Height was measured using a stadiometer and weight was measured using a calibrated electronic scale. Body composition was assessed by a Tanita BC-420 body composition analyzer. Waist and hip circumference were taken at a themed point between the lower rib margin and the iliac crest and at the widest part of the hips, respectively,

with a stretch-resistant tape kept parallel to the ground. Study subjects had their waist uncovered and were asked to stand with their feet close together and their weight equally distributed on each leg.

4.4. Biochemical Measurements

Metabolic parameters were obtained before and after each intervention. The biochemical parameters that were evaluated included glucose, TGs, TChol, HDL, LDL, aminotransferases, alkaline phosphatase (ALP). Moreover, plasma levels of IL-6, IL-17, IL-23, and IL-22 were also evaluated by ELISA kits. Plasma samples were obtained by venipuncture and stored at −80 °C. Concentrations of interleukins were measured using a solid-phase enzyme-linked immunosorbent assay (ELISA) following manufacturer's instructions. Plasma levels of IL23, IL17A, and IL6 were determined using commercially available ELISA kits (Mabtech AB, Nacka Strand, Sweden) (sensitivity, % intra- and inter-precision was 5 pg/mL, 1 pg/mL, and 2 pg/mL, respectively, and <10%) and levels of IL22 (OriGene Technologies, Inc., Rockville, MD, USA) (15 pg/mL sensitivity, % intra- and inter-precision < 10%).

4.5. Assessment of Psoriasis and Psoriatic Arthritis Severity

The severity of the disease was rated using the PASI score [38], which accounts for the extent of psoriatic involvement of the body surface areas on the head, trunk, arms, and legs, in addition to the severity of scale formation, erythema, and plaque indurations on each region of the body (score range: 0–72; higher scores indicate more severe disease). The severity of psoriatic arthritis was assessed by using DAPSA. The DAPSA score was calculated by adding the number of tender and swollen joints, visual analogue scale (VAS) pain, patient's global assessment (PtGA), and CRP (mg/dL).

4.6. Dietary Interventions

Both dietary interventions were designed to provide approximately 1550 (±50) calories per day. The KD provided approximately 34% proteins, 55% fat, and 11% carbohydrates. The macronutrient targets were successfully met by replacing breakfast and two daily snacks with products with the best nutritional value/macronutrient content for a Ketogenic diet, provided by Evivios Med (Athens, Greece). Lunch and dinner were natural protein-rich dishes. Each participant received accurate teaching and a brochure that incorporated a template of a weekly diet plan from an expert dietitian. More specifically, the brochure contained a daily meal plan (each meal had options they could choose from), a portion guide for foods containing protein, a list with the allowed vegetables (based on their carbohydrate content) that could be used in the salad alternatives for olive oil, allowed foods (without calories) such as sweeteners and spices, and other general information. It was proposed that the patients consume 5 meals per day. A typical example of such a meal would be a 120 g oven-baked chicken, 2 cups of salad (only with the allowed vegetables), and 1 tablespoon of olive oil.

The MD provided 20% proteins, 40% fat, and 40% carbohydrates. Each participant received a brochure with a template of a daily five-course (breakfast, lunch, dinner, and two snacks) diet plan that provided several options for each meal and were advised to use it as a guide for the 8-week period. It was proposed that the patients consume 5 meals per day. A typical example of such a meal would bea 90 g oven-baked chicken, salad with 2 teaspoons of olive oil, 1 cup of boiled rice.

The participants met the same expert dietitian every two weeks during the entire study period. They were encouraged to keep a food diary, which assisted them to monitor food consumption and promoted participants' awareness regarding their adherence.

Participants who missed a scheduled visit were advised to attend a make-up counseling session the following week.

Every 2 weeks, participants were asked to attend a face-to-face (F2F) dietetic consultation in an outpatient's clinic. During each follow-up consultation, the expert dietitian assessed participants' food diary and tailored offered advice that aimed to encourage participants' adherence to the dietary intervention they were assigned to.

4.7. Primary and Secondary Endpoints

The primary endpoint was changes in clinical scores of the disease activity (PASI, DAPSA), 8 weeks after KD compared to the 8-week MD diet intervention. Secondary endpoints were biochemical markers of inflammation (IL-6, IL-17, IL-22, IL-23) and alterations in anthropometric parameters 8 weeks after KD compared to the 8-week MD diet intervention.

4.8. Statistical Analysis

The sample size was estimated based on the difference in the DAPSA score after the intervention. DAPSA score improvement $\geq$50 % was considered as clinically significant [44]. To be able to detect a difference of $\geq$50% in the DAPSA score with 80% power at a 5% significance level with a two-sided paired *t*-test, 15 patients should complete this study. To account for possible dropouts, a total of 26 patients were enrolled. The statistical analysis was performed using the SPSS 22.0 statistical software package (SPSS Inc., Chicago, IL, USA). All variables are expressed as the mean $\pm$ SD. Categorical variables are expressed as percentages of the population. Continuous variables were tested by the Kolmogorov–Smirnov test to assess the normality of distribution. Variables with a non-normal distribution were analyzed after transformation into ranks. Categorical data were analyzed using the χ^2 test. All statistical tests were 2-tailed, and $p < 0.05$ was considered to be the level of statistical significance.

5. Conclusions

In our study, we showed that the combined 22-week Mediterranean–Ketogenic diet program for patients with PSO and PSA led to beneficial results in indices of disease activity and pro-inflammatory markers. These beneficial changes were mainly attributed to KD, but MD also showed a beneficial tendency. Although the effects of MD and KD are well established in a range of cardiometabolic diseases, their possible efficacy as an adjunct treatment to conventional pharmacological therapy in patients with psoriasis is a novel clinical implication. These findings further establish the association between dietary interventions and auto-inflammatory disorders and emphasize the need for more, large-scale interventional trials to compare different dietary patterns.

Author Contributions: All authors have made a substantial contribution to the work and approved it for publication, with V.L. being the guarantor for the contents of the article. V.L., P.K. and E.P. were responsible for conceptualization, methodology, supervision, and project administration. V.L., E.P. and D.V. were responsible for the financial support of the study. S.M., E.M., A.K., A.P. and E.K. were responsible for investigation, data curation, and methodology. L.P. was responsible for software. G.P., I.I. and A.K. were responsible for formal analysis. V.L., A.K. and E.K. wrote and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by Ethics Committee of University General Hospital Attikon.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Study data are available upon reasonable request.

Conflicts of Interest: D.V. is general director of Evivios Med. S.M. is a dietician in Evivios Med.

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International Journal of
Molecular Sciences

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Article

β-Carotene Supplementation Improves Pancreas Function during Moderate Ethanol Consumption: Initial Characterization from a Morphological Overview

Cristian Sandoval [1,2,3], Angeles Vera [4], Katherine Birditt [5], Karina Godoy [6], Florencia Carmine [7], José Caamaño [3,8,*] and Jorge Farías [2,*]

1 Escuela de Tecnología Médica, Facultad de Salud, Universidad Santo Tomás, Los Carreras 753, Osorno 5310431, Chile; cristian.sandoval@ufrontera.cl
2 Departamento de Ingeniería Química, Facultad de Ingeniería y Ciencias, Universidad de La Frontera, Temuco 4811230, Chile
3 Departamento de Medicina Interna, Facultad de Medicina, Universidad de La Frontera, Temuco 4811230, Chile
4 Carrera de Tecnología Médica, Facultad de Medicina, Universidad de La Frontera, Temuco 4811230, Chile; a.vera08@ufromail.cl
5 Physiology Development and Neuroscience Department, University of Cambridge, Cambridge CB2 1TN, UK; krb56@cam.ac.uk
6 Núcleo Científico y Tecnológico en Biorecursos (BIOREN), Universidad de La Frontera, Temuco 4811230, Chile; karina.godoy@ufrontera.cl
7 Carrera de Medicina, Facultad de Medicina, Universidad de La Frontera, Temuco 4811230, Chile; f.carmine02@ufromail.cl
8 Laboratorio de Inmunohematología y Medicina Transfusional, Departamento de Medicina Interna, Facultad de Medicina, Universidad de La Frontera, Temuco 4811230, Chile
* Correspondence: jose.caamano@ufrontera.cl (J.C.); jorge.farias@ufrontera.cl (J.F.)

Abstract: Alcohol is believed to harm acinar cells, pancreatic ductal epithelium, and pancreatic stellate cells. After giving ethanol and/or β-carotene to *C57BL/6* mice, our goal was to evaluate their biochemistry, histology, and morpho-quantitative features. There were six groups of *C57BL/6* mice: 1. Group C (control), 2. Group LA (low-dose alcohol), 3. Group MA (moderate-dose alcohol), 4. Group B (β-carotene), 5. Group LA + B (low-dose alcohol combined with β-carotene), and 6. Group MA + B (moderate-dose alcohol combined with β-carotene). After the animals were euthanized on day 28, each specimen's pancreatic tissue was taken. Lipase, uric acid, and amylase were assessed using biochemical assessment. Furthermore, the examination of the pancreatic structure was conducted using Ammann's fibrosis scoring system. Finally, the morpho-quantitative characteristics of the pancreatic islets and acinar cells were determined. In the serum of the MA + B group, there were higher amounts of total amylase (825.953 ± 193.412 U/L) and lower amounts of lipase (47.139 ± 6.099 U/L) ($p < 0.05$). Furthermore, Ammann's fibrosis punctuation in the pancreas revealed significant variations between the groups ($p < 0.001$). Finally, the stereological analysis of pancreatic islets showed that the groups were different ($p < 0.001$). These findings suggest that antioxidant treatments might help decrease the negative effects of ethanol exposure in animal models.

Keywords: alcohol intake; alcohol pancreatitis; antioxidant treatment; chronic alcohol consumption

1. Introduction

The use of alcohol is a significant issue for public health. Based on findings from the United European Gastroenterology Survey of Digestive Health, 155 billion euros per year are spent as a consequence of alcohol intake [1]. The use of alcohol is a well-recognized risk factor for both acute and chronic pancreatitis, accounting for around 50 to 80% of all reported cases [2]. Recent studies have reported that alcohol remains the primary cause of this condition in the United States [3].

In contrast, it has been shown that fewer than 5% of heavy alcohol drinkers experience pancreatitis [4]. The main reason is that showing signs of alcohol toxicity illness requires the presence of other risk factors, which may be caused by the environment or be inherited [5,6].

Acute pancreatitis is a pathological illness defined by the inflammatory response of the pancreas, which can result in local tissue destruction, a systemic inflammatory response, and, ultimately, organ failure [7]. The incidence of this gastrointestinal illness is widespread, and it necessitates prompt hospitalization [8,9].

According to the American College of Gastroenterology guidelines, alcohol can be attributed as the etiological factor for acute pancreatitis when a patient has a documented history of consuming alcohol excessively for a duration beyond five years (>50 g/per day) [10]. But, in some instances, the etiology of acute pancreatitis can be attributed to alcohol drinking when the consumption is infrequent or moderate [11].

The intricate and multifaceted effects of alcohol on the pancreas have left the etiology of alcohol-induced pancreatitis unclear. Alcohol is believed to damage acinar cells, pancreatic ductal epithelium, and pancreatic stellate cells, thereby promoting pancreatic fibrosis [12,13]. Alcohol-induced pancreatitis only occurs when compensatory mechanisms are exhausted or when there is an increased susceptibility to other (genetic and environmental) pancreatic stressors. However, animal models indicate that the pancreas can mitigate alcohol's detrimental effects through an adaptive stress response [14,15].

Pancreatic acinar cells use both oxidative and nonoxidative pathways to metabolize alcohol [16–18]. In fact, previous studies have demonstrated that alcohol probably alters the class II alcohol dehydrogenase (ADH2) [19] and ADH3 isoforms [19,20] to inhibit acinar ethanol oxidation. Some of the bad things that alcohol does to acinar cells are because of how it is broken down and how it makes toxic compounds like fatty acid ethyl esters (FAEEs), acetaldehyde, and reactive oxygen species (ROS) [16,21,22]. Oxidative stress, which is caused by ROS and FAEE, leads to destabilization of zymogen granules and lysosomes and other dysregulations of the cell organelles [4,16,23].

The process of oxidative alcohol metabolism leads to impairment of mitochondrial activity, which serves as a stimulus for the initiation of apoptosis and necrosis [24]. Mitochondrial dysfunction arises due to the permeabilization of membranes caused by oxidative alcohol metabolism [25].

The dysfunction of autophagy is also a prominent symptom of alcohol-induced pancreatitis. The process of autophagy commences by sequestering the material that is intended for destruction into autophagosomes. These autophagosomes subsequently merge with lysosomes, resulting in the formation of autolysosomes. Within these autolysosomes, the cargo undergoes disintegration facilitated by lysosomal hydrolases [26]. One of the early indications of pancreatitis is the presence of acinar cells that have larger autolysosomes, which carry poorly digested cargo [27]. Experimental models of alcohol-induced pancreatitis have shown a considerable drop in levels of lysosome-associated membrane proteins, which play a critical role in preserving the functionality of lysosomes [28,29].

Impairment of the apical secretion of zymogens is an additional significant mechanism that impacts acinar cells and plays a role in the development of alcohol-induced pancreatitis. A block of apical secretion triggers the activation of proteases inside the acinar cells, as well as the release of active zymogens into the interstitial space by basolateral exocytosis via the basolateral plasma membrane of the acinar cell [30–32].

The pathophysiology of alcohol-induced pancreatitis has been described in previous research. Despite this, there is still a lack of conclusive comprehension of this significant issue. Therefore, the aim of this study was to evaluate the biochemical, histological, and morpho-quantitative effects of oral supplementation with β-carotene on the pancreas of *C57BL/6* mice exposed to ethanol consumption.

2. Results

2.1. Biochemical Evaluation

Biochemical analyses for lipase, uric acid, and amylase are shown in Table 1. Briefly, the LA group showed the lowest lipase activity among the experimental groups, whereas the B group showed the highest lipase activity ($p = 0.002$). In relation to uric acid levels, the MA group presented the lowest levels and the LA group the highest levels ($p < 0.001$). Moreover, no significant differences between groups were found in amylase activity ($p = 0.155$).

Table 1. Biochemical analysis of male *C57BL/6* mice exposed to alcohol consumption and oral β-carotene supplementation.

	Media ± SD						
	C	**LA**	**MA**	**B**	**LA + B**	**MA + B**	***p***
Lipase (U/L)	51.315 ± 7.230	43.363 ± 3.377 [a]	55.975 ± 14.098	65.383 ± 6.679 [ab]	64.315 ± 9.555 [ab]	47.139 ± 6.099 [de]	0.002
Uric acid (µmol/L)	99.511 ± 29.729	124.666 ± 24.118	59.983 ± 18.445 [ab]	104.487 ± 28.909 [c]	91.219 ± 26.863 [bc]	113.609 ± 14.520 [ce]	<0.001
Amylase (U/L)	658.420 ± 195.988	705.070 ± 95.701	712.886 ± 134.047	709.438 ± 125.675	735.407 ± 123.837	825.953 ± 193.412	0.155
Lipase/Amylase ratio	0.081 ± 0.018	0.062 ± 0.011	0.085 ± 0.041	0.096 ± 0.027 [b]	0.096 ± 0.021 [b]	0.058 ± 0.011 [de]	<0.001

[a] Significant differences ($p < 0.05$) with the C group. [b] Significant differences ($p < 0.05$) with the LA group. [c] Significant differences ($p < 0.05$) with the MA group. [d] Significant differences ($p < 0.05$) with the B group. [e] Significant differences ($p < 0.05$) with the LA + B group.

2.2. Histological Evaluation

As depicted in Figure 1A, pancreas cells in the control group possessed large nuclei surrounded by a well-defined cytoplasm and cytoplasmic membrane; they also exhibited normal morphology of the islets. The islet appeared less stained than the surrounding acinar cells and was composed of polygonal cells arranged in bundles and separated by a network of blood vessels. Finally, acinar cells were characterized by their basal basophilia and apical acidophilia.

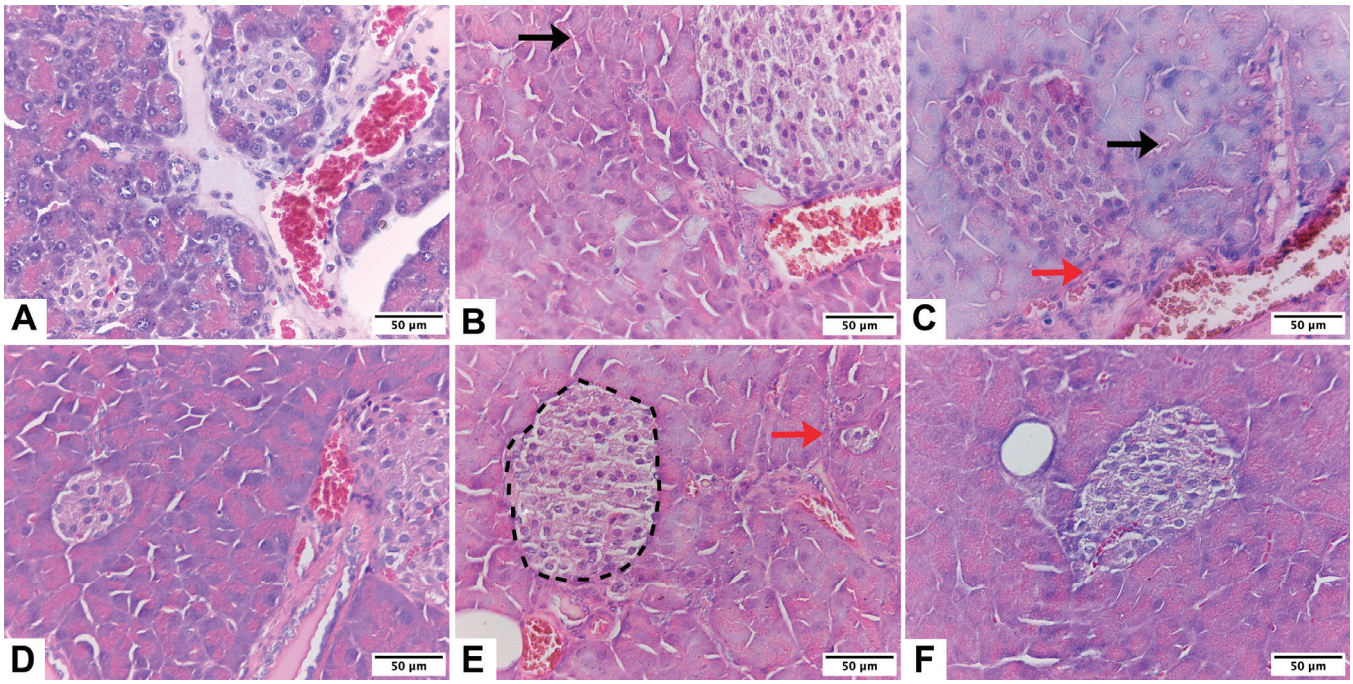

Figure 1. Pancreas of male *C57BL/6* mice. Pancreatic architecture was observed in groups C (**A**), low-dose alcohol (**B**), moderate-dose alcohol (**C**), β-carotene (**D**), low-dose alcohol + β-carotene (**E**), and moderate-dose alcohol + β-carotene (**F**). Interlobular fibrosis (black arrow), perilobular fibrosis (red arrow), and pancreatic islet (segmented line).

Histological examination revealed that islet blood vessels were dilated in alcohol-induced pancreatitis mice (Figure 1B,C). The pancreas of the LA and MA groups, in contrast, displayed abnormal tissue architecture with distinct areas of acinar cell loss and pseudotubular structures. Importantly, the areas of acinar cell injury in the pancreas of alcohol-fed mice were significantly greater than in the C group. When acinar cell loss was assessed, the average area of acinar cell injury was nearly double in the pancreas of the LA and MA groups compared to the C group (Figure 1B,C).

β-carotene was associated with an increase in the number and size of pancreatic islets, as shown in Figure 1D–F. The islets had a lobular aspect, a regular shape, and some fibrotic growth in them. In comparison with groups without β-carotene supplementation, the exocrine pancreas had less cell degeneration and vacuolization, but had eosinophilic cytoplasm.

Table 2 shows the percentages of animals that were categorized to each score of the two histological parameters. The median (lower and upper range) of Ammann's fibrosis score for C was 2.36 (2–4), for LA was 6.44 (6–8), for MA was 10.52 (10–12), for B was 3.84 (2–5), for LA + B was 8.04 (6–10), and for MA + B was 3.88 (2–5). Analysis of peri-lobular parenchyma, intralobular parenchyma, and total score for Ammann's fibrosis punctuation in the pancreas found significant differences between groups ($p < 0.001$; Table 3).

Table 2. Percentages of the histological changes according to Ammann's fibrosis punctuation.

Parameters	Score	Fibrosis Grade	Group Frequency (%)					
			C	LA	MA	B	LA + B	MA + B
Peri-lobular parenchyma	1	Mild	80	0	0	16	0	20
	2	Moderate	20	0	0	76	0	76
	3	Marked	0	80	0	8	20	4
	4	Mild	0	20	0	0	64	0
	5	Moderate	0	0	72	0	16	0
	6	Marked	0	0	28	0	0	0
Intralobular parenchyma	1	Mild	84	0	0	20	0	16
	2	Moderate	16	0	0	68	0	64
	3	Marked	0	76	0	12	20	20
	4	Mild	0	24	0	0	52	0
	5	Moderate	0	0	76	0	28	0
	6	Marked	0	0	24	0	0	0

Table 3. Analysis of peri-lobular parenchyma, intralobular parenchyma, and total score for Ammann's fibrosis punctuation in pancreas.

	Media ± SD						
	C	LA	MA	B	LA + B	MA + B	*p*
Peri-lobular parenchyma	1.200 ± 0.408	3.200 ± 0.408 [a]	5.280 ± 0.458 [ab]	1.920 ± 0.493 [abc]	3.960 ± 0.611 [abcd]	1.840 ± 0.472 [abce]	<0.001
Intralobular parenchyma	1.160 ± 0.374	3.240 ± 0.435 [a]	5.240 ± 0.435 [ab]	1.920 ± 0.571 [abc]	4.080 ± 0.702 [abcd]	2.040 ± 0.611 [abce]	<0.001
Score	2.360 ± 0.568	6.440 ± 0.711 [a]	10.520 ± 0.714 [ab]	3.840 ± 0.850 [abc]	8.040 ± 1.01 [abcd]	3.880 ± 0.781 [abce]	<0.001

[a] Significant differences ($p < 0.05$) with the C group. [b] Significant differences ($p < 0.05$) with the LA group. [c] Significant differences ($p < 0.05$) with the MA group. [d] Significant differences ($p < 0.05$) with the B group. [e] Significant differences ($p < 0.05$) with the LA + B group.

2.3. Morphoquantitative Analysis of the Pancreas

The histological changes in pancreatic islet mass can be coupled with clear qualitative differences in overall cytology, with islets appearing small and well defined in the C, LA, and LA + B groups (Figure 1A,B,E).

In the pancreas of mice exposed to alcohol intake and/or β-carotene supplementation, the N_V and V_V values for pancreatic islets were higher in the LA + B and MA + B groups in comparison with the LA and MA groups, respectively (Table 4). The N_V of acinar cells decreased in the MA + B group in comparison with the MA group ($p = 0.245$). Post hoc tests

showed statistically significant differences in the N_V and V_V of pancreatic islets ($p < 0.001$) and the V_V and S_V of acinar cells ($p < 0.014$).

Table 4. Stereological analysis of mice pancreas exposed to alcohol consumption and oral supplementation of β-carotene.

	Media ± SD						
	C	**LA**	**MA**	**B**	**LA + B**	**MA + B**	***p***
$N_{V\ islets}$ (mm^{-3})	429.183 ± 52.588	604.177 ± 20.168 [a]	651.080 ± 51.059 [a]	672.110 ± 85.790 [a]	715.472 ± 69.053 [a]	707.522 ± 31.115 [a]	<0.001
$V_{V\ islets}$ (%)	23.053 ± 7.539	28.307 ± 7.251	36.167 ± 8.927 [a]	27.595 ± 2.455	28.547 ± 4.488	38.386 ± 8.445 [a]	0.001
$S_{V\ islets}$ (mm^{-1})	7.011 ± 0.219	9.626 ± 1.093	10.077 ± 1.122	9.370 ± 3.840	9.370 ± 3.511	9.182 ± 6.373	0.144
$TM_{\ islets}$	3.270 ± 0.028	4.092 ± 0.052	6.862 ± 0.639 [ab]	6.208 ± 0.701 [a]	4.984 ± 0.460	8.398 ± 0.633 [abd]	<0.001
$N_{V\ acinar\ cells}$ (mm^{-3})	2476.634 ± 854.875	2529.968 ± 890.316	3030.235 ± 283.025	2668.711 ± 907.537	2710.377 ± 466.376	2296.665 ± 756.358	0.245
$V_{V\ acinar\ cells}$ (%)	4.872 ± 1.052	5.228 ± 1.988	4.537 ± 0.295	3.904 ± 0.267 [b]	5.014 ± 0.340	5.452 ± 0.380 [c]	0.014
$S_{V\ acinar\ cells}$ (mm^{-1})	15.266 ± 6.842	15.994 ± 2.595	13.589 ± 3.246	12.043 ± 3.546 [b]	13.698 ± 1.666	14.439 ± 1.017	0.014

$N_{V\ islets}$: number density per area of the pancreatic islets; $V_{V\ islets}$: volume density per area of the pancreatic islets; $S_{V\ islets}$: surface density per area of the pancreatic islets; $TM_{\ islets}$: total mass of the pancreatic islets; $N_{V\ acinar\ cells}$: number density per area of the acinar cells; $V_{V\ acinar\ cells}$: volume density per area of the acinar cells; $S_{V\ acinar\ cells}$: surface density per area of the acinar cells. [a] Significant differences ($p < 0.05$) with the C group. [b] Significant differences ($p < 0.05$) with the LA group. [c] Significant differences ($p < 0.05$) with the B group. [d] Significant differences ($p < 0.05$) with the LA + B group.

3. Discussion

3.1. Summary of Key Findings and Interpretation

Chronic pancreatitis is distinguished by the presence of fibroinflammatory alterations in the pancreatic tissue. The development of this disorder can be observed in conjunction with several factors, including alcohol misuse, smoking, gene mutations, autoimmune syndromes, metabolic changes, environmental conditions, and anatomical anomalies [33–35].

Although acute pancreatitis is a well-recognized cause of hospitalization, accurately diagnosing this condition can be challenging due to the absence of a reliable and straightforward blood test that exhibits high sensitivity. The indicators that are often utilized include serum amylase, lipase, trypsinogen-2, and activation peptide of carboxypeptidase B [36–38].

3.2. Biochemical Evaluation

Standard biochemical indicators employed in clinical settings encompass serum amylase and lipase. However, amylase levels in the serum normalize within 3–5 days, while lipase levels typically normalize between 8 and 14 days [39]. The presence of increased levels of amylase and/or lipase in the blood indicates a higher likelihood of acute pancreatitis, with amylase being the more commonly utilized measurement [38]. Approximately 40% of serum amylase originates from the pancreas, while the majority comes from the salivary glands [40]. Hence, an increase in serum total amylase levels is not exclusive to pancreatitis, and it is important to evaluate other illnesses as well [40]. The L/A ratio is an acceptable indicator of alcohol-induced pancreatitis [41]. In fact, L/A ratio can be used to distinguish between pancreatitis caused by alcohol use and pancreatitis not caused by alcohol [42]. Patients diagnosed with alcohol-induced pancreatitis have a twofold increased likelihood of having an L/A ratio of three or higher with a sensitivity of 75% and a specificity of 56% [43]. Our results show increased lipase levels in the B and LA + B groups in comparison to the C group ($p = 0.002$). In addition, decreased lipase levels and L/A ratio in the MA + B group were found ($p = 0.002$ and $p < 0.001$, respectively). No significant differences in amylase levels were found ($p = 0.155$). These results suggest that β-carotene supplementation prevents acute pancreatitis caused by alcohol and improves pancreas function after moderate alcohol consumption, and they could be an acceptable indicator of alcohol-induced pancreatitis.

Acinar cells in cell culture or isolated pancreatic acini cells have been shown to break down ethanol in two ways: one is by oxidation, and the other is by non-oxidation [44–46]. When it comes to the oxidative pathways, the most common form (called an isoform) of ADH in acinar cells is ADH3. This is an enzyme that cannot reach saturation and has a low

affinity for ethanol and a high Km value. CYP2E1 has been identified in both the human and rat pancreas. As noted before, the expression of this gene can be stimulated in the pancreas of rats that have been fed alcohol [47].

Prolonged exposure to ethanol in mice leads to an increase in aldehyde dehydrogenase (ALDH) activity, suggesting the development of physiological tolerance [48]. However, animals who were exposed to long-term consumption of ethanol and received oral administration of β-carotene showed increased levels of ALDH in their bloodstream. In contrast, the groups that did not receive supplementation exhibited a decrease in the activity of this enzyme [49]. Previous research has confirmed that individuals suffering from chronic liver disease demonstrate diminished ALDH activity in comparison to individuals who do not have the ailment [48]. Multiple studies have noted a substantial decrease in overall ALDH levels among individuals who have progressed to severe liver disease. The decrease is especially evident in individuals with elevated levels of fibrosis and liver damage [50].

3.3. Histological Evaluation

The cardinal histopathologic features of chronic pancreatitis are fibrosis, loss of acinar tissue, and ductal changes [43]. The fibrosis score has been widely utilized in numerous studies to evaluate fibrosis [51]. The evaluation approach initially examines whether perilobular fibrosis is localized or widespread and subsequently categorizes the perilobular fibrosis into one of three degrees: mild, moderate, or severe.

The findings strongly indicate that supplementing mice with β-carotene can ameliorate the pancreatitis associated with prolonged ethanol exposure. The proposed treatment appeared to be more efficacious in animals with moderate alcohol consumption than in the low-alcohol-consumption group. Chronic alcohol intake is responsible for 17% to 25% of acute pancreatitis cases globally and ranks as the second most prevalent cause of acute pancreatitis, following gallstones [52]. Research conducted in recent years has provided evidence indicating that the causes of steatosis induced by ethanol are likely to be complex, involving various factors such as the impact on liver lipid metabolism, hypoxia, oxidative stress, pancreas function, and lipid peroxidation [21,49,53,54]. In accordance with this, our results showed dilated blood vessels in islets, abnormal tissue architecture with distinct areas of acinar cell loss, and pseudotubular structures in mice fed low and moderate alcohol doses (Figure 1B,C). These results agree with previous studies, where long-term ethanol treatment induces pancreatic islet dysfunction and apoptosis [55].

Fruits and vegetables contain large amounts of antioxidant micronutrients, such as vitamins and carotenoids. These micronutrients play a role in protecting the organism against reactive oxygen species [20]. Studies have demonstrated a reduction in antioxidant vitamins and carotenoids in several pancreatic illnesses, leading to a decreased likelihood of pancreatic neoplasia [56,57] and pancreatic cancer [58]. We demonstrated that β-carotene supplementation improved the number, size, and shape of pancreatic islets in alcohol-induced pancreatitis mice with some fibrotic growth (Figure 1D–F). In addition, analysis of peri-lobular parenchyma, intra-lobular parenchyma, and the total score for Ammann's fibrosis punctuation in the pancreas found that the LA + B and MA + B groups showed differences to the C group, while the MA + B group showed differences to the B group ($p < 0.001$; Table 3). At the final assessment, it was discovered that the levels of beta-carotene were significantly elevated in patients with moderate acute pancreatitis compared to those with severe acute pancreatitis [59]. The association between decreased antioxidant levels and increased disease severity indicates the effectiveness of antioxidant supplementation therapy [60].

3.4. Morphoquantitative Analysis of the Pancreas

Pancreatitis has multiple causes, with alcohol and gallstones being the most prevalent etiologies. The exact underlying mechanisms of this disease are not fully understood; nonetheless, it is probable that it originates from the effects of alcohol on the small pancreatic ducts and acinar cells. Alcohol is believed to cause the formation of protein plugs in

the narrow channels of the pancreas by increasing the thickness of pancreatic secretions. The plugs subsequently harden into calculi, resulting in progressive inflammation and fibrosis [4]. Acinar, islet, and ductal cells are lost in the end because of this process [4,61]. Based on our findings, it can be deduced that the ingestion of β-carotene orally counteracts the consequences of prolonged alcohol intake in the pancreatic ducts and acinar cells (Table 4). This accounts for the variations observed in the stereological examination of $N_{V\ islets}$, $V_{V\ islets}$, and $TM_{\ islets}$ ($p < 0.001$, $p < 0.003$ and $p < 0.001$; respectively), but not in $N_{V\ acinar\ cells}$, $V_{V\ acinar\ cells}$, or $S_{V\ acinar\ cells}$ ($p = 0.994$, $p = 0.868$ and $p = 0.987$, respectively) between the MA + B and C groups. In fact, several in vivo and in vitro investigations have demonstrated that antioxidants can effectively suppress pancreatic fibrosis [62–64], where glutathione is a prominent antioxidant found within cells, which serves a crucial function in mitigating the impact of oxidative stress [65].

3.5. Scope and Limitations

The purpose of this study was to ascertain what happened to the biochemical parameters and pancreatic histology of *C57BL/6* mice that received oral beta-carotene after ethanol consumption. Our data provide new evidence of the connection between alcohol-induced pancreatitis and antioxidant therapies, such as β-carotene. Still, this study has some limitations: 1. It does not look at any connections between alcohol metabolism byproducts that are not oxidative, specifically fatty acid ethyl esters, or antioxidant treatments; 2. It does not look at any autophagy- or inflammation-related endpoints; 3. It needs more experiments to look at the substances that hurt pancreatic acinar cells; and 4. It needs collagen immunocytochemistry to confirm the short-term damage caused by heavy alcohol use. These aspects should be addressed in future investigations. However, our data suggest that exposure to β-carotene decreased pancreatic damage during moderate alcohol exposure in *C57BL/6* mice.

4. Materials and Methods

4.1. Animals

Thirty-six male *C57BL/6* mice (*Mus musculus*) aged fifty days were obtained from the Public Health Institute of Chile. In order to help them adjust to their new environment, they were housed for 30 days in the animal facility of the Center of Excellence in Morphological and Surgical Studies (CEMyQ) at the Universidad de La Frontera. Standard laboratory food (AIN-93M) [66] and water were provided, and they were kept in a 12 h light/dark cycle (08:00–20:00/20:00–08:00). All of the mice were given a normal laboratory diet (AIN-93M) during the experimentation period in accordance with the guidelines set forth by the Institute for Laboratory Animal Research's Committee for the Update of the Guide for the Care and Use of Laboratory Animals [67].

On the first day of the experiment (Day 1), the mice were divided into six groups: 1. Group C (control); 2. Group LA (low-dose alcohol): 3% v/v ad libitum alcohol administration for 28 days [68]; 3. Group MA (moderate-dose alcohol): 7% v/v ad libitum alcohol administration for 28 days [68]; 4. Group B (β-carotene): 0.52 mg/kg body weight/day β-carotene administration for 28 days [69]; 5. Group LA + B (Low-dose alcohol + β-carotene): low-dose alcohol plus administration of 0.52 mg/kg body weight/day of β-carotene for 28 days; and 6. Group MA + B (moderate-dose alcohol + β-carotene): moderate-dose alcohol plus administration of 0.52 mg/kg body weight/day β-carotene for 28 days. Body mass was measured at the beginning, during the whole experimental phase, and at the end of the experimental phase. The results are shown in our previous study [49].

The modified liquid diet of Lieber–DeCarli was followed for the administration of oral chronic ethanol in drinking water [68,70]. An oral dose of 0.52 mg/kg body weight/day of β-carotene was given [69]. The experimental model has been used and tested previously [23,54].

4.2. Euthanasia

At the end of the experiment on Day 28, the animals were fasted for 6 h and euthanized with sodium pentobarbital.

4.3. Biochemistry

For the serum analyses, the serum was isolated using centrifugation at a speed of 2058× *g* for a duration of 15 min. The isolated serum was then stored at a temperature of −80 °C until it was ready for analysis. The lipase and amylase enzymes (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA) were used to look at the pancreatic physiology. Furthermore, the quantification of uric acid concentration was performed using a colorimetric kit from Life Technologies, Thermo Fisher Scientific Inc., located in Waltham, MA, USA.

4.4. Processing and Staining of Pancreas

Considering the isotropic properties of the tissue, many sections of each pancreas were obtained to obtain representative characteristics for them. When they were dehydrated, they were embedded in Paraplast Plus (Sigma-Aldrich Co., Ltd., St. Louis, MO, USA). This was done after they had been fixed for 48 h at 4% in buffered formalin (1.27 mol·L^{-1} of formaldehyde in 0.1 M phosphate buffer, pH 7.2). After obtaining the blocks, a microtome (Leica® RM2255, Leica®, Wetzlar, Germany) was used to make cuts that were 5 µm thick. Each block was then divided into five pieces, each of which was stained with hematoxylin and eosin (H&E) for histological analysis.

4.5. Histological Evaluation

Histologic grading was conducted by researchers who had received specialized training using Ammann's fibrosis score [51]. The researcher examined the slides in isolation, without any awareness of the research groups. Fibrosis in the peri-lobular and intralobular parts of the parenchyma was scored using Ammann's Fibrosis System, which runs from 1 to 6. These two elements add up to the final score. The evaluated parameters are shown in Table 5.

Table 5. The scoring system for histological changes according to Ammann's fibrosis punctuation.

Score	Fibrosis Grade	Peri-Lobular Parenchyma	Intralobular Parenchyma
1	Mild	Lobules are separated by fibrous tissue without any changes in structure or atrophy.	Thin fibrous threads that separate the acini within the lobules, but without any substantial changes to the overall structure.
2	Moderate	Lobules are separated by fibrous tissue with changes in structure or atrophy (between 0 and 20%).	Fibrous threads that separate the acini within the lobules, with substantial changes to the overall structure (between 0 and 20%).
3	Marked	Lobules are separated by fibrous tissue with changes in structure or atrophy (between 20 and 40%).	Fibrous threads that separate the acini within the lobules, with substantial changes to the overall structure (between 20 and 40%).
4	Mild	Lobules are separated by fibrous tissue with changes in structure or atrophy (between 40 and 60%).	Fibrous threads that separate the acini within the lobules, with substantial changes to the overall structure (between 40 and 60%).
5	Moderate	Lobules are separated by fibrous tissue with changes in structure or atrophy (between 60 and 80%).	Fibrous threads that separate the acini within the lobules, with substantial changes to the overall structure (between 60 and 80%).
6	Marked	Lobules are separated by fibrous tissue with changes in structure or atrophy (between 80 and 100%).	Fibrous threads that separate the acini within the lobules, with substantial changes to the overall structure (between 80 and 100%).

4.6. Morphoquantitative Analysis of the Pancreas

Five animals from each group were used for the stereological investigation. Using an analytical balance and Scherle's approach, the mass and volume of the pancreas were ascertained [71]. We took five different pieces from each pancreas, dried them, and then embedded them in Paraplast Plus (Sigma-Aldrich Co., Ltd., St. Louis, MO, USA). This was done after 48 h of treatment with 4% buffered formalin (1.27 mol/L of formaldehyde in phosphate buffer 0.1 M, pH 7.2). After obtaining the blocks, 5 μm thick slices were created and stained with H&E.

Ten fields were observed for each region in the stereological research, for a total of 50 fields per group [72,73]. A stereological microscope (Leica® DM2000 LED, Leica®, Wetzlar, Germany) was used to view the slides, and a digital camera (Leica® MC170 HD, Leica®, Wetzlar, Germany) was used to take pictures. Using the STEPanizer® 36-point test method (STEPanizer®, Version 1, Software Eng., Institute of Anatomy, University of Bern, Switzerland), it was possible to determine the pancreatic islets' number density ($N_{V\ islets}$), volume density ($V_{V\ islets}$), and surface density ($S_{V\ islets}$). Furthermore, the pancreatic islets' total mass ($TM_{\ islets}$) was ascertained. The $N_{V\ islets}$ were measured using the following formula: $N_{V\ islets}$ are equal to Q-/($A_T \times t$), where A_T is the entire area of the test system and dissector thickness (*t*) and Q- is the number of observations in a certain area considering the banned lines and the prohibited plane. The formula used to estimate $V_{V\ islets}$ was $V_{V\ islets} = P_{P\ islets}/P_T$ (100%), where P_T is the total number of points in the system and $P_{P\ islets}$ is the number of points that touch the pancreatic islets. The $S_{V\ islets}$ were calculated using the following equation: $S_{V\ islets}$ are equal to $(2 \times I)/L_T$, where L_T is the total length of the lines in the 36-point test system, and I is the number of intersections that touch the structure. The estimation of N_V, V_V, and S_V acinar cells was done in accordance with earlier instructions. By multiplying $V_{V\ islets}$ by the mass of the pancreas, $TM_{\ islets}$ was calculated.

4.7. Statistical Analysis

Levene's test (homoscedasticity of the variances) and the Kolmogorov–Smirnov test (analysis of data normalcy) were used to assess differences in the quantitative data. A one-way ANOVA was used to assess the group differences, and, if necessary, the Dunnett's T3 test or Tukey's post-hoc HSD test was then performed. According to IBM Corp., Armonk, NY, USA, $p < 0.05$ was deemed statistically significant (IBM SPSS Statistics, Version 21, Endicott, NY, USA).

5. Conclusions

In our study, biochemical parameters changed significantly in the MA + B group compared to the C, MA, B, and LA + B groups. Specifically, serum total amylase levels went up and lipase levels went down. In addition, the histological study showed that peri-lobular parenchyma, intralobular parenchyma, and fibrosis score were less punctuated in the MA + B group than in the LA, MA, and LA + B groups. Examination of the islets and acinar cells of the pancreas stereologically after the drinking of moderate amounts of alcohol and β-carotene also showed positive results. These findings suggest that antioxidant therapies may be beneficial in treating ethanol exposure in animal models. Although our understanding of the mechanisms behind antioxidant supplements is extensive, further investigation is required to explore the relationship between alcohol consumption and antioxidant therapies. This entails conducting research using specific cell lines and clinical trials to delve into the signaling pathways as well as the enzyme and non-enzyme mechanisms involved.

Author Contributions: C.S., A.V., K.B., K.G., F.C., J.C. and J.F. carried out the conception and design of the research. C.S., K.G. and J.C. participated in the experimental phase, and they did the biochemical and histological analysis. C.S., A.V. and F.C. did the morpho-quantitative analysis from pancreas tissue. C.S., A.V., K.B., K.G., F.C., J.C. and J.F. wrote the original draft preparation. C.S., A.V., K.B., K.G., F.C., J.C. and J.F. conducted the review and editing. C.S. and J.F. did the supervision and project

administration. C.S. and J.F. participated in obtaining funding. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Universidad de La Frontera, DI22–0007 Project, and Universidad de La Frontera, PDT22-0001 Project.

Institutional Review Board Statement: The animal study protocol was approved by the Scientific Ethics Committee of the Universidad de La Frontera (Nº051/2020).

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are publicly available. This data can be found here: https://doi.org/10.6084/m9.figshare.24988047.v1.

Acknowledgments: The authors thank SmartC-BIOREN (Service Management Analytical Research and Training Center), CCSS210005 Project, Agencia Nacional de Investigación y Desarrollo de Chile (ANID).

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

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International Journal of
Molecular Sciences

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Review

Recent Advances on the Role of B Vitamins in Cancer Prevention and Progression

Zachary Frost, Sandra Bakhit, Chelsea N. Amaefuna, Ryan V. Powers and Kota V. Ramana *

Department of Biomedical Sciences, Noorda College of Osteopathic Medicine, Provo, UT 84606, USA
* Correspondence: karamana@noordacom.org

Abstract: Water-soluble B vitamins, mainly obtained through dietary intake of fruits, vegetables, grains, and dairy products, act as co-factors in various biochemical processes, including DNA synthesis, repair, methylation, and energy metabolism. These vitamins include B1 (Thiamine), B2 (Riboflavin), B3 (Niacin), B5 (Pantothenic Acid), B6 (Pyridoxine), B7 (Biotin), B9 (Folate), and B12 (Cobalamin). Recent studies have shown that besides their fundamental physiological roles, B vitamins influence oncogenic metabolic pathways, including glycolysis (Warburg effect), mitochondrial function, and nucleotide biosynthesis. Although deficiencies in these vitamins are associated with several complications, emerging evidence suggests that excessive intake of specific B vitamins may also contribute to cancer progression and interfere with therapy due to impaired metabolic and genetic functions. This review discusses the tumor-suppressive and tumor-progressive roles of B vitamins in cancer. It also explores the recent evidence on a comprehensive understanding of the relationship between B vitamin metabolism and cancer progression and underscores the need for further research to determine the optimal balance of B vitamin intake for cancer prevention and therapy.

Keywords: B vitamins; cancer; antioxidants; metabolism; nutrients; Warberg effect

1. Introduction

B vitamins are a group of eight water-soluble nutrients essential in maintaining various metabolic processes in the body and are necessary for maintaining human health [1]. The B vitamins include B1 (Thiamine), B2 (Riboflavin), B3 (Niacin), B5 (Pantothenic Acid), B6 (Pyridoxine), B7 (Biotin), B9 (Folate), and B12 (Cobalamin). Each type of B vitamin participates in a specific biochemical reaction as a co-factor and helps with DNA synthesis, repair, methylation, and energy metabolism (Table 1). Since B vitamins play crucial roles in cellular functions and biochemical pathways, it's no surprise that B vitamins are essential for human health and disease [1–4]. The deficiency of these vitamins could lead to multiple complications, including neurological disturbances, anemia, and skin diseases [3,4].

Further, certain drugs and diseases could cause a deficiency of these vitamins and increase complications. For example, prolonged metformin use has been shown to cause thiamine and cobalamin deficiency [5–9]. Similarly, corticosteroids could cause pyridoxine, folate, and cobalamin deficiency [10–12]. Moreover, several drugs, such as proton pump inhibitors [13,14], anticonvulsants [15,16], antibiotics [17], and chemotherapeutic drugs [18–20], have been shown to cause deficiency of specific B vitamins. B vitamins also play a role in cancer development and progression. In certain cancers, the deficiency of B vitamins was also observed.

Table 1. Role of B vitamins in various metabolic and cancer-related pathways.

Vitamin	Key Role	Type of Co-Factor Formed	Key Step in Metabolism	Role in Cancer-Related Pathways
B1 (Thiamine)	Decarboxylation of α-keto acids, transketolase reactions	Thiamine pyrophosphate (TPP)	Pyruvate dehydrogenase complex: Pyruvate → Acetyl-CoA	Thiamine deficiency leads to impaired mitochondrial function and altered glucose metabolism which could promote cancer cell survival.
B2 (Riboflavin)	Redox reactions (electron transfer)	Flavin adenine dinucleotide (FAD), Flavin mononucleotide (FMN)	TCA cycle: Succinate dehydrogenase at step Succinate → Fumarate	Riboflavin deficiency could enhance oxidative DNA damage and increase cancer risk.
B3 (Niacin)	Redox reactions (electron transfer)	Nicotinamide adenine dinucleotide (NAD), Nicotinamide adenine dinucleotide phosphate (NADP)	Glycolysis: Glyceraldehyde-3-phosphate dehydrogenase step (Glyceraldehyde-3-phosphate → 1,3-Bisphosphoglycerate)	Niacin deficiency could impair DNA repair and increase mutation rates.
B5 (Pantothenic Acid)	Acyl group transfer	Coenzyme A (CoA)	Fatty acid synthesis: Formation of Malonyl-CoA from Acetyl-CoA	Altered CoA in cancer cells promote proliferation and membrane biosynthesis.
B6 (Pyridoxine)	Amino acid metabolism (transamination, decarboxylation)	Pyridoxal phosphate (PLP)	Transamination: Conversion of Aspartate to Oxaloacetate	PLP deficiency can increase homocysteine levels and impair DNA synthesis, which contribute to tumorigenesis.
B7 (Biotin)	Carboxylation reactions (gluconeogenesis, fatty acid synthesis)	Biotin-enzymes complex	Gluconeogenesis: Pyruvate carboxylase reaction (Pyruvate → Oxaloacetate)	Biotin is involved in histone modification which affects gene regulation and cancer cell growth.
B9 (Folate)	One-carbon metabolism (DNA synthesis)	Tetrahydrofolate (THF)	DNA synthesis: Methylation of deoxyuridylate to form thymidylate (dUMP → dTMP)	Folate deficiency can cause DNA strand breaks and an aberrant gene expression which increase cancer risk.
B12 (Cobalamin)	Methylation and rearrangement reactions	Methylcobalamin, 5′-deoxyadenosylcobalamin	Methionine synthesis: Conversion of homocysteine to methionine	B12 deficiency can lead to genome instability and increase cancer susceptibility.

The involvement of B vitamins in cancer is a complicated process. Generally, B vitamins play an important role in cancer prevention and progression by altering cellular metabolism and DNA synthesis, repair, and methylation [2,3]. For example, adequate folate, cobalamin, and pyridoxine levels support genomic stability by controlling the DNA strand breaks, mutations, and genomic instability. In addition, niacin contributes to NAD+ produc-

tion, which is essential for DNA repair and energy balance. Although sufficient B vitamin intake could reduce the cancer progression and risk, excessive supplementation, specifically with vitamins B9, B12, and B6, has been associated with accelerated tumor growth in certain cancers, such as colorectal, prostate, and lung cancer. Further, B vitamins could also interact with oncogenic regulators such as HIF-1α, MYC, and AMPK, which influence cancer cell metabolism. Since B vitamins play a key role in normal cellular function, understanding their role in cancer is highly complex and requires a personalized approach to intake and supplementation, especially in cancer patients.

Further, recent studies also suggest that vitamin deficiencies and excessive intake of vitamins could be associated with cancer development and therapeutic resistance [21–24]. The deficiencies seen in B vitamins could disrupt processes such as DNA repair and immune regulation that cause genomic instability and an increased susceptibility to cancer. On the other hand, excessive supplementation of B vitamins has been associated with the risk of cancer progression [23,24]. This suggests that maintaining a balanced intake of vitamins and knowing the vitamin deficiencies are very important for maintaining a healthy lifestyle.

This review discusses the importance of water-soluble B vitamins in cancer prevention and progression. Further, we aimed to provide a comprehensive understanding of how these essential nutrients can influence cancer outcomes. We searched PubMed and Google Scholar to find articles published in the last 10 years or so, using keywords including various types of B vitamins, cancer, melanoma, leukemia, breast cancer, lung cancer, colon cancer, and other cancer types. We included various research articles, comprehensive narrative reviews, meta-analytical studies, systematic reviews, clinical studies, and pre-clinical studies to discuss their findings. We did not include studies on other fat-soluble and water-soluble vitamins, such as A and C. This narrative review article discusses only the role of B vitamins and their therapeutic significance in various cancers.

2. Vitamin B1: Thiamine

Vitamin B1 (thiamine) is a water-soluble vitamin in plant and animal-derived foods, such as meats, eggs, and dairy [25]. Thiamine maintains cellular viability by participating in critical metabolic pathways like glycolysis, the pentose phosphate pathway, and Kreb's cycle. These pathways are essential for producing ATP and NADPH to sustain metabolic functions in the body [26]. Thiamine is particularly significant for aerobic metabolism, as it acts as a primary co-factor for pyruvate dehydrogenase in its active form, thiamine pyrophosphate (TPP). TPP also serves as a co-factor for alpha-keto dehydrogenase and transketolase, which convert ribulose-5-phosphate into RNA and DNA substrates and facilitate the conversion of amino acids and fatty acids into acetyl-CoA. Thus, thiamine directly contributes to ribose synthesis, a necessary substrate for cell proliferation, and provides metabolites for gluconeogenesis and NADPH synthesis [27,28].

The impact of thiamine deficiency can be profound, leading to severe conditions such as Beri-Beri disease and Wernicke-Korsakoff syndrome [29,30]. In cancer patients, thiamine deficiency has been strongly linked to the manifestation of delirium, highlighting its critical role in maintaining nervous system health [31]. Thiamine deficiency can also result in widespread neurological, cardiac, and gastrointestinal dysfunctions, which raises important questions about its role in cancer cell proliferation and progression [32–35].

A metabolic shift known as the Warburg effect (Figure 1) is often observed in the initial stages of cancer cell proliferation. This phenomenon, which has become a focus of alternative chemotherapeutic strategies, describes how cancer cells rely on glycolysis and fermentation of lactic acid for energy production, even in the oxygen presence [36,37]. This preference for glycolysis provides tumor cells with the metabolic intermediates necessary

for rapid cell growth and survival. The role of TPP in the Warburg effect is shown in Figure 1. In addition, the individual roles of B vitamins on the Warburg effect are shown in Table 2. The overexpression of pyruvate dehydrogenase kinase (PDK) in cancer cells inactivates pyruvate dehydrogenase (PDH), effectively disconnecting glycolysis from the Krebs cycle and enhancing glycolytic activity [38]. The inhibition of PDK by TPP has shown promise in triggering apoptosis and reducing tumor cell proliferation by restoring PDH activity [39–41]. Through its role in maintaining PDH complex activity, thiamine has been shown to decrease tumor cell proliferation and glycolytic activity by countering PDK-induced PDH inactivation [42].

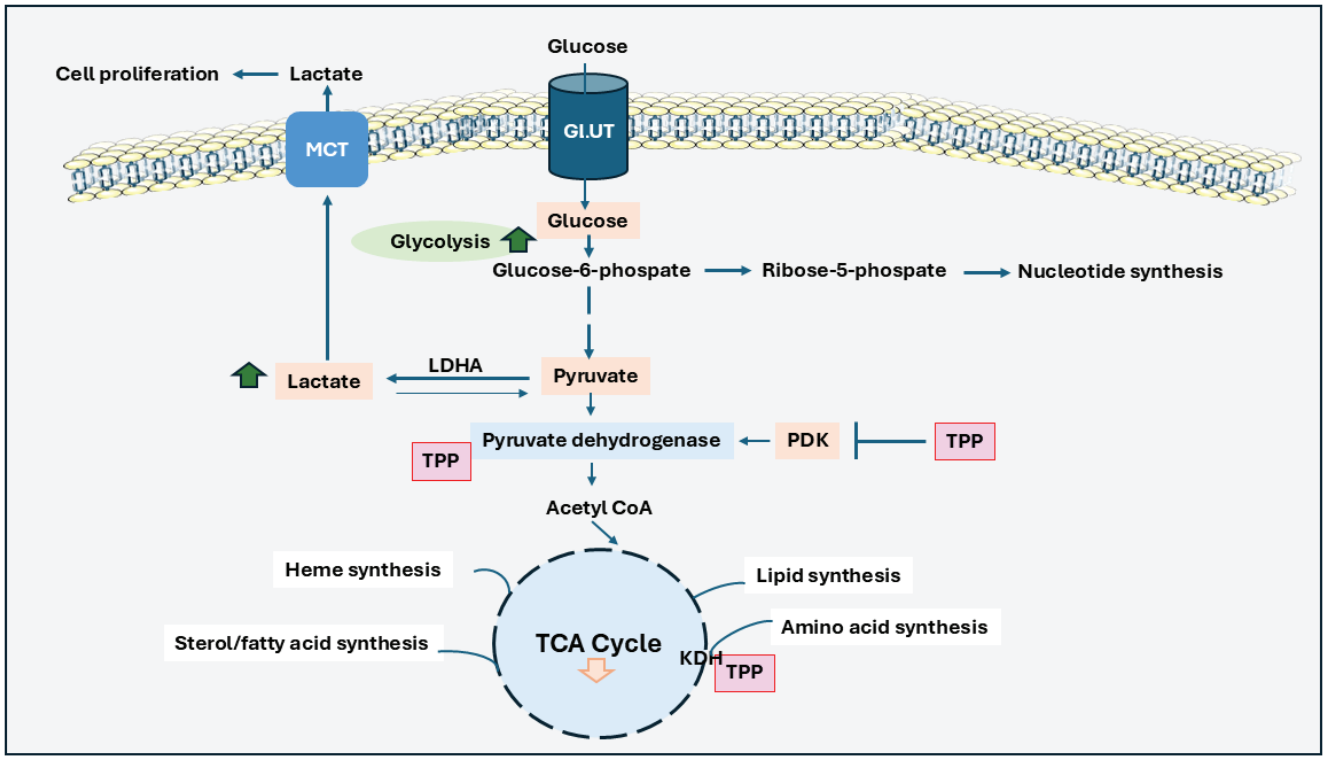

Figure 1. The role of thiamine in Warburg effect and its impact on cancer cell metabolism. Unlike normal cells, cancer cells heavily depend on glycolysis for energy production, even during aerobic conditions, and produce lactic acid instead of relying on oxidative phosphorylation. Glucose is taken up by the cell via GLUT transporters and converted through glycolysis into pyruvate. Instead of entering the TCA cycle within the mitochondria, pyruvate is converted to lactate in the cytoplasm by lactate dehydrogenase A (LDHA). Lactate is transported by monocarboxylate Transporters (MCT) and causes cancer cell proliferation. Further, the pyruvate dehydrogenase complex is inhibited by pyruvate dehydrogenase kinase (PDK), limiting pyruvate's conversion to acetyl-CoA and reducing the flow into the TCA cycle. Thymine pyrophosphate (TPP) plays a critical role in restoring PDH activity and counteracting the inhibitory effects of PDK. This pathway also integrates alternative pathways, such as the pentose-phosphate pathway, hexosamine biosynthesis, and lipid synthesis, further supporting cancer cell growth and survival. The activity of the TCA cycle is generally reduced, which limits energy production from mitochondria. This metabolic adaptation provides rapid energy and intermediates needed for biosynthesis. As a result, cancer cells can grow and divide rapidly, even in challenging conditions like low oxygen levels.

Table 2. Role of B vitamins in Warburg effect. Vitamins B6, B9, and B12 are not directly involved in the Warburg effect. They are directly involved in amino acid metabolism, one-carbon metabolism, and nucleic acid synthesis.

B Vitamin	Key Enzymes & Intermediates	Impact on the Warburg Effect	Interaction with Oncogenic Metabolic Regulators
B1	Pyruvate dehydrogenase (PDH) Transketolase (TKT)	Deficiency: Inhibits PDH, increase pyruvate-to-lactate conversion, reinforce glycolysis. Excess: Enhances PDH activity, promoting oxidative phosphorylation (OXPHOS) and reducing glycolysis.	HIF-1α: Upregulates PDK, which inhibits PDH, favor glycolysis over oxidative phosphorylation. MYC: Activates TKT, promotes pentose phosphate pathway, increases nucleotide synthesis
B2	Succinate dehydrogenase (SDH, ETC Complex II) NADH dehydrogenase (ETC Complex I)	Deficiency: Impairs ETC function, increase glycolysis. Excess: Supports mitochondrial function, promote oxidative phosphorylation.	AMPK: riboflavin deficiency leads to AMPK activation and mitochondrial dysfunction, promotes glycolysis.
B3	NAD+/NADH balance Sirtuins (SIRT1, SIRT3) PARPs	Deficiency: Reduces NAD+/NADH ratio, impairs TCA cycle, promotes glycolysis. Excess: Increases NAD+, enhances mitochondrial function, reverses the Warburg effect.	HIF-1α: NAD+ depletion stabilizes HIF-1α, enhancing glycolysis. SIRT1: Inhibits HIF-1α and promotes oxidative phosphorylation. PARP1: Initiates DNA repair mechanisms, maintains genomic stability.
B5	Acetyl-CoA synthase Fatty acid synthase (FASN)	Deficiency: Reduces acetyl-CoA availability, increases glycolysis dependence. Excess: Enhances lipid metabolism, supports tumor growth	MYC: Increases FASN expression, promotes lipid metabolism for cell proliferation. AMPK: Low acetyl-CoA triggers AMPK activation, promotes glycolysis.
B6	Glutaminase (GLS) Serine hydroxy-methyltransferase (SHMT)	Deficiency: Impairs glutamine metabolism in TCA cycle, reinforces glycolysis. Excess: Enhances one-carbon metabolism, supports oxidative phosphorylation.	MYC: Upregulates GLS, increases glutamine metabolism, promotes cell proliferation.
B7	Pyruvate carboxylase (PC)	Deficiency: Reduces oxaloacetate, impairs TCA cycles, promotes glycolysis. Excess: Promotes mitochondrial metabolism and TCA cycle.	HIF-1α: Suppress PC expression, diverts pyruvate to lactate.
B9	Thymidylate synthase (TS)— Methylenetetrahydrofolate reductase (MTHFR)	Deficiency: Impairs nucleotide synthesis, causes DNA damage and metabolic stress. Excess: Promotes nucleotide biosynthesis, increases tumor growth.	MYC: Enhances folate metabolism, increases cell proliferation. HIF-1α: Induces nucleotide synthesis genes, increases folate demand.
B12	Methionine synthase (MS)	Deficiency: Disrupts methylation, alters metabolic enzyme expression. Excess: Supports epigenetic regulation, promotes tumor survival.	MYC: Upregulates methionine cycle enzymes, promotes epigenetic modifications and oncogene expressions.

Further evidence of thiamine's role in cancer metabolism comes from studies involving dichloroacetate, an analog of acetic acid known for its potential to increase aerobic metabolism. Although typically avoided due to its toxic side effects, dichloroacetate showed the action of reducing colorectal cancer cell growth by enhancing oxidative phosphorylation [43,44]. Thiamine's involvement in cancer is also evident in studies where cancer cells maintained a base level of TPP despite thiamine-depleting vitamin stores in tissues. When keeping TPP levels low, cancer cells could produce the essential intermediates for growth. However, increasing TPP concentrations shifted metabolism towards PDH activation and mitochondrial respiration, stunting cancer cell proliferation [45,46]. Additionally, thiamine analog, oxythiamine has been shown to prevent cancer cell growth [47,48]. Further, it has been shown to reduce DNA and RNA synthesis by inhibiting ribose-5-phosphate and thereby reducing tumor cell growth [49]. Thiamine at high doses has been shown to prevent the growth of MCF-7 breast cancer cells [50]. In these cells, thiamine has also been shown to increase PDH activity and reduce glycolysis in the cancer cells. The expression of thiamine transporter SLC19A3 has been shown to be significantly expressed in chronic hypoxia-induced breast cancer cell lines (BT474) [51]. This increase in the transporter expression is correlated with the increased uptake of thiamine by the breast cancer cells. However, Liu et al. [52] have shown that SLC19A3 mRNA levels were downregulated in human breast cancer tumor tissues compared to normal tissues. They have suggested that decreased expression of this transporter could cause breast cancer cells resistance to apoptosis. Similarly, hypoxia has been shown to inhibit the uptake of microbiota-generated thiamine and TPP by the NCM460 human colon epithelial cells by reducing the expression of transporters such as SLC44A4, SLC19A2, and SLC19A3 [53]. Further, benfotiamine, a lipid-soluble derivative of thiamine, has been shown to control the proliferation of cancer cells and growth in a nude mice xenograft model [54].

Thiamine deficiency is most commonly seen in patients with diabetes [55,56], and diabetic patients are at risk of many complications. Further, some cancer patients have also been shown to have a deficiency of thiamine [57–59]. Therefore, diabetic patients with cancer have a significant risk of developing thiamine deficiency and energy metabolism, and supplementation of thiamine in such patients could prevent the cancer progression. Further, thiamine has been shown to regulate the expression of PKC, NF-κB, and advanced glycation end products (AGEs) in diabetics, and these signaling pathways are also critical in cancer progression [60,61]. Thiamine has been shown to prevent the activation of NF-κB and matrix metalloproteinases (MMPs) [62]. MMP activation is associated with tumor invasion and metastasis by modulating extracellular matrix remodeling [63,64]. Similarly, thiamine could also prevent the expression of prostaglandins and activation of COX-2 [65], which are involved in tumor growth. Similarly, thiamine has been shown to affect tumor growth by inhibiting the generation of reactive oxygen species (ROS) and reactive nitrogen species [66].

Thus, recent studies suggest that the impact of thiamine on cancer progression remains complex and variable (Figure 2). For instance, increased intake of thiamine has been linked with a significant risk of bladder cancer in men, whereas in women, it appears to have a protective effect [67]. This study also suggests that gender differences are influenced by dietary habits, where men typically consume more meat products leading to a risk of developing bladder cancer. On the other hand, women consuming more dairy products have a weaker association with bladder cancer. However, further research is needed to understand the gender-associated thiamine's role in cancer. Thus, depending on the type of cancer and metabolic state, thiamine can have both tumor-promoting and tumor-suppressive effects. For example, thiamine supports tumor growth by enhancing glycolysis, the pentose phosphate pathway, and mitochondrial function and providing ATP, NADPH,

and biosynthetic precursors essential for rapid tumor cell proliferation. However, in some conditions, thiamine deficiency could cause metabolic stress and oxidative DNA damage, potentially suppressing tumor growth. Although adequate dietary thiamine is necessary for normal function, excessive supplementation could promote tumor progression in susceptible cancers, and personalized approaches are needed when using thiamine in cancer therapy.

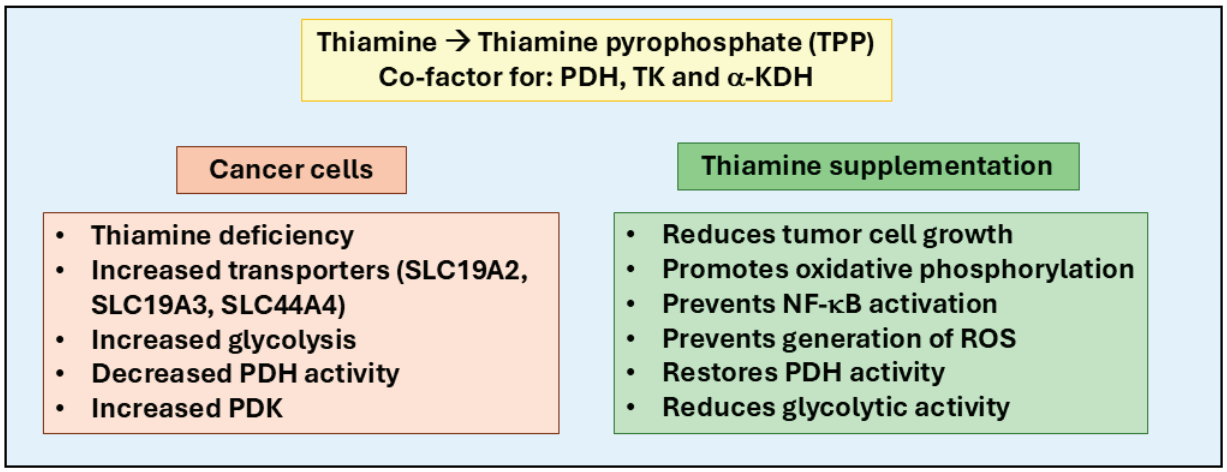

Figure 2. Role of thiamine in cancer progression and therapy. Thiamine (Vitamin B1) plays a crucial role in cellular metabolism by converting into thiamine pyrophosphate (TPP), a co-factor for enzymes like pyruvate dehydrogenase (PDH), transketolase (TK), and alpha-keto dehydrogenase (α-KDH). These enzymes regulate glycolysis, the pentose phosphate pathway, and the Krebs cycle, essential for ATP and NADPH production, DNA/RNA synthesis, and oxidative stress reduction. In cancer cells, thiamine influences the Warburg effect, where cancer cells rely on glycolysis even in oxygen-rich environments, often due to pyruvate dehydrogenase kinase (PDK) overexpression, which inhibits oxidative phosphorylation. Thiamine supplementation counters this by restoring PDH activity, reducing glycolytic dependence, and promoting oxidative metabolism. Cancer cells modulate thiamine uptake via transporters like SLC19A2, SLC19A3, and SLC44A4. Therapeutically, supplementation with thiamine and its derivatives (such as benfotiamine and oxythiamine) could inhibit tumor growth, reduce oxidative stress, prevent NF-κB-mediated inflammatory signaling, reduce glycolytic activity, and promote oxidative phosphorylation.

3. Vitamin B2: Riboflavin

Vitamin B2, or riboflavin, is primarily obtained from food sources like thiamine, with milk and dairy products among the most significant contributors [68,69]. Riboflavin is a coenzyme in various oxidation and reduction reactions in the body, mediating electron transfer and maintaining the integrity of several tissues, particularly neural tissues [70]. Like thiamine, riboflavin plays a critical role in cellular function by helping to keep glutathione in its reduced form, neutralizing reactive oxidative species, and protecting the body from free radical damage. Riboflavin also metabolizes other essential vitamins, including folate, vitamin B6, and B12 [71,72].

Riboflavin deficiency, known as ariboflavinosis, can lead to a marked increase in oxidative stress, with further adverse effects such as migraines, anemia, diabetes mellitus, hyperglycemia, and hypertension [70–72]. In severe cases, deficiency can result in growth retardation, anemia, renal damage, and degenerative effects on the nervous system [73]. While the extent of riboflavin's influence on various cancer types varies, it is well-established that riboflavin is a crucial precursor to the coenzymes FAD and FMN, which regulate redox reactions in the TCA cycle and control levels of reactive oxygen species (ROS) in the body [70]. Riboflavin has been shown to regulate cellular ROS levels and increase the therapeutic efficacy of anti-cancer drugs [74–76]. A few studies also

suggest that moderate riboflavin levels could trigger the extrinsic pathway of apoptosis and autophagy [77–79]. At the same time, higher doses could also activate specific apoptotic mechanisms by downregulating anti-apoptotic factors and promoting pro-apoptotic factors [80,81].

Further, the anti-inflammatory properties of riboflavin also help in cancer therapy. Notably, when combined with the chemotherapeutic drug cisplatin, riboflavin has been shown to mitigate cisplatin's toxic effects by reducing the inflammatory response and replenishing antioxidant enzymes and cellular reductants [82,83]. Regulation of FAD/FMN levels, control of ROS production, and induction of apoptosis are major pathways where riboflavin is associated with increased cancer risk (Figure 3). Although some processes have been established, further research is needed to understand riboflavin's dose-dependent and cancer-specific effects. However, few studies have indicated a correlation between riboflavin intake and cancer development [84–86]. Like thiamine, overconsumption and underconsumption of riboflavin can alter cancer risk, with the impact varying depending on the cancer type and affected region [86–89]. Riboflavin has been shown to prevent the growth of MCF-7 and MDA-MB-231 breast cancer cells, but not normal cells (L929) growth under the influence of visible light [90]. Similarly, Sturm et al. [91] have shown that riboflavin in combination with gemcitabine decreases the growth of bladder cancer cells growth in the presence of blue light at a wavelength of 453 nm. Another study by Chiu et al. [92] has shown that blue light and violet light illumination of riboflavin prevents the growth of colon cancer cells. In addition, radiated riboflavin prevents growth and increases apoptosis of C6 glioblastoma cells when compared to non-radiated riboflavin. Further, a few studies also indicate the role of riboflavin in prostate cancer. A recent study by Lv et al. [93] has shown a relationship between riboflavin intake and prostate-specific antigen (PSA) levels detected in American men. They have shown that there is an inverse relationship between riboflavin intake and PSA detection levels. Another study by Zhao et al. [94] has shown that riboflavin, by inhibiting the TGF-β signaling, could prevent pancreatic cancer metastasis. These studies suggest that a higher riboflavin intake may suggest a greater risk of diagnosing patients with advanced prostate cancer. Similarly, Gunathilake et al. [95], in a Korean population-based study, indicates that a higher riboflavin intake lowers the risk of developing colorectal cancer. Specifically, they have shown that males homozygous for the major alleles of MTRR rs1801394 and MTR rs1805087 polymorphisms and who had a higher intake of vitamin B2 had a significantly lower colorectal cancer risk. In a Chinese health study, Paragomy et al. [96] have shown that the increased riboflavin levels in the serum are correlated with the increased risk of pancreatic cancer. Similarly, Ma et al. [97] have also shown that increased riboflavin levels in the serum are associated with increased colorectal cancer.

As a precursor to FAD and FMN, riboflavin is linked to folate metabolism. Consequently, low riboflavin intake can exacerbate the effects of low dietary folate, which is necessary for DNA methylation and purines and pyrimidine synthesis. These factors are needed for cell growth and repair mechanisms [98,99]. Further, FAD also serves as a co-factor for the folate-metabolizing enzyme methylenetetrahydrofolate reductase (MTHFR). It catalyzes the one-carbon metabolism of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, eventually converting homocysteine to methionine [98]. Few studies have also shown that riboflavin deficiency is associated with reduced MTHFR activity and elevated homocysteine levels, which could also serve as a risk factor for cancer development [100,101].

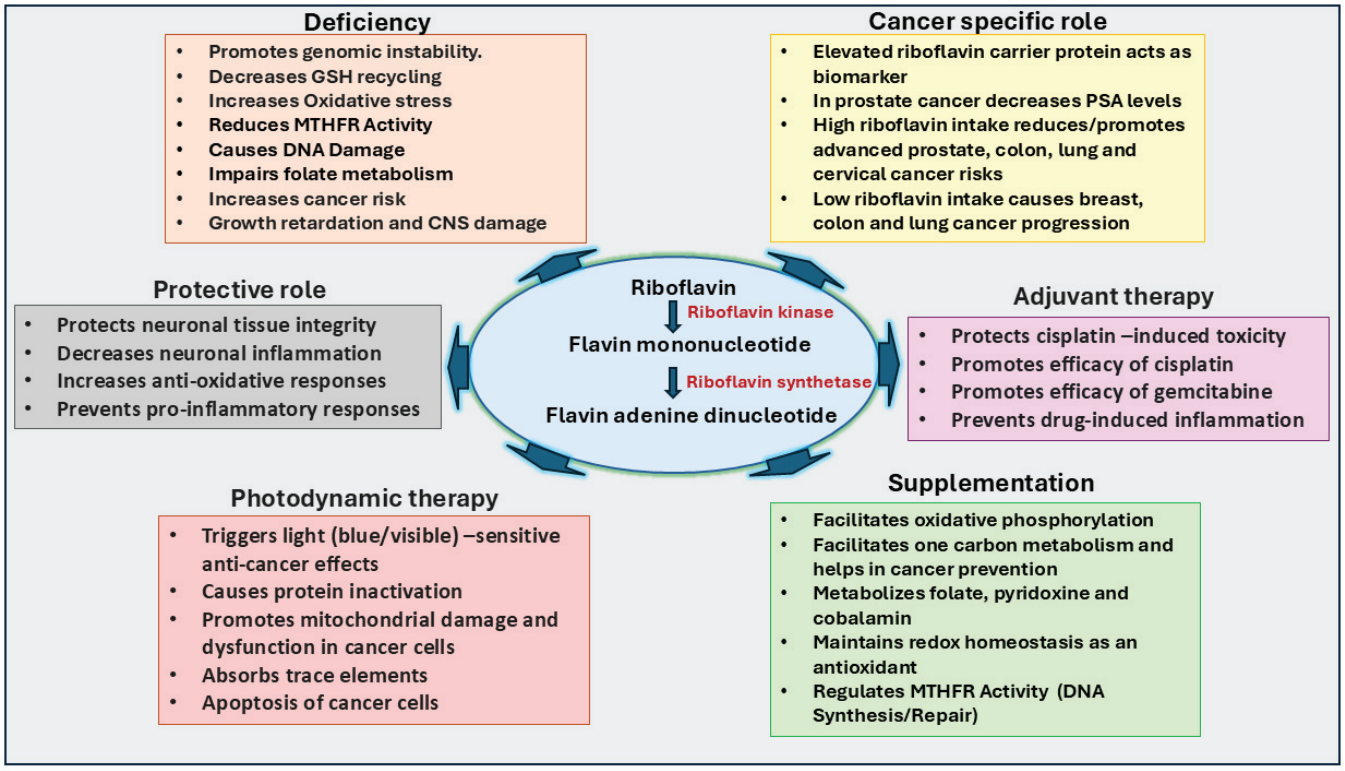

Figure 3. Significance of riboflavin in cancer growth and therapy. Riboflavin functions as a precursor for flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), essential coenzymes in redox reactions, the TCA cycle, and reactive oxygen species (ROS) regulation. Riboflavin supports neural health, vitamin metabolism, and glutathione maintenance. Its deficiency, termed ariboflavinosis, can increase oxidative stress and promote cancer risk by impairing folate metabolism and DNA synthesis. In cancer therapy, riboflavin regulates ROS to inhibit tumor growth, promotes apoptosis, and reduces inflammation, enhancing the efficacy of cisplatin and gemcitabine. Photosensitive properties of riboflavin increase its anti-cancer potential. Elevated riboflavin intake correlates with reduced risks for colorectal, lung, and cervical cancers, while high serum riboflavin levels may increase risks for pancreatic and colorectal cancers.

Further exploration of riboflavin's role in cancer in the context of breast cancer has shown elevated riboflavin carrier protein (RCP) levels in patients with breast adenocarcinoma [102]. Since RCP is estrogen-induced, its elevated levels in breast cancer patients suggest that RCP could serve as a predictive marker for breast cancer [103] and may be involved in prostate cancer [104]. Additionally, riboflavin intake has been shown to decrease the risk of colorectal cancer in women, where deficiency disrupts MTHFR enzyme activity, contributing to cancer progression [86].

In esophageal cancer, riboflavin deficiency has been linked to changes in the esophageal epithelium, increasing the risk of cancer development [105,106]. Supplementation with riboflavin and niacin has shown effectiveness in reducing esophageal cancer incidence in regions with high rates of the disease [107]. Higher riboflavin intake, especially among female non-smokers, has also been associated with a reduced risk of lung cancer [108]. Riboflavin deficiency is also linked to cervical dysplasia, a precursor to cervical cancer [109]. In addition, riboflavin, combined with other B vitamins, such as thiamine, folate, or cobalamin, could offer protection against the progression of cancer [110–112]. Indeed, a recent study suggests that intake of riboflavin at a dose of 1.2 to 2.4 mg/day could contribute to a reduced risk of cervical cancer in Korean women [112]. Thus, recent studies suggest that riboflavin could be adjuvant therapy to increase the efficacy of anti-

cancer drugs. However, its individual effects on cancer prevention and treatment need additional studies.

4. Vitamin B3: Niacin

Vitamin B3, known as niacin, is primarily derived from nicotinic acid, nicotinamide, and tryptophan. It is essential for generating coenzymes such as nicotinamide adenine dinucleotides (NAD and NADPH). These molecules are necessary for several metabolic pathways, including glycolysis, the Krebs Cycle, oxidative phosphorylation, and the hexose monophosphate (HMP) shunt [113]. The conversion of tryptophan to niacin, and subsequently to NAD, is catalyzed by a dioxygenase enzyme, which is upregulated by cortisol and tryptophan in the liver [114]. However, only a small amount of dietary tryptophan is typically converted to niacin, with most niacin obtained through other methods (Figure 4).

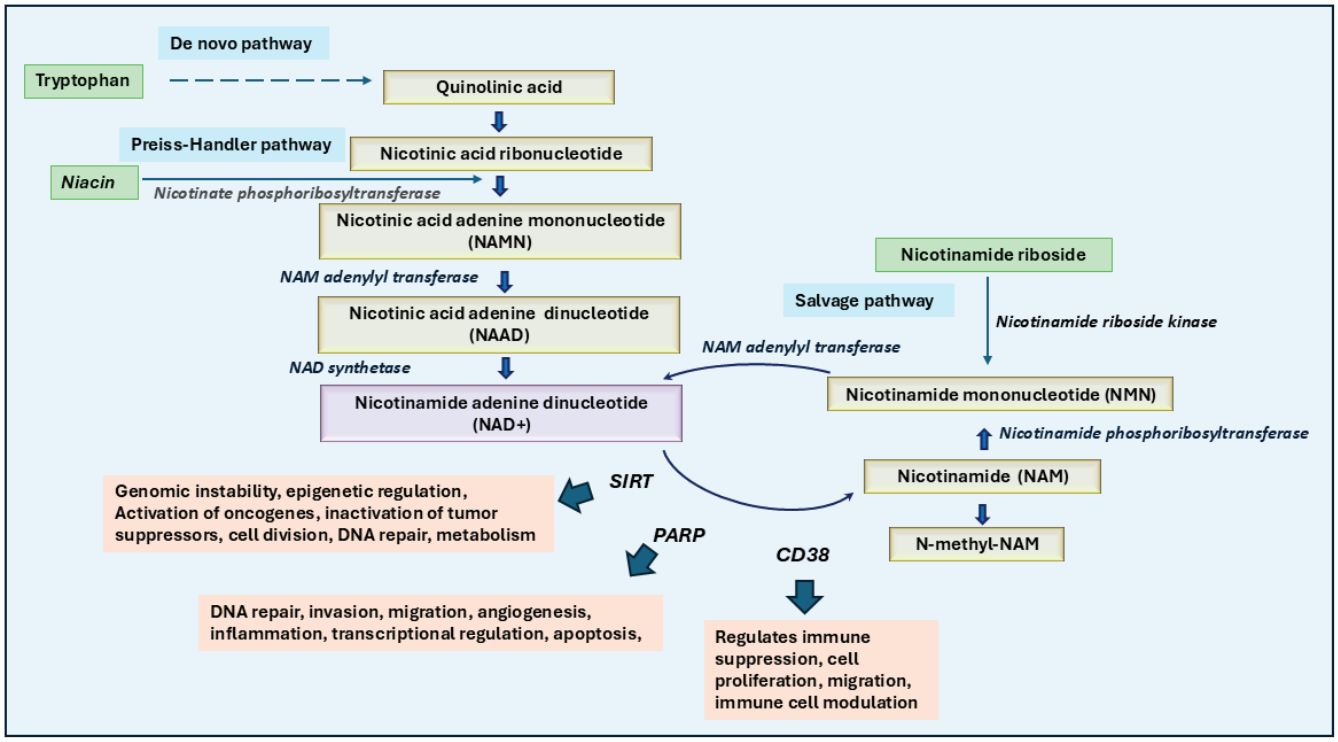

Figure 4. Role of niacin and its cofactors in cancer growth and therapy. The biosynthesis and metabolism of NAD+ (nicotinamide adenine dinucleotide) occur through three main pathways: the de novo pathway (from tryptophan to quinolinic acid and eventually NAD+), the Preiss-Handler pathway (from niacin to NAD+ via intermediates like NAMN and NAAD), and the salvage pathway (from nicotinamide riboside or nicotinamide to NAD+ via NMN). Enzymatic reactions, such as those catalyzed by nicotinamide riboside kinase and nicotinate phosphoribosyltransferase, play critical roles in these conversions. NAD+ serves as a cofactor for key enzymes like sirtuins (SIRTs), Poly (ADP-ribose) polymerases (PARPs), and CD38, which are involved in genomic stability, DNA repair, cell proliferation, and immune suppression, which eventually lead to cancer growth. NAMPT inhibitors, when combined with niacin, effectively block NAD salvage pathways crucial for tumor growth. High dietary niacin intake could correlate with improved survival and lower mortality in cancer patients, supporting its potential as a chemopreventive agent.

Further, niacin, through its precursor nicotinamide (NAM), contributes to the production of NAD+, which in turn acts as a substrate for key enzymes like sirtuin 1 (SIRT1) and poly ADP-ribose polymerase 1 (PARP1) [115]. These enzymes play a role in DNA repair, apoptosis, and carcinogenesis [116–118]. Thus, the therapies targeting NAD+ metabolism, such as PARP and NAMPT inhibitors, could enhance cancer cell death by blocking NAD+

dependency. Thus, the modulation of NAD+/NADH balance demonstrates a promising therapeutic strategy, but its effects depend on the specific cancer type and treatment approach. As with all essential nutrients, niacin deficiencies can significantly affect human health. The most severe effects of niacin deficiency are manifest in Pellagra, a disease characterized by the "three D's": dermatitis (often presenting as a casal necklace), diarrhea, and dementia [119]. Pellagra can also occur in Hartnup disease, where the failure to absorb amino acids like tryptophan through the intestine and kidneys leads to a secondary niacin deficiency [120].

Nikas et al. [121] have shown that PARP1 is activated by DNA strand breaks, initiates DNA repair mechanisms, and maintains genomic stability. However, excessive DNA damage can over-activate PARP1, leading to the depletion of NAD+ and other metabolic factors, which may result in necrosis. NAM is notable for inhibiting SIRT1 and PARP1 through negative feedback mechanisms, thus preventing over-regulated replication processes [121,122].

NAM has been shown to prevent carcinogenic events in melanoma [123,124]. For example, NAM has been shown to prevent UV-radiation-induced damage to the keratinocytes [125]. It promotes DNA repair and cellular energy production and decreases inflammation and cell death. In a randomized phase-III clinical trial, Chen et al. suggest that oral NAM reduces the risk of developing new nonmelanoma skin cancers and actinic keratoses in high-risk patients [126]. Similarly, Carneiro et al. [127] have also suggested that NAM is preventive to the development of non-melanoma skin cancers in cancer patients. However, a meta-analytical systemic study by Tosti et al. [128] suggests no significant chemopreventive effect of NAM supplementation in skin cancers. Along similar lines, a comprehensive epidemiological study indicates that niacin supplementation provided partial benefit from developing SCC but had no effect on BCC and melanoma [124]. However, a recent study indicates that NAM prevents melanoma cell growth in culture as well as in mice models [123].

Recent studies also suggest that niacin deficiency regulates DNA damage repair caused by nicotine-derived nitrosamine ketones by modulating the genes involved in the cancer progression [129]. This study indicates that niacin deficiency could be a risk for cigarette smoke-induced genomic instability and cancer development. Moreover, Nicotinamide phosphoribosyltransferase (NAMPT) is a key enzyme involved in the production of NAD in the salvage pathway required for cancer cell growth and progression [130] (Figure 3). Overexpression of NAMPT has been shown in many cancer types and inhibitors of NAMPT have been shown to prevent cancer growth [131]. Cole et al. have also indicated a possible treatment option of coadministration of NAMPT along with niacin for small-cell lung cancer and neuronal cancers [132]. Similarly, Nomura et al. [133] have shown that reducing the levels of nicotinic acid riboside could enhance the efficacy of NAMPT inhibitors in treating neuroendocrine cancers.

In addition, Tabrizi and Abyar [134] have shown that copper-based compounds containing vitamins B3 and B4 prevent the growth of breast cancer cells such as MCF7 and MDA-MB-231. Comparably, Abdel-Mohsen et al. [135] have also suggested that the copper(I) nicotinate complex, by regulating Notch1 signaling, could prevent triple-negative breast cancer cell growth. NAM has been shown to prevent triple-breast cancer cells by reducing the mitochondrial membrane potential and ATP production and increasing reverse electron transport, lipid metabolism, and reactive oxygen species [136]. Interestingly, a curcumin derivative containing two molecules of niacin has been shown to prevent the growth of colon, breast, and nasopharyngeal cancer cells by promoting P53-mediated apoptosis and cell cycle arrest [137]. In addition, Kim et al. [138] have also shown that niacin prevents TRAIL-induced apoptotic cell death by activating the autophagy flux in human colon cancer cells.

Similarly, niacin has been shown to prevent glioblastoma in pre-clinical studies. Sarkar et al. [139] have shown that niacin-stimulated monocytes produced by interferon-alpha14 inhibit the growth of brain tumor-initiating cells. In the same study, authors have found that in a mouse model of glioblastoma, niacin prevents tumor growth. NAM has been shown to prevent melanoma growth in culture as well as in mouse models, maybe by inhibiting the activation of SIRT2 [123,140]. Selvanesan et al. [141] have also shown in a mouse model that a combination of gemcitabine with nicotinamide (NAM) prevents pancreatic cancer by decreasing the tumor-associated macrophages and myeloid-derived suppressor cells. Similarly, Shu et al. [142] have shown that the niacin-ligated platinum (iv) and ruthenium (ii) chimeric complex prevents metastasis as well as the growth of cancer cells in vivo.

Accordingly, current evidence suggests that niacin could be a potent chemopreventive agent in controlling the growth of various cancer types [124,143]. Further, niacin, along with chemotherapeutic drugs, has been shown to increase overall survival and efficacy. Indeed, a recent National Health and Nutrition Examination Survey from 1999–2014 conducted by Ying et al. [144] has suggested that higher dietary vitamin B3 intake is associated with an improved survival rate and lowered mortality in cancer patients.

5. Vitamin B5: Pantothenic Acid

Pantothenic acid (vitamin B5 or pantothenate) in its anionic form, is a precursor to coenzyme A (CoA) and is an essential micronutrient found generally in a wide variety of foods, including animal products, mushrooms, potatoes, and oats [145]. A portion of pantothenic acid is also synthesized de novo by the gut microbiota after a three-step enzymatic reaction, which concludes with an adenosine triphosphate (ATP)-dependent condensation of β-alanine and pantoate by pantothenate synthetase [146]. Along with cysteine and four ATP molecules, pantothenic acid is necessary for the biosynthesis of CoA in a five-step enzymatic reaction [146]. CoA is then utilized as a fundamental coenzyme in numerous metabolic processes, most notably in the pyruvate oxidation in the Krebs cycle for energy production and during both the synthesis and oxidation of fatty acids.

Despite its essential role in metabolism, pantothenic acid could have multiple effects on cancer progression and prevention. Few studies have shown its cancer-promoting actions. For example, a recent study by Heckmann et al. [147] has demonstrated that pantothenic acid increases the levels of CoA in fibroblasts. [147]. Murine models, including TLX-5 lymphoma, sarcoma 180, and fibrosarcoma, also demonstrated lower levels of pantothenate, CoA, and acetyl-CoA compared to healthy mice. Miallot et al. [148] have indicated that pantothenic acid increases the polarization of myeloid and dendritic cells and antigen presentation in sarcoma. Interestingly, these tumor models showed an increase in 4-phosphopantothenate levels, an intermediate in CoA synthesis catalyzed by pantothenate kinase. This abnormal level may result from CoA degradation or increased pantothenate kinase activity rather than an increase in CoA itself [149].

However, some human studies have shown no significant association between a high pantothenic acid-containing diet and the risk of esophageal squamous cell carcinoma [150]. Similarly, studies on urothelial cell carcinoma and gastric cancer revealed no statistically significant correlation between pantothenic acid intake and cancer risk [151,152]. In breast cancer, a cohort study involving 27,853 women aged 45 years or older, with 462 cases of diagnosed breast cancer, found no association between pantothenic acid intake over 12 months and breast cancer development [153]. Furthermore, in a study involving five patients with advanced carcinomas (three with stage 4 cervical carcinoma and two with recurrent carcinoma of the floor of the mouth), a liquid diet deficient in pantothenic acid for 4 to 10 weeks did not alter the tumor growth rate compared to periods when the diet

included pantothenic acid. Although urinalysis confirmed a decrease in pantothenic acid excretion, suggesting lower body levels, no symptoms of pantothenic acid deficiency were observed, likely due to endogenous production from intestinal microbiota [154].

Further, tumor-promoting effects of pantothenic acid have been shown in certain cancer cell types. For example, in breast cancer, increased pantothenic acid concentrations correlated with enhanced glycolytic activity and cell migration, particularly in the MCF-7 luminal gene cluster cell line, the JIMT-1 basal A cell line, and the MDA-MB-231, MDA-MB-435, and MDA-MB-436 basal B cell lines [155]. Similarly, Kreuzaler et al. [156] have shown that pantothenic acid is involved in the upregulation of c-MYC and SLC5A6, which promote cancer growth. Further, dietary restriction of pantothenic acid prevents tumor growth by reversing the c-MYC-modulated metabolic changes. Similarly, pantothenic acid and its metabolites have demonstrated protective qualities in specific cancer cells. For example, in Ehrlich ascites tumor cells grown in the peritoneal cavity of Swiss female mice, pantothenic acid and its derivatives (pantothenol and pantethine) decreased plasma membrane and mitochondrial outer membrane permeability. This protective effect is thought to result from the biosynthetic increase in cholesterol, which counters the leakage induced by digitonin, a compound that disrupts membrane integrity by complexing with cholesterol [157]. Additionally, pantothenic acid and its derivatives protected Ehrlich ascites tumor cells against lipid peroxidation induced by free oxygen radicals, further supporting its role in promoting cellular repair mechanisms [158].

Elevated levels of pantothenic acid levels were also found in the MKN-28 and AGS cell lines of gastric cancer cells, suggesting that increased phospholipid production from reactive oxygen species could contribute to both the rise in pantothenic acid levels and the proliferation of these cells [159]. A case-control study by Secchi et al. [160] in Cordoba, Argentina, revealed that high dietary intake of pantothenic acid was statistically significant in increasing the risk of oral squamous cell carcinoma.

Pantothenic acid and its metabolites have also been implicated in treating various cancers in some clinical studies. For example, higher pre-treatment plasma levels of pantothenic acid in patients with Stage III or IV melanoma correlated with an increased response to anti-programmed cell death protein 1 (PD1) antibody therapy. Similarly, pre-treatment with pantothenate injections enhanced the efficacy of anti-PD1 antibody therapy in the murine colon carcinoma model MC38 [161]. In cancers that rely heavily on glycolysis and lactic acid fermentation for growth, such as soft tissue sarcoma (STS), targeting pantetheine, a degradative product of CoA, has shown promise in reducing tumor growth. Treatment with Vnn1 pantetheinase, which degrades pantetheine into pantothenate and cysteamine, led to increased mitochondrial CoA levels and a move from glycolysis to oxidative phosphorylation, thereby suppressing tumor growth (Figure 5) [162]. Pantethine, a disulfide bridge-linked molecule of two pantetheine molecules, has also shown potential in treating multidrug-resistant cancers by inhibiting the release of microparticles that protect cancer cells from treatment [163]. In mouse models of ovarian tumors derived from human ovarian tumor xenografts, pantetheine treatment decreased metastasis and ascites formation [164].

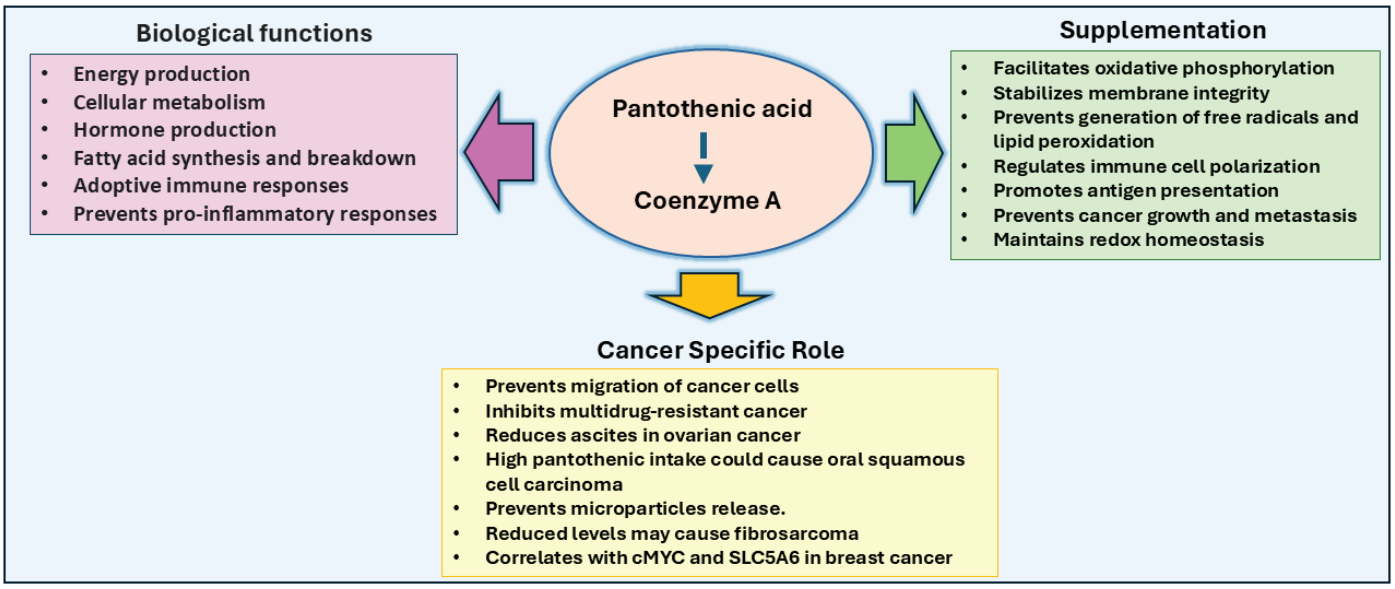

Figure 5. Pantothenic acid in cancer: Pantothenic acid is a precursor to Coenzyme A (CoA) and is vital for numerous metabolic processes, including the citric acid cycle, fatty acid metabolism, and hormone production. Increased pantothenic acid levels correlate with enhanced glycolytic activity and cancer cell migration, particularly in breast and gastric cancer cells, and its association with c-MYC and SLC5A6 expression promotes breast cancer growth. Pantothenic acid supplementation could enhance immune responses by increasing antigen presentation, shifting tumor metabolism from glycolysis to oxidative phosphorylation, and preventing tumor growth. High intake has been linked to oral squamous cell carcinoma risk, and pantothenate supplementation has been shown to treat multidrug-resistant cancers and reduce metastasis.

6. Vitamin B6: Pyridoxine

Vitamin B6 is an essential water-soluble vitamin that plays a role in over 140 different biochemical reactions within the body. These reactions include the synthesis of heme precursor δ-aminolevulinic acid, sphingoid bases essential for myelin production, and several neurotransmitters such as serotonin, norepinephrine, epinephrine, and γ-aminobutyrate (GABA) [165]. Vitamin B6 is also a cofactor for several enzymatic reactions involved in amino acid metabolism, glycolysis, gluconeogenesis, glycogenesis, trans-sulfuration, immune response, and polyamine biosynthesis [166]. Structurally, vitamin B6 encompasses six chemically similar compounds, all containing a pyridine ring with varying groups at the 4′ position, and these compounds are interconvertible. The most bioactive form is pyridoxal 5′-phosphate (PLP), with other forms including pyridoxal, pyridoxamine, pyridoxamine 5′-phosphate, pyridoxine, and pyridoxine 5′-phosphate [167]. Failure to consume adequate amounts of vitamin B6, as well as conditions such as alcoholism and renal or liver complications, can lead to various health issues, including sideroblastic anemia, seizures, peripheral neuropathy, mood changes, and seborrheic dermatitis [165].

Further, pyridoxine also plays an important role in amino acid metabolism. It acts as a coenzyme in transamination, decarboxylation, and one-carbon metabolism, which supports nucleotide biosynthesis and cellular redox balance [166]. Further, PLP is crucial for protein synthesis, neurotransmitter production, and glutathione-mediated antioxidant defense. Pyridoxine influences cell proliferation in cancer cells by increasing amino acid metabolism and nucleotide synthesis. It also modulates apoptosis by regulating oxidative stress, redox balance, and epigenetic alterations. Thus, the deficiency of pyridoxine could promote DNA damage and tumorigenesis, while an excess of pyridoxine could promote tumor growth by modulating the redox-mediated metabolic pathways. Therefore, pyridoxine seems to

play a major role in cancer progression as solid tumors rely on high amino acid turnover for survival and growth.

On the other hand, vitamin B6 has also been linked to a reduced risk of developing and proliferating diverse types of cancer. A meta-analysis study that monitored the dietary intake of vitamin B6 across 1,959,417 individuals, including 98,975 cancer cases, revealed that high intake of vitamin B6 was associated with a reduced risk of cancer at multiple sites, including the breast, colorectal area, ovary, prostate, immune system, endometrium, lung, stomach, esophagus, pancreas, kidney, bladder, oral cavity, nasopharynx, larynx, cervix, liver, and brain [168]. Specifically concerning breast cancer, a case-control study nested in the Multiethnic Cohort in Hawaii and Southern California indicates that increased circulating levels of PLP are associated with a reduced risk of invasive postmenopausal breast cancer [169]. Another cohort study suggested that vitamin B6 intake over 12 months had a protective effect against breast cancer development [153]. Additionally, a meta-analysis of eighteen studies (11 prospective studies and seven case-control studies) on the association of dietary vitamin B6 intake with pancreatic cancer showed that high blood levels of pyridoxal 5′-phosphate might protect against the development of pancreatic cancer [170]. While vitamin B6 supplementation has shown inconsistent and mostly nonsignificant associations with colorectal cancer development, high blood levels of pyridoxal 5′-phosphate have consistently demonstrated a 30–50% reduction in colorectal cancer risk [171]. Another meta-analysis found variability in the association between dietary vitamin B6 intake and colorectal cancer risk across nine studies but confirmed an inverse relationship between blood pyridoxal 5′-phosphate levels and colorectal cancer risk across four studies [172]. A recent meta-analysis study also indicates the risk of developing colon cancer is negatively correlated with the vitamin B6 and PLP levels [173]. Interestingly, Holowatyj et al. [174] have indicated that high preoperative vitamin B6 is linked to increased survival in stage 1-III colorectal cancer patients. Similarly, Li et al. [175] have also indicated that high PLP levels but not Par could be associated with the increased survival of colon cancer patients. Along similar lines, Xu et al. [176] have also suggested that increased PLP and pyridoxal are associated with colorectal cancer risk in the Chinese population. Thus, the protective effects of pyridoxal 5′-phosphate are thought to be due to its involvement in one-carbon metabolism, which is related to DNA synthesis and methylation, and its role in reducing inflammation, proliferation, and oxidative stress [171]. Indeed, Wu et al. [177] have indicated that Vitamin B6 is a cofactor associated with enzymes, like serine hydroxymethyltransferase (SHMT), methionine synthase reductase (MTRR), and methionine synthase (MS) and that it also plays a regulatory role in regulating genomic stability and cell viability in breast cancer patients.

The ability of vitamin B6 to interact with nuclear receptors and express antioxidant, pro-apoptotic, and anti-angiogenic effects in cells has been further elucidated. Vitamin B6 conjugates with RIP140, a receptor-interacting protein, enhancing its transcriptional co-repressive activity by increasing its interaction with histone deacetylases and retaining RIP140 in the nucleus [178].

Further, vitamin B6 deficiency has been linked to an increased risk of various cancers. For example, a recent study indicates that PLP deficiency leads to malignant tumors in a Drosophila model [179]. Similarly, Yasuda et al. [180] have shown that vitamin B6 deficiency is common in patients with primary and secondary myelofibrosis. Moreover, vitamin B6 deficiency has been linked to sarcopenia, a condition in which muscle loss is due to factors such as aging or immobility [181]. Spinneker et al. [182] have suggested an increase in colon tumorigenesis in vitamin B6-deficient mice, while vitamin B6 deficiency in rats showed signs of chronic pancreatitis, a condition known to increase the risk of pancreatic cancer. Several mechanisms have been proposed to explain the correlation

between vitamin B6 deficiency and carcinogenesis. In DNA synthesis, vitamin B6 deficiency reduces serine hydroxy-methyltransferase activity, leading to a lack of methylene groups for 5,10-methylenetetrahydrofolate. This deficiency causes inadequate methylation of deoxy-uridylate to deoxy-thymidylate, resulting in the misincorporation of uracil into DNA instead of thymidine, which leads to chromosome strand breaks and impaired DNA excision repair. Another proposed mechanism involves DNA hypomethylation, given that vitamin B6 is associated with DNA methylation. A third mechanism suggests that vitamin B6 deficiency impairs the activity of detoxifying enzymes such as glutathione S-transferase and glutathione peroxidase, which detoxify carcinogenic compounds. Pyridoxal 5′-phosphate is required for enzymes in the trans-sulfuration pathway to generate cysteine, which is essential for glutathione synthesis (Figure 6). A fourth mechanism proposes that vitamin B6 deficiency increases steroid hormone sensitivity, potentially contributing to the development of breast, uterine, and prostate tumors [182].

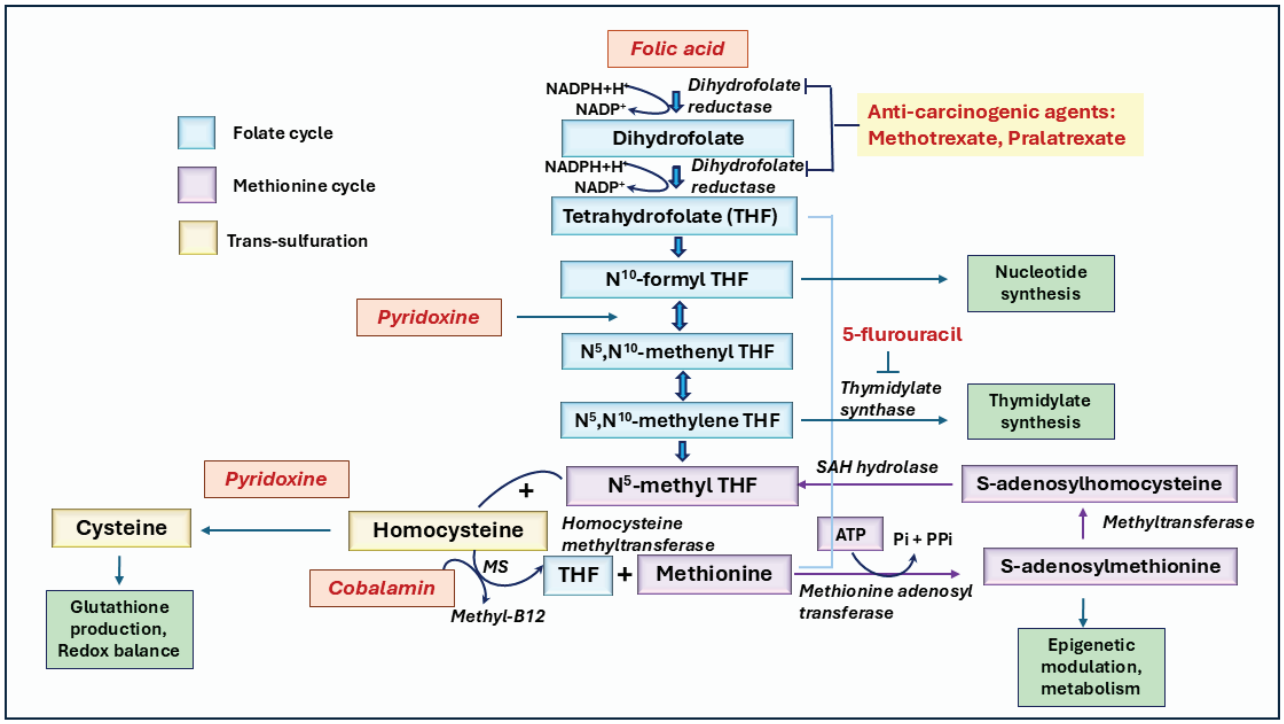

Figure 6. The role of vitamins B6, B9, and B12 mediated one-carbon metabolism in cancer. Folate (vitamin B9) is converted to dihydrofolate and then to tetrahydrofolate (THF), which undergoes further modifications to form derivatives like N^5, N^{10}-methylene THF, and N^5-methyl THF. These derivatives are essential for methylation processes, and nucleotide synthesis requires cancer cell growth. The pathway highlights the involvement of pyridoxine (vitamin B6) in converting homocysteine to cysteine via the trans-sulfuration pathway and cobalamin (vitamin B12) in the re-methylation of homocysteine to methionine, which subsequently forms S-adenosylmethionine (SAM), a key methyl donor in several biochemical and epigenetic methylations. Anti-carcinogenic agents, such as methotrexate and pralatrexate, inhibit steps in folate metabolism, while 5-fluorouracil blocks nucleotide synthesis. This pathway underscores the interdependence of folate, pyridoxine, and cobalamin in one-carbon metabolism in essential biological functions like methylation, DNA synthesis, and cellular regulation.

Increased vitamin B6 catabolism, marked by a rise in the PAr index (the ratio of 4-pyridoxic acid, a catabolic product, to the sum of pyridoxal and pyridoxal 5′-phosphate), has been associated with a higher risk of lung cancer. A study of 20 cohorts across four continents found that an increase in the PAr index could serve as a pre-diagnostic marker for lung cancer [183]. Additionally, a survey of 5364 matched case-control pairs showed

that impaired vitamin B6 status was significantly associated with an increased risk of lung cancer, with squamous cell carcinoma being the most common form [184]. In patients with non-small cell lung cancer, treatment with cytotoxic agents such as cisplatin was less effective if the patient had low expression of pyridoxal kinase, which converts pyridoxal to its more active form, pyridoxal 5′-phosphate [185]. Huang et al. [186] have also shown that low serum B6 vitamers are linked with increased pancreatic cancer risk in the Asian population. Similarly, lower levels of PLP have been associated with the development of pancreatic ductal adenocarcinoma [187]. These studies suggest that high levels of vitamin B6 could provide protection against various cancers, while low levels or deficiency are a major risk factor for promoting cancer growth.

7. Vitamin B7: Biotin

Vitamin B7, known as biotin, is water-soluble and typically obtained through dietary sources such as beef, eggs, salmon, pork chops, and vegetables like broccoli and spinach [188]. It acts as a cofactor for various carboxylase enzymes involved in metabolic pathways, including fatty acid synthesis, glucose metabolism, and amino acid metabolism [189]. Additionally, biotin plays a role in epigenetics and influences gene silencing, DNA repair, and chromatin remodeling [189]. The importance of biotin is further highlighted by the substantial energy required to produce a single molecule for participation in these biochemical reactions [190].

While biotin is a vital cofactor in many biochemical reactions, its broader roles in other pathways are less understood than other B vitamins. However, evidence suggests that biotin may be necessary for skin maintenance. Ogawa et al. [191] observed that acrodermatitis enteropathica (AE), partly due to biotin deficiency, led to symptoms similar to those in zinc-deficient diets. Further, biotin deficiency results in more pronounced irritant contact dermatitis, mirroring the effects of zinc deficiency. Therefore, biotin may play a key role in regulating and maintaining skin health, especially in individuals with skin conditions that impact their quality of life [191,192].

Biotin has also been associated with effects on glycolytic and gluconeogenic enzyme activity. Sugita et al. [193] have shown that in biotin treatment in streptozotocin-induced rats, the mRNA levels of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase were decreased, and glucokinase was increased. Similarly, McCarty [194] has suggested that a high dose of biotin also alters the expression of glycolytic enzymes. In studies on chronic kidney disease (CKD), the regulation of cGMP, particularly when complexed with specific protein kinases, showed promise for reno-protective functions, potentially aiding in the prevention of CKD [195]. Moreover, inhibitors of the cGMP pathway, especially when part of the cGMP-cGK1-PDE complex, have shown potential for anti-clotting and anticancer properties. These findings could help to develop new cancer treatment modalities. However, further studies are needed to demonstrate biotin's role in cGMP activity, signaling, coagulation, and thrombotic events, as well as its potential contributions to cancer cell proliferation.

Indeed, Maiti and Paira [196] have indicated that sodium-dependent multivitamin transporter (a biotin transporter) is overexpressed in various cancer cells, including breast, colon, lung, ovarian, and leukemia. Thus, biotin is used in the development of various drug delivery methods to treat cancers. For example, Tang et al. [197] have developed biotin-modified liposomes for breast cancer. Raza et al. [198] have indicated that the selenobiotin compound induces apoptosis in ovarian cancer cells. Similarly, Kundu et al. [199] have shown the potential use of Chitosan-biotin-conjugated nanoparticles for increasing the chemotherapy potential.

Biotin is well-tolerated, with few noted adverse effects, toxicities, or contraindications. However, biotin deficiency can arise from insufficient dietary intake or an inability to utilize the vitamin within the body. Individuals deficient in biotin typically present symptoms such as skin rashes, hair loss, and fragile nails, along with neurological deficits like depression and paresthesias [188]. Recent studies mostly concentrated on developing drug delivery methods using biotin conjugates to prevent cancer. However, the connection between biotin deficiency and cancer is not clear, and additional studies are needed to understand the significance of biotin in cancer development and therapy.

8. Vitamin B9: Folate

Vitamin B9, known as folate, is an essential water-soluble vitamin found in legumes and green leafy vegetables. Folate deficiency is associated with developmental and degenerative conditions such as neural tube defects in embryos [200]. Consequently, vitamin B9 supplementation is recommended for women of childbearing age, starting one month prior to conception to the first trimester of pregnancy. Folate is involved in one-carbon metabolism and has been studied for its role in cancer development [201,202]. It is required in the methionine pathway to convert homocysteine to methionine, which is converted to S-adenosylmethionine (SAM). SAM plays a fundamental role in DNA and RNA methylation, affecting gene transcription and the expression of tumor suppressors and proto-oncogenes (Figure 6). Additionally, folate is vital in converting deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), which is essential for DNA synthesis and repair. Therefore, folate deficiency can lead to uracil misincorporation instead of thymine, resulting in DNA repair dysfunction, unstable DNA, and strand breaks [203,204]. On the other hand, excessive folate can also cause tumor progression by supporting rapid cell proliferation. Thus, deficiency and oversupply of folate can influence tumor development and progression.

Epigenetic changes caused by chronic inflammation can also be a factor in cancer development. Further patients with inflammatory bowel diseases are at a higher risk of developing colorectal cancer. Tumors of the gastrointestinal tract, prostate, and liver have also been linked to sites of chronic inflammation. B vitamins, including folate, regulate inflammation and the immune response due to their involvement in nucleic acid, protein synthesis, and methylation. Disruptions in the immune system can occur due to improper cytokine production, faulty antigen presentation, and an unregulated immune response. Inefficient methylation, often caused by vitamin B deficiencies, can lead to hyperhomocysteinemia, a condition associated with oxidative stress and chronic inflammation [205]. Elevated homocysteine levels and low folate have shown an increased risk of lung, breast, and colorectal cancers [206,207]. Further, elevated homocysteine and methionine have been suggested to disrupt the epigenetic modification of specific genes that regulate breast cancer progression and initiation, such as RASS-F1 and BRACA1 [208,209].

Moreover, some case-control studies have shown an inverse correlation between dietary folate intake and the risk of pharyngeal, oral, esophageal, colorectal, pancreatic, laryngeal, and breast cancers [210–215]. Additionally, risk estimates for cancers of the endometrium, ovary, prostate, and kidney were below unity, indicating a potential protective effect, while stomach cancer showed no relation to dietary folate intake [216,217]. Studies using colon cancer cells have demonstrated that folic acid supplementation can inhibit cell proliferation. This inhibition occurs through activating the c-SRC-mediated pathway and amplified levels of the cyclin-dependent kinase (CDK) inhibitor and tumor suppressor p53, leading to G0/G1 cell cycle arrest [218]. Folate has been shown to be critical in regulating the cancer growth [210–215,219–222]. Specifically, colorectal cancer has been one of the most extensively studied cancers concerning folate's role in carcinogenesis. Most

studies have shown a significant inverse relationship between folate status and colorectal cancer risk [220–222]. Epidemiologic studies comparing subjects with the highest dietary folate intake to those with the lowest have suggested a reduction of about 40% in the risk of colorectal neoplasms [223]. Few studies also suggest that even a modest decrease in folate levels can enhance colorectal cancer risk without clinical evidence of folate deficiency [224,225]. Randomized intervention studies in humans have also shown that folate supplementation in patients with resected colonic adenomas can reverse colorectal cancer biomarkers, including genomic DNA hypomethylation in the rectal mucosa and decreased proliferation of rectal mucosal cells [226,227].

Although these studies provide evidence of an inverse association between folate levels and colon cancer risk, they are still insufficient to draw definitive conclusions. However, a safe and effective dosage for folate has not been established in humans. Some studies have suggested that excessive folate supplementation could increase cancer risk and promote tumor progression [219]. Folate supplementation is available in various forms, including tablets, mixtures, and intravenous preparations. Recently, several countries have implemented food fortification programs in grains and cereals to prevent neural tube defects in embryos [228].

Further, few studies suggest that the protective effects of folate plateau are beyond a certain level and do not increase indefinitely with higher intake [219,222]. Colorectal cancer studies observed a decreased cancer risk with moderate folate status but an increased risk with excessive intake [223]. Similar findings were observed in breast cancer, where high plasma folate concentrations were associated with premenopausal breast cancer. A double-blind study on colorectal polyp recurrence showed that individuals supplemented with 1 mg/day of folate had more multiple and advanced adenomas than the placebo group [229]. These results suggest that people with prior adenomas might be at a higher risk of developing various or advanced polyps due to folic acid supplementation. The study also indicated a higher rate of invasive prostate cancer among the folic acid group [229]. Multiple in vitro and animal studies have summarized that increased folate supplementation may promote the progression of established tumors, while folate deficiency might contribute to tumorigenesis [230].

9. Vitamin B12: Cobalamin

Vitamin B12 (Cobalamin) is found in animal-derived products such as dairy, red meat, and eggs [231]. It is not produced by plants, making vegans and vegetarians at a higher risk for B12 deficiency unless they rely on supplementation, as they depend solely on microbial sources. Like folate, vitamin B12 is necessary due to its role in one-carbon metabolism (Figure 6), which involves homocysteine, methionine, and B vitamins [232]. One-carbon metabolism directs the one-carbon units toward the folate cycle, essential for DNA and RNA synthesis, or toward methionine regeneration, where methionine acts as a precursor to S-adenosylmethionine (SAM). SAM is vital for the methylation of histones, proteins, and DNA. Homocysteine generates methionine through a reaction catalyzed by the enzyme methionine synthase (MeS). The activation of MeS requires cobalamin as a cofactor for converting 5-methyl tetrahydrofolate (THF) to THF. Further, SAM is a potential methyl group donor for DNA, RNA, and protein methylations. Thus, SAM-mediated methylation is also significant for gene expression regulation, genomic stability, and epigenetic modifications, which influence cancer progression. Cobalamin is also vital for nucleotide synthesis, which is required for DNA replication and repair. Thus, a deficiency of cobalamin could lead to DNA damage, uracil misincorporation, and hypomethylation, which increases genomic instability and promotes the risk of developing

cancer-causing mutations. Similarly, an excess of B12 could promote tumorigenesis by increasing cell proliferation and modulating cancer cell metabolism.

Further, both vitamin B12 and folate deficiencies are associated with a reduction in a significant antioxidant protein called glutathione, a product of the trans-sulfuration pathway. Glutathione acts as an inhibitor of reactive oxygen species (ROS)-induced oxidative stress and a mediator of redox balance. The impact of folate and vitamin B12 was examined in a study in which mice were treated with Azoxymethane (AOM), a compound known to induce tumors and oxidative stress. The study showed that both vitamins reduced the cytotoxic effects of AOM and mitigated oxidative stress [233]. A recent study by Obeid et al. [234] has indicated high plasma levels of vitamin B12 in various cancer patients. However, the role of B12 in promoting or preventing cancer is still not clear.

Moreover, a few studies also indicated a significant role of vitamin B12 intake and risk of developing colorectal, breast, prostate, malignant melanoma, or squamous cell carcinoma [235–238]. However, the study did reveal a positive correlation between vitamin B12 and the risk of esophageal cancer [239]. Another nested case-control study examined the association between serum concentrations of one-carbon nutrients, including vitamin B12, and upper gastrointestinal cancers. A statistically significant correlation was noticed between low vitamin B12 serum concentration and an increased risk of non-cardia gastric adenocarcinoma (NCGA). This association is mechanistically understandable due to the growth of the intestinal type of gastric cancer model. Chronic superficial gastritis often progresses to chronic atrophic gastritis and eventually to adenocarcinoma. Chronic atrophic gastritis leads to decreased gastric acid secretion, which diminishes vitamin B12 absorption. This study suggested that vitamin B12 deficiency in these cases was due to malabsorption rather than insufficient dietary intake [240].

While vitamin B12 deficiency can contribute to tumor development, over-supplementation may also increase cancer risk. For example, Collin et al. [241] have shown that increased intake of folate and vitamin B12 increases the risk of prostate cancer. Similarly, use of vitamin B12 supplements, excluding those from multivitamins, has been associated with a 30–40% increase in lung cancer risk. In contrast, no association was found between folic acid supplementation and cancer risk [242,243]. Similarly, a Norwegian randomized controlled trial has found an enhanced risk of lung cancer development in individuals who have taken both vitamin B12 and B9 supplements [244]. Additionally, Fanidi et al. [245] have also suggested increased lung cancer risk with high supplementation of vitamins B12 and B6. To further confirm the role of vitamin B12 in lung cancer, various population-based cohort studies measured circulating vitamin B12 concentrations in pre-diagnostic samples [246–249]. These studies found a positive correlation between cancer risk and the circulating concentration of vitamin B12 in cancer patients' blood samples.

10. Conclusions and Future Perspectives

B vitamins are essential in maintaining cellular functions and metabolic processes. Their balance is very important for cell growth, proliferation, differentiation, and survival. Thus, understanding how B vitamins imbalance could lead to cancer growth and spread will help to control unnecessary dietary intake of excessive vitamin supplements. Further, identifying the role of vitamins is also crucial for developing chemopreventive strategies not only to maximize benefits but also to minimize potential risks. This review focused specifically on the relationship between B vitamins and cancer development or prevention. Several studies provided considerable evidence indicating both beneficial and harmful effects depending on the type of vitamin and cancer type [250–252]. While deficiencies in water-soluble B vitamins, such as thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, biotin, folate, and cobalamin, are generally associated with an increased risk of cancer due

to impaired metabolic and cellular functions, emerging research highlights the potential dangers related to excessive intake supplementation of these vitamins. Further, over-supplementation of B vitamins could enhance cancer cell metabolism, DNA repair, and genomic instability which can accelerate tumor progression in cancers with strong metabolic flexibility (Table 3).

Table 3. Effects of B Vitamin Deficiencies and Over-Supplementation on Cancer Progression and Prevention.

B Vitamin	Effects of Deficiency	Effects of Over-Supplementation
B1 (Thiamine)	Impairs mitochondrial function, decreases oxidative phosphorylation, increases oxidative stress and metabolic dysfunction, inhibits tumor growth [32–35,42,45–49].	Enhances cancer cell metabolism by promoting glycolysis and the pentose phosphate pathway and accelerates cancer progression in specific cancers [50–52,67].
B2 (Riboflavin)	Reduces flavoprotein activity, increases oxidative stress and DNA damage; impairs Krebs cycle; inhibits tumor progression in some cancers [70–73,86–89].	Enhances Krebs cycle and oxidative phosphorylation, reduces free radical production, promotes cancer cell growth, and inhibits oxidative stress-induced apoptosis [74–81,93,94].
B3 (Niacin)	Reduces NAD+ levels, impairs DNA repair, inhibits PARP and SIRT activation, increases genomic instability and promotes tumorigenesis [113–116,129].	Increases NAD+ levels, enhances cancer metabolism and DNA repair; may prevent cancer progression in some cases but also promote tumor survival in therapy-resistant cancers [123–126,144].
B5 (Pantothenic Acid)	Disrupts Coenzyme A (CoA) production, impairs fatty acid synthesis and energy metabolism, and prevents tumor growth [147–149,155].	Enhances lipid metabolism, promotes cancer cell proliferation and progression [150–156,160].
B6 (Pyridoxine)	Reduces amino acid metabolism and neurotransmitter synthesis; increases homocysteine levels which lead to inflammatory responses that may promote cancer progression [165–168,177–180].	Stimulates angiogenesis and increases cancer cell survival in specific conditions [169–176,182–187].
B7 (Biotin)	Decreases biotin-dependent carboxylase activities, limits fatty acid synthesis and prevents tumor growth [188,189,193,194].	Promotes biotinylation, affects oncogene expression, and contributes to tumor growth [196–199].
B9 (Folate)	Impairs one-carbon metabolism, hinders DNA synthesis and repair, increases mutation rates and cancer risk [201–204,207–212].	Enhances one-carbon metabolism, promotes tumor progression by providing nucleotides for DNA replication [219–230].
B12 (Cobalamin)	Causes genomic instability due to impaired methylation and DNA synthesis, increases cancer risk and progression [231–234,239].	Supports cancer cell metabolism and promote the risk of certain cancers [235–246].

For example, vitamins like folate and B12, which are essential for DNA synthesis and repair, and their excessive supplementation have been linked to increased risks of certain cancers, such as colorectal and lung cancer [247–249]. A recent case-control study by Le et al. [253] has suggested the risk of esophageal, lung, and breast cancers in patients with low intake of cobalamin and gastric cancer in patients with high intake in Vietnamese population. On the other hand, riboflavin and niacin have been shown to enhance the efficacy of cancer therapies [254,255]. Premkumar et al. [255] have shown that supplementation of riboflavin and niacin along with tamoxifen reduced tumor burden in breast cancer patients. Similarly, rapamycin and niacin combination increases apoptotic cell death in acute myeloid leukemia cells. [256]. Yuvaraj et al. [257] have also shown that combination

treatment of Coenzyme-Q, riboflavin and niacin with tamoxifen e increases blood parameters in postmenopausal breast cancer women. However, the impact of vitamins B2 and B3 on cancer prevention and progression depends on dosage and cancer type.

Some risks exist in taking these B vitamins without proper guidance, as they can interact with certain medications and potentially alter their effectiveness. In addition to enhancing the chemotherapeutic effects of some drugs, some B vitamins could also improve the therapeutic effects of general medicines. For example, vitamin B12 supplementation can counteract the deficiency caused by long-term use of metformin [258]. Similarly, pyridoxine can reduce the efficacy of the anti-epileptic drug levodopa by increasing its metabolism [259]. Additionally, high doses of niacin have been shown to increase the risk of liver toxicity when taken along with cholesterol-lowering statins [260]. High niacin levels are also shown to be a risk for cardiovascular complications [261]. In addition, folic acid can interfere with methotrexate and reduce its effectiveness [262].

Further, recent studies also indicated several limitations and knowledge gaps that warrant attention on vitamin supplementation. A significant concern is the potential side effects of high-dose supplementation. A cohort study by Meyer et al. [263] has found that a combined high intake of pyridoxine and cobalamin was associated with an increased risk of hip fracture. Similarly, B vitamins might prevent cardiovascular diseases by lowering homocysteine levels; however, they can also alter the efficacy of statin drugs. Another limitation is the inconsistent results from clinical trials. While most observational studies have suggested the protective roles of certain B vitamins in preventing cancer growth, clinical trials have not consistently supported this, and additional studies are required. Further, there is a lack of comprehensive studies understanding the synergetic interactions between different B vitamins in human diseases. For example, taking folic acid alone could mask vitamin B12 deficiency, which could lead to neurological problems [264]. Thus, some of the discussed limitations point towards the need for more rigorous and well-designed studies to investigate the significance of B vitamins in human health and disease.

The interplay of B vitamins in cancer biology emphasizes the importance of maintaining an equilibrium in vitamin consumption. Careful attention is required to identify the cancer risk in patients with vitamin deficiency. Additional extensive population-based studies are needed to understand the dietary intake of vitamins and cancer progression and therapy. Understanding the role of vitamins in combination with chemotherapeutic drugs and immunotherapeutic drugs is required to enhance the therapeutic efficacy and increase the survival rate.

Further, identifying the molecular mechanisms of how the vitamins-mediated cellular and metabolic pathways are involved in cancer initiation and progression will help identify potential therapeutic strategies. Recent proteomic, metabolomic, and genomic studies will help identify novel biomarkers. Next-generation gene sequencing will also help to identify potential carcinogenic genes regulated by vitamin supplementation. Further studies are also needed to understand the interactions between different B vitamins and their combined effects on cancer progression, which could lead to more targeted and personalized approaches in cancer therapy.

Thus, future research should focus on elucidating the precise mechanisms by which these vitamins influence cancer pathways and identify safe and effective dose levels. Taking over-the-counter vitamin supplementation without a physician's recommendation is sometimes a risk of developing unnecessary complications and side effects [265]. Since the requirement of vitamin supplementation depends on age, body metabolic rate, gender, and any prescribed medications, one should not self-diagnose and take supplements that unnecessarily can mask underlying health issues and complicate the disease progression [266]. Thus, physician input ensures vitamin supplements are safe, effective,

and tailored to a patient's needs. Therefore, always consult a healthcare provider before starting vitamin supplementation, who can recommend suggested doses based on specific medical conditions.

Author Contributions: Z.F., S.B., C.N.A., R.V.P. and K.V.R. drafted the initial manuscript, and analyzed, reviewed, and edited the final draft. K.V.R. conceptualized the idea and topic of the review, drafted, reviewed, and edited. Grammarly online software was used for language editing and improvement. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: All the authors declare no conflicts of interest.

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International Journal of
Molecular Sciences

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Review

Flavonoids and Their Role in Preventing the Development and Progression of MAFLD by Modifying the Microbiota

Aneta Sokal-Dembowska [1], Sara Jarmakiewicz-Czaja [1] and Rafał Filip [2,3,*]

[1] Institute of Health Sciences, Medical College of Rzeszow University, 35-959 Rzeszow, Poland; asokal@ur.edu.pl (A.S.-D.).; sjczaja@ur.edu.pl (S.J.-C.)
[2] Institute of Medicine, Medical College of Rzeszow University, 35-959 Rzeszow, Poland
[3] Department of Gastroenterology with IBD Unit, Clinical Hospital No. 2, 35-301 Rzeszow, Poland
* Correspondence: r.s.filip@wp.pl

Abstract: With the increasing prevalence and serious health consequences of metabolic-associated fatty liver disease (MAFLD), early diagnosis and intervention are key to effective treatment. Recent studies highlight the important role of dietary factors, including the use of flavonoids, in improving liver health. These compounds possess anti-inflammatory, antioxidant, and liver-protective properties. Flavonoids have been shown to affect the gut microbiota, which plays a key role in liver function and disease progression. Therefore, their role in preventing the development and progression of MAFLD through modulation of the microbiome seems to be of interest. This narrative review aims to consolidate the current evidence on the effects of selected flavonoids on MAFLD progression, their potential mechanisms of action, and the implications for the development of personalized dietary interventions for the management of liver disease.

Keywords: antioxidants; flavonoids; inflammation; liver disease; microbiota

1. Introduction

Liver disease is responsible for approximately 2 million deaths annually and approximately two thirds of all liver-related deaths occur in men [1]. The most common causes of chronic liver disease are hepatitis B and C virus infection, autoimmune diseases, alcohol consumption, steatosis associated with metabolic dysfunction (MAFLD), and cirrhosis, which is the final stage of various dysfunctions of this organ [2]. In addition, polymorphisms of certain genes can affect the occurrence of liver fibrosis, e.g., PNPLA-3 and MBOAT7 [3]. MAFLD accounts for 50% of cases of chronic liver disease [4] and cirrhosis is the 11th leading cause of death worldwide [5]. Chronic liver disease is characterized by progressive deterioration of liver function [6]. Therefore, the therapeutic approach is largely dependent on the degree of organ function and not only the type of disease itself [5]. Given the magnitude of the problem and the prognosis associated with chronic advanced liver disease and hepatocellular carcinoma, early diagnosis appears to be very important for the rapid implementation of appropriate treatment [7]. As MAFLD may become one of the most important chronic liver diseases, it is important to understand the phenotypes of the disease in order to be able to implement effective, individualized therapeutic therapies in individual patients [8].

Modern therapeutic approaches, depending on the cause of the disease, include drug therapies, including antivirals and immunosuppressants, and surgical treatment if liver transplantation is required. However, the critical importance of lifestyle changes in the prevention and treatment of liver disease cannot be overlooked [5]. Current nutritional recommendations focus primarily on preventing malnutrition and sarcopenia. Therefore, ensuring an adequate supply of protein and energy in patients' diets is considered a cornerstone. Another important element is an adequate intake of fruits and vegetables in the diet [9], which are a source of valuable compounds, such as phytonutrients, that have

protective, antioxidant, and anti-inflammatory effects [10,11]. Consumption of vegetables in general has been shown to be associated with a lower risk of liver cancer. In turn, increased consumption of cruciferous vegetables and legumes, among others, has been associated with a lower risk of mortality from chronic liver disease [12].

Widespread in nature, flavonoids are known for their many valuable biological properties [13] and their role in the treatment of MAFLD has been increasingly discussed by researchers in recent years. Flavonoids with potential beneficial effects on liver health include quercetin, rutin, luteolin, epigallocatechin-3, silibinin, puerarin naringenin, apigenin, genistein, and resveratrol, among others [14].

Previous data from the National Health and Nutrition Examination Survey (NHANES) and the Food and Nutrient Database for Dietary Studies have already suggested that using flavonoids offers a potential opportunity to prevent MAFLD [15]. Flavonoids may be a promising therapeutic component in the treatment of MAFLD and its complications, as a recent meta-analysis of randomized controlled trial data showed that flavonoids improve MAFLD through beneficial effects on liver function, lipid profile, and inflammation [16]. A flavonoid-rich diet was associated with a lower risk of MAFLD and lower imaging biomarkers of MAFLD in a prospective cohort study by Bell et al. [17].

In addition to anti-inflammatory or antioxidant effects, they can exert beneficial effects on the intestinal microbiota, modulating it by promoting or inhibiting specific microbial species in the intestinal tract, and modifying their metabolites [13]. Flavonoids may contribute to an increase in the relative abundance of intestinal probiotics such as *Bifidobacterium* and *Lactobacillus* while decreasing the relative abundance of *Lachnoclostridium* or *Bilophila* [18,19] In addition, they exert a protective effect on the intestinal epithelial barrier through the production of short-chain fatty acids (SCFAs) or lithocholic acid [20]. According to current evidence, flavonoids are characterized by their ability to inhibit lipid metabolism disorders and therefore can have an effect on reducing oxidative stress, inflammation, and disrupting the intestinal microbiota [13]. Flavonoids possess potent pharmacological activity, mitigating the effects of MAFLD and metabolic dysfunction-associated steatohepatitis (MASH). This is likely related to the effects of flavonoids on the gut microbiota, regulation of lipid metabolism, autophagy, oxidative stress, and inflammation in the body [21].

On this basis, this narrative review aims to update reports on the effects of selected flavonoids with potential applications in the treatment of chronic liver disease. The tables include detailed findings from studies over the past 5 years that analyzed the simultaneous effects of individual flavonoids on liver health, metabolic status, inflammation, and gut microbiota.

1.1. Chronic Liver Disease Pathogenesis

Hepatitis, steatosis, or cholestatic liver diseases cause continuous damage to liver cells, leading to organ fibrosis, its failure, and cirrhosis [22,23]. It has been shown that the onset and regression of fibrosis through various pathways is multidirectional and nonunilateral, for example, changing the MMP/TIMP-1 ratio and accelerating ECM degradation, inhibiting the TGF-β1 signaling pathway, reducing IL-6 and types I and III collagen expression [23]. The result of cellular injury is a naturally occurring process of the healing of damaged tissue, during which fibrogenic pathways are activated. The production of myofibroblasts (MFBs) that promote scar formation by activated hepatic stellate cells (HSCs) occurs [24]. Various cells, including Kupffer cells (KCs), hepatocytes, and numerous signaling pathways, can regulate HSC activation and are potential therapeutic targets that can influence the reversal of fibrosis [25]. For repair, fibrogenic components of the extracellular matrix (ECM) are secreted into the Disse space. During organ damage, the composition and density of the ECM are altered [26].

1.2. The Role of Inflammation and Oxidative Stress in Disease Progression

Damage to hepatocytes results in the release of threat-associated molecular patterns (DAMPs) or, in the case of infection, pathogen-associated molecular patterns (PAMPs).

Both DAMPs and PAMPs signal to pattern recognition receptors (PRRs) located on the surface of non-parenchymal cells and resident resistance cells. It is the activation of these cells through the interaction of PRRs and the aforementioned patterns that is responsible for the production of cytokines and chemokines that promote inflammation [24]. In addition, the inflammatory response can be sustained by the excessive production of reactive oxygen species (ROS). ROS can affect the activation and proliferation of HSCs, thus initiating and exacerbating fibrosis [25]. They are also responsible for the apoptosis and necrosis of hepatocytes, leading to the release of inflammatory mediators including transforming growth factor (TGF)-β and tumor necrosis factor (TNF)-α. Furthermore, they stimulate KCs to produce profibrogenic mediators and accelerate the recruitment of circulating inflammatory cells to the liver [26].

1.3. Intestinal Microbiota Disorders in the Development of Liver Diseases: Gut–Liver Axis

It has been shown that the gut–liver axis is essential for maintaining the normal physiological state of the body and plays an important role in the prognosis of many diseases, including in patients with MAFLD [27]. Liver diseases, including MAFLD and MASH, have been associated with intestinal dysbiosis and small intestinal bacterial overgrowth (SIBO) [28]. Yang et al. identified the characteristic microbial taxa of patients with MAFLD. They observed that a decrease in *Alistipes* is characteristic of this group of patients and that this decrease is negatively associated with glucose, gamma-glutamyltransferase (GGT), and alanine aminotransferase (ALT) levels. On the contrary, *Dorea*, *Lactobacillus*, and *Megasphaera* were enriched in the MAFLD group [29]. An analysis by Qi et al. showed a decrease in *Bacteroidetes* in patients with liver disease, including MAFLD and cirrhosis [30]. According to Boicean et al., fecal microbiota transplantation may become one of the treatments for cirrhosis [31]. Promising results in the treatment of MAFLD have also been associated with the use of rifaximin, prebiotics, probiotics, and glucagon-like peptide 1 (GLP-1) agonists [32]. However, well-designed clinical trials in this area are still lacking.

Changes in the microbiota and thus a decrease in intestinal mucosal epithelial barrier permeability and changes in metabolite release can promote liver regeneration. The gut microbiota can modulate the release of inflammatory factors including interleukin (IL)-6, interferon (IFN)-γ, TGF-β, TNF-α, and hepatocyte growth factor (HGF), thus regulating the immune microenvironment of the liver [33]. The gut microbiome can control hepatocytes via Gram-negative bacteria/lipopolysaccharide (LPS), which interacts with toll-like receptors (TLRs), especially TLR4, in KCs and HSCs [34].

Intestinal dysbiosis can lead to impaired gut–liver circulation, the action of farnesoid X receptor (FXR) agonists, and the production of BA itself [35]. Increased levels of total fecal BA, including cholic acid and chenodeoxycholic acid, are observed in patients with MAFLD [32]. In addition, similar changes are observed in patients with cirrhosis. Cholic acid and chenodeoxycholic acid are 7α-dehydroxylated exclusively by *Clostridium* bacteria, the amount of which is reduced due to reduced substrate levels. This leads to reduced conversion of primary BA to secondary BA in more pathogenic families such as *Enterobacteriaceae*, causing dysbiosis [36].

Recent evidence suggests that targeting the gut microbiota may help treat and mitigate the progression of liver disease. Thus, the microbiota taxa may be important therapeutic targets in this patient population.

2. The Effect of Flavonoids on Chronic Liver Diseases through the Modulation of Gut Microbiota

The available scientific data suggest that flavonoids may improve liver health by reversing the adverse effects associated with dysregulation of the gut microbiota. Flavonoid supplementation has been shown to increase SCFA production, reduce the *Firmicutes*/*Bacteroidetes* ratio, improve the intestinal barrier, and inhibit the growth of harmful bacteria [21]. Through interactions with the intestinal microbiota, flavonoids may influence lipid metabolism by modulating BA excretion and promoting the growth of microorganisms that play a positive

role in lipid metabolism, such as *Akkermansia muciniphila*, *Bifidobacterium*, and *Lactobacillus*. Reducing lipogenesis and genes related to inflammation and increasing the expression of genes related to fatty acid oxidation and fatty acid oxidase activity may lead to a reduction in fat accumulation and weight gain [13].

2.1. Silymarin

Silymarin is a mixture of polyphenolic molecules, more precisely flavonolignans, among which we can distinguish silibinin A, silibinin B, isosilibin A, isosilibin B, silicristin, isosilicristinm, the flavonoid tacphylline, and silidanin [37], an extract of spotted thistle that is considered a hepatoprotective compound [38]. Existing data highlight the role of the gut microbiota in the hepatoprotective effects of silymarin on the liver. However, it is still the case that most of the scientific evidence comes from animal model studies (Table 1) [32–35].

2.1.1. Effect of Silymarin on Liver Metabolic Status, Inflammation, and Gut Microbiota

Silymarin, due to its antioxidant properties, has been demonstrated to attenuate lipid peroxidation and free radical production. [38]. Moreover, it has been shown to have anti-inflammatory and anti-fibrotic effects [39]. Silybin has also shown promise in lowering glycemia and improving insulin sensitivity through peroxisome proliferator-activated receptor (PPAR)-γ activation [40,41]. In addition, silymarin may stimulate the endogenous production of antioxidants such as glutathione (GSH), catalase (CAT), glutathione peroxidase (GPX), and superoxide dismutase (SOD), and reduce inflammation by inhibiting the nuclear factor (NF)-κB and lowering TNF-α levels [40].

In an animal model study, Wang et al. confirmed that silymarin or silymarin with the salvianolic acid B and puerarin formula improved liver steatosis induced by a high-fat diet (HFD). Supplementation with these compounds improved liver function, including lowering pro-inflammatory cytokines and enabling more cholesterol to be metabolized to bile acids in the liver. Additionally, improvements in changes in the intestinal ecosystem contributed to increased SCFA production, which relieved MAFLD [42]. Wen-Long et al. also showed that silymarin supplementation contributed to microbiological changes. In addition, the study authors observed that these changes were associated with increased vitamin B12 production, which was associated with improved lipid metabolism and decreased pro-inflammatory cytokines [43]. Improvements in liver function were also observed by Guo et al. in a study conducted in laying hens. Supplementation of the diet with silymarin at a dose of 500 mg/kg resulted in a significant reduction in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels and improved the lipid metabolism of the cecal microbiota function. Therefore, it is likely that silymarin can alleviate bile acid stasis and increase the ability of metabolic microbes to metabolize bile acids [44]. Ralli et al. showed that silymarin can also potentiate the effects of probiotics alone in the treatment of MAFLD. In combination with piperine, fulvic acid, and probiotics, silymarin can significantly reduce liver steatosis by modulating the microbiota [45].

The first population-based study by Jin et al. showed that silymarin supplementation at a dose of 103.2 mg silibin per day for 24 weeks could have a significant protective effect against liver stiffness in patients with MAFLD by modulating the microbiota. Analysis of the gut microbiome showed that silymarin supplementation effectively modulated the composition and abundance of microbial populations [46].

Furthermore, results from a randomized placebo-controlled trial by Anushiravani et al. showed that silymarin supplementation at a dose of 140 mg/d for 3 months significantly reduced BMI and waist circumference in patients with MAFLD. Improvements in TGs, HDL, LDL levels, and the liver enzymes ALT and AST were also observed [47].

Mediterranean Diet Containing Flavonolignans in the Treatment of MAFLD

There has been evidence of a negative association between adherence to the Mediterranean diet and the degree of liver damage [48]. This dietary model is characterized by the consumption of foods rich in polyphenolic compounds, such as vegetables, fruit, whole-

grain cereals, olive oil, nuts, and red wine [49]. The use of a hypocaloric Mediterranean diet (MED) combined with supplementation with hepatoactive compounds, including silymarin, in overweight/obese patients for 3 months resulted in significant improvements in nutritional status, body weight composition, biochemical parameters, and degree of liver steatosis (LS) compared to the baseline. The supplementation group showed a significantly greater improvement in liver steatosis [50]. The results of a randomized controlled trial by Abenavoli et al. showed that an MED with an antioxidant complex containing milk thistle extracts can improve anthropometric parameters but can also reduce insulin resistance and fat accumulation in the liver [51].

The use of the MED is known to have a beneficial effect on the gut microbiota, as it contains many substrates necessary for the conversion of bioactive compounds. In addition, the MED diet can be tailored and personalized based on the individual's microbiome profile for optimal results [52].

This dietary model has applications in the treatment of chronic liver disease. A cross-sectional analysis of the RaNCD cohort showed that adherence to a MED diet, characterized by a high intake of fruits, vegetables, whole grains, legumes, nuts, and also fish, was associated with a lower risk of liver fibrosis in patients with MAFLD [53].

Table 1. Simultaneous effects of silymarin on the liver, metabolic state, inflammation, and gut microbiota.

Name of the Active Compound	Results of the Study	Reference
Animal studies		
Silymarin	• reduced accumulation of lipid droplets in the liver, reduced levels of liver TGs and serum TC; • improved glucose tolerance; • improvement in infulino resistance; • decreased levels of the liver inflammatory cytokines TNF-α and IL-6; • increase in *Akkermansia* and *Blautia*; • inhibition of proliferation of *Lactobacillus*, *Bacteroides*, *Clostridium*, and *Ileibacterium*.	Wang et al. (2024) [42]
	• improving liver function, lowering ALT and AST levels; • lowering levels of the liver inflammatory cytokines TNF-α and IL-6; • improving lipid metabolism, preventing accumulation of TGs and TC accumulation; • increase bacterial richness, increase ASV5 (*Akkermansia muciniphila*).	Sun et al. (2023) [43]
	• improving liver function, lowering ALT and AST levels; • decreased expression of genes related to lipid metabolism FXR, CYP7A1, BSEP, and MRP2; • significant reduction in TG and TC levels and lower serum LDL-C and HDL-C; • decreased endogenous bile acid synthesis and accelerated enterohepatic circulation; • increased expression of the bile acid receptor FXR and decreased expression of CYP7A1; • increasing the abundance of *Flavonifractor*; • improving the relative abundance of the genus *Phocaeicola*.	Guo et al. (2024) [44]

Table 1. *Cont.*

Name of the Active Compound	Results of the Study	Reference
Studies involving humans		
	• a significant decrease in liver stiffness measurement from baseline; • a significant difference in the change in serum GGT levels was found between the two groups (silymarin group and placebo) at 24 weeks; • no significant changes in mean CAP, serum ALT and AST levels, AST/ALT ratio, total bilirubin concentrations, or APRI or FIB-4; • AST/ALT, ApoA1, and SOD concentrations, ApoB levels significantly improved; • no statistically significant differences in the concentrations of TC, TGs, HDL-C, LDL-C, and ApoA1 and the ratio of ApoA1/ApoB between the groups; • no statistically significant differences in fasting blood glucose and insulin concentrations, HOMA-IR, and UA, SOD, and hsCRP concentrations before and after intervention between the two groups; • no statistical differences in physical parameters between the groups (DBP, SBP, BMI, WHR, BF% and BMR); • the abundance of *Selenomonadaceae* in the silymarin group decreased and *Oscillospiraceae* were significantly enriched.	Jin et al. (2024) [46]

Abbreviations: AST—aspartate aminotransferase, ALT—alanine aminotransferase, hsCRP—high-sensitivity C-reactive protein; LPS—lipopolysaccharide; MDA—malondialdehyde; GSH—glutathione; TC—total cholesterol; TG—triglycerides; HDL-C—high-density lipoprotein; LDL-C—low-density lipoprotein cholesterol; TNF-α—tumor necrosis factor-α, IL-6—interkulin-6; MPO—myeloperoxidase; HFD—high-fat diet.

2.2. Genistein

Genistein is an isoflavone widely distributed in legumes, especially in soybeans, but also in broccoli, cauliflower, and sunflower [54]. It is known for its antioxidant, anticancer, and cardioprotective properties. Genistein is used in the treatment of menopause due to its estrogenic effects. It is also considered an important modulator of various types of signaling pathways at the translational and transcriptional levels [55]. There are also data suggesting that genistein may reduce lipid accumulation, inflammation, insulin resistance, liver steatosis, and even liver fibrosis [56]. Furthermore, genistein supplementation has been associated with changes in the gut microbial community [57].

Obesity and visceral fat accumulation promote the development of both type II diabetes and MAFLD [58]. As current data indicate, the common characteristics of the aforementioned metabolic disorders are intestinal dysbiosis and chronic inflammation [59–61].

Effect of Genistein on Liver, Metabolic Status, Inflammation, and Gut Microbiota

Hou et al. showed that dietary genistein modulates homeostasis in the ageing gut and extends the health and life span of ageing mammals [62]. There are also data supporting the anti-inflammatory properties of genistein, although mostly from in vivo and in vitro studies. Genistein can exert such effects on the body, among other things, by inhibiting prostaglandins, nitric oxide synthase (iNOS), NF-κB or ROS, and scavenging free radicals [63]. Ortega-Santos et al. reached an interesting conclusion, investigating how genistein supplementation combined with a high-fat diet and exercise can affect inflammation and the gut microbiota. The intervention had a significant effect on increasing the abundance of *Ruminococcus*. Total levels of fecal bile acid and secondary fecal bile acid were significantly higher during exercise and as a result of the combination of exercise and supplementation with genistein. In addition, combining supplementation and exercise

helped prevent the negative effects of HFD and combined treatment resulted in lower serum IL-6 concentrations [57].

A high-fat diet not only causes obesity. It can also increase intrahepatic lipid accumulation and disrupt the integrity of the intestinal barrier [64]. It is likely that genistein has protective effects against high-fat diet-induced insulin resistance and liver steatosis also by maintaining glucose homeostasis through modulation of the insulin signaling pathway and liver energy status [65]. In a study by Guevara-Cruz et al., two months of genistein supplementation at a dose of 50 mg/day reduced insulin resistance in obese subjects, accompanied by a reduction in gut microbiome dysbiosis and metabolic endotoxemia. In particular, an increase in the type of *Verrucomicrobia* and in particular the abundance of *Akkermansia muciniphila* were observed [66]. Supplementation of mice with ulcerative colitis (UC) with genistein accelerated SCFA production (at doses of 20 mg/kg and 40 mg/kg for 10 days). Inhibition of weight loss, increased colon length, increased expression of mucus components, and decreased disease activity index values were also observed [67].

SCFAs are known for their ability to modulate inflammation and immune responses [68]. Furthermore, they play an important role in protecting the intestinal barrier by inhibiting IL17 production and promoting IL-10 production [68,69]. The supply of genistein to mice consuming a normal diet resulted in better glucose tolerance and greater expression of *UCP1* and *PGC1α* in white adipose tissue compared to mice that received no supplementation. An enrichment of the microbiota with bacteria of the genus *Blautia*, *Ruminiclostridium_5*, and *Ruminiclostridium_9* was also observed. Interestingly, changes in the microbiota were correlated with markers of browning of adipose tissue and glucose tolerance. On the contrary, in obese mules, genistein supplementation alleviated the effects of HFD and increased the expression of *UCP1* and *PGC1α* expression in brown adipose tissue [70]. A study by Ahmed et al. showed that low BAT activity was associated with greater visceral fat accumulation, but did not observe that this process was mediated by the gut microbiota [71].

A meta-analysis of 12 randomized clinical trials confirmed that genistein supplementation can significantly increase high-density lipoprotein (HDL-C) and lower serum levels of fasting blood glucose, insulin, triglycerides (TGs), and homocysteine [72]. In a randomized clinical trial by Neshatbini Tehran et al., the administration of genistein in combination with other isoflavones (genistin, daidzin, daidzein, glycitin, glyciteini) for 12 weeks at a dose of 100 mg/d significantly reduced TGs, LDL, TC, and waist and hip circumference in patients with MAFLD [73]. Possibly, genistein can improve liver lipid metabolism by activating the estrogen receptor β and further modulating Akt/mTOR signaling. Therefore, it may have applications in the prevention and treatment of hepatic steatosis in postmenopausal women [74]. Pummoung et al., in an animal model study, showed that MASH can be worsened by estrogen deficiency and genistein supply can alleviate liver steatosis and apoptosis by decreasing PPARγ expression and increasing adiponectin expression [75].

The results of available research on genistein supply for the prevention and treatment of chronic liver disease are promising. However, there is still a lack of data that examine the effects of its supply simultaneously on liver health, inflammation, metabolic status, and microbiota composition. This potential impact is shown in Figure 1.

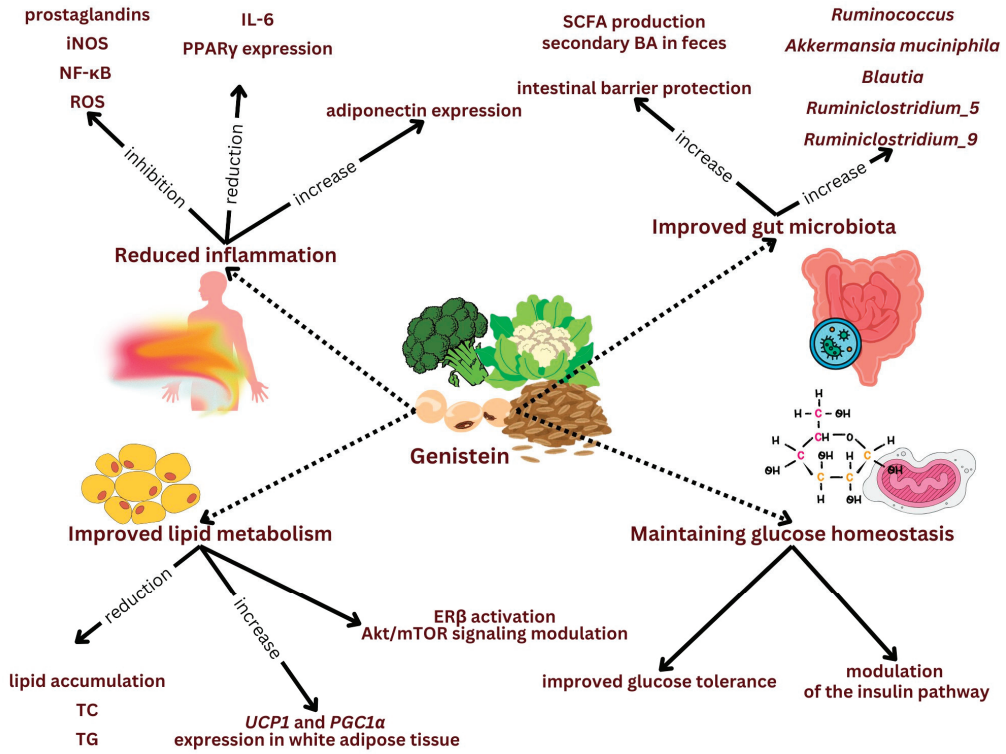

Figure 1. The potential effect of genistein in mitigating MAFDL and preventing its further development. Genistein exerts its effect through several mechanisms. It may influence the improvement of the intestinal microbiota, helps maintain proper glucose homeostasis, improves lipid metabolism, and contributes to reducing inflammation in the body.

2.3. Narginin and Naringenin

Naringin (NAR) and its aglycone form naringenin belong to the flavones from the flavonoids group and are mainly found in citrus fruits, including lemon, tangerine, grapefruit, or orange, as well as bergamot and tomatoes. Numerous articles point to their potential anticancer and neurodegenerative disease risk reduction effects [76,77]. The results of an exploratory randomized clinical trial conducted by Notarnicola et al. showed that orange consumption may be helpful in the adjunctive treatment of MAFLD. Consumption of oranges at 400 g per day for 4 weeks led to a reduction in hepatic steatosis and a decrease in plasma gamma glutaryl transferase [78].

Recently, the use of these substances in alleviating the symptoms of COVID-19 has also been studied due to their anti-inflammatory and antiviral properties [79].

Effect of Narginin and Naringenin on Liver, Metabolic Status, Inflammation, and Gut Microbiota

Currently available data suggest that NAR can attenuate the activity of ulcerative colitis, among other things, by reducing the secretion of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β), activating PPARγ, and inhibiting NF-κB. Similar effects have been shown to improve MAFLD. NAR supply can result in improved lipid profile parameters and reduced liver inflammation (inhibition of IL-1β, IL-6, IL-12, TNF-α, and IFN-γ). NAR has also been shown to inhibit NF-κB and MAPK signaling pathways in both UC and MAFLD [80].

The supply of NAR in mice fed HFD diets in a study by Mu et al. attenuated HFD-induced hepatic lipid accumulation and weight gain and affected the structure of the gut bacterial community [81]. Similar results were obtained by Yu et al. with the use of naringenin. The authors of the study concluded that it could effectively alleviate liver and intestinal inflammation in the course of MAFLD. The results of the study showed that naringenin can effectively reduce MASH by improving steatosis, inflammation, and

swelling of liver cells. In addition, it can protect the intestinal barrier by regulating tight junction proteins [82]. According to Cao et al., the supply of naringenin can effectively inhibit the progression of MASH, reversing adverse changes by mitigating cellular damage, lipid deposition, and reducing oxidative stress in the liver. Furthermore, the authors speculate that naringenin may inhibit the development of steatosis and inflammation by lowering levels of lysophosphatidylethanolamine (LPE) [83]. The consumption of bioactive compounds, including narginine, can favorably influence intestinal bacterial growth and gene expression and increase the abundance of bacteria responsible for SCFA production [84,85]. The supply of orange juice to a group of 10 women for 2 months positively modulated the composition and metabolic activity of the microbiota, increasing the population of fecal *Bifidobacterium* spp. and *Lactobacillus* spp. In addition, changes in blood biochemical parameters such as a reduction in LDL and glucose levels and an improvement in insulin sensitivity were observed [85]. Results from a randomized, double-blind, placebo-controlled clinical trial by Namkhah et al. showed that the daily administration of 200 mg of naringenin for 4 weeks had a beneficial effect on lipid profile and the percentage of MAFLD grade as an indicator of the severity of liver steatosis [86]. Furthermore, according to Naeini et al., naringenin supplementation may be a promising strategy for the treatment of cardiovascular complications in patients with MAFLD [87].

The current scientific reports on the simultaneous effects of naringin and naringenin on microbiota composition and liver health are presented in Table 2.

Table 2. Simultaneous effects of narginine and nirginine on the liver, metabolic state, inflammation, and gut microbiota.

Name of the Active Compound	Results of the Study	Reference
Animal studies		
Naringin	• reduction in serum lipids, ALT, AST, glucose, hsCRP, and LPS; • attenuation of liver inflammation; • negative correlation of TC, HDL-C, and LDL-C parameters with *Allobaculum*, *Alloprevotella*, *Butyricicoccus*, *Lachnospiraceae_NK4A136_group*, *Parasutterella*, and *uncultured_bacterium_f_Muribaculaceae*; • *Campylobacter*, *Coriobacteriaceae_UCG-002*, *Faecalibaculum*, and *Fusobacterium* were positively correlated with serum lipids; • reduction in ALT levels; no differences in AST levels; • restoration of normal serum glucose and hsCRP levels.	Mu et al. (2020) [81]
Naringenin	• less weight gain; • reduction in fat mass and liver mass; • reduction in serum TC and TG levels; • reduction in inflammatory factors (TNF-α, IL-6 and inflammatory marker F4/80) and MPO activity; • inhibition of infiltration of colon macrophages and dendritic cells; • improvement in intestinal permeability; • alleviation of the decrease in colonic protein levels (ZO-1, claudin-1, and Occludin); • increase in claudin-2 levels; • inhibition of the downward trend induced by HFD in gene expression; • decreasing the abundance of *Proteobacteria* and increasing the abundance of *Bacteroidetes*; • increasing the levels of *Epsilonproteobacteria* and *Deltaproteobacteria*; • inhibition of cecal bacterial overgrowth.	Yu et al. (2023) [82]
	• lowering TC and TG levels; • improving AST, ALT, MDA, and GSH levels; • increasing KN-93 levels and inhibiting hepatic stellate cell proliferation; • restoring favorable levels of *Akkermansia* and *Enterobacter*; • increasing the [*Eubacterium*] *nodatum* group.	Cao et al. (2023) [83]

Abbreviations: AST—aspartate aminotransferase, ALT—alanine aminotransferase, hsCRP—high-sensitivity C-reactive protein; LPS—lipopolysaccharide; MDA—malondialdehyde; GSH—glutathione; TC—total cholesterol; TG—triglycerides; HDL-C—high-density lipoprotein; LDL-C—low-density lipoprotein cholesterol; TNF-α—tumor necrosis factor-α, IL-6—interkulin-6; MPO—myeloperoxidase; HFD—high-fat diet.

2.4. Resveratrol

Resveratrol is a polyphenol known for its powerful antioxidant, anti-inflammatory, anti-cancer, neuroprotective, and anti-diabetic properties. In addition, it is believed to be an aid in the treatment of cardiovascular disease or obesity. The main sources of this compound are blueberries, grapes, peanuts, and red wine [88,89]. A recent meta-analysis of preclinical and clinical studies demonstrated that resveratrol at a dose of 50–200 mg/kg and a time interval of 4–8 weeks showed an effect in alleviating MAFLD in preclinical studies [90]. It is likely that the hepatoprotective effect of resveratrol is related to inhibition of the NF-κB pathway and activation of the SIRT-1 (sitrulin 1) and AMPK (activated protein kinase) pathways, although the results of clinical trials are still inconclusive [90,91].

Asghari et al. found that resveratrol supplementation could affect weight loss in patients with MAFLD, but the benefits of calorie restriction were greater and were associated with reductions in body weight, BMI, waist circumference, and ALT and AST activity [92]. Different results were obtained by Chen et al. Supplementation with resveratrol at a dose of 150 mg twice daily for 3 months resulted in a reduction in AST, ALT, glucose, LDL cholesterol, TC, and the insulin resistance index [93]. Nevertheless, there are also data from clinical trials that do not support the efficacy of RSV supplementation in the treatment of MAFLD [94–100].

Effect of Resveratrol on the Liver, Metabolic Status, Inflammation, and Gut Microbiota

Resveratrol (RSV) and its derivatives appear to also modulate the composition of the intestinal microbiota and to affect the intestinal barrier function and intestinal tight junction [101,102]. Cai et al. showed that resveratrol improves intestinal barrier function and attenuates intestinal permeability and inflammation in mice with diabetic nephropathy. Furthermore, the supply of resveratrol reversed dysbiosis characterized by a low abundance of the genera *Alistipes*, *Alloprevotella*, *Bacteroides*, *Odoribacter*, *Parabacteroides*, and *Rikenella* [103]. Interestingly, resveratrol has been shown to alleviate liver fibrosis precisely by affecting the composition of the microbiota. Li et al. observed that this could occur by inhibiting the growth of *Staphylococcus_xylosus* and *Staphylococcus_lentus* [104]. Nevertheless, the evidence for this is still inconclusive. In a study in an animal model, resveratrol administration alleviated steatosis and hepatitis induced by high-fat and high-fructose diets. However, no differences in microbiota composition were observed, although the abundance of *UBA-1819* was associated with a lower liver weight in animals that received resveratrol concurrently [105], although an earlier observation by this team showed a greater presence of *Lactococcus* and *Blautia* bacteria in the microbiome of animals receiving resveratrol [106]. Similarly, Du et al. also observed differences in the gut microbiota of mice with MAFLD even with a low RSV supply. At the type level, the predominant bacteria were *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*. A tendency was also observed to promote the growth of *Erysipelotrichaceae* and inhibit the growth of *Ruminococcaceae*. It has also been shown that RSV can restore the expression of genes related to lipid metabolism to normal levels and repair the damaged insulin signaling pathway [107]. In a study by Wang et al., resveratrol supply attenuated weight gain in HFD-fed mice and inhibited fat accumulation without affecting energy intake. Resveratrol treatment improved glucose tolerance and insulin resistance. Improvements in intestinal permeability, intestinal villi length and density, and effects on uniform distribution were also observed. Increased mRNA expression of tight junction proteins has also been reported [108].

Current scientific reports on the simultaneous effects of resveratrol on microbiota composition and liver health are presented in Table 3.

Table 3. Simultaneous effects of resveratrol on the liver, metabolic state, inflammation, and gut microbiota.

Name of the Active Compound	Results of the Study	Reference
Animal studies		
Resveratrol	• lowering liver weight; • lowering serum TC and non-HDL cholesterol levels, and increasing serum HDL cholesterol levels; • reduction in AST and ALT levels; • no significant changes in the gut microbiota.	Milton-Laskibar et al. (2022) [105]
	• downward trend in ALT, AST, TG, cholesterol, and LDL-C levels and body weight; • reduction in TG levels and fasting blood glucose and HOMA-IR insulin levels; • increased relative abundance of *Actinobacteria*; • increased levels of zo-1 and occludin protein and zo-1 gene expression; • decreased levels of signaling molecules; • TLR4 and MyD88 and inflammatory IL-1 and TNF-α.	Du et al. (2021) [107].
	• complete inhibition of HFD-induced MDA, ROS, and GSH-Px, and restoration of SOD and T-AOC activity in the liver of HFD-fed mice; • restoration of normal HDL, LDL, ALT, and AST levels; • reduction in TC and TG levels (including lower liver concentrations); • improvement in fasting glucose and insulin values; • increased mRNA expression of tight junction proteins (ZO-1, ZO-2, and occludin) and interstitial adhesion molecule A (JAM-A); • restoration of the expression of TFF3, Muc1, Relmβ and Reg3γ; • increase in relative abundance of *Allobaculum*, • *Bacteroides*, and *Blautia* in mice fed an HFD diet; • increased mRNA expression of the cytokine IL-10 and decreased mRNA expression of pro-inflammatory cytokines such as IL-16, IL-6, and IL-1β; • increased mRNA expression of genes related to • mitochondrial function, such as NRF-1, Tfam, UCP3, PGC-1α, and SIRT1.	Wang et al. (2020) [108]
	• decreased collagen deposition in the liver; decreased expression of α-SMA and Collagen I in liver tissues; • inhibition of ZO-1 loss; • partial improvement in ALT, AST, and ALP.	Li et al. [45]

Abbreviations: TC—total cholesterol; HDL—high-density lipoprotein; AST—aspartate aminotransferase, ALT—alanine aminotransferase; TG—triglycerides; LDL-C—low-density lipoprotein cholesterol; HOMA-IR—homeostasis model assessment of insulin resistance; TFF3—trefoil factor family protein 3; Relmβ—resistin-like molecule β; NRF-1—nuclear respiratory factor 1; Tfam—mitochondrial transcription factor A; UCP3—uncoupling protein 3; PGC-1α—peroxisome proliferator-activated receptor gamma coactivator 1-alpha; SIRT1—sirtuin 1; α-SMA—α-alpha-smooth muscle actin; TLR4—toll-like receptor 4; MDA—malondialdehyde; SOD—superoxide dismutase; T-AOC—total antioxidant capacity; HFD—high fat diet; ROS—reactive oxygen species; ZO-1—zonula occludens-1; ZO-2—zonula occludens-2; ALP—alkaline phosphatase.

2.5. Quercetin

Quercetin is an agglucon or aglycone that does not contain any carbohydrate molecules in its structure. Rich dietary sources of quercetin include capers, rocket, dill, coriander, fennel, juniper berries, elderberries, field poppy, bee pollen, and muskmelon [109]. Quercetin is characterized by its anti-inflammatory, anti-cancer, and antiviral properties and has been used in the treatment of age-related diseases, diabetes, cardiovascular disease, and hypertension [110]. Recent data show that it can prevent the onset of Alzheimer's disease by inhibiting amyloid β aggregation [111]. The molecular structure of quercetin allows it to eliminate oxygen-free radicals and produce metal-chelating compounds [112]. In addition, quercetin may have a role in the reduction in abdominal obesity through its effects on the gut microbiota [113].

Effect of Quercetin on Liver, Metabolic Status, Inflammation, and Gut Microbiota

There are a number of data supporting the effect of quercetin on the development of liver disease through various mechanisms. Analysis by Zhao et al. showed that quercetin can inhibit liver inflammation primarily through NF-κB/TLR/NLRP3 and can also reduce phosphatidylinositol 3-kinase (PI3K)/nuclear factor erythroid 2-related factor 2 (Nf2)-dependent oxidative stress. In addition, it can activate mTOR in autophagy and inhibit the expression of apoptotic factors associated with the development of liver diseases. Interestingly, the results indicate that quercetin has an effect on the regulation of PRAR, UCP, and protein perlipin (PLIN) factors through activation of brown adipose tissue in liver steatosis [114]. Increased UCP1 expression is associated with increased WAT browning and BAT activity, by activating the AMPK/PPARγ pathway, which can help prevent obesity and metabolic complications [115]. Pei et al. observed that quercetin could upregulate UCP1 gene expression in HFD-fed mice, leading to the bronzing of retroperitoneal WAT and weight reduction. Quercetin also decreased the ratio of *Firmicutes* to *Bacteroidetes* and increased SCFA production [116].

In a study by Feng et al. involving laying hens in which mucosal damage, necrosis, and exfoliation were induced, quercetin markedly increased total antioxidant capacity and glutathione peroxidase activity. Quercetin supplementation restored goblet cell density, mucin2 expression levels, and Claudin1 and Occludin mRNA expression in the intestinal mucosa. The expression of IL-1 β i TLR-4 in the intestinal mucosa and an increase in bacteria responsible for SCFA production, such as *Negativicutes*, *Phascolarctobacterium*, *Megamonas*, *Prevotellaceae*, *Bacteroides_salanitronis*, and *Selenomonadales*, were also observed [117]. Transplantation of the gut microbiota of donors who respond to the HFD diet predisposed germ-free mice to MAFLD and the supply of quercetin had a protective effect on the development of metabolic changes by blocking the gut–liver axis. Moreover, a protective role for *Akkermansia* genus bacteria was observed in the development of MAFLD [118]. In a study by Juárez-Fernández et al., the simultaneous administration of *Akkermansia muciniphila* and quercetin resulted in steatosis remission, associated with modulation of liver lipogenesis. Synbiotic supply increased the plasma levels of unconjugated hydrophilic bile acids and increased liver expression of BA synthesis and transport genes [119]. Zhao et al. also confirmed the existence of the gut–hepatic axis. The supply of *Lactobacillus plantarum NA136* corrects gut microbiota disorders caused by a fat- and fructose-rich diet by strengthening the intestinal barrier and reducing inflammation in the liver [120].

A meta-analysis of 16 case–control studies in recent years showed that quercetin supplementation is associated with lower levels of total cholesterol (TC), low-density lipoprotein (LDL), and C-reactive protein (CRP) in patients with metabolic syndrome and related disorders [121]. The results of in vivo and in vitro studies indicate that quercetin and its derivatives exert hepatoprotective effects against MAFLD by regulating oxidative stress and inflammation, and also through changes in the gut microbiota [122]. In Porras et al.'s study, quercetin supplementation reduced intrahepatic lipid accumulation through the modulation of lipid metabolism gene expression, cytochrome P450 2E1 (CYP2E1)-dependent lipoperoxidation, and associated lipotoxicity. Thus, it reduced insulin resistance and MAFLD activity and reversed dysbiosis [123]. The results of a randomized, double-blind, placebo-controlled, crossover study showed that a 500 mg dose of quercetin for 12 weeks was associated with reductions in intrahepatic lipids, body weight, and BMI [124].

The result of a study by Abd El-Emam et al. showed that the administration of quercetin liposome (QR-lipo) can protect against Co-Amox-induced hepatotoxicity. The combined administration of Co-Amox and QR-lipo significantly improved antioxidant status. Furthermore, a protective effect of QR-lipo against antibiotic-induced intestinal dysbiosis was observed [125]. Combination therapy with quercetin and anti-PD-1 antibodies altered necrosis, fibrosis, and PD-L1 expression in liver tissues, thus reducing the tumor microenvironment of HCC in mice, while upregulating the gut microbiota and macrophage immunity. An increase in the abundance of *Firmicutes*, *Actinobacteria*, and *Verrucomicbiota* has also been reported at the type level, as well as an increase in *Dubosiella* and *Akkermansia*

at the genus level [126]. Similarly, in a study by Xie et al., the administration of buckwheat quercitin to rats at a dose of 200 mg/kg bw had a positive effect on the gut microbiota and significantly reduced body weight, the liver index, and lipid levels, thus reducing oxidative stress and positively influencing the repair of damaged liver [127].

The current scientific reports on the simultaneous effects of quercetin on microbiota composition and liver health are presented in Table 4.

Table 4. Simultaneous effects of quercetin on the liver, metabolic state, inflammation, and gut microbiota.

Name of the Active Compound	Results of the Study	Reference
Animal studies		
Quercitin	• reduction in ALT and AST concentrations; • no effect on albumin concentrations was noted; • decrease in MDA levels and increase in GSH, CAT, and TAC levels; • activation of IL-10 gene expression; • increased expression of SIRT1 and Nrf2; • decreased expression of pro-inflammatory liver factors, including IL-6, IL-1β, TNF-α, NF-κB and iNOS, • reduction in Keap1 levels; • increase in the abundance of *Bifidobacterium*, *Bacteroides*, and *Lactobacillus*; • reduction in *Clostridium* and *Enterobacteriaceae*.	Abd El-Emam et al. (2023) [125].
	• stunted weight gain; • lowering liver index; • lowering TC, TG, and LDL-C levels and increasing HDL levels; • alleviation of lipid peroxidation (higher levels of GSH-PX, lower CHE levels); • lowering the levels of NF-kB, TLR4; • lowering the ratio of *Firmicutes* to *Bacteroidetes*; • reduction in the abundance of proteobacteria; reduction in the growth of *Lachnospiraceae*; • increased the amount of relative abundance of *Oscillospiraceae*, *Ruminococcaceae*, *Christensenellacea*, and *Eubacterium*_co-prostanoligenes_group.	Xie et al. [127].

Abbreviations: AST—aspartate aminotransferase, ALT—alanine aminotransferase; MDA—malondialdehyde; GSH—glutathione; CAT—catalase; TAC—total antioxidant capacity; IL-10—interleukin 10; SIRT1—sirtuin 1; Nrf2—nuclear factor erythroid 2-related factor 2; IL-6—interleukin-6; IL-1β—interleukin 1β; TNF-α—tumor necrosis factor α; NF-κB—nuclear factor kappa B; iNOS—inducible nitric oxide synthase; TC—total cholesterol; TG—triglycerides; LDL-C—low-density lipoprotein cholesterol; GSH-PX—plasma glutathione peroxidase; CHE—cholinesterase; TLR4—toll-like receptor 4.

3. Long-Term Use Safety of the Flavonoids Discussed in the Review

According to Soleimani et al., silymarin is safe for human consumption at therapeutic doses and can be well tolerated, even at a high dose of 700 mg given three times daily for 24 weeks [128]. Toxicity studies in animals treated long-term with silymarin confirmed its high tolerability and studies with humans provided data on the most common side effects such as headaches and itching, which can occur at high doses [129].

The threshold for no observed adverse effects from genistein is considered to be 50 mg/kg/day, based on the presence of mild liver effects at the high dose of 500 mg/kg/day [130]. Genistein is generally considered to be well tolerated and safe. Minimal toxicity may include symptoms of nausea, leg cramps, or tenderness and may occur at a dose of 16 mg/kg body weight [131].

According to Rebello et al., naringenin doses of 150–900 mg are safe in healthy adults, and serum concentrations are proportional to the dose administered. Naringenin metabolites present in the circulation are eliminated within 24 h [132]. Toxicity studies have

confirmed the safety of naringenin in animal models, suggesting its potential for safe administration to humans. However, controlled clinical trials are needed to confirm its safety for long-term use [133].

Current data suggest that quercitin supplementation is safe, and adverse effects in humans have been rare in the studies that have been conducted. However, some results from animal studies suggest that its long-term use may have nephrotoxic effects, especially in cases of pre-existing kidney damage [121].

Randomized clinical trials suggest that resveratrol supplementation is safe in both adults and adolescents [94,134]. Short-term resveratrol supplementation at doses of 300 mg/day and 1000 mg/day does not adversely affect blood chemistry and is well tolerated by overweight older adults [135]. RSV has shown beneficial effects on cognitive function in the elderly, but at high doses increased some biomarkers of cardiovascular risk (at a dose of 1000 mg for 90 days). Therefore, it is considered that optimal dosing, long-term effects, and potential drug interactions need to be investigated in well-designed randomized clinical control trials [135,136].

4. Conclusions

Liver diseases, including MAFLD, are a serious health problem, leading to high mortality rates and the risk of developing cirrhosis and hepatocellular carcinoma. Flavonoids, natural compounds present in a variety of plant products, have shown promising therapeutic properties in the context of liver health. Numerous studies show that flavonoids can improve the lipid profile of the liver, reduce inflammation and oxidative stress, and modulate the composition of the gut microbiota. In particular, constituents such as silymarin, genistein, quercetin, and narginine/nirginine have shown the ability to reduce lipid accumulation in the liver, improve glucose metabolism, and alleviate inflammation.

Additionally, their effects are often associated with improved intestinal barrier function and the modulation of gut microbiota activity, which may contribute to protection against MAFLD progression. Supplementation and dietary changes based on flavonoid sources can lead to significant improvements in health parameters for patients with liver disease. As a result, understanding the role of flavonoids in the management of MAFLD opens new opportunities for nutritional therapies that can be customized to optimize liver health and reduce the risk of severe complications associated with chronic liver disease. Still, most of the scientific evidence comes from preclinical studies, which is why well-designed long-term clinical trials are so important.

Author Contributions: Conceptualization, A.S.-D., S.J.-C. and R.F.; writing—original draft preparation, A.S.-D. and S.J.-C.; writing—review and editing, A.S-D., S.J.-C. and R.F.; supervision, R.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

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

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Review

The Bioactivity and Phytochemicals of *Muscari comosum* (*Leopoldia comosa*), a Plant of Multiple Pharmacological Activities

Varun Jaiswal [1,*,†] and Hae-Jeung Lee [1,2,3,†]

[1] Department of Food and Nutrition, College of BioNano Technology, Gachon University, Seongnam 13120, Republic of Korea; skysea@gachon.ac.kr or skysea1010@gmail.com
[2] Institute for Aging and Clinical Nutrition Research, Gachon University, Seongnam 13120, Republic of Korea
[3] Department of Health Sciences and Technology, GAIHST, Gachon University, Incheon 21999, Republic of Korea
* Correspondence: compuatationalvarun@gmail.com or varunjais1@gachon.kr; Tel.: +82-1040709935 or +91-9667526228
† These authors contributed equally to this study.

Abstract: *Leopoldia comosa* (LC), popularly known as *Muscari comosum*, spontaneously grows in the Mediterranean region and its bulbs are used as a vegetable. Traditionally, they are also used to treat various diseases and conditions, which has inspired the study of the pharmacological activities of different parts of LC. These studies revealed the numerous biological properties of LC including antioxidant, anti-inflammatory, anti-diabetes, anti-obesity, anti-cancer, anti-Alzheimer's disease, antibacterial, and immune stimulant. High antioxidant activity compared to other non-cultivated plants, and the potential role of antioxidant activity in other reported activities make LC an excellent candidate to be developed as an antioxidant plant against important associated diseases. The presence of a diverse class of phytochemicals (n = 85), especially flavonoids and homoisoflavones, in LC, also imparts significance to the nutraceutical candidature of the plant. However, limited animal studies and the lack of a directional approach have limited the further design of effective clinical studies for the development of LC. The current study is the first attempt to comprehensively compile information regarding the phytochemicals and pharmacological activities of LC, emphasize the targets/markers targeted by LC, important in other activities, and also highlight the current gaps and propose possible bridges for the development of LC as a therapeutic and/or supplement against important diseases.

Keywords: pharmacological activities; cancer; phytochemicals; *Muscari comosum*; obesity; diabetes; antioxidants; *Leopoldia comosa*

1. Introduction

Leopoldia comosa (L.) Miller (LC), popularly known as *Muscari comosum*, is a vegetable spontaneously growing in the Mediterranean region, including southern and central Europe, northern Africa, and southern-western Asia [1]. The bulbs of LC are commonly called lampascioni or cipudizze (in Italy) and Bassila (in Morocco) and are known to have been used as food for a long time. People from Egypt, Greece, and the Mediterranean region have been habituated to eating LC bulbs [1,2]. LC also has economic significance, as the trade in the plant bulbs is an important income source for collectors in Morocco through export, especially to Italy [2]. Nowadays, the bulbs are used in a variety of recipes, including boiled bulbs served with sweets; peeled, cut, and fried in olive oil and served with cheese or eggs; boiled after 24 h of soaking in water and dressed with vinegar, oil, salt, and pepper; pickled bulbs in the Benevento region; and in the Murge region, cooked covered in hot ash, and dressed with oil, salt, and pepper after removing the outer layer. Bulbs are also used as an alternative to onions and are sometimes eaten raw but it is not

common practice, as cooking is required to neutralize their characteristic bitter taste [1]. Despite various uses, LC is generally not cultivated because it is easy to find it growing in wild conditions.

LC is also a rich source of various phytochemicals, which give its unique taste. Several phytochemicals (n = 85) have been reported in LC, mostly identified in bulbs. These diverse phytochemicals belong to different classes, such as phenolic acids, fatty acids, flavonoids, triterpenes, phytosterols, and homoisoflavones. Among the phytochemicals, flavonoids and homoisoflavones are more emphasized for the reported pharmacological activities of the bulbs [3].

Traditionally, LC has been used to treat various diseases and conditions, such as dermatological affections, digestive disorders, toothache, pus discharge from the lungs, and spots; it has also been used as a diuretic and emollient [1,2,4]. Inspired by the traditional uses, the medicinal properties of LC have been investigated in several pharmacological studies, especially in recent years. In these studies, LC was found to have different pharmacological properties including antioxidant, anti-inflammatory, anti-diabetes, anti-obesity, anti-cancer, anti-Alzheimer's disease (AD), antibacterial, and immune stimulant in various in vitro and in vivo experiments.

The antioxidant activity of LC was investigated in the majority of studies, and it was found to be the highest in screening experiments among other plant extracts [5]. One of the primary reasons for focusing on LC's antioxidant activity is that its antioxidant properties may also contribute to the majority of other reported LC activities, such as anti-inflammatory, anti-diabetes, anti-obesity, anti-cancer, anti-AD, and immune stimulant [6–9]. The presence of various phytochemicals, especially polyphenols such as flavonoids and homoisoflavones, in different parts of the plant such as bulbs, leaves, and inflorescence also supports the antioxidant activities of these parts.

Despite its gastronomical importance, high mineral contents, the presence of numerous biologically active phytochemicals, and several potential biological properties, the complete compilation of the phytochemicals and pharmacological activities of LC is still absent in the literature [10]. This may be an important reason for the underutilization of this vegetable. Hence, in the current study, the comprehensive compilation of phytochemicals as well as pharmacological activities of LC has been attempted for the first time, which also highlights the current gaps and proposes possible bridges for the development of LC as a therapeutic and/or supplement against important diseases. Another novel aspect of this study is that it also points out imported drug targets and markers modulated by LC, and emphasizes their importance in other known pharmacological activities to promote them in future research and development studies.

2. Literature Search

The systematic literature search was carried out against important scientific literature databases such as Web of Science, Scopus, and PubMed. The keywords utilized to search the databases were *Muscari comosum*, *Leopoldia comosa*, and their combinations with phytochemicals, phytoconstituent, disease, obesity, diabetes, cancer, antioxidant activities, and inflammation and pharmacological activities. The relevant research and review articles, books, book chapters, and other documents from the search results published in English until December 2023 were considered in this study.

3. Botanical Description

LC is a perennial bulbous flowering plant that belongs to the family Asparagaceae (Figure 1). The LC bulbs are pink to reddish, spherical to oval in shape [1]. The leaves of the plant are linear, with a size of around 7–40 × 0.5–1.7 cm. The leaves are mostly shorter than the scape which is 80 cm long. The scapeis erect and has a cylindrical and glabrous shape. The raceme of the plant is also cylindrical in shape; it can be pyramidal and lax. The fertile and sterile flowers are pale brown and bright violet and later become smaller. The fertile flowers are urceolate and patent at the anthesis, with pedicel lengths

between 4 and 10 mm. The sterile flowers are from globose to obovoid in shape and they are assembled in a corymbose terminal tuft with pedicels between 2 and 6 mm long, fleshy, ascending, and violet in color. The obovoid-shaped capsule of LC is the size of around 10–15 × 6–8 mm. The pollen grains are monads, monosulcate, ellipsoidal, isopolar, and bilaterally symmetrical. Flowering usually occurs in March and April in the Mediterranean region, and pollination happens through insects [11].

Figure 1. Image of *Leopoldia comosa* (*Muscari comosum*) [image by Emilian Robert Vicol from Pixabay].

4. Toxicity and Adverse Reactions

Limited toxicity studies on LC have been conducted in the literature, as LC bulbs have been used as a vegetable in different populations. The toxicity of the different types of extracts from the bulbs was around 20% in HepG2 cells at up to a 600 µg/mL dose. In the same study, the 70% methanol extract of the bulbs helped in the proliferation of cells in 24-h treatment even at the highest concertation of the extract. However, a reduction in cell viability was observed in 72-h treatment. In a similar study, the extracts of raw and cooked bulbs showed a different inhibition activity against the MCF-7 cell line. The extract from the cooked bulb had an IC_{50} value of 669.3 µg/mL while the extract from the raw bulb had 10.27 µg/mL [12]. In animal experiments, up to 60 mg/kg of the dose has been used comfortably on rats in models as a treatment against obesity [13]. Allergy to LC bulbs is rare: only one case has been reported. In this rare example, IgE-mediated allergies to LC bulbs were observed in a 32-year-old patient after ingestion of a very low quantity of it [14].

5. Phytochemicals Reported in LC

Several phytochemicals have been reported in the bulbs and other aerial parts of LC such as leaves and inflorescence (Table 1). Most of these studies were focused on bulbs, as the bulb of the plant is the part that is used as a dietary vegetable. The prime objective behind the phytochemical identification in LC bulbs was the discovery of phytochemicals responsible for the reported biological activities and taste of the plant bulbs. Hence, in several studies, further biological properties were also analyzed after the phytochemical studies. Various important phytochemicals discovered in these studies may explain the reported biological properties of LC.

In the initial study, inspired by the antitumor activity of LC bulbs, the mixture of glycosides from the bulbs was isolated through extraction [15,16]. After the acidic methanolysis, through nuclear magnetic resonance (NMR), one important aglycone moiety was identified as 27-norlanostane triterpene eucosterol [15] (Table 1). The researchers also identified two more compounds (ketotriol and diketotriol), based on the same 27-norlanostane skeleton from the mixture [17] (Table 1). Later, the researchers isolated the nortriterpene fraction from the extract of LC bulbs, and a series of nortriterpenes were identified, including

(23*R*)-17,23-epoxy-33,31-dihydroxy-27-nor-5α-lanost-8-ene-15,24-dione and (23*R*)-17,23-epoxy-31-hydroxy-2 7-nor-5α-lanost-8-ene-3,15,24-trione [18,19]. In a series of studies, researchers also isolated and elucidated the structure of various glycosides, i.e., muscarosides A, B, C, D, E, and F [15,20–23].

Later, the presence of a new class of natural phytochemical homoisoflavonoids (3-benzylidenechroman-4-ones) in LC bulbs prompted research to identify the new compounds of this class. Like glucosides, in a series of studies, the researchers identified compounds of this class including muscomosin, comosin, 8-*O*-demethyl-8-*O*-acetyl-7-*O*-methyl-3,9dihydropunctatin, 3′-hydroxy-3,9dihydroeucomin, 4′demethyl-3,9-dihydroeucomin, 7-*O*-methyl-3,9_dihydropunctatin, 8-*O*-demethyl-7-*O*-methyl-3,9_dihydropunctatin, and 3,9-dihydroeucomnalin in the bulbs [3,24,25]. NMR imaging was used for the spectral characterization of the compounds [24].

The researchers also conducted a study for the identification of compounds that are important for the taste of LC bulbs (taste-active compounds). A series of solvents (hexane, ether, dichloromethane, ethanol, and water) according to polarity was used to extract and identify the compounds present in the bulbs. Among these solvents, ether solvent was used to identify phytochemicals responsible for the taste, as the taste of this fraction was selected by the sensory evaluation team. ^{1}H-NMR spectroscopy was used to identify the pure component of the extract separated through column chromatography, followed by preparative thin layer chromatography (TLC). Three identified compounds, muscomin, 8-*O*-demethyl-7-*O*-methyl-3,9-dihydropunctatin, and 3,9-dihydroeucomnalin, were suggested to be the contributors to the taste of LC bulbs in the study. The study also suggested taking these molecules for further experiments for biological activities [26].

In several studies, the initial interest was to analyze the total phenolic and flavonoid content of the extract from LC, as compounds from both chemical classes are considered to be responsible for the biological activities of medicinal plants.

For the first time, the lipophilic profile of the extract from the bulbs of LC was investigated after measuring the total phenolic and flavonoid amounts [27]. To analyze the nonpolar components of the extract, the *n*-hexane extract was utilized for gas chromatography coupled with mass spectrometry analysis (GCMS). The major constituents of the *n*-hexane extract were fatty acids and their ethyl ester, which comprised more than half percent of the total components of the *n*-hexane extract. Among fatty acids, palmitic acid and ethyl palmitate comprised more than 37% of the extract [27].

In the initial studies, the ethanol extract from the bulbs of LC was found to have flavonoid and phenolic contents equivalent to 23.4 mg of quercetin and 56.6 mg of chlorogenic acid per gram of extract [27,28]. Considering the use of bulbs as edible vegetables, the total phenolic and flavonoid content of cooked (traditionally boil-cooked and steam-cooked) was also studied along with raw bulbs [12]. The phenolic and flavonoid contents were found to decrease in both types of cooking. The maximum phenolic content was present in the raw extract (92.47 ± 0.020), and it decreased to 49.80 ± 0.012 and 39.53 ± 0.027 mg chlorogenic acid equivalents (CAE) per g FW in steam-cooked and boiled extract, respectively. Similarly, the maximum flavonoid content was present in the raw extract (4.57 ± 0.003), which decreased to 1.63 ± 0.010 and 0.635 ± 0.026 mg quercetin equivalents (QE) per g in steam-cooked and boiled extract, respectively [12].

Because LC bulbs from both wild and cultivated plants are used as a vegetable, the phenolic and flavonoid contents have been studied in both types. A high difference in phenolic and flavonoid contents was found to be present in the bulbs of cultivated and wild plants. Both phenolic and flavonoid contents were found to be higher in the bulbs of wild plants (264.33 and 10.40 mg/g, respectively), compared with bulbs from the cultivated plants (42 and 5.74 mg/g, respectively) [29]. Afterward, phytochemical profiling to identify the individual phytochemicals present in the extract of the bulbs from both cultivated and wild-grown plants was conducted in the *n*-hexane fraction of the extract through GC-MS analysis. A total of 12 compounds were identified in the bulbs of wild-grown plants and 22 compounds were found to be present in the bulbs of cultivated plants (Table 1). Later, a

methanolic (70%) extract of LC bulbs was utilized to study the presence of phytochemicals after total phenolic and flavonoid analysis. The total phenolic and flavonoid contents were found to be 57.67 mg Gallic acid equivalents (GAE)/g and 18.79 mg QE/g in the extract. Further, HPLC analysis compared with external standards revealed the presence of 12 different compounds in the extract. Out of these compounds, seven were phenolic acids and five were flavonoids. The most abundant phenolic acid and flavonoid were p-coumeric acid and catechin, respectively (Table 1) [30].

Researchers have also used the aerial parts of LC such as leaves and inflorescence to analyze phytochemical components. The total phenolic and flavonoid contents of the hydrochloric extract of leaves were found to be 50.50 mg/g and 4.59 mg/g, respectively. Similarly, the total phenolic and flavonoid contents of the hydrochloric extract of inflorescence were found to be 47.67 mg/g and 5.61 mg/g, respectively. The identification of phytochemicals of leaves and inflorescence was carried out on *n*-hexane and dichloromethane fractions of the hydrochloric extract of both leaves and inflorescence, considering the polarity of the phytochemicals present in the extracts. In the *n*-hexane fraction, three unique phytosterols (two in inflorescence and one in leaves) and eighteen unique fatty acids (sixteen in leaves and nine in inflorescence) were identified in GCMS analysis (Table 1). Similarly, in the dichloromethane fraction, nine unique phytocompounds (seven in inflorescence and five in leaves) were identified in the study (Table 1) [31]. Researchers also reported the high content of important minerals in the bulbs of LC. The mineral content of the bulbs was studied through inductively coupled plasma atomic emission spectroscopy, which revealed high contents of important minerals such as iron, potassium, phosphorus, sodium, copper, magnesium, and calcium, i.e., 33,552, 1843.14, 756.36, 439.65, 303.9, 272.37, and 20.55 mg/kg, respectively. Low content (<3 mg) of minerals such as selenium, strontium, and zinc was also found to be present in LC bulbs [32]. Later, researchers prepared different types of aqueous and organic extracts to achieve the optimal concentration of bioactive phytochemical class of compounds such as phenolic, flavonoids, and tannins [32]. Among three aqueous and six organic extracts, the phenolic, flavonoid, and tannin content was found to be maximum in diethyl ether extract, i.e., 129.75 ± 0.29 µg GAE/mg, 988.26 ± 0.18 µg QE/mg, and 30.22 ± 0.15 µg CE/mg, respectively [32]. Recently, aqueous and methanolic (50 and 70%) extracts of LC bulbs were used to study the total phenolic and flavonoid contents before the evaluation of their biological properties. The maximum phenolic and flavonoid contents were found to be present in the 70% methanolic extract, i.e., 58.72 mgGAE/g and 20.37 mg QE/g, respectively [33].

Table 1. Different phytochemicals reported in *Leopoldia comosa*.

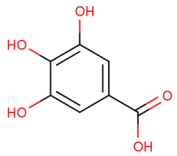
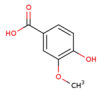
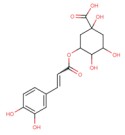
Sr. No.	Compound Name	Amount (mg/g)	Compound Type	Type of Extract/Fraction	Part of Plant	Ref
1	 Gallic acid	0.31	Phenolic acid	Methanol extract	Bulbs	[30]
2	 Vanillic acid	0.28	Phenolic acid	Methanol extract	Bulbs	[30]
3	 Chlorogenic acid	0.85	Phenolic acid	Methanol extract	Bulbs	[30]

Table 1. *Cont.*

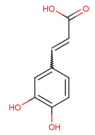
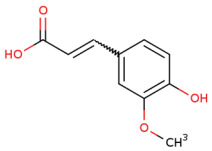
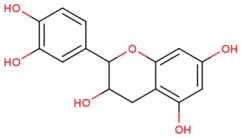
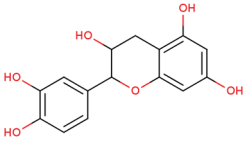
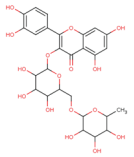
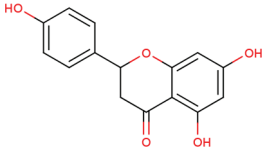
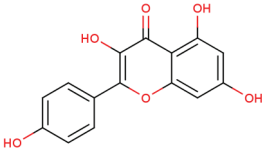
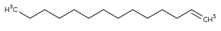
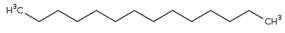
Sr. No.	Compound Name	Amount (mg/g)	Compound Type	Type of Extract/Fraction	Part of Plant	Ref
4	 Caffeic acid	0.45	Phenolic acid	Methanol extract	Bulbs	[30]
5	 Ferulic acid	1.32	Phenolic acid	Methanol extract	Bulbs	[30]
6	 Catechin	1.17	Flavonoids	Methanol extract	Bulbs	[30]
7	 Epicatechin	0.36	Flavonoids	Methanol extract	Bulbs	[30]
8	 Rutin	0.11	Flavonoid glycoside	Methanol extract	Bulbs	[30]
9	 Naringenin	0.17	Flavonoids	Methanol extract	Bulbs	[30]
10	 Kaempferol	0.24	Flavonoids	Methanol extract	Bulbs	[30]
11	 1-Tetradecene	0.04	Hydrocarbon	*n*-hexane fraction of extract from cultivated bulbs	Bulbs	[30]
12	 Tetradecane	0.03	Hydrocarbon	*n*-hexane fraction, cultivated bulbs	Bulbs	[29]
13	Pentadecane	0.10	Hydrocarbon	*n*-hexane fraction of extract from cultivated bulbs	Bulbs	[29]

Table 1. *Cont.*

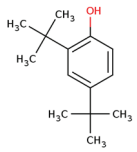
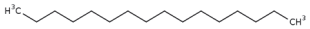
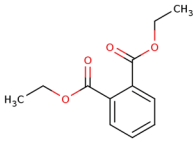
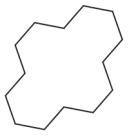
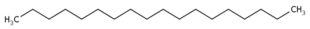
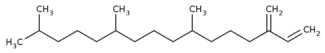
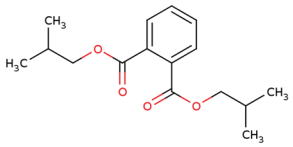
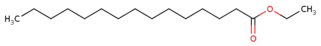
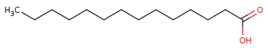
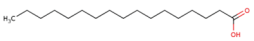
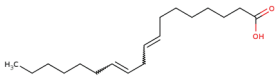
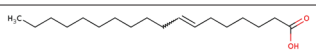
Sr. No.	Compound Name	Amount (mg/g)	Compound Type	Type of Extract/Fraction	Part of Plant	Ref
14	 Phenol, 2,4-bis(1,1-dimethylethyl)-	0.13	Other	*n*-hexane fraction of extract from cultivated bulbs	Bulbs	[29]
15	 Hexadecane	0.14	Hydrocarbon	*n*-hexane fraction of extract from cultivated bulbs	Bulbs	[29]
16	 1,2-Benzenedicarboxylic acid, diethyl ester	0.04	Phthalate ester.	*n*-hexane fraction of extract from cultivated bulbs	Bulbs	[29]
17	 Cyclotetradecane	0.01	Other	*n*-hexane fraction of extract from cultivated bulbs	Bulbs	[29]
18	 Octadecane	0.13	Hydrocarbon	*n*-hexane fraction of extract from cultivated bulbs	Bulbs	[29]
19	 Neophytadiene	0.14	Hydrocarbon	*n*-hexane fraction of extract from cultivated bulbs	Bulbs	[29]
20	 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	0.32	Other	*n*-hexane fraction of extract from cultivated bulbs	Bulbs	[29]
21	 Pentadecanoic acid, ethyl ester	1.27	Fatty acid ester	*n*-hexane fraction of extract from cultivated bulbs	Bulbs	[29]
22	 Myristic acid	17.52	Fatty acid	*n*-hexane fraction of extract from wild bulbs	Bulbs	[29]
23	 Margaric acid	1.20	Fatty acid	*n*-hexane fraction of extract from cultivated bulbs	Bulbs	[29]
24	 8,11-Octadecadienoic acid	1.34	Fatty acid	*n*-hexane fraction of extract from wild bulbs	Bulbs	[29]
25	 7-Octadecenoic acid	1.30	Fatty acid	*n*-hexane fraction of extract from wild bulbs	Bulbs	[29]

Table 1. *Cont.*

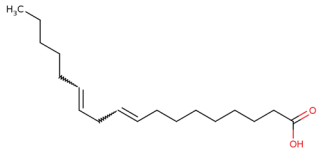
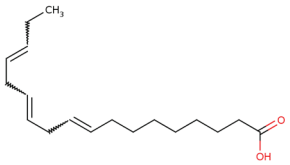
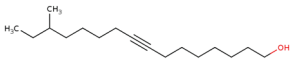
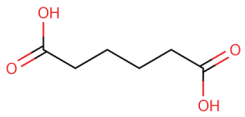
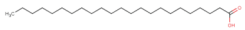
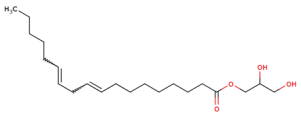
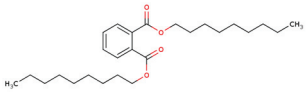
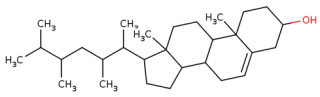
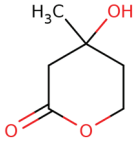
Sr. No.	Compound Name	Amount (mg/g)	Compound Type	Type of Extract/Fraction	Part of Plant	Ref
26	 Linoleic acid	12.92	Fatty acid	*n*-hexane fraction of extract from cultivated bulbs	Bulbs	[29]
27	 Linolenic acid	4.22	Fatty acid	*n*-hexane fraction of extract from cultivated bulbs	Bulbs	[29]
28	 (R)-(−)-14-Methyl-8-hexadecyn-1-ol	0.50	Other	*n*-hexane fraction of extract from cultivated bulbs	Bulbs	[29]
29	9,17-Octadecadienal, (Z)-	0.10	Other	*n*-hexane fraction of extract from cultivated bulbs	Bulbs	[29]
30	 Adipic acid	1.5	Other	*n*-hexane fraction of extract from wild bulbs	Bulbs	[29]
31	 Tricosanoic acid	0.03	Other	*n*-hexane fraction of extract from wild bulbs	Bulbs	[29]
32	 β-Monolinolein	2.30	Other	*n*-hexane fraction of extract from cultivated bulbs	Bulbs	[29]
33	 1,2-Benzenedicarboxylic acid, dinonyl ester	5.62	Phthalic acid monoester	*n*-hexane fraction of extract from wild bulbs	Bulbs	[29]
34	 (22R, 24S)-22,24-Dimethylcholesterol	0.50	Sterol	*n*-hexane fraction of extract from cultivated bulbs	Bulbs	[29]
35	 Mevalonic acid lactone	3.7	Other	Dichloromethane fractions	Leaves	[31]

Table 1. *Cont.*

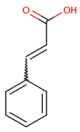
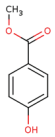
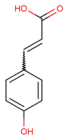
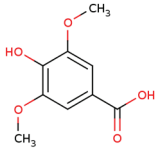
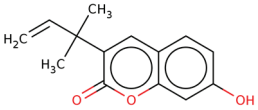
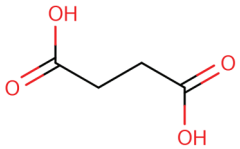
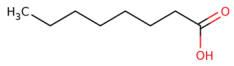
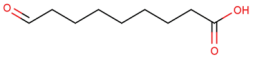
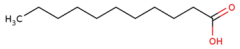
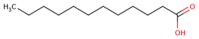
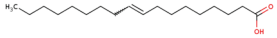
Sr. No.	Compound Name	Amount (mg/g)	Compound Type	Type of Extract/Fraction	Part of Plant	Ref
36	 Cinnamic acid	Trace	Carboxylic acid	Dichloromethane fractions	Inflorescences	[31]
37	 Benzoic acid, 4-hydroxy-, methyl ester	2.2	Other	Dichloromethane fractions	Leaves	[31]
38	 p-Hydroxycinnamic acid	0.9	Phenolic acid	Dichloromethane fractions	Leaves	[31]
39	 Syringic acid	1.9	Phenolic acid	Dichloromethane fractions	Inflorescences	[31]
40	 7-Hydroxy-3-(1,1-dimethylprop-2-enyl)coumarin	0.2	Other	Dichloromethane fractions	Leaves	[31]
41	 Butanedioic acid	TA	Carboxylic acid	*n*-hexane	Inflorescences	[31]
42	 Caprylic acid	0.3	Carboxylic acid	*n*-hexane	Leaves	[31]
43	 Azelaaldehydic acid	0.6	Carboxylic acid	*n*-hexane	Leaves	[31]
44	 Undecanoic acid	0.1	Carboxylic acid	*n*-hexane	Leaves	[31]
45	 Lauric acid	0.5	Fatty acid	*n*-hexane	Leaves	[31]
46	 Oleic acid	0.1	Fatty acid	*n*-hexane	Inflorescences	[31]

Table 1. *Cont.*

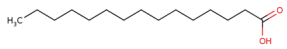
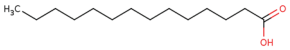
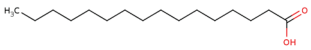
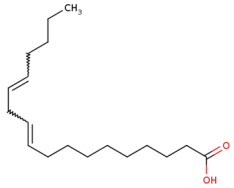
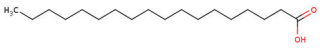
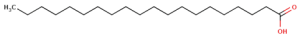
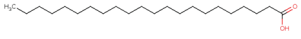
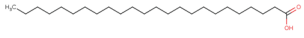
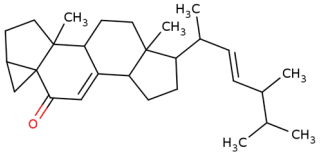
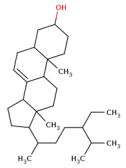
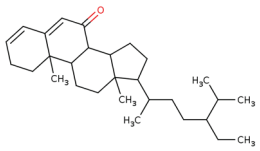
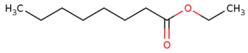
Sr. No.	Compound Name	Amount (mg/g)	Compound Type	Type of Extract/Fraction	Part of Plant	Ref
47	 Pentadecanoic acid	0.5	Fatty acid	*n*-hexane	Leaves	[31]
48	 Tetradecanoic acid	0.2	Fatty acid	*n*-hexane	Leaves	[31]
49	 Palmitic acid	19.8	Fatty acid	*n*-hexane	Leaves	[31]
50	 10,13-Octadecadienoic acid	0.2	Fatty acid	*n*-hexane	Leaves	[31]
51	 Stearic acid	7.9	Fatty acid	*n*-hexane	Inflorescences	[31]
52	 Eicosanoic acid	1.2	Fatty acid	*n*-hexane	Inflorescences	[31]
53	 Behenic acid	0.9	Fatty acid	*n*-hexane	Leaves	[31]
54	 Lignoceric acid	0.6	Fatty acid	*n*-hexane	Inflorescences	[31]
55	 3α, 5-cyclo-ergosta-7,22-dien-6-one	0.3	Phytosterols	*n*-hexane	Inflorescences	[31]
56	 Stigmast-7-en-3-ol, (3β, 5α)-	0.3	Phytosterols	*n*-hexane	Leaves	[31]
57	 Stigmasta-3,5-dien-7-one	0.3	Phytosterols	*n*-hexane	Inflorescences	[31]
58	 Ethyl caprylate	0.9% [a]		*n*-hexane	Bulbs	[27]

Table 1. *Cont.*

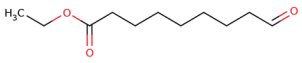
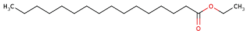
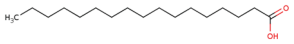
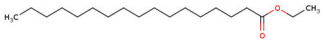
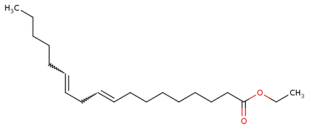
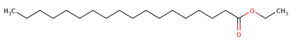
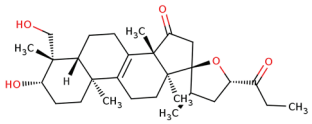
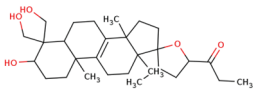
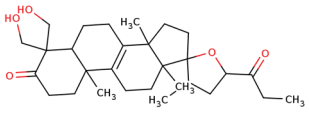
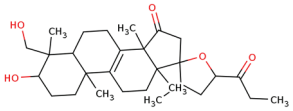
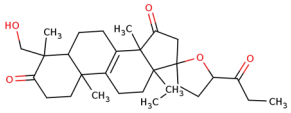
Sr. No.	Compound Name	Amount (mg/g)	Compound Type	Type of Extract/Fraction	Part of Plant	Ref
59	 9-oxo-Nonanoic acid, ethyl ester	2.7% [a]		*n*-hexane	Bulbs	[27]
60	 Ethyl palmitate	19.7% [a]		*n*-hexane	Bulbs	[27]
61	 Heptadecanoic acid	2.8% [a]	Fatty acid	*n*-hexane	Bulbs	[27]
62	 Ethyl heptadecanoate	1.4% [a]		*n*-hexane	Bulbs	[27]
63	 Ethyl linoleate	4.9% [a]		*n*-hexane	Bulbs	[27]
64	 Ethyl stearate	1.5% [a]		*n*-hexane	Bulbs	[27]
65	 Eucosterol	NP	Phytosterols	Acetone	Bulbs	[15]
66	 Ketotriol	NP	Phytosterols	Acetone	Bulbs	[17]
67	 Diketotriol	NP	Phytosterols	Acetone	Bulbs	[17]
68	 (23*R*)-17,23-epoxy-33,31-dihydroxy-27-nor-5α-lanost-8-ene-15,24-dione	NP	Phytosterols	Acetone	Bulbs	[18]
69	 (23*R*)-17,23-epoxy-31-hydroxy-2 7-nor-5α-lanost-8-ene-3,15,24-trione	NP	Other	Acetone	Bulbs	[18]

Table 1. *Cont.*

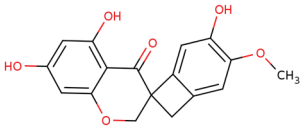
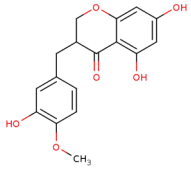
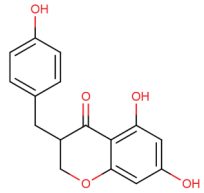
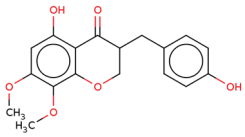
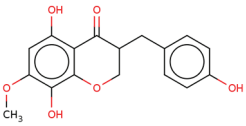
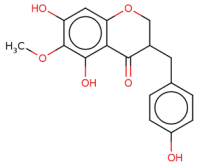
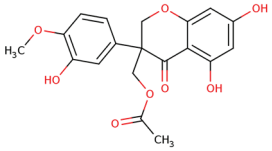
Sr. No.	Compound Name	Amount (mg/g)	Compound Type	Type of Extract/Fraction	Part of Plant	Ref
70	 Muscomosin	NP	Homoisoflavonoids	Ether	Bulbs	[25]
71	 3′-hydroxy-3,9dihydroeucomin	NP	Homoisoflavonoids	Ether	Bulbs	[25]
72	 4′demethyl-3,9-dihydroeucomin	NP	Homoisoflavonoids	Ether	Bulbs	[25]
73	 7-*O*-methyl-3,9_dihydropunctatin	NP	Homoisoflavonoids	Ether	Bulbs	[24]
74	 8-*O*-demethyl-7-*O*-methyl-3,9_dihydropunctatin	NP	Homoisoflavonoids	Ether	Bulbs	[24]
75	 3,9-dihydroeucomnalin	NP	Homoisoflavonoids	Ether	Bulbs	[24]
76	 Comosin	NP	Homoisoflavonoids	Ether	Bulbs	[3]

Table 1. *Cont.*

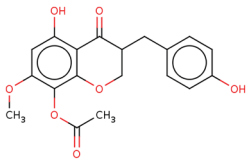
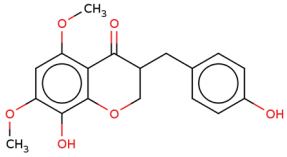
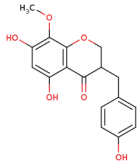
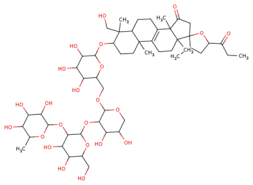
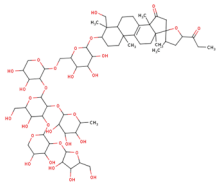
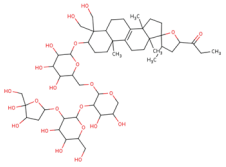
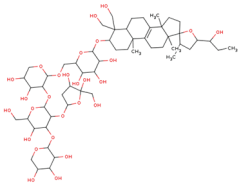
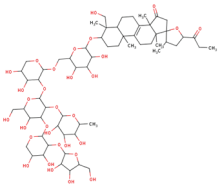
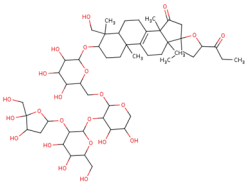
Sr. No.	Compound Name	Amount (mg/g)	Compound Type	Type of Extract/Fraction	Part of Plant	Ref
77	 8-*O*-demethyl-8-*O*-acetyl-7-*O*-methyl-3,9dihydropunctatin	NP	Homoisoflavonoids	Ether	Bulbs	[3]
78	 H2	NP	Homoisoflavonoids	Ether	Bulbs	[34]
79	 H5	NP	Homoisoflavonoids	Ether	Bulbs	[34]
80	 Muscaroside A	NP	Glycoside	Acetone	Bulbs	[20]
81	 Muscaroside B	NP	Glycoside	Acetone	Bulbs	[21]
82	 Muscaroside C	NP	Glycoside	Acetone	Bulbs	[22]
83	 Muscaroside D	NP	Glycoside	Acetone	Bulbs	[23]

Table 1. *Cont.*

Sr. No.	Compound Name	Amount (mg/g)	Compound Type	Type of Extract/Fraction	Part of Plant	Ref
84	 Muscaroside E	NP	Glycoside	Acetone	Bulbs	[23]
85	 Muscaroside F	NP	Glycoside	Acetone	Bulbs	[23]

[a] Peak area relative to total peak area percentage (relative area percentage); NP: not provided.

6. Bioactivities

The early pharmacological activity of plants is known from its traditional use in society [1]. The different bioactivity studied for LC has primarily been focused on the bulbs of plants, as they have been used as a vegetable since ancient times. However, some activities have also been studied in other aerial parts of the plant such as leaves and inflorescence [31]. The important biological activities of LC plants are reported in the literature, including antioxidant, anti-obesity, anti-cancer, anti-AD, anti-inflammatory, antibacterial, and immune enhancement activities. Among these, the antioxidant activity of the plant has the potential to contribute to other important pharmacological activities such as anti-obesity, anti-cancer, anti-inflammatory, and anti-diabetes [9,35–37]. Hence, the antioxidant activity of plants has been analyzed in numerous experimental studies.

6.1. Antioxidant Activity

Natural antioxidants are considered a potential pharmacological intervention against deadly diseases and disorders including cancer and neurodegenerative disorders [38–40]. Consequently, the research on natural antioxidants for their health benefits has been increasing. Initially, the antioxidant activity of LC was premeditated in a study conducted to evaluate the antioxidant properties of non-cultivated vegetables from the southern part of Italy [5]. The antioxidant potential of all selected vegetable plants was screened through the 1,1-diphenyl-2-picrylhydrazil (DPPH) radical screening assay, which is based on free radical scavenging activity. The extract from the LC bulbs had the highest antioxidant activity among all 27 selected extracts and even more than the reference extract from *Rhodiola rosea* used in the study. Further antioxidant activity of the LC bulb extract was found to be highest in the in vitro non-enzymatic inhibition of the lipid peroxidation in liposomes, which is equivalent to the reference compound quercetin. Similarly, the effective antioxidant activity of LC in xanthine oxidase (XO) inhibition was also observed [5].

Later, metal chelating activity through Fe2+ chelating activity assay, and antioxidant activity through DPPH, ferric-reducing ability power (FRAP), and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays of LC bulb extract were carried out in ethanolic and *n*-hexane fractions. In the FRAP assay, the antioxidant activity of LCB extract in the ethanolic fraction was comparable with the positive control (butyl hydroxytoluene) used in the study [27]. ABTS and DPPH assay again revealed that both extract fractions have reducing power (Table 2).

In a further study, the antioxidant activity of LC bulb extracts extracted through different solvents including ethanol and *n*-hexane was evaluated [28]. The antioxidant activity of the ethanol extract activity was slightly higher than that of the *n*-hexane extract (Table 2).

Similarly, the antioxidant activity of LC bulb extract from wild and cultivated LC was examined through the DPPH assay and β-carotene bleaching test. The antioxidant activity of wild plant bulbs was higher than the cultivated plant bulb in the DPPH assay. Further, the highest antioxidant activity was achieved by the dichloromethane fraction from the bulbs of the cultivated plants. Similar results were obtained in the β-carotene bleaching test: the antioxidant activity of the raw extract from bulbs of wild-grown plants was higher than that from the cultivated plants. The highest antioxidant activity in the β-carotene bleaching test was achieved by ethyl acetate fraction from the bulbs of the cultivated plants [29].

In another study, the antioxidant activities of LC bulbs grown in southern Italy were studied through DPPH, nitric oxide, and superoxide radicals scavenging assays. The strong superoxide anion scavenging activity of LC bulb extract was calculated and found to be better than the positive control (ascorbic acid) used in the study [30]. Similarly, a dose-dependent nitric oxide radical scavenging activity was observed but it was lesser than the positive control [30]. The antioxidant activity in DPPH assays was also identified and it was found to be similar to that reported in the previous studies [27,28].

Considering the dietary aspect of LC bulbs, the extracts from the traditional boiled and steam-cooked bulbs were also studied for their antioxidant properties in the study [12]. In both of the studied methods (i.e., DPPH assay and β-carotene bleaching test) for antioxidant activity, the raw extract was found to have the highest antioxidant activities, which might suggest a small loss of antioxidant activity during the cooking process. In other studies, researchers analyzed the antioxidant activity of extract from the bulbs of LC before animal studies for the anti-obesity properties. In this study, strong antioxidant activity of extract from the bulbs of LC was observed in both experiments, i.e., the DPPH assay and the β-carotene bleaching test [13] (Table 2).

Later, researchers studied the antioxidant properties of the extract from the different aerial parts of LC, which included leaves and inflorescences [31]. In this study, the highest antioxidant activity was found in leaves as compared to inflorescences in both the studied antioxidant assays, i.e., the DPPH assay and the β-carotene bleaching test (Table 2 and Figure 2). Among the fractions and the raw extract, the antioxidant activity was maximum in ethyl acetate fractions in the case of both leaves and inflorescences [31].

Recently, the antioxidant activities of aqueous (decocted, infused, and macerated) and organic (ethanolic, macerated ethanolic, acetone, macerated acetone, diethyl ether, and macerated diethyl ether) extracts through different polarities have been studied for their antioxidant activities through five different methods, i.e., hydrogen peroxide scavenging assay (HPSA), Trolox equivalent antioxidant capacity, ferric-reducing antioxidant power assay (FRAP), reducing power assay, and DPPH [32]. In HPSA, similar antioxidant activities were observed for all three types of aqueous extracts, but the maximum was in the case of the decocted extract. Among organic extracts, the highest activities were observed for hot diethyl ether extract in all five different methods used in the studies (Table 2).

The antioxidant activity of bulbs extracted through aqueous and other concentrations of methanol has been evaluated with in vitro assays and cell line experiments. Antioxidant assays used in the study include DPPH, total reducing power, nitric oxide, and superoxide radicals scavenging properties. In the cell line study, the antioxidant activities of LC bulb extracts were evaluated by measuring intracellular reactive oxygen species stimulated through tert-butyl hydroperoxide (t-BOOH) and expression of antioxidant-related genes in the HepG2 cells. Pretreatment with extract at low doses decreased the production of ROS, which had been enhanced due to the t-BOOH treatment in the HepG2 cells [33]. Additionally, expression of several genes/markers related with antioxidant activities, such as nuclear factor-erythroid 2 p45-related factor 2 (*NRF2*), superoxide dismutase (*SOD-2*), glutathione peroxidase (*GPX1*), catalase (*CAT*), NADPH quinone oxidase-1 (*NQO1*), ATP

binding cassette subfamily C member (*ABCC6*), and ATP binding cassette subfamily G member (*ABCG2*) were studied through RT-PCR experiments. Aqueous and 50% methanolic extracts of LC bulb treatment enhanced the expression of NRF2 in HepG2 cells. Similarly, the expression of *SOD2* was enhanced with a high dose of aqueous and 70% and 50% methanolic extracts. The expression of *GPX1* was increased in the treatment with a high dose of aqueous and a low dose of 70% methanolic extracts. The expression of *NQO1* was enhanced with a low dose of 50% methanolic extract, and the expression of *ABCC6* was increased in the treatment with a high dose of aqueous (Table 2).

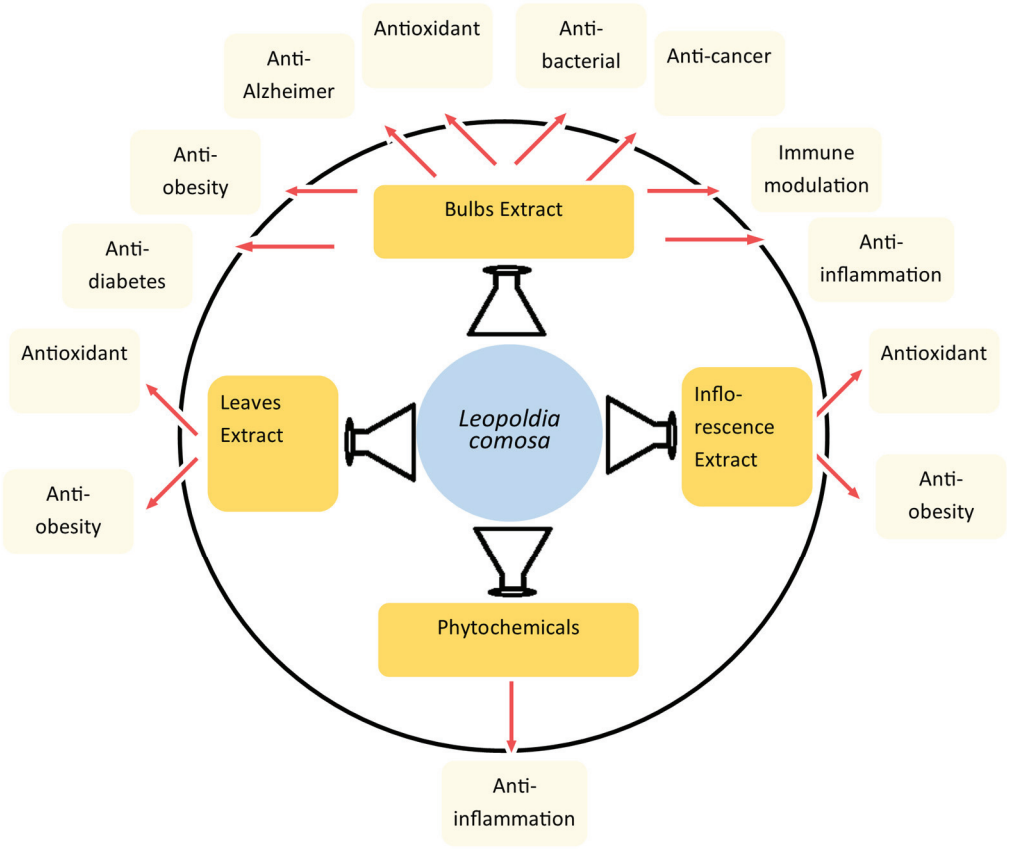


Figure 2. Pharmacological activities of different parts and forms of LC reported in literature.

Table 2. Different biological activities of LC.

Activity	Dose	Method	Result	Refs
Antioxidant activity	Extract from bulbs in ethanol	DPPH	>80%	[5]
		Lipid peroxidation in liposome inhibition assay	>85%	
		XO inhibition	>35%	
	Extract from bulbs in ethanol and *n*-hexane	Metal chelating assay	IC_{50} values of 78.8 (ethanol) and 113.6 (*n*-hexane) µg/mL	[27]
		FRAP	66.7 ± 3.1 (ethanol) and 112.4 ± 2.5 (*n*-hexane) µM Fe/g	
		ABTS	1.7 ± 0.7 and 3.8 ± 0.9 TEAC value	
		DPPH of ethanol and *n*-hexane extracts	IC_{50} values of 40.9 (ethanol) and 46.6 (*n*-hexane) µg/mL,	[28]

Table 2. *Cont.*

Activity	Dose	Method	Result	Refs
Antioxidant activity	Extract from bulbs of wild and cultivated plants (raw, dichloromethane, and ethyl acetate fractions)	DPPH	IC_{50} values of raw, dichloromethane, and ethyl acetate fractions were 152.90, 38.97, and 31.57 μg/mL, respectively, for the bulbs of wild plants; and the IC_{50} values of dichloromethane and ethyl acetate fractions were 11.25 and 53.24 μg/mL, respectively, for the bulbs of cultivated plants.	[29]
		β-carotene-linoleic acid bleaching assay	IC_{50} values of raw, dichloromethane, and ethyl acetate fractions were 24.68, 17.30, and 57.71 μg/mL, respectively, for the bulbs of wild plants; and the IC_{50} values of dichloromethane and ethyl acetate fractions were 8.54 and 4.51 μg/mL, respectively, for the bulbs of cultivated plants.	
	Methanolic (70%) extract from LC bulbs	DPPH, nitric oxide, and superoxide radicals scavenging assay	IC_{50} = 36.73 μg/mL (DPPH) IC_{25} = 144.13 μg/mL (NO) IC_{50} = 54.15 μg/mL (superoxide anion radical)	[30]
	Ethanolic extracts from bulbs from raw, boiled, and steam-cooked bulbs	DPPH	IC_{50} = 1.34 mg/mL (raw) IC_{50} = 3.59 mg/mL (steam-cooked) IC_{50} = 9.63 mg/mL (boiled)	[12]
		β-carotene-linoleic acid bleaching assay	IC_{50} = 9.13 mg/mL (raw) IC_{50} = 17.37 mg/mL (steam-cooked) IC_{50} = 14.81 mg/mL (boiled)	
	Extract from LC bulbs obtained using a dynamic extractor	DPPH	The IC_{50} = 10.2 ± 0.2 μg/mL (n = 5).	[13]
		BCB assay (The IC_{50} of antilipoperoxidant activity, measured by BCB assay, for ascorbic acid was 1.50 ± 0.10 μg/mL.)	IC_{50} = 10.80 ± 0.74 μg/mL (30 min) and 81.4 ± 1.28 μg/mL (60 min) (n = 5 for each test).	
	Extract of leaves	DPPH	The IC_{50} values of raw, n-hexane, dichloromethane, and ethyl acetate fractions were 154.8, >1000, >1000, and 86.09 μg/mL, respectively, for the extract from leaves; and the	[31]
		BCB assay	dichloromethane and ethyl acetate fractions were 44.46, >100, >100, and 23.73 μg/mL, respectively, for the extract from leaves.	
	Extract from inflorescences	DPPH	The IC_{50} values of raw, n-hexane, dichloromethane, and ethyl acetate fractions were 316.6, >1000, 472.1, and 102.4 μg/mL, respectively, for the extract from inflorescences.	
		BCB assay	The IC_{50} values of raw, n-hexane, dichloromethane, and ethyl acetate fractions were 84.42, >1000, >1000, and 53.35 μg/mL, respectively, for the extract from inflorescences.	

Table 2. *Cont.*

Activity	Dose	Method	Result	Refs
Antioxidant activity	Aqueous and organic extract from bulbs	HPSA	The percentages of scavenging activities were 62.12 ± 0.2, 61.89 ± 0.3, 61.72 ± 0.1, 61.3 ± 0.16, 61.09 ± 0.05, 61.24 ± 0.2, 60.94 ± 0.2, 62.67 ± 0.06, and 61.91 ± 0.1 for decocted aqueous, infused aqueous, macerated aqueous, ethanolic, macerated ethanolic, acetone, macerated acetone, diethyl ether, and macerated diethyl, respectively.	[32]
		Trolox equivalent antioxidant capacity (µg of Trolox equivalent per mg of dry plant extract)	The Trolox equivalent (µg TE/mgE) antioxidant capacity values were 27.46 ± 0.69, 17.18 ± 0.17, 6.63 ± 0.31, 225.86 ± 1.04, 89.47 ± 0.68, 364.96 ± 0.28, 343.02 ± 1.44, 381.63 ± 0.63, and 360.93 ± 0.25 for decocted aqueous, infused aqueous, macerated aqueous, ethanolic, macerated ethanolic, acetone, macerated acetone, diethyl ether, and macerated diethyl, respectively.	
		DPPH	The IC_{50} values were 1011.33 ± 4.37, 1089.33 ± 0.92, 1140 ± 20.64, 139.4 ± 6.93, 220.5 ± 2.91, 99.76 ± 0.04, 100 ± 0.03, 10.08 ± 0.01, and 10.15 ± 0.04 µg/mL for decocted aqueous, infused aqueous, macerated aqueous, ethanolic, macerated ethanolic, acetone, macerated acetone, diethyl ether, and macerated diethyl, respectively.	
		FRAP assay (µg of Trolox equivalent per mg of dry plant extract)	The Trolox equivalent (µg TE/mgE) antioxidant activity values were 12.9 ± 0.1, 11.16 ± 0.52, 15.27 ± 0.1, 131.55 ± 0.26, 49.24 ± 0.13, 277.74 ± 0.67, 225.77 ± 0.15, 394.77 ± 0.74, 358.77 ± 0.74 for decocted aqueous, infused aqueous, macerated aqueous, ethanolic, macerated ethanolic, acetone, macerated acetone, diethyl ether, and macerated diethyl, respectively.	
		Reducing power assay (µg of ascorbic acid equivalent per mg of dry plant extract)	The Trolox equivalent (µg TE/mgE) antioxidant activity values were 8.36 ± 0.06, 7.91 ± 0.14, 10.68 ± 0.13, 59.40 ± 0.21, 18.86 ± 0.05, 147.39 ± 1.07, 133.32 ± 0.8, 356.7 ± 0.92, and 283.95 ± 0.59 for decocted aqueous, infused aqueous, macerated aqueous, ethanolic, macerated ethanolic, acetone, macerated acetone, diethyl ether, and macerated diethyl, respectively.	
	Aqueous and methanolic (50 and 70%) extracts from bulbs	DPPH	IC_{50} = 38.02 µg/mL (aqueous) IC_{50} = 29.43 µg/mL (50% methanolic) IC_{50} = 24.60 µg/mL (70% methanolic)	[33]
		Nitric oxide scavenging	IC_{50} = 269.21 µg/mL (aqueous) IC_{50} = 168.52 µg/mL (50% methanolic) IC_{50} = 122.94 µg/mL (70% methanolic)	
		Superoxide radicals scavenging	IC_{50} = 51.43 µg/mL (aqueous) IC_{50} = 50.10 µg/mL (50% methanolic) IC_{50} = 36.50 µg/mL (70% methanolic)	
		Reducing power	37.93 (mg GAE/g) (aqueous) 44.51(mg GAE/g) (50% methanolic) 47.52(mg GAE/g) (70% methanolic)	

Table 2. *Cont.*

Activity	Dose	Method	Result	Refs
Antioxidant activity	1, 5, 10, 50, 100, and 600 μg/mL	In HepG2 cells the intracellular ROS level was measured with DCFH-DA.	Pre-treatment with low doses (100–1 μg/mL) of the extracts for 24 h protected cells from oxidative stress and ROS ↓.	[33]
	Aqueous and methanolic extracts from bulbs	RT-PCR	*NRF2* ↑ (aqueous and 50% methanolic extracts), *SOD-2* ↑ (aqueous and methanolic extracts), *GPX1* ↑ (aqueous and 70% methanolic extracts), *NQO1* ↑ (50% methanolic extract), *ABCC6* ↑ (aqueous extract)	
Anti-obesity	Extracts from bulbs of wild and cultivated plants in raw, dichloromethane, and ethyl acetate fractions	Lipase inhibition assay (orlistat is taken as positive control IC_{50} = 0.018 mg/mL)	The IC_{50} values of raw, dichloromethane, and ethyl acetate fractions were 0.166, 0.290, and 0.153 mg/mL, respectively, from the bulbs of wild plants; and the IC_{50} values of dichloromethane and ethyl acetate fractions were 0.218 and 0.469 mg/mL, respectively.	[29]
	Leaves and inflorescences of wild and cultivated plants in raw, dichloromethane, and ethyl acetate fractions		The IC_{50} values of raw, *n*-hexane, dichloromethane, and ethyl acetate fractions were 3.819, 0.369, 1.409, and 0.336 mg/mL, respectively, for the leaves of wild plants; and the IC_{50} values of raw, *n*-hexane, dichloromethane, and ethyl acetate fractions were 6.561, 0.736, 1.570, and 0.780 mg/mL, respectively, for the inflorescences of wild plants.	[31]
	Ethanolic extract from LC bulbs	Inhibition assays for pancreatic lipase (positive controls, i.e., orlistat IC_{50} values = 0.19 μg/mL)	IC_{50} values of 70.5 ± 0.89 μg/mL for extract and 57.20 ± 0.19 μg/mL for positive control drug	[13]
		Inhibition assays for pancreatic α-amylase (positive controls i.e., acarbose IC_{50} = 36.50 ± 0.32 μg/mL)	IC_{50} values of 46.3 ± 0.23 μg/mL for extract and 36.50 ± 0.32 μg/mL for positive control drug	
	Wistar rats oral administration of 20 or 60 mg/die for 12 weeks	Anthropometric and metabolic variables	Body weight ↓, circumference of waist ↓ perirenal ↓, retroperitoneal ↓, epididymal ↓, and abdominal fat ↓ weight ↓ and CSI ↓ values of heart and liver, ROS ↓	
		Blood biochemical measurements and HOMA-IR index	ROS production ↓, triglycerides ↓, LDL cholesterol ↓, LDL-cholesterol-ox ↓, and total cholesterol ↓; similarly, the level of plasma insulin ↓, basal glycemia level ↓, and HOMA-IR index ↓	
		Tissue histology	In abdominal fat, the areas of adipocytes ↓. In liver samples, the presence of fat vacuoles ↓, the triglyceride content of liver ↓.	
		WB analysis of key enzymes of gluconeogenesis	The expression of PEPCK ↓ and G6Pase ↓	
Anti-diabetes activity	Extract from LC bulbs in ethanol and *n*-hexane	α-amylase inhibition assay (positive controls i.e., acarbose IC_{50} = 50.0 ± 0.9 μg/mL)	IC_{50} = 81.3 ± 2.7 μg/mL (ethanol) IC_{50} = 166.9 ± 3.4 μg/mL (*n*-hexane)	[27]
		α-Glucosidase (positive controls, i.e., acarbose IC_{50} = 35.5 ± 1.2 μg/mL)	IC_{50} = 112.8 ± 3.3 μg/mL (ethanol) IC_{50} = 200.8 ± 2.8 μg/mL (*n*-hexane)	

Table 2. *Cont.*

Activity	Dose	Method	Result	Refs
	Ethanolic extracts from raw, boiled, and steam-cooked bulbs	α-amylase inhibition assay	IC_{50} values of 0.16 ± 0.03, 0.73 ± 0.13, and 0.69 ± 0.02 mg/mL in RB, SB, and BB, respectively	[12]
	Methanolic extract from LC bulbs	α-amylase (positive control acarbose IC_{50} = 47.33 µg/mL)	IC_{50}: 75.17 µg/mL	[30]
		α-glucosidase (positive control acarbose IC_{50} = 33.72 µg/mL)	IC_{50}: 85.33 µg/mL	
	Aqueous (decocted, infused, and macerated) and organic (ethanolic, macerated ethanolic, acetone, macerated acetone, diethyl ether, and macerated diethyl) extract from bulbs	α-amylase (positive control acarbose IC_{50} = 616.33 µg/mL)	The IC_{50} values were 1200, 2880, 2752, 2264 2384, 2219, 2289, 2512, and 2897 µg/mL for decocted aqueous, infused aqueous, macerated aqueous, ethanolic, macerated ethanolic, acetone, macerated acetone, diethyl ether, and macerated diethyl, respectively.	[32]
		α-glucosidase inhibition assay (positive control acarbose IC_{50} = 195 µg/mL)	The IC_{50} values were 238.5, 258.9, 268.2, 257.9, 162.7, 85.4, 85.9, 136, and 130.8 µg/mL for decocted aqueous, infused aqueous, macerated aqueous, ethanolic, macerated ethanolic, acetone, macerated acetone, diethyl ether, and macerated diethyl, respectively.	
		β-galactosidase inhibition assay (positive control quercetin IC_{50} = 171.16 µg/mL)	The IC_{50} values were 216, 205, 245, 182, 196, 163, 200, 240, and 291 µg/mL for decocted aqueous, infused aqueous, macerated aqueous, ethanolic, macerated ethanolic, acetone, macerated acetone, diethyl ether, and macerated diethyl, respectively.	
Anti-AD	*n*-hexane and ethanolic extract from bulbs	Acetylcholinesterase and butyrylcholinesterase inhibiting activities	IC_{50} = 131 µg/mL (ethanol) AChE IC_{50} = 282.9 µg/mL (ethanol) BChE IC_{50} = 104.9 µg/mL (*n*-hexane) AChE IC_{50} = 128.1 µg/mL (*n*-hexane) BChE.	[28]
	Methanolic extract from LC bulbs	Acetylcholinesterase inhibition assay (positive control, i.e., galantamine IC_{50} = 8.9760.15 µg/mL)	IC_{50} = 107.6465.38 µg/mL	[30]
Anti-cancer	100, 200, and 400 mg/kg 50% ethanol fraction of aqueous bulb extract	Walker-256 (intramuscular) carcinosarcoma	Weight of tumor decreased	[16]
	Ethanolic extracts from raw and cooked bulbs	MTT assay of MCF-7 cell line	IC_{50} = 10.27 µg/mL (ethanol) raw IC_{50} = 669.3 µg/mL (ethanol) cooked	[12]
Anti-inflammation	Five homoisoflavones and fraction at 100 µg/ear	Croton oil-induced mouse ear dermatitis	27–41% ↓ (in inflammation)	[34]
	Methanolic extract from LC bulbs (25, 50, and 75 mg/mL)	MMP-2 and MMP-9 derived from the primary culture of rat astrocytes activated with LPS detected through gelatin gel zymography and 1,10 phenanthroline used as a positive control	MMP-2 ↓ and MMP-9 ↓ (completely inhibited the activity of MMP-9 at 50 mg/mL)	[30]

Table 2. *Cont.*

Activity	Dose	Method	Result	Refs
Antibacterial activity	Aqueous and ethanolic extracts from LC bulbs	Inhibition of biofilm formation through methicillin-resistant *Staphylococcus aureus* studied through staining with crystal violet	IC_{50} = 8 µg/mL (aqueous) raw IC_{50} = 16 µg/mL (ethanol) cooked	[41]
	Extract of LC bulbs in different solvents: ethanolic, macerated ethanolic, acetone, macerated acetone, diethyl ether, and macerated diethyl.	Agar disc diffusion assay measuring the zone of inhibition formed around the discs against selected bacterial species (*Bacillus subtilis*, *Staphylococcus aureus*, *Listeria innocua*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Escherichia coli*).	Inhibition was observed against *Listeria innocua* and *Proteus mirabilis*.	[32]
Immune stimulation	0.1 mL of the alcoholic plant extract in two different concentrations (0.5 mg/fish and 2 mg/fish)	*Sparus aurata* NBT-positive cells count	NBT-positive cells count ↑	[42]
		Specific growth rate	Growth ↑	
		Lysozyme activity of serum samples	Lysozyme activity ↑	
		Total and differential leukocyte count	level of total leukocyte ↑, neutrophils ↑, monocytes ↑, and eosinophils counts ↑	

ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); BB: boiled bulbs; BCB: β-carotene-linoleic acid bleaching; DCFH-DA: 2′,7′-dichlorodihydrofluorescein diacetate; DPPH: 1,1-diphenyl-2-picrylhydrazil; ferric-reducing ability power (FRAP); GAE: gallic acid equivalents; HPSA: hydrogen peroxide scavenging assay; LC: leopoldia comosa; LPS: lipopolysaccharide; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBT: nitroblue tetrazolium; RB: raw bulb; ROS: reactive oxygen species; SB: Steamed bulbs; TE: Trolox equivalent; WB, Western blot; XO: Xanthine oxidase; ↑: up-regulation; ↓: down-regulation/inhibition.

6.2. Anti-Diabetes

Over 529 million people were found globally to have diabetes in 2021, which indicates that the prevalence of diabetes is increasing worldwide; this poses a substantial challenge to public health [43]. Natural compounds are believed to have the potential to be developed against diabetes [44–46]. The anti-diabetes potential of LC bulbs has been studied by analyzing the inhibition of two important enzymatic drug targets of diabetes, i.e., α-glucosidase and α-amylase. Extract from the bulbs of LC was able to effectively inhibit both drug targets, and in the case of both targets, the inhibition was higher for ethanol extract as compared to *n*-hexane [27]. The relatively low activity of the non-polar extract (*n*-hexane) indicated the important role of the polar component of LC bulbs in anti-diabetes activity [27]. The identification of components important for the activity may be helpful in optimizing the anti-diabetes activity of LC bulbs in further studies.

Considering the dietary use of LC, the extracts from the traditional boiled and steam-cooked bulbs have also been studied for their antidiabetic potential [12]. The extracts from traditionally cooked, steam-cooked, and raw bulbs were studied for their inhibitory activity against α-amylase. A higher inhibition activity was observed in the case of the extract from raw bulbs. The significant difference between the properties of the cooked and raw extracts indicated that cooking slightly reduced the α-amylase inhibition activity of the extract from the cooked bulbs (Table 2). Later, the researcher studied the anti-diabetes potential of methanolic extract from LC bulbs along with other biological properties. Anti-diabetes activity of the extract against α-amylase and α-glucosidase was studied using acarbose as a positive control.

In a recent study, the anti-diabetes investigation was conducted with the inclusion of an inhibitory assay of one more important target in diabetes, i.e., β-galactosidase along

with α-glucosidase and α-amylase [32]. Different extracts of aqueous (decocted, infused, and macerated) and organic (ethanolic, macerated ethanolic, acetone, macerated acetone, diethyl ether, and macerated diethyl ether) LC bulbs were used to study their activity against all three selected drug targets. Strong inhibition was observed for organic extracts, particularly the acetone extract, against β-galactosidase and α-glucosidase enzymes, which was better than their control compounds (acarbose and quercetin for α-glucosidase and β-galactosidase, respectively) used in the experiments. In the case of α-amylase, the most active extract was decocted aqueous extract, but its activity was lower than the positive control (acarbose) used in the experiments (Table 2).

6.3. Anti-Obesity

The worldwide occurrence of overweight and obesity is worryingly growing and also leading to several other serious public health concerns [47]. As with diabetes, the natural compounds are believed to have the potential to be developed against obesity [48,49]. The accumulation of excessive fat and its inappropriate storage is considered as obesity; hence, fat and the dysregulation of its metabolism can be considered as the primary factors responsible for obesity in the population. Pancreatic lipase is the enzyme that helps with the digestion and absorption of fat from food in the intestine. Inhibiting pancreatic lipase is one of the proven strategies for the treatment of obesity. Orlistat is the only conventional drug used for obesity treatment through the inhibition of pancreatic lipase, but the side effects of the drug have ignited the hunt to develop plant-based lipase-inhibiting drugs for obesity [50]. The pancreatic lipase inhibition activity of LC bulb extract from wild and cultivated plants has been studied to explore its anti-obesity potential (Figure 2). Importantly, raw and polar fractions of extract from wild-grown plants showed good lipase-inhibiting activity in the assays, which clearly indicated the anti-obesity potential of LC bulb extract. The study also suggests that the polyphenols, which can be considered bioactive metabolites of the plant, might be responsible for the inhibition of pancreatic lipase activity, but further research may be required to confirm this [29]. Similarly, anti-obesity potential through the inhibition of pancreatic lipase has also been studied for the extract from the leaves and inflorescence of LC [31]. Lipase-inhibition activities were found to be higher in the leaves than in the inflorescence in the raw extract and different fractions used in the study (Table 2). Significant lipase-inhibition activities of both of these aerial parts of LC suggest the anti-obesity potential of these parts along with bulbs. Further studies aimed at the identification of components important for anti-obesity activity would help to optimize and develop LC as an anti-obesity therapeutic [31]. Encouraging results of anti-obesity activities from previous in vitro studies inspired further in vivo anti-obesity studies using a rat model [13,31]. Two different doses of extract from LC bulbs and a positive control drug (orlistat) were used in animal studies in Wistar rats. The doses were selected according to the results from pancreatic lipase and pancreatic α-amylase inhibition assays, conducted before the animal study. In both enzyme inhibition studies, the extract from LC bulbs achieved comparable activities with positive controls, i.e., orlistat and acarbose in the case of pancreatic lipase, and pancreatic α-amylase inhibition assays, respectively. In the animal study, the body weight and circumference of the waist of rats in both treatment groups were lower than in the high-fat diet (HFD) group after 12 weeks of the oral administration of extract from LC bulbs. Additionally, the perirenal, retroperitoneal, epididymal, and abdominal fat in the rats in both treatment groups was lower than in the high-fat diet group after treatment [13]. Similarly, the weight and CSI values of the heart and liver in the high-fat diet group were enhanced, which was then reversed by the treatment with LC bulb extract. In blood, ROS production, triglycerides, LDL cholesterol, LDL-cholesterol-ox, and total cholesterol were increased in the high-fat diet group compared to the control group, while being significantly reduced in both low and high-dose treatment groups. Similarly, the level of plasma insulin, basal glycemia level, and homeostatic model assessment-estimated insulin resistance (HOMA-IR) index of the

high-fat diet group were enhanced in comparison with the control group, which was also reversed by the treatment with LC bulb extract in both treatment groups [13].

In tissue histology of abdominal fat, the areas of adipocytes were increased in the HFD group compared to the control group, while being significantly reduced in the high-dose (HFD + Lc (60 mg)) treatment group (Table 2). Similarly, in liver samples, the presence of fat vacuoles was strongly increased in the HFD group compared to the control group, while being significantly reduced in the high-dose (HFD + Lc (60 mg)) treatment group. The triglyceride content of the liver was also increased in the HFD group and significantly reduced in both low- and high-dose treatment groups. Additionally, the expression of key enzymes of gluconeogenesis PEPCK and G6Pase was also found to be increased in the HFD group compared to the control group, while being significantly reduced in the high-dose (HFD + Lc (60 mg)) treatment group. The in vitro antioxidant activity and reduction of antioxidant factors such as ROS and LDL-cholesterol-ox in the high-fat diet animal model observed in the study suggested that the antioxidant property of the extract may be an important factor in the anti-obesity effect in both metabolic and anthropometric observations. It was also suggested that the anti-obesity activity of LC may be due to the antioxidant activity of the extract, as factors such as ROS and LDL-cholesterol-ox were found to be reversed in the HFD animals with extract treatment [13].

6.4. Antibacterial

Antimicrobial resistance is one of the current important public health concerns that demands the rapid discovery of new/novel antibacterial drugs [51]. Different plants were selected from southern Italy to study their potential to inhibit growth and biofilm formation against methicillin-resistant *Staphylococcus aureus*. A total of 168 extracts from the plants, including aqueous and ethanolic extracts from LC bulbs, were studied [41]. Both extracts ofromLC bulbs showed strong inhibition of biofilm formation, and LC was among the top 10 biofilm-inhibiting extracts with IC50 $\leq$ 32 μg/mL.

The antibacterial activity of extract from LC bulbs was carried out against different gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*, and *Listeria innocua*) and negative bacteria (*Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Escherichia coli*) causing common infectious diseases. The agar disc diffusion assay was used to study the inhibition of selected bacterial strains with different doses of extract. The organic extract from LC bulbs showed antibacterial activity against both gram-positive and negative bacteria but it was limited to only two bacterial species selected in the study, i.e., *Proteus mirabilis* and *Listeria innocua*, and it was lower than the positive control used in the study [32]. Importantly, the inhibition of *Listeria innocua* was only observed in the diethyl extracts, which suggests the importance of the components present in these extracts. These initial antibacterial studies highlighted the importance of components imperative for antibacterial activity and the identification of these components may be useful for the optimization of the antibacterial properties of the extract for further development as an antibacterial therapeutic candidate.

6.5. Immune Enhancement Effect of LC Bulb Extracts

Several herbal products are considered to have potential immune modulation properties [52,53]. Studies have shown that immune stimulation is not only found to be useful in fighting against infectious diseases but it may also help against other important noninfectious diseases such as cancer [8]. Considering the importance of immune stimulation in aquaculture, the immune-stimulating effect of the extract from LC bulbs was studied in gilt-head seabream, *Sparus aurata* [42]. The extract was investigated at two different doses (0.5 and 2 mg) through intraperitoneal injection, and both doses were found to enhance different parameters associated with immune stimulation. Blood parameters that were found to be increased in the study were nitroblue tetrazolium (NBT)-positive cell count, lysozyme activities of serum samples level of total leukocyte, neutrophils, monocytes, and eosinophils counts [42]. The growth of the fish was also found to be increased after the administration of extracts. Additionally, there was no negative effect of the extract

treatment observed on the hematocrit level of blood in the study. The study concluded that the extract from LC bulbs may enhance the nonspecific immune system of *Sparus aurata*, which can be further studied on other fishes in aquaculture.

6.6. Anti-Cancer Activity

Like diabetes and obesity, the global burden of cancer is also increasing, rapidly surpassing the control capacity [54]. Natural products are considered important potential anti-cancer agents due to their accessibility, applicability, and reduced cytotoxicity [55]. In the initial study, the anticancer activity of ethanol-precipitated fraction of extract from LC bulbs was studied on Walker 256 (intramuscular) carcinosarcoma [16]. Before this experiment, the toxicity study on the DBA strain of mice was carried out. The daily dose of 2 g/kg was found to be tolerated by all the mice used in the study, and no side effects were observed. It was concluded that the water-soluble part of the extract had less toxicity. Different fractions of the extract were prepared according to the volume of ethanol used for the precipitation. Among these, the most active fraction demonstrated a reduction in tumor volume at 1:5 (treatment/control) compared with control (Table 2) [16].

Later, the anti-tumor activity of LC bulbs was studied on human breast adenocarcinoma-derived MCF-7 cell lines. The effect of the extract on cell viability was studied through a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The extracts from the raw and cooked bulbs were examined for anti-proliferative activities. Effective dose-dependent anti-proliferative activity of extract from the raw bulbs was observed in the study [12]. In the case of cooked bulbs, the activity dropped and was only observed at a high dose, which suggested that cooking reduced the anti-proliferative activity of the extract such as antioxidant and anti-diabetes activities, which were reported to be reduced in the cooked bulbs in comparison with raw bulbs. Thus, cooking may reduce the content of biologically active components of the extract.

6.7. Anti-Inflammatory

Inflammation may lead to other lethal diseases or conditions including cancer, cardiovascular disease, diabetes, non-alcoholic fatty liver disease, and neurodegenerative and autoimmune disorders [56]. Hence, anti-inflammatory activity can be considered an important activity, as it may be preventive as well as therapeutic in other deadly diseases, including cancer and AD [57–59]. In the initial study, the presence of newly discovered homoisoflavanones that were structurally similar to flavonoids was also speculated to have biological activity like flavonoids. Therefore, the researcher studied the anti-inflammatory activity of homoisoflavanones containing a fraction of extract from LC bulbs through croton oil-induced dermatitis in a mouse model [34]. Effective anti-inflammatory activity (comparable with indomethacin-positive control drug) observed for the fraction inspired the further isolation of 5 homoisoflavanones from the fraction for anti-inflammatory activity (Figure 2). The anti-inflammatory activity was observed in all five compounds in a dose-dependent manner, and the inhibition of inflammation ranged from 21 to 41% at 100 mg/ear (Table 2). Among them, 4′demethyl-3,9-dihydroeucomin was the most active and lightweight (lowest molecular weight) homoisoflavanone, which may be further studied for anti-inflammatory activities in in vivo experiments (Table 1).

The inflammatory activity of the extract from LC bulbs was also evaluated along with antioxidant and other activities (Figure 2). This activity was studied through the inhibition of MMP-9 and MMP-2 enzymes, which are considered important markers in inflammation response (Figure 3). These enzymes are considered important therapeutic targets, as they are found to be up-regulated in inflammatory conditions. The inhibition of MMP-2 and MMP-9 derived from the primary culture of rat astrocytes activated with lipopolysaccharide (LPS) was examined through gelatin gel zymography [30]. In the study, the strong inhibitory activity of the extract from LC bulbs was able to entirely inhibit MMP-9 at 50 mg/mL concentration. In the case of MMP-2, the same concentration of extract was able to reduce it to 55.664.3% (Table 2).

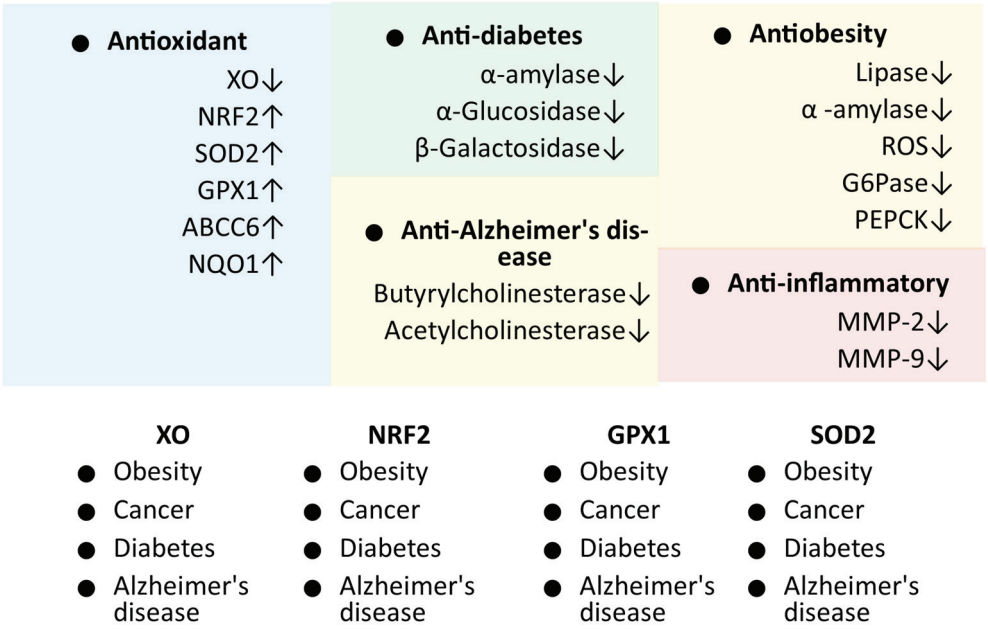


Figure 3. Markers/targets associated with the pharmacological activities of LC. Genes/markers found to be linked with other diseases are listed on the white background. Abbreviations: ↑, up-regulation; ↓, down-regulation/inhibition.

6.8. Anti-Alzheimer's Disease

Alzheimer's disease (AD) is the most common neurodegenerative disorder with no cure, and its incidence is alarmingly high in old age. In recent years, several natural compounds have shown strong potential against AD [60,61]. The anti-AD activity of LC bulbs was studied through the inhibitory potential against the two main types of cholinesterase, including acetylcholinesterase and butyrylcholinesterase (Figure 3), which are the known drug targets in AD and other neurodegenerative disorders [62]. The *n*-hexane and ethyl alcohol extracts from LC bulbs inhibited acetylcholinesterase and butyryl cholinesterase enzymes in the study, and *n*-hexane extract had better inhibition in both drug target enzymes, compared to ethyl alcohol extract [28]. Later, in a different study, the methanolic extract from LC bulbs was prepared to study its different biological properties, including anti-AD activity through the inhibition of acetylcholinesterase [30]. Similar to the previous study, the inhibition of acetylcholinesterase in terms of IC_{50} was observed in this study (Table 2). The study suggested that the inhibition of acetylcholinesterase may improve neuromuscular signaling and cognitive functions, which might be helpful in neurodegenerative disorders such as Parkinson's disease and schizophrenia, along with AD [30].

7. Discussion and Future Directions

Along with traditional gastronomy use as a vegetable, LC has shown promising results in several diseases and conditions, which makes it a wonderful nutraceutical candidate that can be developed in further studies. However, different challenges and gaps persist for its different pharmacological properties, which may require systematic and directional research efforts.

Early phytochemical studies of LC were focused on the isolation and identification of individual or several compounds from the extract [15,20–23]. In later studies, the quantification of the whole class of phytochemicals such as phenolic acid, tannins, and flavonoids was conducted [27–29,31]. In most of these studies, the profiling of several phytochemicals present in the LC extracts was also conducted, which resulted in the identification and quantification of possible phytochemicals in these extracts [29,31]. In recent studies, the optimal extract types and conditions were studied to maximize the concentration of phytochemicals

responsible for the reported and possible biological activities. The antioxidant properties of LC were found to be highest in comparison with reference and other 27 extracts of different plants [5], and they can be considered among the most prospective properties, as they can support other important properties of LC such as anticancer, anti-obesity, and antidiabetic [9]. Furthermore, the antioxidant properties of LC were strongly validated through various antioxidant assays and methods including DPPS, ABTS, lipid peroxidation, metal chelating, FRAP, HPSA, superoxide radicals, and NO scavenging assays (Table 2). Additionally, LC exerted an antioxidant effect through the enhanced expression of *NRF*, *SOD*, and glutathione peroxidase in cell line studies, and the reduction of ROS in the cell line and blood of rat models used in the anti-obesity study [13]. Importantly, most of the genes (*Nrf2* [63–66], XO [67–70], *GpX1* [7,70–72], and *SOD2* [6,70,73,74]) targeted through LC for antioxidant activity are also associated with all other diseases studied for LC impact, i.e., diabetes, cancer, AD, and obesity (Figure 3). Studies to validate these targets strongly suggested that it would not only establish the antioxidant potential of LC but also the development of LC against these diseases. However, in numerous studies, the excellent antioxidant activity of LC was established through cell culture and in vitro experiments. Still, an effective study through in vivo experiments for the antioxidant effect of LC is missing in the scientific literature. An analysis of the antioxidant activity of LC in different organs in animal studies is suggested, which might be helpful to utilize the optimal antioxidant potential of LC, especially in the brain.

Similarly, the anti-diabetes activity of LC has not yet been conducted with an animal model, which is strongly suggested for future studies. Furthermore, antidiabetic activity was observed by inhibiting important target enzymes including α-amylase, α-glucosidase, and β-galactosidase. The maximum inhibition activities of α-glucosidase and β-galactosidase were better than the control compounds in organic extract, but the maximum inhibition in the case of α-amylase was observed in aqueous extract [32]. The difference in the inhibition effects of extracts on different target enzymes indicated that the different phytochemicals may be responsible for the anti-diabetes effect of LC. These results emphasize the importance of knowledge of the phytochemical profile of the extracts, as they can be responsible for anti-diabetes activity in LC and its development as a candidate against diabetes. Hence, the identification of phytochemicals present in the (aqueous and organic) extracts is proposed for further studies.

Similarly, the effective inhibition of pancreatic lipase was observed through in vitro experiments, which supported the anti-obesity effects of LC, also confirmed through animal studies. Further clinical studies may be suggested for LC after the safety studies with other animal models. The study also suggested that polyphenol present in the plant may be responsible for the anti-obesity activity of the plant, but further studies would be required to confirm and identify the phytochemicals responsible for it.

Like antioxidant activity, immune modulation can also be considered as a prospective property of LC, as it can help to fight against various infectious and important non-infectious diseases, including cancer [8]. So far, the immune modulation activity of CL has been observed in fish only; therefore, further studies for immune modulation must be conducted on other animal models before its further consideration in clinical studies.

The anti-AD potential of LC is also in its early phase. However, in different studies, the anti-AD potential of extracts from the bulbs of LC was observed against two important target enzymes of AD, i.e., acetylcholinesterase and butyrylcholinesterase. These targeted enzymes are also important in other neurological disorders, including PD and schizophrenia, which suggests possible positive outcomes of LC use in other neurodegenerative disorders. However, neurodegenerative disorders are complex diseases of the brain, and crossing the blood–brain barrier may also be the decisive factor for candidate drugs against AD. Hence, it is suggested that anti-AD activity is studied in further animal models, along with pharmacokinetics and bioavailability studies.

Anti-cancer activities of LC extracts were observed in both in vitro cell lines and animal experiments focusing on different cancers, which creates the possibility of LC

being used against other important cancers such as lung and gastric cancer. However, more studies are required to establish the anti-cancer properties of LC in other cancer cell lines and animal models. To optimize the anti-cancer activity, further exploration of phytochemical constituents of the extracts, responsible for the anticancer activity, needs to be conducted. No effective mechanistic study to identify the action mechanism of anti-cancer activities of LC is available. Hence, it is proposed to conduct studies to identify the action mechanism behind the anti-cancer activity of LC in further studies, which would be helpful in developing LC as an intervention against cancer.

Like the limited use of LC bulbs as vegetables, their pharmacological potential is also underutilized. The compiled information on phytochemicals and pharmacological activities, along with highlighted gaps and suggested precise directions, may be helpful in the development of LC as a therapeutic or supplement against the above-discussed important diseases or conditions. The high mineral content and presence of health-promoting phytochemicals highlighted in the current review may also encourage the use of LC as a vegetable.

Author Contributions: Study conceptualization, H.-J.L. and V.J.; original draft preparation, V.J.; data arrangement, figures, and tables, V.J.; review and editing, H.-J.L. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Cooperative Research Program for Agriculture Science and Technology Development (project No. RS-2022-RD010230), Rural Development Administration, Republic of Korea.

Data Availability Statement: Data are contained within this article.

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

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MDPI AG
Grosspeteranlage 5
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ISBN 978-3-7258-4670-2