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Special Issue Reprint

Nutraceuticals, Bioactive Compounds and Functional Foods

Their Role in Chronic Diseases

Edited by
María D. Navarro-Hortal and Alfonso Varela-López

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Nutraceuticals, Bioactive Compounds and Functional Foods: Their Role in Chronic Diseases

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Guest Editors

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About the Editors

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Preface

Population aging is associated with a growing burden of chronic diseases, including cardiometabolic disorders, neurodegeneration, liver disease and other conditions that compromise quality of life and functional capacity. Nutrition has emerged as a key modifiable factor that may help prevent or mitigate many of these conditions. In particular, increasing attention has been given to food nutrients and bioactive compounds derived from natural sources, as growing evidence suggests that these molecules can influence several biological pathways involved in disease development and progression.

This Reprint of the Special Issue published in *Nutrients* brings together a collection of original research articles and reviews that explore the role of dietary compounds and natural bioactives in the modulation of metabolic, cardiovascular, neurological and inflammatory processes. The contributions included address a wide range of compounds and nutritional strategies, such as polyphenols, short chain fatty acids, phytochemicals from medicinal plants, and other food derived bioactives. Several studies examine molecular and cellular mechanisms, including the regulation of oxidative stress, inflammation, endoplasmic reticulum stress, endothelial function and metabolic signaling pathways, while others provide comprehensive overviews of the potential of specific foods or compounds in the context of diseases such as metabolic dysfunction associated steatotic liver disease, chronic kidney disease, hypertension, neurodegenerative disorders and reproductive dysfunction.

Together, the articles collected in this Reprint provide updated insights into how dietary components and nutraceutical approaches may contribute to the prevention or management of chronic conditions. The aim of this Reprint is to present current advances in the field and to encourage further interdisciplinary research on the role of nutrition and food derived bioactive compounds in human health. It is addressed to researchers, nutrition scientists, clinicians and health professionals interested in the biological and clinical implications of nutrition-based strategies for disease prevention and health promotion.

María D. Navarro-Hortal and Alfonso Varela-López
Guest Editors

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Article

Ginsenoside Rh2 Mitigates Endoplasmic Reticulum Stress-Induced Apoptosis and Inflammation and Through Inhibition of Hepatocyte–Macrophage Inflammatory Crosstalk

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Abstract: Background/Objectives: Endoplasmic reticulum stress (ERS) contributes to hepatocyte inflammation, triggered by prolonged exposure to lipotoxicity, and promotes non-alcoholic fatty liver disease (NAFLD) progression by recruiting and activating hepatic macrophages, which accelerate fibrosis and exacerbate disease progression. Here, we aimed to evaluate the therapeutic potential of ginsenoside Rh2 (Rh2) in a cell model of NAFLD induced by the ERS inducer thapsigargin (THA). **Methods:** HepG2 cells were treated with THA to induce ERS and mimic NAFLD conditions. The effects of Rh2 on ERS, lipid accumulation, and apoptosis were assessed in HepG2 cells. Additionally, THP-1 cells were used to investigate macrophage activation upon exposure to conditioned medium (CM) from THA- and Rh2-treated HepG2 cells. Gene and protein expression of inflammatory and lipid synthesis markers were analyzed, as well as M1/M2 macrophage polarization markers. **Results:** Rh2 inhibited THA-induced apoptosis, ERS, and lipid accumulation in HepG2 cells. It also reduced the expression of lipid synthesis genes (SREBF1, FAS) and inflammatory markers (IL-6, IL-1β, TNF-α, MCP-1). CM from Rh2-treated HepG2 cells suppressed macrophage activation in THP-1 cells, decreased M1 polarization markers (CD80, CD86), and increased M2 markers (CD163, Arg1, MRC-1). **Conclusions:** These results suggest that Rh2 effectively suppresses inflammation and lipid storage in ERS-induced HepG2 cells while modulating the crosstalk between hepatocytes and macrophages. These findings underscore the potential of Rh2 as a promising therapeutic agent for the prevention and early intervention of NAFLD progression.

Keywords: endoplasmic reticulum stress; ginsenoside Rh2; NAFLD; macrophage

1. Introduction

The rapid changes in dietary habits and lifestyles have contributed to the rapid increase in metabolic disorders worldwide. Among them, nonalcoholic fatty liver disease (NAFLD) has emerged as a major public health problem, with a global prevalence of approximately 30% [1], and approximately 20% of them progress to nonalcoholic steatohepatitis (NASH) [2,3]. Endoplasmic reticulum stress (ERS), in particular, is frequently observed in the livers of patients with NAFLD [4], and is considered a key contributor to the progression from steatosis to NASH. Experimental evidence demonstrates that the treatment of hepatocytes with the ERS inducer thapsigargin (THA) upregulates the expression of key regulators such as XBP1 and FOXA3, along with genes involved in lipid

synthesis [5]. Moreover, ERS activates lipid synthesis pathways [6–10] including that of SREBP1c/ACC/FAS, leading to triglyceride accumulation and the subsequent activation of inflammatory pathways such as JNK and NF-κB [11–13]. These processes collectively establish a chronic inflammatory environment, further amplifying hepatocellular damage.

Emerging evidence highlights the critical role of hepatocyte–macrophage crosstalk in the progression of NASH. In particular, inflammation induced by fatty liver exacerbates liver damage and fibrosis by secreting various inflammatory cytokines and promoting macrophage recruitment and infiltration into liver tissue [14–17]. These activated macrophages differentiate into classical macrophages (also known as M1 macrophages) or alternative macrophages (also known as M2 macrophages). In the early stage of NAFLD, M1 macrophages stimulate hepatic stellate cells (HSCs), contributing to fibrosis [18–21]. These pathological interactions accelerate NAFLD progression and increase the risk of liver-related morbidity and mortality [22]. Given these findings, therapeutic strategies aimed at attenuating ERS, reducing inflammation, and inhibiting macrophage activation and infiltration are considered crucial for effectively managing and preventing NAFLD progression.

In recent years, natural plant chemical extracts have been identified as promising alternatives for alleviating the side effects and complications of existing disease-treating drugs [23]. Ginseng, a root plant cultivated in several countries in East Asia and North America, has been used for thousands of years in traditional Asian medicine to prevent and treat various physiological and pathological conditions. Notably, the level of ginsenoside Rh2 (Rh2), which is present only in trace amounts or is undetectable in unprocessed ginseng, is markedly increased in red ginseng. Rh2 has been reported to provide various health benefits, including anticancer [24,25], antidiabetic, anti-Alzheimer's disease [26], and anti-inflammation effects [27,28]. However, research on the role of Rh2 in the progression of NAFLD, particularly in relation to macrophage recruitment and polarization, remains limited. Further mechanistic investigations are warranted to elucidate its therapeutic potential in this context. Recent studies have shown that Rh2 effectively reduces ERS-related proteins and alleviates ERS induced by ERS inducers. Additionally, Rh2 has been shown to attenuate adipocyte differentiation in both rodent and human preadipocyte cell lines, and dietary Rh2 intake has been observed to inhibit adipogenesis in obese mice [29,30]. Therefore, this study aimed to investigate whether Rh2, recognized for its anti-inflammatory properties [31,32], could regulate lipogenesis and mitigate inflammation in ERS-induced HepG2 cells. Furthermore, it examined whether Rh2-treated hepatocytes could modulate hepatic macrophage infiltration and activation, thereby alleviating cellular damage and preventing the progression of NAFLD.

2. Materials and Methods

2.1. Reagents

Rh2 (purity $\geq$ 97%), Compound K (purity $\geq$ 96%), Panaxadiol (PD) (purity $\geq$ 96%), Palmitic acid (PA), and Ethanol (EtOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Protopanaxadiol (PPD) (purity $\geq$ 90%) was purchased from PytoLab (Vestenbergsgreuth, Germany). Thapsigargin (THA) was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Cell Culture and Stimulation

HepG2 cells and THP-1 cells were purchased from ATCC. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco). THP-1 cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640; Gibco) supplemented with 10% heat-inactivated FBS (Gibco) and 1% penicillin/streptomycin

(Gibco). The cells were always washed with FBS before experiments to reduce interference from unknown substances in the serum and to ensure a homogeneous cell population. All cells were cultured in a humidified 5% CO_2 environment at 37 °C. To induce stress in HepG2 cells, the cells were treated with 60 μM PA, 1000 μM EtOH, and 2 μM THA. After incubation for 42 h or 48 h, the cells were harvested and used for experiments. Cells at passages 12–16 were used for all experiments.

2.3. Cell Viability Assay

HepG2 cells were seeded in 96-well plates (Falcon) at a density of 3×10^4 cells per well. For the ginsenoside compound cell viability assay, HepG2 cells were treated with both 2 μM THA and Rh2 (2.5–10 μM), Compound K (0–3 μM), PD (0–15 μM), and PPD (0–15 μM) for 48 h. After washing the cells, 200 μL of fresh medium was added per well. Cell viability was assessed using a water-soluble tetrazolium salt-based EZ-Cytox assay kit (Dogenbio, Seoul, Republic of Korea) according to the manufacturer's instructions.

2.4. Apoptosis Assay

Apoptotic cells were detected using Annexin V (FIFC; BD bio, San Jose, CA, USA) and propidium iodide (PI; BD bio). HepG2 cells were stained with 5 μL of Annexin V and PI for 15 min at RT while protected from light. Stained cells were analyzed with the CytoFLEX flow cytometer (Bexkman Coulter, Brea, CA, USA) within 1 h.

2.5. Oil Red O Staining

ERS-induced lipid accumulation in HepG2 cells was visually confirmed by staining lipid droplets using Oil Red O solution (Sigma-Aldrich, St. Louis, MO, USA). The Oil Red O working solution was diluted with distilled water at a 4:6 ratio and filtered through a 0.2 μM filter for 20 min, followed by washing twice with DW. The cells were incubated with 60% isopropanol for 5 min, removed by suction, and completely left alone for 1 h in the dark. After washing 4 times with DW, the stained lipid droplets were photographed using a microscope. The stained lipid droplets were extracted by adding 100% isopropanol and quantified by measuring the absorbance at 510 nm. The values were corrected based on cell viability. All experiments were performed at room temperature.

2.6. Triglycerides Assay

HepG2 cells were harvested after washing with PBS. To quantify intracellular triglycerides, 5% NP-40 was added to the harvested cells. The mixture was heated at 80–100 °C for 5 min, cooled, and then heated again for 5 min. Then, the mixture was centrifuged at 3000× *g* for 20 min at 4 °C. Subsequently, the supernatant was separated, diluted 10 times in DW, and used for experiments. Intracellular triglyceride accumulation was quantified according to the protocol of the PicoSens™ Triglyceride Assay Kit (Biomax, Guri, Republic of Korea). The values were corrected based on cell viability.

2.7. Protein Extraction and Immunoblotting

Protein extraction was performed using RIPA lysis buffer (Rockland, Pottstown, PA, USA) containing protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). After centrifugation at 15,000× *g* for 20 min at 4 °C, the resulting supernatant was collected. Protein quantification was performed by Bradford protein assay (Bio-Rad, Hercules, CA, USA), and equal amounts of protein were subjected to SDS-PAGE. The proteins were subsequently transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween® 20 detergent (TBST) at room temperature for 1 h, followed by overnight incubation with primary antibodies against XBP1s (Cell signaling, Beverly, MA, USA), FAS (Cell

signaling), and β-actin (Cell signaling) at 4 °C. Then, the membranes were incubated with horseradish peroxidase-linked secondary antibodies (Bio-Rad) at RT for 1 h. Enhanced chemiluminescence (Bio-Rad) was employed for blot visualization using the ChemiDoc Imaging System (Bio-Rad). The resulting bands were quantified using National Institutes of Health Image J software (v1.53e).

2.8. Harvest of Conditioned Medium (CM) from HepG2

HepG2 cells were stimulated with 2 μM THA and either 2.5 or 5 μM Rh2 for 48 h. After washing with PBS, fresh culture medium was added and incubated for 24 h to collect the medium. Then, the CM was obtained after centrifugation (200× *g*, 10 min, 20 °C).

2.9. Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of IL-6, TNF-α, IL-1β, and MCP-1 in the CM obtained from HepG2 cells were determined using ELISA kits (R&D Systems, Minneapolis, MN, USA; DY206, DY210, DY201, and DCP00). CM and standards were dispensed into 96-well plates pre-coated with capture antibodies. The levels of each cytokine and chemokine were then measured following the standard sandwich ELISA protocol provided by the manufacturer.

2.10. CM from HepG2 Cells Treatment in THP-1 Cells

Undifferentiated THP-1 cells were cultured at a density of 1×10^5 cells in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin, mixed with conditioned medium (CM) at a 1:1 ratio. A maximum of 1 mL of CM was collected from each sample for use in the mixture, and FBS was present throughout the incubation. The medium was replenished every 48 h. On the third day of culture, the number of attached cells was counted using a microscope. Cells were harvested after 48 or 96 h of culture.

2.11. RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA extraction was performed using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA quantification was conducted using NanoDrop (Quawell Technology, Palo Alto, CA, USA), and complementary DNA (cDNA) was synthesized from 100 ng or 50 ng of purified RNA using the iScript cDNA synthesis kit (Bio-rad). The reaction volume was adjusted to 20 μL following the protocol for iQ™SYBR® Green Supermix (Bio-Rad). Each sample was subjected to PCR amplification using an equal amount of the cDNA template with the following steps: initial denaturation (95 °C, 3 min), followed by 40 cycles of denaturation (95 °C, 10 s) and annealing/extension (58 °C, 30 s). The CFX96 Touch Real Time-PCR Detection System (Bio-Rad) was utilized. RNA expression was normalized to β-actin expression, and relative RNA expression was calculated using the 2-ΔΔCT method. The custom-designed primers are presented in Table 1.

Table 1. Primer sequences used in RT-PCR.

Gene		Primer Sequences	Accession Number
β-actin	Forward (5′-3′) Reverse (5′-3′)	GATTCCTATGTGGGCGACGA TCTCCATGTCGTCCCAGTTG	NM_001101.5
IL-1β	Forward (5′-3′) Reverse (5′-3′)	CTCTGTCATTCGCTCCCACA ACACTGCTACTTCTTGCCCC	XM_054341810.1
IL-6	Forward (5′-3′) Reverse (5′-3′)	AGTGAGGAACAAGCCAGAGC ATTTGTGGTTGGGTCAGGGG	NM_000600.5
TNF-α	Forward (5′-3′) Reverse (5′-3′)	GTCCTCTTCAAGGGCCAAGG GGCTCTTGATGGCAGAGAGG	NM_000594.4

Table 1. *Cont.*

Gene		Primer Sequences	Accession Number
FAS	Forward (5′-3′) Reverse (5′-3′)	GGCCCACAAGAGCTACATCA GGAGCGAGAAGTCAACACGA	XM_054315477.1
SREBF1	Forward (5′-3′) Reverse (5′-3′)	TGACCGACATCGAAGGTGAA AAAGTGCAATCCATGGCTCC	NM_001005291.3
FOXA3	Forward (5′-3′) Reverse (5′-3′)	TCTTGGGGCCTGATCCTTCT GGATCAACACCATGCCCACT	NM_004497.3
BiP	Forward (5′-3′) Reverse (5′-3′)	TGAAAGAAACCGCTGAGGCT TCTTTGGTTGCTTGGCGTTG	NM_005347.5
MCP-1	Forward (5′-3′) Reverse (5′-3′)	GCAGTAAGTGTCCCAAAGAAGC TGGGTTTGCTTGTCCAGGTG	NM_002982.4
COL1A1	Forward (5′-3′) Reverse (5′-3′)	GCTGGTGCTCGTGGAAAT ACCCTTAGCACCAACAGC	NM_000088.4
CD86	Forward (5′-3′) Reverse (5′-3′)	CTTCCTGCTCTCTGGTGCTG GCTCACTCAGGCTTTGGTTC	NM_176892.2
CD80	Forward (5′-3′) Reverse (5′-3′)	CCACAACCTTTGGAGACCCA AGGCAGGGCTGATGACAATC	NM_005191.4
IL-12A	Forward (5′-3′) Reverse (5′-3′)	GCTCCAGAAGGCCAGACAAA TAAACAGGCCTCCACTGTGC	NM_000882.4
CD163	Forward (5′-3′) Reverse (5′-3′)	GGACCCACTTCCTGTTCTGG TGACACACCACCTGAGCATC	XM_054373862.1
ARG1	Forward (5′-3′) Reverse (5′-3′)	GGGTTGACTGACTGGAGAGC CGTGGCTGTCCCTTTGAGAA	NM_000045.4
MRC1	Forward (5′-3′) Reverse (5′-3′)	GGGACGTGGCTGTGGATAAA TCCAAAACCCAGAAGACGCA	NM_002438.4
IL-10	Forward (5′-3′) Reverse (5′-3′)	CGAGATGCCTTCAGCAGAGT GGCAACCCAGGTAACCCTTA	NM_000572.3

2.12. Statistical Analysis

The results are expressed as the mean ± standard deviation (SD). Statistical analysis for two-sample comparisons was performed by Student's t-test with the 95% confidence interval (SPSS PASW Statistic 23.0; SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Inhibitory Effect of Rh2 Treatment on Apoptosis and Lipid Accumulation in ERS-Induced HepG2 Cells

HepG2 cells were exposed to various stress inducers, including PA, EtOH, and THA, for 42 h, and then Oil Red O staining and RT-PCR analysis were performed (Figure 1A). The results demonstrated elevated expression of ER stress markers (BiP and FOXA3) and inflammatory markers (IL-6, TNF-α, and IL-1β) (Figure 1B–F). Moreover, the expression of lipogenic genes and triglyceride accumulation were significantly higher in the THA-treated group compared to other conditions (Figure 1G–I). This confirms that THA-induced ER stress is an important factor in promoting hepatic lipogenesis. Additionally, the therapeutic effects of ginsenoside compounds, including Rh2, Compound K, PD, and PPD, were evaluated. Among these compounds, Rh2 showed the most significant restoration of cell viability reduced by ER stress (Figure 2A–D, $p < 0.001$).

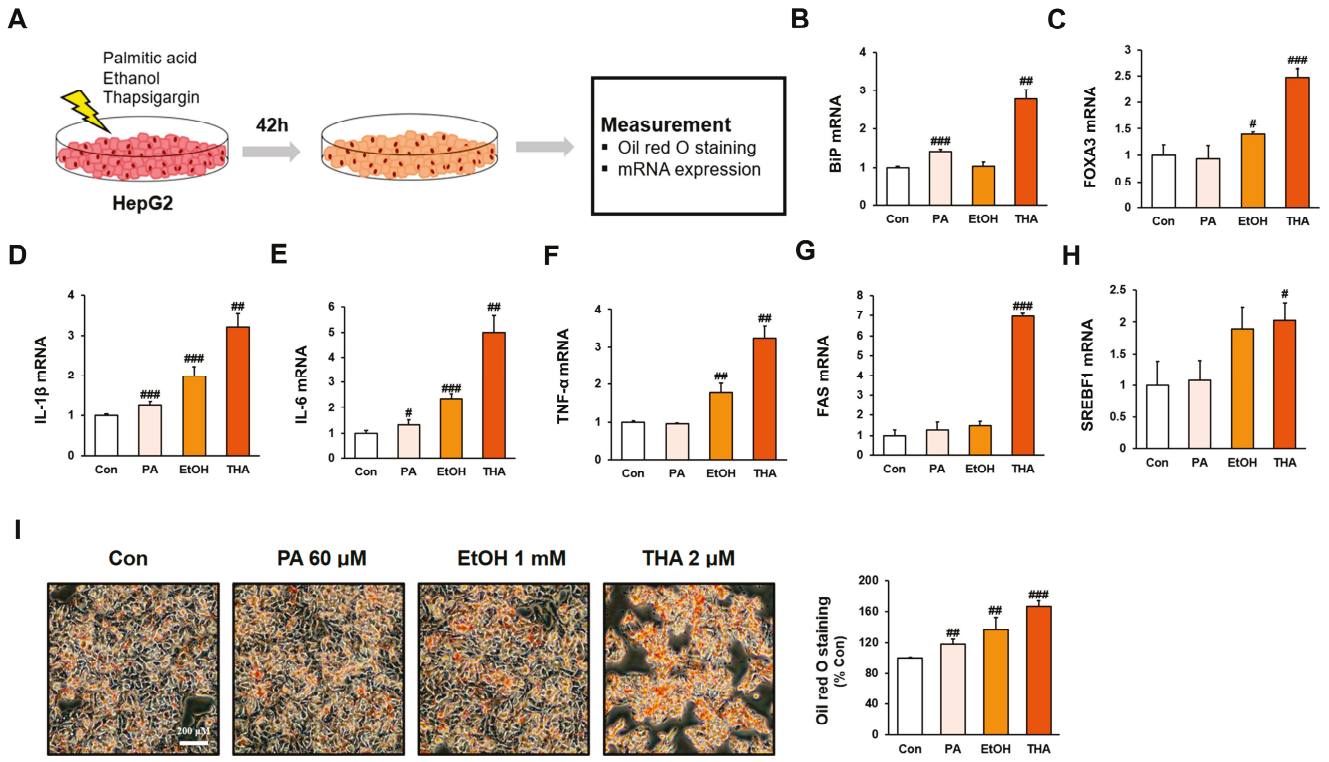


Figure 1. Inflammation responses and lipogenesis in HepG2 cells following treatment with PA, EtOH, and THA. HepG2 cells treated with PA, EtOH, and THA. The Con group was treated with vehicle (PBS). The identified non-cytotoxic concentrations (60 μM PA, 1000 μM EtOH, and 2 μM THA) were used in subsequent experiments. (**A**) The experiment was conducted after treating HepG2 cells with PA, EtOH, and THA, followed by incubation for 42 h. (**B**,**C**) mRNA expression of ERS markers BiP and FOXA3. (**D**–**F**) mRNA expression of inflammation markers IL-1β, IL-6, and TNF-α. (**G**,**H**) mRNA expression of FAS and SREBF1. (**I**) Comparison of lipid accumulation in HepG2 cells based on Oil Red O staining. Values are shown as mean ± SD (n = 3). Con vs. PA, Con vs. EtOH, Con vs. THA, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$.

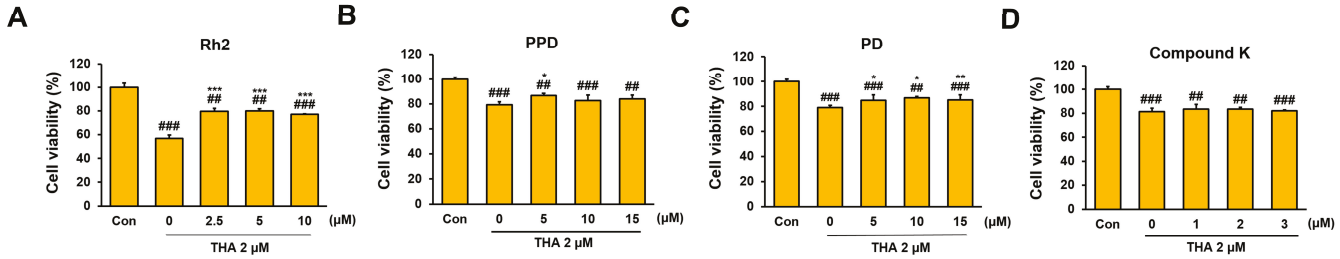


Figure 2. Comparison of the cytoprotective effects of ginsenosides against THA-induced ERS in HepG2 cells. Cell viability of HepG2 cells treated with 2 μM THA and ginsenosides Rh2 (0–10 μM; **A**), PPD (0–15 μM; **B**), PD (0–15 μM; **C**), and Compound K (0–3 μM; **D**) for 48h. The Con group was treated with vehicle (PBS). Values are shown as mean ± SD (n = 3). Con vs. 0, Con vs. 1, Con vs. 2, Con vs. 2.5, Con vs. 3, Con vs. 5, Con vs. 10, Con vs. 15, ## $p < 0.01$, ### $p < 0.001$. 0 vs. 1, 0 vs. 2, 0 vs. 2.5, 0 vs. 3, 0 vs. 5, 0 vs. 10, 0 vs. 15, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Rh2 was selected for further investigation because of its excellent cytoprotective effect. Annexin V/PI staining further confirmed that Rh2 at concentrations of 2.5 and 5 μM effectively reduced cell apoptosis (Figure 3A). Next, we examined the ERS-related factors BiP and XBP1s in HepG2 cells treated with THA and exposed to Rh2. BiP was markedly increased in HepG2 cells treated with THA (0 group) compared with the Con group. However, Rh2 treatment effectively reduced BiP mRNA expression (Figure 3B). Additionally, the protein level of XBP1s was increased in the 0 group compared with the

Con group, and it was reduced by Rh2 treatment (Figure 3C). The findings indicated that Rh2 treatment reduced ERS-induced cell damage.

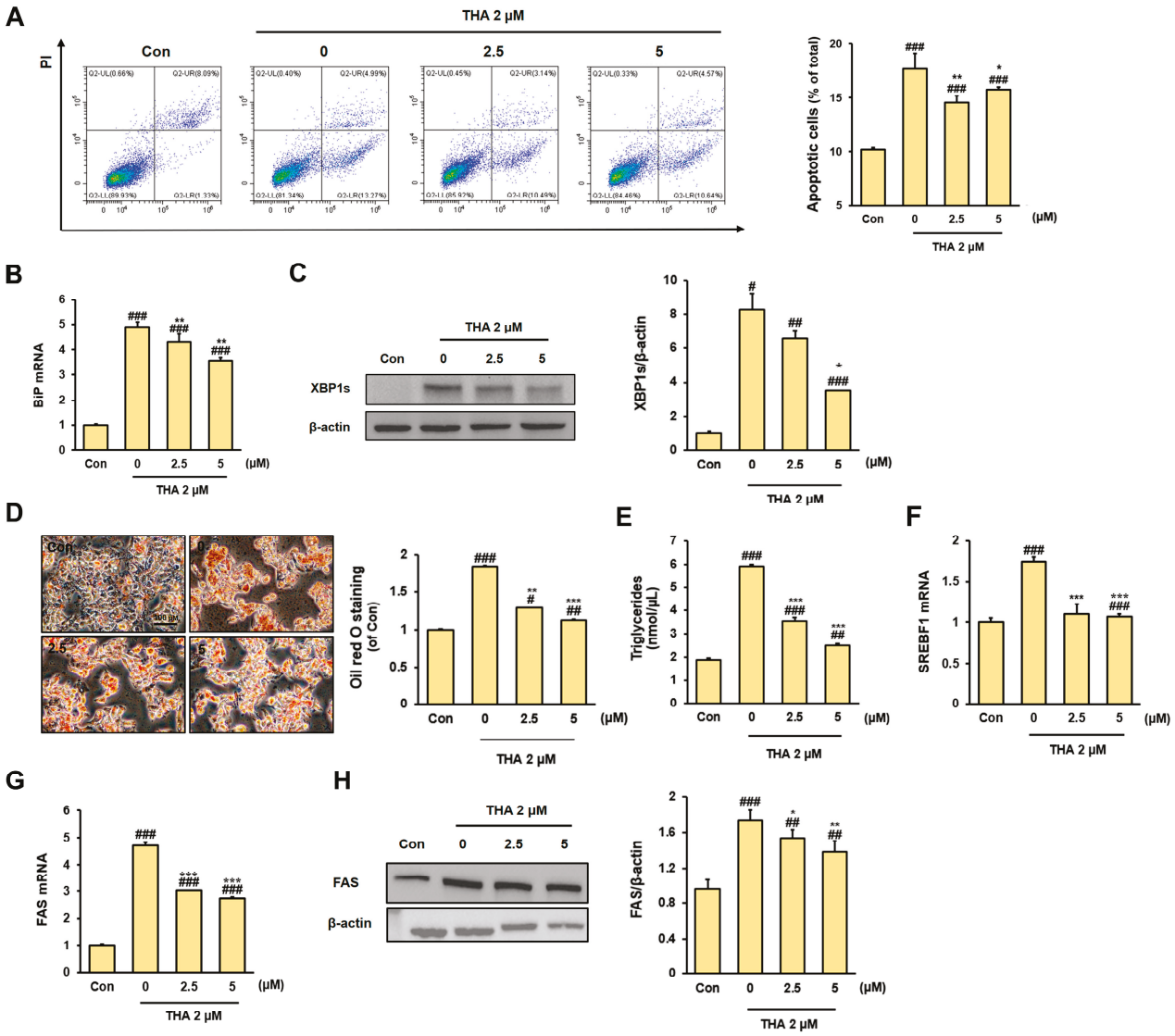


Figure 3. Effect of Rh2 treatment on lipogenesis and apoptosis in ERS-induced HepG2 cells. (**A**) Apoptosis of HepG2 cells treated with 2 μM THA, followed by treatment with (0 μM, 2.5 μM, 5 μM) Rh2 for 48 h. The apoptosis rate was measured by Annexin V/PI staining. mRNA expression of ERS marker (**B**) BiP, (**C**) XBP1s protein expression in HepG2 cells. (**D**) Comparison of lipid accumulation in HepG2 cells based on Oil Red O staining. (**E**) Measurement of triglycerides in HepG2 cells. mRNA expression of (**F**) SREBF1 and (**G**) FAS and (**H**) protein expression of FAS in HepG2 cells. The Con group was treated with DMSO, and the x-axis of the graph represents the Rh2 concentration. Values are shown as mean ± SD (n = 3). Con vs. 0, Con vs. 2.5, Con vs. 5, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$. 0 vs. 2.5, 0 vs. 5, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

As ERS could induce lipid accumulation in hepatocytes, we investigated whether Rh2 could exert protective effects. As expected, stronger Oil Red O staining was observed in the ERS-induced 0 group compared with the Con group. However, compared with the 0 group, the Rh2-treated group showed a weaker stained area. The results were confirmed by the quantitative measurement of Oil Red O (Figure 3D), and the triglyceride assay further supported the findings (Figure 3E). We investigated whether these effects were caused by changes in the lipid synthesis pathway. The expression level of SREBF1 mRNA in the 0 group, which was increased more than two-fold compared with the level of the Con

group, was decreased following Rh2 treatment (Figure 3F). Additionally, the expression level of FAS mRNA in the 0 group, which was increased approximately five-fold compared with the level of the Con group, was significantly decreased with Rh2 treatment (Figure 3G, $p < 0.001$). Similar results were observed for the FAS protein level (Figure 3H). Overall, the results demonstrated that Rh2 treatment suppressed upregulated lipid synthesis induced by ERS, thereby preventing excessive lipid accumulation.

3.2. Inhibitory Effect of Rh2 Treatment on Inflammation and Fibrosis in ERS-Induced HepG2 Cells

When ERS occurs in hepatocytes, various inflammatory cytokines and chemokines are released. Therefore, we performed RT-PCR to monitor the mRNA expression of inflammatory factors in ERS-induced HepG2 cells. Upon ERS induction, the mRNA expression levels of IL-6, TNF-α, and IL-1β were significantly increased; however, these levels were markedly decreased following Rh2 treatment (Figure 4A–C, $p < 0.001$). Similar results were observed in the ELISA performed using CM derived from HepG2 cells. The levels of inflammatory cytokines were very high in the THA-treated group and significantly decreased when Rh2 was treated (Figure 4E–G). Interestingly, the mRNA expression of mcp-1, a chemokine important for recruiting macrophages and monocytes, was upregulated during ERS but significantly downregulated after Rh2 treatment (Figure 4D, $p < 0.01$, $p < 0.001$). This was similarly observed for MCP-1 levels in CM (Figure 4H). In addition, we confirmed that the mRNA expression of COL1A1 and TGF-b, factors affecting the liver fibrosis process, was upregulated by THA treatment but decreased by Rh2 treatment (Figure 4I,J). These results indicate that Rh2 can reduce the inflammatory response of hepatocytes and inhibit the secretion of inflammatory cytokines, thereby inhibiting macrophage recruitment and fibrosis progression.

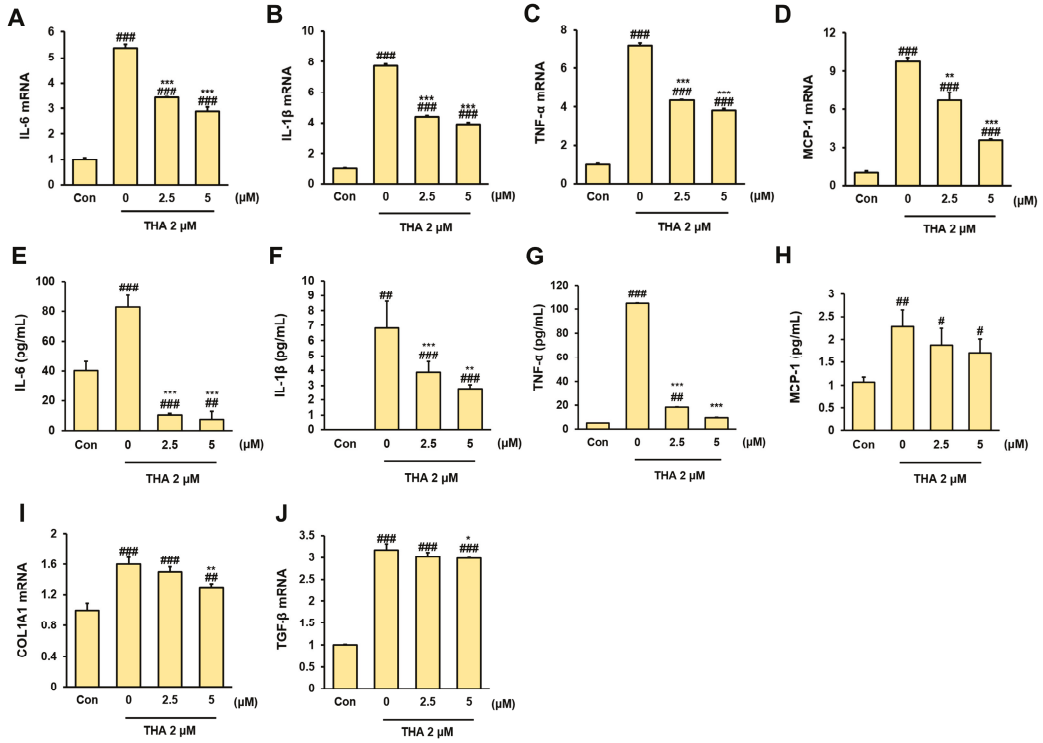


Figure 4. Anti-inflammatory effects of Rh2 treatment on ERS-induced HepG2 cells. mRNA expression of (**A**) IL-6, (**B**) IL-1β, (**C**) TNF-α, and (**D**) MCP-1 in HepG2 cells untreated (Con) or treated with 2 μM THA and (0 μM, 2.5 μM, 5 μM) Rh2 for 48 h. Quantitative analysis of CM (**E**) IL-6, (**F**) IL-1β, (**G**) TNF-α, and (**H**) MCP-1 from HepG2 cells was performed using a sandwich ELISA. (**I**,**J**) mRNA

expression of fibrosis-related factors. The Con group was treated with DMSO, and the x-axis of the graph represents the Rh2 concentration. Values are shown as mean ± SD (n = 3). Con vs. 0, Con vs. 2.5, Con vs. 5, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$. 0 vs. 2.5, 0 vs. 5, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3. Inhibition of THP-1 Cell Activation by CM Derived from ERS-Induced HepG2 Cells Treated with Rh2

To determine whether factors released by ERS-induced HepG2 cells treated with Rh2 could affect THP-1 cells, we conducted an experiment in which CM from HepG2 cells was extracted and applied to THP-1 cells (Figure 5A). THP-1 cells were divided into four groups: a group treated with CM extracted from the Con group (CCM); a group treated with CM extracted from the 0 group (0CM); a group treated with CM extracted from the 2.5 group (2.5CM); a group treated with CM extracted from the 5 group (5CM). When observed under a microscope on the third day of culture, THP-1 cells in the CCM group were found to be floating in clumps with minimal attachment to the bottom, whereas cells in the 0CM group were attached to the bottom and differentiating into macrophages (Figure 5B). In addition, differentiation into macrophages was observed in the 2.5CM and 5CM groups; however, differentiation was reduced in these groups compared with the 0CM group. Similar results were obtained when the number of cells attached to the bottom was counted (Figure 5C). Therefore, Rh2 treatment inhibited the induction of THP-1 differentiation into macrophages by HepG2 cells.

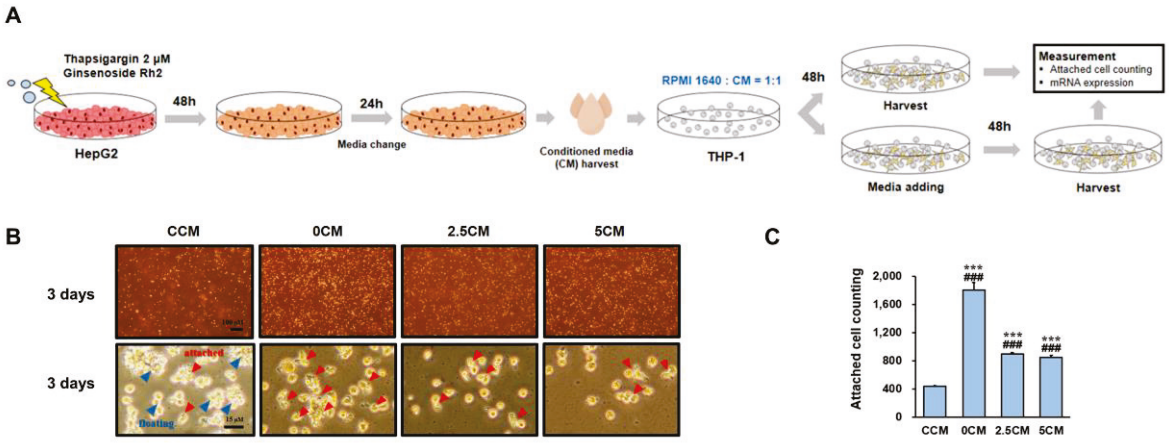


Figure 5. Inhibitory effect of conditioned medium (CM) from ERS-induced HepG2 cells treated with Rh2 on THP-1 cell differentiation. (**A**) The experiment where CM collected from HepG2 cells was treated to THP-1 cells is depicted. (**B**) Images of THP-1 cells cultured for 3 days under various medium conditions: Control CM from untreated HepG2 cells (CCM), CM from only ERS-induced HepG2 cells (0CM), and CM from ERS-induced HepG2 cells treated with 2.5 μM or 5 μM Rh2 (2.5CM and 5CM). Blue arrows indicate floating cells, and red arrows indicate attached cells. (**C**) Quantification of attached THP-1 cells. Values are shown as mean ± SD (n = 3). CCM vs. 0CM, CCM vs. 2.5CM, CCM vs. 5CM, ### $p < 0.001$. 0CM vs. 2.5CM, 0CM vs. 5CM, *** $p < 0.001$.

3.4. Regulation of THP-1 Cell Polarization by CM Derived from ERS-Induced HepG2 Cells Treated with Rh2

To investigate the effect of CM from HepG2 cells on the differentiation phenotype, THP-1 cells cultured with CM for 2 or 4 days were harvested for RT-PCR analysis. The expression levels of M1 markers, including CD80, CD86, IL-1β, TNF-α, and IL-12α, were significantly decreased in the 2.5CM and 5CM groups on day 2 compared with the 0CM group on day 4 (Figure 6A,B). However, IL-6 was slightly increased in the 2.5CM group on day 4. The results demonstrated that CM from ERS-induced HepG2 cells treated with Rh2 inhibited

the differentiation of liver-infiltrating macrophages into the M1 phenotype. The expression levels of M2 markers, such as MRC-1, CD163, Arg1, IL-10, and TGF-1β, were examined by RT-PCR. The levels of Arg1, TGF-1β, IL-10, and MRC-1 were significantly increased ($p < 0.05$, $p < 0.01$) when treated for 4 days compared with 2 days with CM (Figure 6C,D). However, the overall expression of CD163 was decreased in the 2.5CM and 5CM groups when treated for 4 days with CM, in contrast to 2 days. The results demonstrated that CM from ERS-induced HepG2 cells treated with Rh2 induced differentiation into the M2 phenotype.

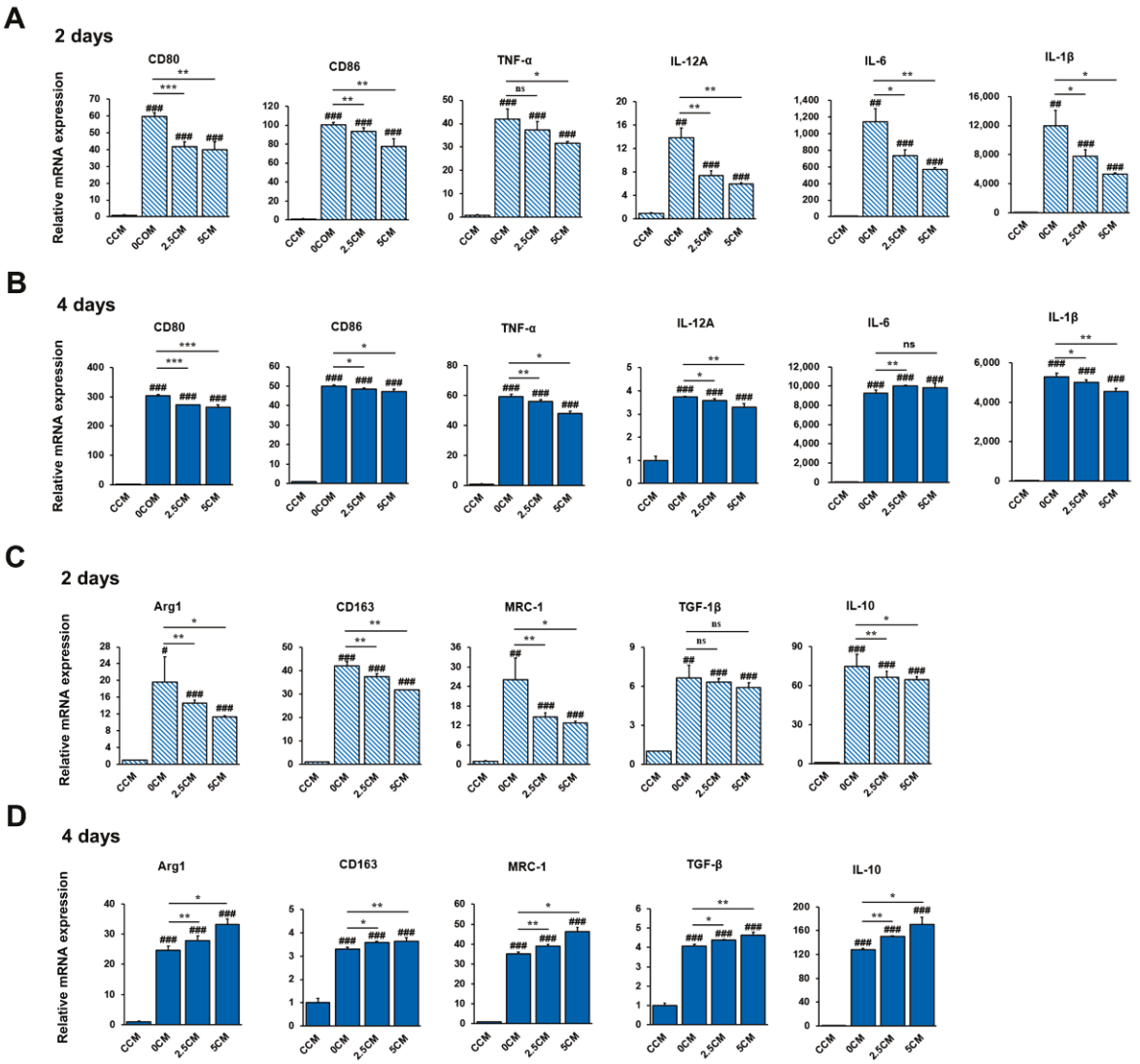


Figure 6. Effect of conditioned medium (CM) from ERS-induced HepG2 cells treated with Rh2 on the differentiation of THP-1 cells. mRNA expression of CD80, CD86, IL-1β, IL-12A, IL-6, and TNF-α in THP-1 cells treated with CM control CM from untreated HepG2 cells (CCM), CM from ERS-induced HepG2 cells (0CM), and CM from ERS-induced HepG2 cells treated with 2.5 μM or 5 μM Rh2 (2.5CM and 5CM) for (**A**) 2 days or (**B**) 4 days. mRNA expression of CD163, Arg1, TGF-1β, IL-10, and MRC1 in THP-1 cells treated with CM control CM from untreated HepG2 cells (CCM), CM from ERS-induced HepG2 cells (0CM), and CM from ERS-induced HepG2 cells treated with 2.5 μM or 5 μM Rh2 (2.5CM and 5CM) for (**C**) 2 days or (**D**) 4 days. Values are shown as mean ± SD ($n = 3$). CCM vs. 0CM, CCM vs. 2.5CM, CCM vs. 5CM, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$. 0CM vs. 2.5CM, 0CM vs. 5CM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

NAFLD is caused by a combination of factors, making it difficult to identify a therapeutic target [14]. Notably, although NAFL is characterized by excess lipid accumulation without inflammation, NASH involves hepatic inflammation, increasing the risk of progression to hepatocellular carcinoma [33]. Given the limited understanding of the mechanisms underlying the transition from NAFL to NASH, effective preventive and therapeutic strategies remain difficult to establish. Consequently, further research is essential to elucidate the pathogenesis of this transition.

Yao HR et al. [34] and Wang Q et al. [2] demonstrated that free fatty acids and ERS accelerate fatty liver formation and trigger inflammatory responses, which significantly contribute to the progression of NASH. Therefore, to establish a cell model of NAFLD, we first treated HepG2 cells with PA, EtOH [35], and THA (an ERS inducer) to identify substances that induce lipid accumulation. Among them, THA effectively induced ERS in HepG2 cells. Furthermore, THA treatment upregulated the inflammatory markers IL-1β, IL-6, and TNF-α, as well as key genes involved in the lipid biosynthesis pathway, including SREBP1 and FAS, leading to increased lipid accumulation.

In our search for a natural compound that could inhibit and treat the ERS-accelerated progression from NAFL to NASH, we focused on ginsenosides, known for their anti-inflammatory effects [27,28]. Notably, previous research demonstrated that Rh2 could reduce ERS in lung cancer cells [36]. Similarly, in our study, Rh2 treatment reduced apoptosis, downregulated ERS-related factors, and suppressed pro-inflammatory cytokines in THA-treated HepG2 cells. These findings suggest that Rh2 treatment may mitigate hepatocyte damage associated with liver inflammation during the early transition from NAFL to NASH. Although recent research has demonstrated that Rh2 can inhibit adipocyte differentiation [10], its effect on reducing lipid accumulation in hepatocytes remains unexplored. In our study, morphological and quantitative assessments via Oil Red O staining and triglyceride assay, as well as evaluation of SREBF1/FAS mRNA expression, demonstrated that Rh2 significantly inhibited the expression of lipid synthesis-related genes and mitigated lipid accumulation in HepG2 cells.

Interestingly, we also observed that MCP-1 expression, a key factor in macrophage recruitment and infiltration [37], was more than 10-fold higher in the CM of THA-treated HepG2 cells than in controls and was significantly reduced by Rh2 treatment. These findings suggest that ERS may facilitate macrophage recruitment in hepatocytes, whereas Rh2 treatment may counteract this effect. A critical aspect of macrophage function in NASH development is polarization toward either the M1 or M2 phenotype. Recent studies have shown that macrophage infiltration and activation, particularly polarization toward the M1 phenotype, increase during the transition from NAFL to NASH [38]. M1 macrophages are pro-inflammatory, releasing cytokines such as TNF-α, IL-1β, and IL-6 [39], which exacerbate liver damage and promote the progression to NASH. In contrast, M2 macrophages are associated with anti-inflammatory functions, secreting cytokines such as TGF-1β and IL-10, which help to resolve inflammation and facilitate tissue repair [40,41]. During the transition from NAFL to NASH [42], an imbalance occurs with increased M1 polarization, leading to sustained chronic inflammation and enhanced fibrogenesis. Therefore, modulation of the M1/M2 macrophage balance has been identified as a potential therapeutic strategy to mitigate NASH progression. In our study, the treatment of THP-1 cells with CM significantly reduced the expression of M1 phenotype markers in the 2.5CM and 5CM groups compared with the 0CM group. This reduction was more pronounced after 4 days of treatment than after 2 days. In contrast, the expression of M2 phenotype markers increased in the 2.5CM and 5CM groups, with a more pronounced effect after 4 days than after 2 days. These results suggest that Rh2 regulates ERS-induced HepG2 cells, promoting M2 differentiation

while suppressing M1 differentiation. These findings are partially consistent with previous findings showing that Rh2, in combination with MSC-derived exosomes, suppressed M1 polarization and promoted M2 differentiation in inflammatory disease models [43].

Collectively, our findings suggest that Rh2 treatment of HepG2 cells could inhibit lipid synthesis and inflammatory responses, thus protecting hepatocytes and potentially preventing NAFLD progression by modulating the interaction between HepG2 cells and THP-1 monocytes. Although a limitation of this study is the inability to determine the precise ratio of M1 to M2 macrophages in CM-treated THP-1 cells, our results suggest that Rh2 treatment could promote the expression of M2 macrophage-related factors, which are associated with anti-inflammatory and tissue repair functions, rather than M1 macrophage-related pro-inflammatory factors that exacerbate inflammation. Therefore, Rh2 may have therapeutic potential in mitigating NAFLD progression.

Moreover, our study emphasizes the importance of hepatocyte-derived inflammatory signals in macrophage polarization. The marked reduction in MCP-1 and other pro-inflammatory cytokines following Rh2 treatment indicates that Rh2 may suppress paracrine signaling that recruits and activates monocytes. Given the central role of chronic inflammation in NAFLD progression, the ability of Rh2 to modulate immune cell responses indirectly through hepatocyte signaling provides valuable insight into its therapeutic potential.

Although our findings highlight the promising therapeutic effects of Rh2 on NAFLD in vitro, its pharmacokinetic limitations must be acknowledged. According to pharmacokinetic studies, many ginsenosides, including Rh2, exhibit very low oral bioavailability (less than 5%) due to poor gastrointestinal absorption [44,45]. Despite this limitation, Rh2 has demonstrated significant pharmacological activity in various in vitro models, and in vivo studies have reported promising outcomes, such as reduced tumor incidence in mice following oral administration [46,47]. These findings motivated our investigation into Rh2, and additional studies focusing on its oral absorption and systemic bioavailability could further support its development as a therapeutic agent and its potential translation into clinical use.

While our findings are based on an in vitro model, they suggest that Rh2 could be effective in mitigating early inflammatory responses and preventing the fibrotic remodeling observed in NASH. Future studies employing in vivo NAFLD models are warranted to validate these findings and assess the long-term metabolic and histological benefits of Rh2 administration.

5. Conclusions

This study demonstrates that the ginsenoside Rh2 alleviates hepatic lipotoxicity and inflammation by attenuating ERS and modulating hepatocyte–macrophage interactions in an in vitro model of NAFLD. Rh2 suppressed THA-induced lipid accumulation, ERS markers, and inflammatory cytokine expression in HepG2 cells, while the conditioned medium from Rh2-treated hepatocytes inhibited THP-1 differentiation into M1-type macrophages and promoted M2 polarization. These findings suggest that Rh2 not only protects hepatocytes from ER stress-induced damage, but also indirectly regulates immune responses in the liver microenvironment. Therefore, Rh2 holds therapeutic potential as a preventive or adjunctive agent in the early management of NAFLD, and possibly its progression to NASH.

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TOTUM-854 Human Circulating Bioactives Preserve Endothelial Cell Function

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Abstract: Background: TOTUM-854 is a patented plant extract blend characterized by its components that have previously been described for their potential health benefits in limiting hypertension onset. However, most of the literature data remain descriptive regarding the mode of action at the cellular level, especially in humans, and further investigations are required for optimized therapeutic strategies. **Methods**: We first demonstrated in an L-NAME mouse model that TOTUM-854 supports the prevention of hypertension in vitro and in vivo. Then, we designed an ex vivo clinical innovative approach considering the circulating metabolites produced by the digestive tract upon TOTUM-854 ingestion in humans. Human serum was collected in healthy volunteers before and after the acute intake of 3.71 g of TOTUM-854. The bioavailability of circulating metabolites was confirmed and characterized by UPLC-MS. Human serum containing TOTUM-854-derived metabolites was further processed for incubation with human endothelial cells (HUVECs), in the absence or presence of palmitate (200 μM). **Results**: HUVEC protection against lipotoxicity was characterized by (1) decreased ACE-1 activity (−32% $p < 0.0001$); (2) the inhibition of oxidative stress with decreased ROS (−12% observed by DCFDA and DHE fluorescent microscopy) and decreased Nox2 gene expression (−6.7 fold change vs. palmitate, $p < 0.01$); and (3) the inhibition of an inflammatory response, with a decrease in IL-1β release (−37% compared to palmitate, $p < 0.001$) and decreased MCP-1 and VCAM-1 gene expression (−93% $p < 0.001$ and −77% $p < 0.001$, respectively). **Conclusions**: Overall, this study provides insightful data regarding the protective role of TOTUM-854 in human

endothelial cells. Using an innovative clinical ex vivo approach, our data support the role of TOTUM-854 circulating metabolites in vascular protection in humans.

Keywords: clinical trial; ex vivo; hypertension; plant extract; oxidative stress; lipotoxic stress; human metabolites; human endothelial cells

1. Introduction

Blood pressure is highly correlated with both cardiovascular morbidity and mortality [1]. To date, hypertension affects over 1.2 billion people worldwide and critically impacts public health systems [2]. Hypertension is currently defined as systolic or diastolic blood pressure above 140 mmHg and 90 mmHg, respectively, alone or in combination [3]. Ninety percent of diagnosed patients suffer from essential hypertension, the etiology of which remains to be determined. Hypertension is a multifactorial disease involving environmental, genetic, and behavioral factors [4]. Family-based studies have shown that genetic factors contributing to the variability of either systolic and/or diastolic blood pressure represent 30 to 60% [5]. Risk-conferring behaviors such as smoking, drugs, a lack of physical activity, obesity, and diet also potently contribute to hypertension onset [6].

Hypertension relies on an angiotensin-related mechanism that is widely supported by a large amount of experimental, clinical, and genetic evidence. Indeed, blood pressure is tightly regulated by the renin angiotensin aldosterone system (RAAS). Among the key protagonists, ACE-1 (angiotensin converting enzyme-1) converts angiotensin I into angiotensin II (AngII) that binds its cognate receptors (AGT1R and AGT2R) on target tissues, including the brain (thirsty and vasopressin), the kidneys (aldosterone and reabsorption), and vessels (arteriolar vasoconstriction), leading to the increase in both the extracellular water volume and the total peripheral resistance [7,8].

Along with this "angiotensin-focused" point of view, a few original studies have proposed that the skin, the immune system, and the peripheral vascular resistance could fulfill the global picture, all of them sharing inflammation, immune cell infiltration, ROS activation, or loss of vascular capabilities as a common starting point [4,9]. Obesity and diet are risk factors that potently contribute to endothelial cell dysfunction and further hypertension onset [6]. To date, hypercholesterolemia and hypertriglyceridemia have been shown to drive atherosclerosis onset, promoting local inflammation and oxidative stress that finally alter endothelial cells' abilities to regulate vessel tone and peripheral resistance [10,11].

In this context, lifestyle interventions such as dietary manipulation, including salt or calorie restrictions, represent the initial step in the treatment of hypertensive patients, regardless of the grade of HP [12]. In cases where BP remains above 150/95 mmHg, these lifestyle interventions may be complemented with immediate drug treatment such as diuretics and/or angiotensin-converting enzyme (ACE) inhibitors. However, the side effects reported for both and ACE inhibitors are widely associated with dry cough (mostly), hypotension, hyperkalemia, and angioedema [13]. Thus, dietary interventions remain the first-line strategy, and nutritional strategies have become a major field for therapeutic innovation.

Nutrition is our first source of bioactive compounds and therefore may play a key role in maintaining or preserving body homeostasis. Among those bioactives, polyphenols, which are phenolic molecules belonging to the plant immune system, have widely recognized antioxidant and anti-inflammatory properties [14]. Their health benefits have particularly been reported in the cardiovascular system [15]. Regarding their role in chronic

vascular diseases, in the last five years, nearly one-third of the total articles related to this theme demonstrated the dynamics of nutritional approaches in the field.

To date, ex vivo studies have evidenced that the phenolic compounds present in fruit and vegetable juices could induce potent vasorelaxation in aorta segments in rats [16]. Earlier, in a preclinical model of L-NAME-induced hypertension, garlic consumption completely prevented the increase in systolic blood pressure [17]. Bilberry extract also demonstrated an inhibitory effect on the establishment of induced pulmonary arterial hypertension in Wistar rats [18]. In a spontaneous model of hypertension in rats, the consumption of chlorogenic acid reduced hypertension and led to an improvement in endothelial function [19]. Likewise, in a model of cyclosporine-induced hypertension, both chlorogenic acid and caffeic acids limited angiotensin-converting enzyme activity and oxidative stress [20,21]. However, clinical evidence on how it may work in humans at the cellular level is lacking.

TOTUM-854 is a polyphenol-rich blend composed of six different plant extracts. Each compound has been previously shown, separately, to impact inflammation and oxidative stress. The potential synergistic properties still need to be deciphered. The aim of this study was to demonstrate if and how this polyphenol combination can maintain, improve, or preserve endothelial function in order to support preventive strategies in the mid to moderately hypertensive population.

The potential health value of TOTUM-854 was first evidenced both in vitro and in a preclinical model of L-NAME-induced hypertension. Then, according to our ex vivo clinical approach [11,22–28], we collected human bioactive metabolites in the bloodstream resulting from TOTUM-854 ingestion and examined their influence on human endothelial cells' behavior and functions in the context of a lipotoxic environment.

2. Materials and Methods

2.1. Study Product

VALBIOTIS has formulated TOTUM-854 (Patent: FR1460064), a food supplement resulting from the combination of six plant extracts (chrysanthellum indicum, artichoke leaves (*Cynara scolymus* L.), olive leaves (*Olea europaea* L.), blueberry (*Vaccinium myrtillus* L.), *Piper nigrum* L., and garlic (*Allium sativum* L.)). The blend was formulated according to previously described clinical data and the traditional uses for their supposed benefits in preventing hypertensive conditions. The formulation of the product meets the requirements of the European regulations relating to food supplements (European directive n°2002/46/EC and French decree n°2006-352 of 20 March 2006, which transposes it).

2.2. Ethics Preclinical Study

All animal experiments were performed according to the European Parliament Directive 2010/63/EU and were approved by the local research ethics committee (C.E. Languedoc-Roussillon, France, under the number CEEA-00322.03).

2.3. Ethics Clinical Trial

This study was conducted in accordance with the Declaration of Helsinki of 1975 (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki (accessed on 12 November 2021)) revised in 2013. The human study was approved by the French Ethical Committee (N° SI RIPH: 21.03989.000023/N° ID RCB: 2021-A02695-36/Comité de Protection des Personnes CPP Est III; approved 8 December 2021). Before they participated in the project, all volunteers were informed of the objectives and the potential risks of the present study and provided their written informed consent.

2.4. In Vitro ACE Inhibition Assays

Reagents: angiotensin-converting enzyme from rabbit lung (ACE), Hippuryl-His-Leu acetate salt, Hippuric acid, sodium hydroxide, sodium chloride, potassium chloride, boric acid, trifluoroacetic acid, captopril, and dimethyl sulfoxide (DMSO) were obtained from Merck/Sigma-Aldrich (Darmstadt, Germany). Acetonitrile was obtained from Carlo-Erba (Val-de-Reuil, France). Pure water was obtained using an Evoqua ultra-pure water production system (Water Technologies, Günzburg, Germany). *ACE inhibition assay:* ACE hydrolytic activity was assessed by monitoring the conversion of Hippuryl-His-Leu (HHL) into Hippuric acid (HA) in a borate buffer (0.05 M, pH 8.3) using an HPLC-UV. A commercial reference inhibitor, captopril, was used as a positive control.

The relative ACE activity was calculated as follows: ACE activity (%) = 100 × (A2 − A0)/(A1/A0) where A0 is the Hippuric acid peak area in Blank, A1 is the Hippuric acid peak area in the negative control, and A2 is the Hippuric acid peak area in the sample with the potential inhibitor.

2.5. Preclinical Protocol

Twelve-week-old C57Bl6/J mice were divided into three groups: a control group, drinking tap water (n = 11); an L-NAME group, receiving L-NAME-supplemented drinking water (100 mg/kg/day) (n = 11); and an L-NAME + TOTUM-854 group, receiving L-NAME-supplemented drinking water (100 mg/kg/day) and TOTUM-854 administration (500 mg/kg/day) via daily oral gavage (n = 11). The control and L-NAME groups received the vehicle via daily oral gavage. Body weights were recorded weekly at the same time as bedding changes. Arterial blood pressure was measured using the CODA Tail-Cuff System (Kent Scientific Corporation, Torrington, CT, USA) after one week of acclimation, at the beginning (T0), and once a week for 3 weeks. All measurements were performed by the same trained operator and in the same time frame (from 9 a.m. to 1 p.m.). All blood pressure data were obtained by taking the average of at least 7 measurements (from 15 measuring cycles per machine).

2.6. Human Study Design and Pharmacokinetic of Absorption

A total of 10 healthy volunteers (age: 23.7 years old, +/−3.97; BMI: 22.29 kg/m^2, +/−1.18; >60 kg; absence of pathologies or drug treatment was required; and no distinction in ethnicity) were enrolled in this study. They were monitored for normal blood formulation and renal (urea and creatinine) and liver functions (including aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyltransferase (GGT) activity).

The first phase of the clinical approach characterized TOTUM-854's metabolite absorption profile. Volunteers fasted for at least 12 h prior to the ingestion of 3710 mg of TOTUM-854. TOTUM-854 was provided as eight capsules. Serum samples (from venous blood) were collected before ingestion and every 20 min for 240 min after the ingestion of TOTUM-854. Samples were then aliquoted and stored at −80 °C until analyses. Blood sample collection was conducted by the Clinical Investigation Center—Inserm 1405, University Hospital of Clermont-Ferrand (ethical clinical certification NF S 96900).

Human circulating polyphenolic bioactives were characterized by ultra-high-performance liquid chromatography combined with tandem mass spectrometry (UPLC-MS/MS).

In the second phase of the clinical approach, volunteers were recalled to the clinical center for the collection of the metabolites' enriched serum fractions. They were asked to fast for 12 h prior to being given 3710 mg of TOTUM-854. The collection consisted of two serum samples. One sample was collected before ingestion as the naïve serum fraction (baseline) and the second sample was collected after ingestion at Cmax (absorption profile's peak) as the enriched serum fraction. Serum aliquots were stored at −80 °C until analyses.

2.7. Phenolic Compounds Extraction from Serum

The serum was mixed with 100% methanol for 1 min (1:3 v/v). The mixture was then centrifuged at 20,000× g for 15 min. The supernatant was then evaporated and dried. The dried material was dissolved in methanol/water (50:50 v/v). After agitation (1 min), ultrasonication (1 min), and centrifugation (20,000× g for 15 min), the supernatant was stored at −20 °C until analysis by ultra-high-performance liquid chromatography combined with tandem mass spectrometry.

2.8. Ultra-High-Performance Liquid Chromatography–Mass Spectrometry (UPLC-MS/MS)

Analyses were carried out using a 1260 Infinity UHPLC (Agilent Technologies, Santa Clara, CA, USA) system coupled to a 6430 triple quadrupole mass spectrometer from Agilent Technologies. Solvent A (water/formic acid 99.9:0.1, v/v) and solvent B (acetonitrile/formic acid 99.9:0.1, v/v) were used as a mobile phase at a flow rate of 0.3 mL/min, and the gradient in solvent A was as follows: 0 min 1% B, 2 min 5% B, 3 min 25% B, 6 min 25% B, 8 min 40% B, 11.5 min 95% B, 14 min 95% B, and 16 min 1% B. The MS/MS parameters were as follows: negative ion mode, capillary tension of 3000 V, nebulizer at 15 psi, dry gas of 11 L/min, a dry temperature of 350 °C, and acquisition in multiple reaction monitoring (MRM); see Supplementary Figure S1 for further details. The data were processed using MassHunter software (V.12) Agilent Technologies, Santa Clara, CA, USA).

2.9. Human Umbilical Vein Endothelial Cell Cultures

The primary human umbilical vein endothelial cells (HUVECs) were obtained from Thermo Fisher Scientific (C0155C, Villebon sur Yvette, France). During the maintenance phase, HUVECs were cultured in Complete EGM^{TM}-2 Endothelial Cell Growth Medium-2 (CC-3162, Lonza, Levallois-Perret, France). All cell cultures were performed at 37 °C in an atmosphere of 5% CO2/95% air. To analyze the effects of TOTUM-854 metabolites, the cells were preincubated for 24 h in basal EGM^{TM}-2 in the presence of 10% of human serum (naïve or containing circulating metabolites) according to the Clinic'n'Cell methodology (DIRV INRAE 18-0058), prior to an additional 24 h incubation in a palmitate-induced lipidic stress environment (palmitate 200 µM).

2.10. Preparation of Palmitate Solution

Palmitate (Sigma, Saint-Quentin-Fallavier, France) dissolution proceeded as follows: incubation with bovine serum albumin (BSA; Saint-Quentin-Fallavier, France, Sigma) and dissolution in pure ethanol at 70 °C. The final concentration was set to 500 mmol/L. A pre-warmed BSA solution (10% w/w, 37 °C) was mixed with this stock solution to reach a final concentration of 5 mmol/L. Mixture clarification occurred via two sessions of incubation at 55 °C for 15 min. The final molar ratio was set at 3.2:1 (palmitate–BSA). The control vehicle proceeded in the same conditions.

2.11. Cell Viability

An XTT-based method was used to determine the cell viability (Cell Proliferation Kit II, Sigma-Aldrich, Saint-Quentin-Fallavier, France). The experimental procedures followed the supplier's recommendations. Measurements were performed in hexaplicate for each sample condition of the ten volunteers. OD was measured at 450 nm.

2.12. Angiotensin-Converting Enzyme 1 Assays

ACE-1 activity was tested in endothelial cells using ACE1 assay kits from Abcam (ab273308, Abcam, Paris, France). The experimental procedures were set according to the supplier's recommendations. The kit utilizes the ability of ACE1 to hydrolyze a synthetic

substrate, which results in a decrease in optical density at 345 nm. Measurements were performed in quadruplicates for each sample condition of the ten volunteers.

2.13. Dihydroethidium (DHE) Staining

The DHE (Dihydroethidium) Assay Kit (ab236206, Abcam, Paris, France) was used to measure reactive oxygen species (ROS) directly in live cells. This kit uses DHE as a fluorescent probe for the detection of ROS generation, notably superoxide, hydrogen peroxide, and non-specific oxidation by other sources of ROS to form ethidium (excitation at 480–520 nm and emission at 570–600 nm) [29]. At the end of the incubation period, with palmitate in the presence of naïve or enriched serum, cells were incubated with 5 µM of the DHE probe for 30 min at 37 °C and washed prior to observation. Live cells were imaged using a ZEISS optical microscope (ZEISS, Rueil Malmaison, France) with a B/W cooled camera and analyzed using ImageJ software (NIH) (version 1.54f). Measurements were performed for each sample condition of the ten volunteers.

2.14. Measurement of Nitric Oxide (NO)

A Nitrate/Nitrite (final products of NO) colorimetric assay kit was obtained from Cayman Chemical (references 780001, Cayman Chemical, Ann Arbor, MI, USA). Assays were conducted according to the manufacturer's instructions. The levels of NO were examined in culture supernatants. Measurements were performed in quadruplicate for each sample of the ten volunteers.

2.15. IL-1β Release

IL-1β levels were evaluated in the cell culture supernatant using Human IL-1 beta ELISA Kit (abcam—ab214025, Paris, France) according to the manufacturer's recommendations. Measurements were performed in quadruplicate for each sample condition of the ten volunteers.

2.16. Real-Time RT-qPCR

mRNA from HUVECs was isolated using TRIzol™ Reagent (Ambion—Life Technologies) according to the supplier's recommendations. VCAM-1 (vascular cell adhesion molecule 1), MCP-1/CCL2 (monocyte chemoattractant protein 1), and NOX2 (NADPH oxidase 2) mRNA expression levels were measured by RT-qPCR (PowerUp SYBRgreen, Applied Biosystems). β-Actine was used as a housekeeping gene. Primers were designed as follows: VCAM-1-F: 3′- ACT TGA CTG TGA TCG GCT T -5′; VCAM-1-R: 3′- TGA CTC CGT CTC ATT GAC TTG -5′; MCP-1-F: 3′- GCC TCT GCA CTG AGA TCT TC -5′; MCP-1-R: 3′- AGC AGC CAC CTT CAT TCC -5′; NOX2-F: 3′- ATT CCT GTC CAG TTG TCT TCG -5′; NOX2-R: 3′- CTG ATT CTC TTG CCA GTC TGT′; ACTβ-F: 3′-ATT GGC AAT GAG CGG TTC-5′; ACTβ-R: 3′-GGA TGC CAC AGG ACT CCA-5′.

2.17. Statistics

Prism V.7.0, 8.0, and V.9.4.1 (GraphPad Software) were used to run statistical tests and draw figures. The following statistical plan was applied: a Shapiro–Wilk normality test was used to determine whether the data were consistent with a Gaussian distribution. If the data were not distributed according to a normal distribution, a Kruskal–Wallis non-parametric test was used, followed by the Dunn test for post hoc comparison. When a normal distribution and equal variance were assumed, the measures were subjected to a one-way ANOVA followed by Tukey's test for multiple comparisons. In the case of measurements repeated over time, the differences between groups and time points were tested using a repeated-measures two-way ANOVA followed by Sidak's post hoc test for multiple comparisons. If a piece of data was missing, making it impossible to run a repeated-

measures two-way ANOVA, mixed-effect analysis was used instead. In Figures 1 and 2, the values are expressed as mean ± SEM. In Figure 3, data are expressed as mean ± SD. In Figures 4–7, values are presented by boxes that indicate the median and interquartile range (lower and upper) and by whiskers that indicate the minimum and maximum. Differences were considered statistically significant at $p < 0.05$, with * representing $p < 0.05$, ** representing $p < 0.01$, *** representing $p < 0.001$, and **** representing $p < 0.0001$. Finally, ns represents $p > 0.05$.

3. Results

3.1. In Vitro and In Vivo First Evidences of the TOTUM-854 Potential in Regulating Blood Pressure

To test the potential benefit of TOTUM-854 on blood pressure, we first questioned its capacity to inhibit ACE activity in vitro. As shown in Figure 1, TOTUM-854 dose-dependently decreased ACE activity. This inhibition nicely paralleled the effect of Captopril, a synthetic analog of the ACE-inhibiting peptide found in the Jararaca snake venom and a drug of reference that is widely used to treat hypertension. TOTUM-854's IC50 was about 9.869 g/L, while Captopril had an IC50 of 10.31 nM (2.2 µg/L). Thus, the pharmacological capacity of Captopril to inhibit ACE activity was found to be much higher than that of TOTUM-854 (about 5.10^6 times); still, these data were very encouraging from a nutritional point of view.

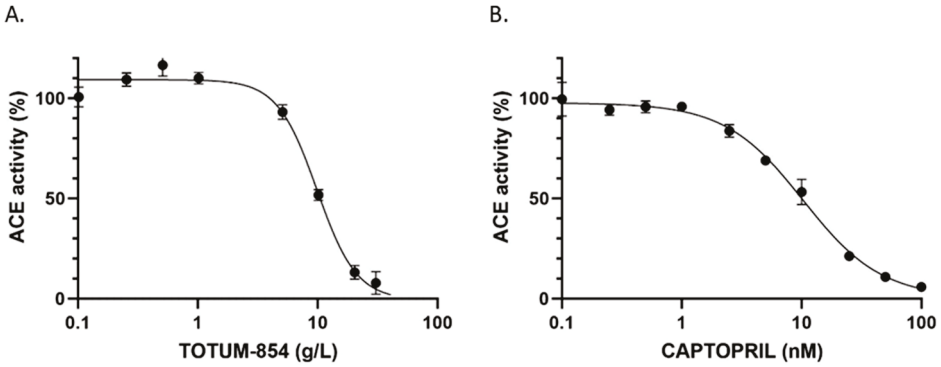


Figure 1. In vitro ACE inhibition assays by TOTUM-854 (**A**) and Captopril (**B**).

A commercial reference inhibitor, captopril, was used as a positive control. The IC50 of each compound corresponds to the lowest concentration at which ACE activity was halved (n = 2–6). Data are expressed as mean ± SEM.

To further investigate the relevance of such ACE inhibition in a more physiological model, TOTUM-854 was subjected to in vivo investigations (Figure 2A–D). L-NAME is a convenient analog to inhibit NO synthetase and block the production of NO, thus impairing endothelial function and driving hypertension onset. In our preclinical model, mice receiving L-NAME consistently showed an increase in blood pressure. Blood pressure increased over time during the 3 weeks in the L-NAME group (compared to the group's baseline at day 0) to reach 27.8% and 24.9% for diastolic and systolic blood pressure, respectively. In contrast, when mice were given L-NAME in combination with TOTUM-854 supplementation, the rise in blood pressure at 3 weeks was constrained and limited to 7.9% and 8.7% for diastolic and systolic blood pressure, respectively (compared to the group's baseline at day 0). The variation in blood pressure in the control group was up to 4.3% and 0.9% for diastolic and systolic blood pressure, respectively (compared to the group's baseline at day 0), and there was no significant difference compared to the L-NAME+TOTUM-854 group.

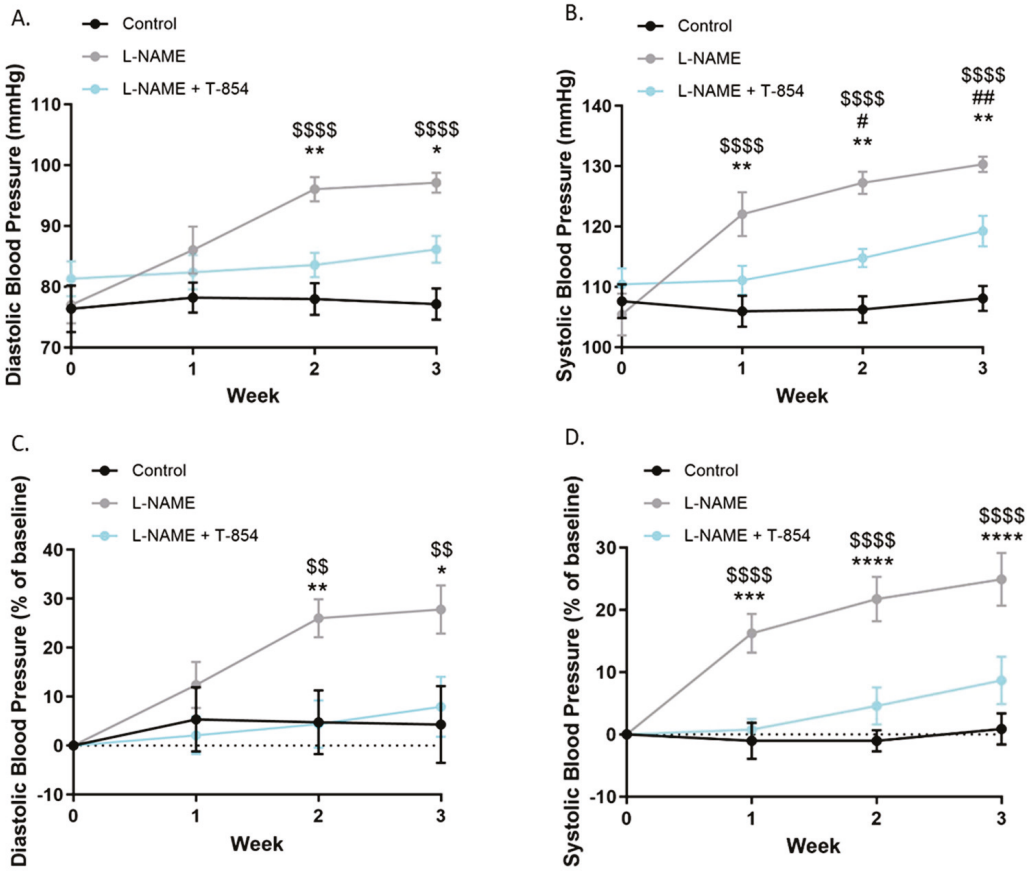


Figure 2. In vivo investigation of TOTUM-854 influence on blood pressure in mice. L-NAME was used as a convenient analog to blunt NO release and drive hypertension onset. Panels represent raw blood pressure values ((**A**): diastolic blood pressure; (**B**): systolic blood pressure) and blood pressure percentage of baseline ((**C**): diastolic blood pressure; (**D**): systolic blood pressure) over three weeks of L-NAME and T-854 administration. Data are expressed as mean ± SEM. L-NAME vs. L-NAME + T-854: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Control vs. L-NAME + T-854: # $p < 0.05$; ## $p < 0.01$; L-NAME vs. Control: $$ $p < 0.01$; $$$$ $p < 0.0001$ (n = 11).

3.2. Kinetic of Apparition of Circulating Metabolites Resulting from TOTUM-854 Ingestion in Humans

TOTUM-854 was ingested and digested by fasted volunteers, and the occurrence of circulating metabolites in the blood was monitored using a kinetic approach to determine the time frame of the absorption peak. We found nine detectable circulating human metabolites, including two oleuropein glucuronides isomers, three luteolin glucuronides isomers, one hydroxytyrosol sulfate isomer, one ferulic acid sulfate isomer, one homovanillic acid sulfate isomer, and one dihydrocaffeic acid sulfate isomer (Figure 3). The chromatograms of the detected metabolites in serum are presented in the supplemental data section (Figure S1). These data evidence the efficient absorption and bioavailability of the product. The Tmax (time to reach the maximum concentration observed in serum) ranged from 40 min to 180 min post-absorption, depending on the type of metabolites. According to the global absorption curves, and in order to obtain the most diverse enrichment of serum in TOTUM-854 metabolites, blood was collected before ingestion (naïve fraction) and at 60 min post-ingestion (enriched fraction) for the ex vivo investigation of HUVECs.

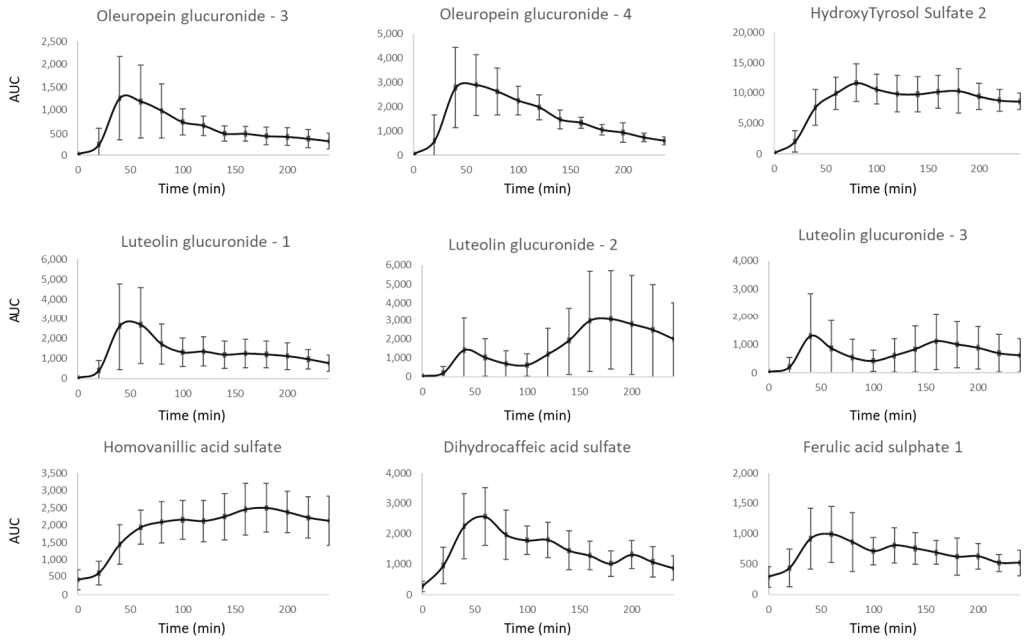


Figure 3. Metabolomic profiles in human serum following TOTUM-854 ingestion. Circulating metabolites resulting from TOTUM-854 ingestion were determined using ultra-high-performance liquid chromatography–mass spectrometry (UPLC-MS/MS) (AUC: Area under curve). Humans n = 10 volunteers.

3.3. Absence of Impact on Cell Culture Growth Following Incubation of HUVECs with Either Naïve or Metabolite-Enriched Human Serum

To avoid any bias in our conclusions, we first ensured that human serum (either naïve or containing circulating metabolites resulting from TOTUM-854 ingestion) had no detrimental effect on HUVEC growth and viability (Figure 4). HUVECs were pre-incubated for 24 h in the presence of either naive serum or TOTUM-854-enriched serum 24 h prior to lipotoxic stress induction by palmitate (200 µM). The data showed no differences between the different serum conditions. Neither naïve nor enriched human serum modified cell viability compared to each other or compared to the control FCS treatment. In contrast, the presence of palmitate slightly but significantly reduced cell growth. Interestingly, palmitate had no effect on cells when HUVECs were pre-incubated with the circulating metabolites from TOTUM-854.

3.4. TOTUM-854 Human Metabolites Decrease Angiotensin-Converting Enzyme 1 Activity Under Palmitate Stress

Angiotensin-converting enzyme 1 is a key player in the regulation of endothelial function. In this model, the presence of lipotoxic stress induced by the presence of palmitate consistently tends to increase ACE-1 activity without reaching significance. Metabolites from TOTUM-854 had no impact on ACE-1 activity on their own, but interestingly, in the presence of palmitate, they significantly decreased ACE-1 activity (−31% $p < 0.0001$) in HUVECs, decreasing ACE-1 activity to baseline in this lipotoxic stress environment (Figure 5).

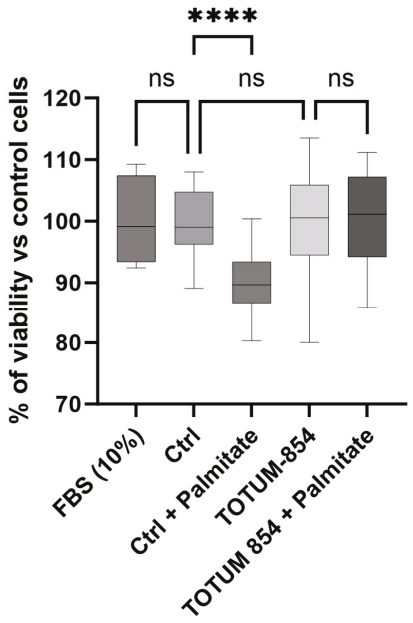


Figure 4. Cell viability (human umbilical vascular endothelial cells (HUVECs)). Cell viability was determined using an XTT-based method. Fetal Bovine Serum (FBS) was used as the reference (100% viability). Control groups (Ctrl) consist of human naïve serum exposition with or without palmitate. TOTUM-854 groups consist of human serum containing circulating metabolites from TOTUM-854 ingestion with or without palmitate. Measurements were realized in hexaplicate for each volunteer (n = 10 volunteers). Boxes indicate median and interquartile range (lower and upper), while whiskers indicate minimum and maximum ****: $p < 0.0001$; ns: $p > 0.05$.

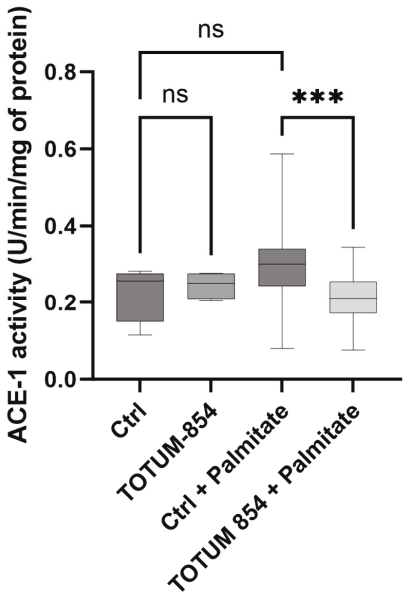


Figure 5. Influence of TOTUM-854 human metabolites on ACE-1 activity in human umbilical vascular endothelial cells (HUVECs). Control groups (Ctrl) consist of human naïve serum exposition with or without palmitate. TOTUM-854 groups consist of human serum containing circulating metabolites from TOTUM-854 ingestion with or without palmitate. Measurements were realized in triplicate for each volunteer (n = 10 volunteers). Boxes indicate median and interquartile range (lower and upper), while whiskers indicate minimum and maximum ***: $p < 0.001$; ns: $p > 0.05$.

3.5. TOTUM-854 Metabolites Limit Oxidative Stress in a Lipotoxic Environment

Oxidative stress, and more specifically the production of reactive oxygen species, is a driving force of endothelial tissue dysfunction. Using a DHE probe, we investigated the

production of ROS in our different conditions (Figure 6A,B). Metabolites from TOTUM-854 had no impact on their own in the absence of palmitate. As expected, DHE staining increased in the presence of palmitate, evidencing a consistent increase in ROS production in a lipotoxic context (+130%). However, the presence of TOTUM-854 metabolites limited the increase in DHE staining (−25% compared to palmitate alone or −46% for the combination). In other words, cells were protected from this palmitate-induced oxidative stress when they were pre-incubated with TOTUM-854 metabolites (DHE's increase was almost halved). NADPH oxidases, and most notably NOX2, are responsible for ROS production in endothelial tissues. Interestingly, NOX2 expression nicely correlates with DHE staining. Although the decrease in NOX2 expression by TOTUM-854 metabolites alone did not reach significance, in the presence of palmitate, they significantly reduced its expression by 0.58 $\log^{10}$ (−3.8 fold) (Figure 6C). We also investigated NO release as a master endothelium hypotensive mechanism. Neither palmitate nor TOTUM-854 metabolites had any impact on NO production by HUVECs (Figure 6D).

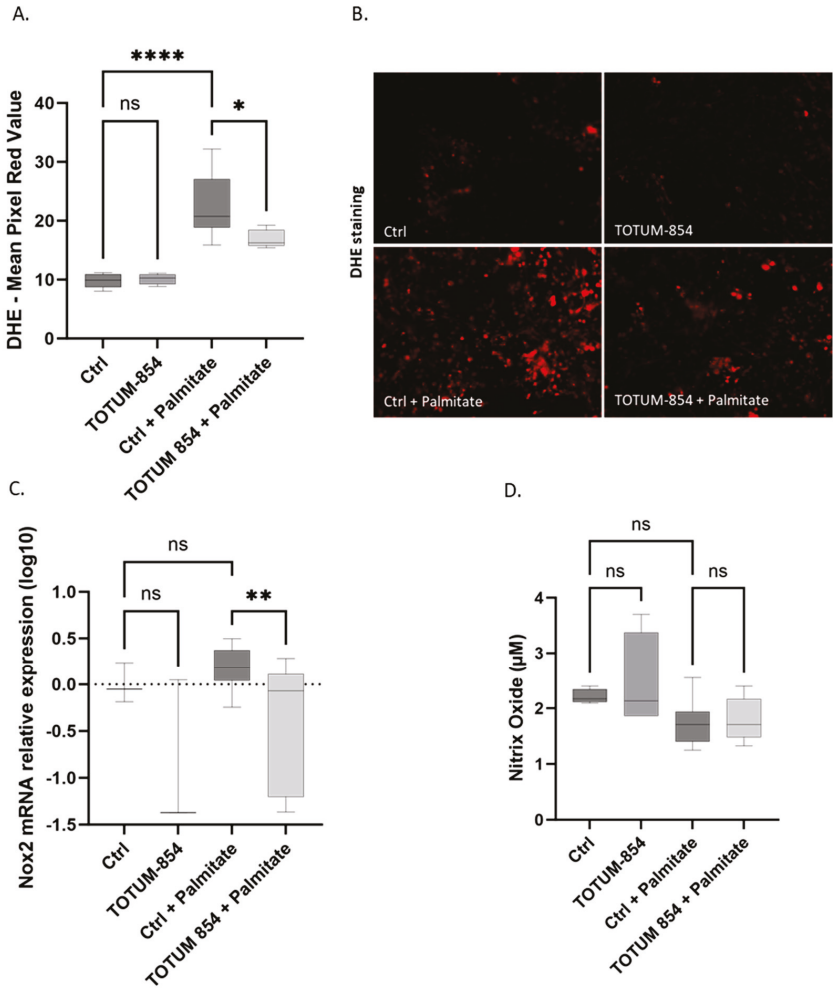


Figure 6. Influence of TOTUM-854 human metabolites on redox status in human umbilical vascular endothelial cells (HUVECs). DHE staining: (**A**) quantification expressed as the mean red pixel value; (**B**) inverted fluorescence microscopy; (**C**) Nox2 mRNA relative expression; (**D**) Nitric Oxide release in cell culture media. Control groups (Ctrl) consist of human naïve serum exposition with or without palmitate. TOTUM-854 groups consist of human serum containing circulating metabolites from TOTUM-854 ingestion with or without palmitate. Measurements were realized in triplicate for each volunteer (n = 10 volunteers). Boxes indicate median and interquartile range (lower and upper), while whiskers indicate minimum and maximum *: $p < 0.05$; **: $p < 0.01$; ****: $p < 0.0001$; ns: $p > 0.05$.

3.6. Inhibition of the Inflammatory Response

Hypertensive and lipotoxic context fuels inflammation by stimulating pro-inflammatory cytokine release and promoting the recruitment of immune cells. We investigated IL-1b production and release under different conditions. As for previous parameters, metabolites alone had no impact on IL-1b release. Consistently, the induction of lipotoxic stress by palmitate doubled IL-1b production (+129%, $p = 0.0524$), while pre-incubation with TOTUM-854 metabolites potently abolished it, returning IL-1b release values close to the baseline values (−41% compared to palmitate, $p < 0.001$) (Figure 7A). We also examined MCP-1 (Figure 7B) and VCAM-1 (Figure 7C) expression, two markers known for their involvement in immune cell recruitment. Their expression profiles nicely paralleled IL-1b release. Palmitate dramatically induced the expression of both MCP-1 ($p = 0.0506$) and VCAM-1. In contrast, even if the decrease in the expression of MCP-1 and VCAM-1 by TOTUM-854 metabolites alone did not reach significance, in the presence of palmitate, TOTUM-854 metabolites successfully blunted the increased expression of both markers (−86% $p < 0.001$ and −70% $p < 0.001$, respectively).

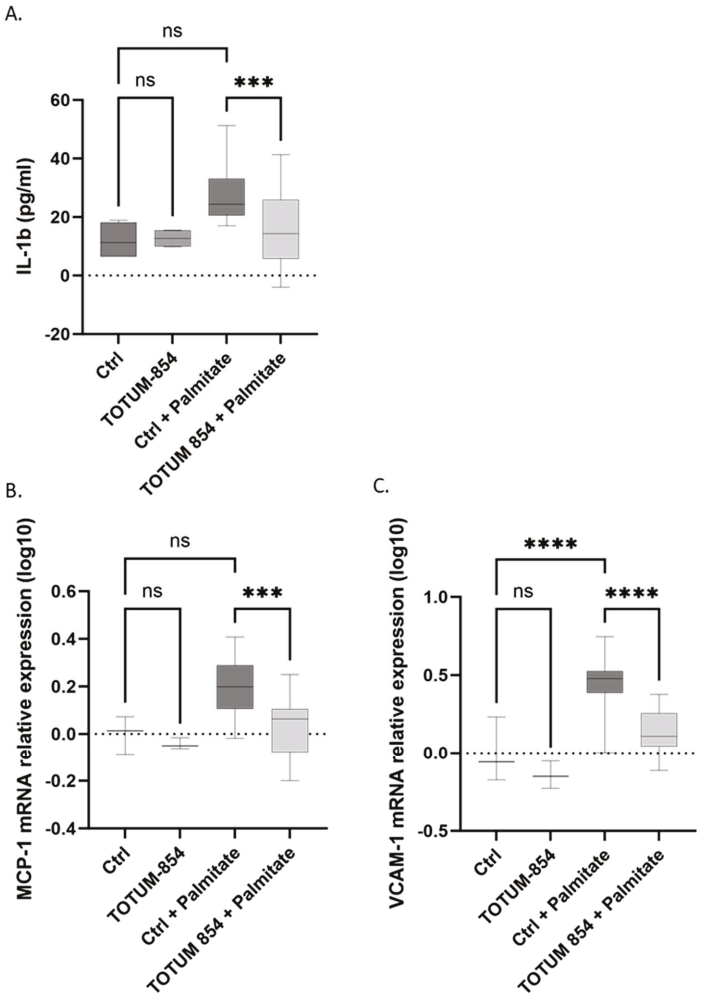


Figure 7. Influence of TOTUM-854 human metabolites on inflammatory mediators and cell adhesion in human umbilical vascular endothelial cells (HUVECs). (**A**) IL-1β release (pg/mL). (**B**) MCP-1 mRNA relative expression. (**C**) VCAM-1 mRNA relative expression. Control groups (Ctrl) consist of human naïve serum exposition with or without palmitate. TOTUM-854 groups consist of human serum containing circulating metabolites from TOTUM-854 ingestion with or without palmitate. Measurements were realized in triplicate for each volunteer (n = 10 volunteers). Boxes indicate median and interquartile range (lower and upper), while whiskers indicate minimum and maximum ***: $p < 0.001$; ****: $p < 0.0001$; ns: $p > 0.05$.

4. Discussion

In this manuscript, we first demonstrated that TOTUM-854, a plant extract blend, was able to inhibit ACE activity in vitro. Its potential health benefit was then further explored and validated in vivo as a candidate for regulating blood pressure in a mouse model of hypertension. Upon those encouraging preliminary data, we investigated the nutraceutical potential of TOTUM-854 in humans. Using an ex vivo clinical approach, we characterized the bioavailability of the blend and demonstrated that the circulating metabolites resulting from TOTUM-854 ingestion in humans were able to maintain endothelial cell function in a lipotoxic environment by (1) inhibiting ACE-1 activity, (2) limiting oxidative stress, (3) preventing the release of IL-1β, and (4) reducing the expression of MCP-1 and VCAM-1, which are both involved in inflammation and the recruitment of immune cells that drive endothelial dysfunction onset.

The relevance of each model used in this work may be questioned, including the nutritional dimensions of the dose used for the clinical investigation. Each volunteer was exposed once per clinical phase to a total of 3170 mg of TOTUM-854. If we take into consideration the amount of oleuropein, the most abundant polyphenol in the blend, the exposure dose was about 334 mg, which is consistent with other clinical studies. In Lockyer's 2017 study, the dose of oleuropein given daily to volunteers for 6 weeks was 136 mg/d [30]. Overall, the total quantity of polyphenols provided by 3170 mg of TOTUM-854 is estimated to be around 560 mg. Since the recommended daily dose of polyphenols is about 1 g [21,31], the dose used in this study was relevant from a nutritional point of view.

In this context, about nine circulating human metabolites were detected following TOTUM-854 ingestion in humans. Among them, two were oleuropein glucuronides isomers, three were luteolin glucuronides isomers, one was a hydroxytyrosol sulfate isomer, one was a ferulic acid sulfate isomer, one was a homovanillic acid sulfate isomer, and one was a dihydrocaffeic acid sulfate isomer.

As expected, these metabolites mainly originate from olives and artichokes, which are the most abundant extracts of the blend. Olive and artichoke extracts were previously and consistently reported for their positive influence on both lipid metabolism and blood pressure. In the early 2000s, a clinical study on hyperlipidemic patients showed that daily supplementation with artichoke juice for six weeks improves arterial compliance, with increased brachial flow-mediated vasodilation [32]. More recently, in a double-blind randomized study, an olive leaf extract, characterized by its oleuropein and hydroxytyrosol content, was given to hypertensive patients for 6 weeks and led to a reduction in blood pressure and inflammatory status and improved the lipid profile [30]. One homovanillic acid sulfate isomer was detected in the serum. Interestingly, this tyrosol-derived metabolite has been known for decades to be a catecholamine metabolite involved in the reduction in blood pressure in hypertensive patients [33]. Altogether, these data on bioavailability are quite consistent with the literature and with both our preclinical and clinical observations, providing strong support for the potential health benefits of TOTUM-854 in hypertension.

Conversely, and despite their interest previously described in treating hypertension, we did not detect either garlic-, bilberry-, or piperin-derived metabolites. In an intervention study conducted over 12 weeks in hypercholesterolemic subjects, the daily supplementation of bilberry extract (320 mg/day, n = 75) led to a significant improvement in arterial compliance [34]. The same conclusions were obtained in a more recent study that was twice as long (6 months) in subjects with metabolic syndrome [35]. Piperin, administered by gavage (20 mg/kg/day) for 3 weeks, limited the increase in blood pressure induced by L-NAME in rats [36]. Additionally, two recent clinical studies reported the benefit of garlic extract on endothelial function and subsequent blood pressure in obese [37] and type-2 diabetic patients [38]. In our study, volunteers were exposed once to 3 mg of black pepper,

which is lower than previous studies on the biological activity of long-term exposure to piperin (7.5 mg/day to 16 mg/day for 4 to 8 weeks) [39,40]. In fact, in this blend, piperin was used for its ability to improve the bioavailability and bioefficacy of the product with which it is associated and thus optimize the potential synergistic effects [41,42]. Regarding garlic, alliin is one of its most interesting bioactive but only represents 1% of garlic components [43]. In TOTUM-854, the quantity of alliin found in the raw blend was about 2000 times lower than oleuropein, which may contribute to explaining why its circulating metabolites remained under the detection threshold.

One of the main results of this study is the ability of TOTUM-854 human metabolites to inhibit ACE-1 activity both in vitro and ex vivo. As previously mentioned, despite several side effects, ACE inhibitors have been successfully used for decades to treat hypertension [4,13]. In this light, a few preclinical studies have shown that the efficacy of nutritional strategies in the management of hypertensive conditions may involve ACE inhibition. In a model of spontaneous hypertension in rats, a diet enriched with 3% freeze-dried blueberries for 4 or 8 weeks improved systolic blood pressure [44]. This protection involved the inhibition of the angiotensin-converting enzyme [45]. In a rat L-NAME-induced model of hypertension, garlic consumption completely prevented the increase in systolic blood pressure [17]. The hypotensive action of garlic was partly proven to rely on its ability to inhibit the angiotensin-converting enzyme [46–48]. Regarding isolated polyphenols, both chlorogenic acid and caffeic acids were reported to inhibit angiotensin-converting enzyme activity in a preclinical model of cyclosporine-induced hypertension [20,21]. Bilberries and garlic extracts are part of the blend, and TOTUM-854 also contains a wide range of polyphenols including chlorogenic and caffeic acids. Even though we could not detect all the specific metabolites of the different extracts, in line with the literature data, the bulk of the human metabolites derived from TOTUM-854 ingestion decreased ACE-1 activity in human endothelial cells in a lipotoxic context, likely contributing to explaining the observed benefit on endothelial cell function.

Among the molecular mechanisms that drive hypertension onset, oxidative stress and inflammation are major triggers. Endothelial inflammation stimulates pro-inflammatory cytokine production and monocyte recruitment and adhesion. These events promote the initiation of atherosclerosis, leading to hypertension development [49]. IL-1β (a pro-inflammatory cytokine), MCP-1 (a chemo-attractant cytokine), and VCAM-1 (an adhesion molecule) have been particularly highlighted in the context of endothelial cell dysfunction and hypertension [49,50]. Here, we demonstrated that human metabolites from TOTUM-854 consistently and potently reduced the release of IL-1β and the expression of both MCP-1 and VCAM-1 in a lipotoxic environment. Interestingly, in a recent randomized, double-blind, placebo-controlled study on patients with high cardiovascular risk, daily supplementation with 1200 mg of garlic extract for 4 weeks led to a potent reduction in both vascular inflammation and oxidative stress [38]. The same observations were made when the dose was reduced to 400 mg/d for a longer period of time (3 months) in obese patients [37]. Consistent with this, it has recently been reported that the hypotensive properties of olive leaf extract occur via the suppression of oxidative stress [51]. In our study, TOTUM-854 metabolites dramatically reduced the generation of ROS in HUVECs in a lipotoxic environment, as shown by DHE staining. This inhibition of DHE fully matches the observed inhibition of NOX2 expression and its role in hypertension establishment. A clinical study focused on the role of dark chocolate polyphenols on endothelial function in non-alcoholic steatohepatitis patients demonstrated a correlation between the reduction in NOX2 and the improvement of flow-mediated dilatation in the brachial artery (an index of endothelium-dependent vasodilation) [52]. The same trend was observed in children and adults with obstructive sleep apnea. NOX2 expression was associated with oxidative

stress-mediated arterial dysfunction, and the decreased expression of NOX2 was inversely correlated with the improvement in FMD [53,54].

Hence, these data further support the health benefits of TOTUM-854 supplementation in the management of moderate hypertension and highlight how TOTUM-854 metabolites contribute to maintaining normal endothelial cell function in a stress context.

This study provides important initial insights and promising avenues for understanding the mechanisms of action of TOTUM-854. By integrating a clinical study with preliminary ex vivo experiments on a cellular model and an in vivo proof-of-concept in a rodent model, we offer the first characterization of TOTUM-854's potential effects. However, additional complementary experiments are needed to fully dissect its mechanisms of action. Future studies should explore alternative in vitro models, particularly those with pre-existing NO production impairment, to further assess the potential direct or indirect effects of TOTUM-854 on NO synthesis. Moreover, investigations into additional in vivo models of hypertension will be essential to confirm and extend our findings, as well as to better understand the broader physiological relevance of TOTUM-854's effects on vascular function. Ultimately, clinical efficacy studies in humans, specifically in populations with elevated blood pressure, will be necessary to fully establish the therapeutic potential of TOTUM-854.

5. Conclusions

In this study, we showed that TOTUM-854 was able to protect against hypertension in vivo and further demonstrated how such benefits could occur ex vivo at a clinical level. The bio-available human metabolites from TOTUM-854 protected HUVECs from induced lipotoxic stress. HUVEC protection was characterized by (1) decreased ACE-1 activity, (2) decreased oxidative stress, (3) the inhibition of the inflammatory response, and (4) decreased marker expression of endothelial reactivity. Using both a relevant in vivo model and a pioneering clinical ex vivo approach, altogether these data support hypertension prevention and enlighten the bio-availability of TOTUM-854 human metabolites and their role in endothelial cell protection.

Two phase II/III randomized clinical trials (NCT05469503 and NCT05370625) are ongoing to fulfill the clinical evidence of the health benefits of TOTUM-854 in maintaining endothelial cell function and managing moderate hypertension.

6. Patents

The human ex vivo methodology used in this study has been registered as a written invention disclosure by the French National Institute for Agronomic, Food, and Environment Research (INRAE) (DIRV#18-0058). Clinic'n'Cell® has been registered as a trademark [11,22–28].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu17081331/s1, Figure S1: Extracted MRM chromatograms of the main circulating bioactive metabolites found in the serum of volunteers.

Author Contributions: Conceptualization, F.W. and Y.W.; design, D.R., F.L.J., Y.F.O., P.S., M.B., M.C. and V.S.; methodology, Y.W., L.B.-W., M.M., G.B., G.M., C.R. and F.W.; formal analysis, D.R., S.K., J.V. and F.W.; clinical investigation, V.R., N.M., G.P. and L.B.-W.; writing F.W. and Y.W.; data interpretation and reviewing, D.R., F.L.J., Y.F.O., P.S., T.M., B.P., M.B., M.C., V.S., F.W. and Y.W.; project administration, S.P., L.B.-W. and F.W. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki of 1975 (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/, accessed on 1 April 2021) revised in 2013. The human study was approved by the French Ethical Committee (N° SI RIPH: 21.03989.000023/N° ID RCB: 2021-A02695-36/Comité de Protection des Personnes CPP Est III; approved 8 December 2021).

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

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to ethical restrictions.

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Conflicts of Interest: F.W. and L.W.-B. work for Clinic'n'Cell SAS (Faculty of Medicine and Pharmacy Clermont-Ferrand- France). D.R., Y.F.O., J.L.B., M.M., P.S., S.P., M.B., M.C., and V.S. work for VALBIOTIS and provided the ingredients. Y.W. provides scientific consulting for Clinic'n'Cell SAS. S.K., J.V., T.M., G.B., G.M., C.R., V.R., N.M., and G.P. have no conflicts of interest to declare.

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Dose-Dependent Effects of Short-Chain Fatty Acids on 3T3-L1 Adipocyte Adipokine Secretion and Metabolic Function

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Abstract: Background: Short-chain fatty acids (SCFAs) produced from microbial fermentation of non-digestible carbohydrates and protein have been shown to modulate adipocyte adipokine secretion and metabolic function, which has implications for mitigating dysfunction in obese adipose tissue; however, the individual effects of different SCFAs and the optimal concentration required is unknown. The purpose of this study was to dose-dependently determine the effects of individual SCFAs on adipocyte adipokine secretion and metabolic function. Methods: We recapitulated the obese adipocyte inflammatory conditions using mature 3T3-L1 adipocytes and a physiological concentration of lipopolysaccharide (LPS) ± individual SCFAs, namely acetate, propionate, and butyrate, in a dose-dependent manner (0.25 mM, 0.5 mM, and 1 mM) for 24 h. Results: SCFAs dose-dependently affected inflammatory adipokine secretion, wherein at 1 mM, all three SCFAs reduced the secretion of leptin, IL-6 and IL-1β, but only propionate and butyrate reduced MCP-1/CCL2 and MIP-1α/CCL3 compared to control ($p < 0.05$). Interestingly, 1 mM acetate increased RANTES/CCL5 secretion versus control, whereas propionate and butyrate decreased RANTES/CCL5 secretion, and only 1 mM propionate reduced MCP-3/CCL7 secretion ($p < 0.05$). At the lower 0.5 mM concentration, both propionate and butyrate reduced IL-6 and IL-1β secretion compared to control ($p < 0.05$), and there was no difference in adipokine secretion between groups at the 0.25 mM SCFA concentration ($p > 0.05$). Intracellular protein expression in the ratio of phosphorylated–to–total STAT3 was reduced by all SCFAs at 1 mM and by propionate and butyrate at 0.5 mM versus control ($p < 0.05$). The ratio fo phosphorylated–to–total NFκB p65 expression was reduced by propionate and butyrate at 1 mM and by butyrate alone at 0.5 mM compared to control ($p < 0.05$). Basal (no insulin stimulation) and insulin-stimulated glucose uptake did not differ between control and any 1 mM SCFA treatment conditions ($p > 0.05$). Conclusions: Individual SCFAs exert different dose-dependent effects on LPS-stimulated adipocyte function.

Keywords: short-chain fatty acids; acetate; propionate; butyrate; adipocytes; inflammation; adipokines; lipopolysaccharide; insulin-stimulated glucose uptake

1. Introduction

The local (i.e., within adipose tissue) and systemic obese phenotype is associated with chronic low-grade inflammation which perpetuates metabolic dysfunction including insulin resistance and dyslipidemia [1–5]. Dietary interventions that can attenuate the severity of the obese inflammatory phenotype provide an alternative approach for obesity management [6,7]. These approaches include the role of dietary non-digestible carbohydrates (NDCs) that are fermented by the gastrointestinal microbiome to produce short-chain fatty acids (SCFAs); predominantly acetate (or acetic acid), propionate (or propionic acid),

and butyrate (butyric acid), although SCFAs with longer carbon chains such as valerate (or valeric acid) or caproate (or caproic acid) are also produced in lower amounts [8,9]. Additionally, SCFAs can be produced in lower amounts from the fermentation of undigested protein [10]. SCFAs have been shown to exert extraintestinal effects, although circulating concentrations are lower than fecal or gastrointestinal luminal concentrations [11–16].

With respect to modulating the severity of the obese phenotype, NDC supplementation in both humans and obese rodents has been shown to improve critical elements of the obese phenotype, including reducing weight gain [17–29], reducing adipose tissue mass [18,19,28–32], and improving blood glucose regulation and/or insulin resistance [26,33–39]. Production of SCFAs from dietary NDC intake likely underlies the beneficial effects, and SCFAs have been shown to modulate host metabolic function extra-intestinally in adipose tissue, skeletal muscle, liver, and the pancreas [10,40–47]. Specifically, within adipose tissue or adipocyte cultures, SCFAs have been shown to decrease lipolysis and increase triglyceride accumulation, along with increasing glucose uptake and adipogenesis [48–54]. Investigations into the metabolic effects of SCFA in adipose have primarily focused on select individual SCFAs, such as only acetate [49,50,53], only butyrate [48], or studies assessing differing outcomes between acetate, propionate, and butyrate [47,55–57], or SCFAs with a longer carbon chain length, such as valerate [54]. Although frequently considered to exert similar biological effects, in part due to their shared signaling through G protein-coupled receptors (GPCR, mainly GPR41, GPR43, and GPR109a), individual SCFAs have different binding affinities for these receptors [58]. Moreover, SCFAs are produced through microbial fermentation at different amounts (typically a 3:1:1 molar ratio of acetate–propionate–butyrate, respectively) that collectively account for 90–95% of colonic SCFA [9,59–61]. Furthermore, the amount and types of SCFA produced will vary based on multiple factors including the amount and type(s) of NDC in the diet (i.e., SCFA precursors including, but not limited to, soluble fibers, resistant starches, oligosaccharides, etc.), the relative fermentability of each type of NDC by the microbiota (i.e., fast versus slow fermentation), the microbiota composition of SCFA-producing microbial species, and intestinal SCFA absorptive capacity [10,62–67]. Previous comparative studies have reported that individual SCFAs exert differential effects on metabolic function and/or inflammatory mediator secretion in skeletal muscle cells [68,69] and adipocytes or adipose tissue [70,71].

The effect of SCFA on adipokine (i.e., adipose tissue-derived hormones, cytokines, and chemokines) gene expression or protein secretion from adipose tissue or adipocyte cultures has been focused on leptin and adiponectin, with some studies demonstrating the ability of select SCFAs to stimulate leptin gene expression or secretion from obese adipose tissue, with the effects of acetate differing from SCFAs with a longer carbon chain length [71,72], or having no effect of SCFA on leptin secretion [12]. In contrast, select SCFAs had no effect on adiponectin gene expression in obese primary adipose tissue; however, adiponectin gene expression increased with SCFA treatment or adipose tissue from obese donors with concurrent type 2 diabetes, thereby indicating that the adipose tissue microenvironment and metabolic status may impact how the tissue responds to SCFA signaling [72]. Conversely, acetate provided at higher doses (10 mM) has been shown to stimulate adiponectin secretion from primary brown adipose tissue and white adipose tissue [12], which indicates that the specific effects of SCFA may be dose dependent. The variations in SCFA dose and experimental conditions may account for differing outcomes. Moreover, an array of other cytokines and chemokines that can influence adipose tissue function remain under investigation, particularly at concentrations that mimic circulating SCFA levels in humans. What has been shown is the capacity for a 3 mM concentration of propionate to reduce inflammatory cytokine (TNFα) and chemokine (regulated upon activation, normal T cell expression, and secreted (RANTES)/chemokine ligand (CCL)5, macrophage inflammatory protein (MIP)-1α/CCL3 and MIP-1β/CCL4) secretion from

obese primary adipose tissue cultures [73]. Separately, a lower and more physiologically relevant concentration (1 mM) of butyrate was shown to reduce adipocyte secretion of multiple inflammatory mediators including IL-6 IL-1β, MIP-1α/CCL3, RANTES/CCL5, macrophage chemoattractant protein (MCP)-1/CCL2, and MCP-3/CCL7 in response to combined normoxic and lipopolysaccharide stimulated conditions, whereas valerate only reduced IL-6 secretion under the same environmental conditions [70]. Furthermore, under hypoxic conditions, butyrate prevented and valerate increased adipocyte secretion of inflammatory mediators [70], thereby highlighting the different effects of individual SCFAs and the differing effects of SCFAs within unique obesity-associated adipose tissue microenvironments [72].

Since the range of SCFA concentrations in the systemic circulation is considerably lower than fecal levels [11–16,59–61], and the range of individual SCFAs evaluated at various concentrations differs between studies [57,72,74–82], and frequently, but not always, exceeds circulating concentrations, the purpose of this study was to assess the effects of the main SCFAs (acetate, propionate, and butyrate) [9,59–61] on adipocyte adipokine secretion and metabolic function in a dose-dependent manner.

2. Materials and Methods

2.1. T3-L1 Cell Culture and Differentiation

3T3-L1 murine pre-adipocytes (CL-173; American Type Culture Collection, Manassas, VA, USA) were cultured and maintained according to the manufacturer's instructions in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) supplemented with 4 mM L-glutamine, 4500 mg/L glucose, 10% (*v*/*v*) low endotoxin sterile-filtered fetal bovine serum (FBS; Millipore-Sigma, Oakville, ON, Canada) and 1% (*v*/*v*) penicillin-streptomycin (Fisher Scientific, Mississauga, ON, Canada), as described previously [70,83]. Pre-adipocytes were differentiated into adipocytes using DMEM containing 1 μmol/L dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 10 μg/mL insulin (all from Millipore-Sigma), and matured in DMEM supplemented with 10 μg/mL insulin, as described previously [70,83]. Media were changed every two days. On day 8 post-differentiation, adipocytes were incubated for 12 h in serum-free DMEM containing 1% (*v*/*v*) penicillin-streptomycin prior to the addition of the experimental treatments.

2.2. Experimental Treatment Conditions

Adipocytes were incubated for 12 h in serum-free DMEM containing % (*v*/*v*) penicillin-streptomycin before they were used in any experiment. Subsequently, the cells were treated for 24 h with DMEM media alone (control, CON) or with media dose-dependently (0.25 mM, 0.5 mM, or 1 mM) containing each SCFA, namely sodium acetate (ACE), sodium propionate (PRO), and sodium butyrate (BUT) (all from Millipore-Sigma), concentrations that have been used previously [48,51,52,54,71,84]. These CON and SCFA-containing experimental treatment groups were either unstimulated (n = 6/experimental group) or treated with an inflammatory stimulus of 10 ng/mL lipopolysaccharide (LPS, from *Escherichia coli* 055:B5 (Millipore-Sigma); n = 9/experimental group). The dose of LPS utilized in these experiments recapitulates the circulating endotoxin levels reported in obese humans [85] and rodent high-fat-diet-induced obesity models [86,87]. Cell viability in response to all treatment conditions was assessed using Trypan blue exclusion and exceeded 90%, as seen previously [70]. After 24 h, culture supernatant was collected and stored at −80 °C to await secreted adipokine analysis, and cells were lysed using the lysis buffer from the RNA/Protein Purification Plus Kit (Norgen Biotek Corp., Thorold, ON, Canada), which was collected and stored at −80 °C.

2.3. Secreted Adipokines

Adipocyte secretion of adipokines in culture supernatant was measured by Bio-Plex using the Bio-Plex 200 system and accompanying Plex Manager software, version 6.0 (Bio-Rad, Mississauga, ON, Canada). All adipokines were measured following the manufacturers' protocol, wherein adiponectin was measured separately using the Bio-Plex Pro Mouse Diabetes Adiponectin Assay (Bio-Rad, #171F7002M), and leptin and resistin were measured together using the Bio-plex Pro Reagent kit V (Bio-Rad, #12002798). Finally, secreted cytokines (IL-1β, IL-6, IL-10, and TNFα) and chemokines (MCP-1/CCL2, MCP-3/CCL7, MIP-1α/CCL3, MIP-1β/CCL4, and RANTES)/CCL5) were simultaneously measured using the mouse Bio-Plex Pro kit (Bio-Rad). IL-10 was below the assay limit of detection in all samples. Secreted protein levels for all other adipokines assessed in unstimulated 3T3-L1 cell cultures ± SCFA did not differ between experimental groups (i.e., CON, ACE, PRO, and BUT) and were significantly lower than LPS-stimulated secretion levels ($p < 0.05$; results not shown).

2.4. Intracellular Protein Analysis

Total intracellular protein was quantified using the bicinchoninic assay according to the manufacturer's instructions (Thermo-Fisher Scientific, Mississauga, ON, Canada). An equal amount of protein (10 μg/sample/assay) was used to measure the ratio of phosphorylated-to-total expression of transcription factors STAT3 (phosphorylated STAT3 [Tyr705]–total STAT3) and NFκB p65 (phosphorylated NFκB p65 [Ser536]–total NFκB p65) using an enzyme-linked immunosorbent assay, as per the manufacturer's instructions (Thermo-Fisher Scientific). The final absorbance was measured at 450 nm using SpectraMax M5e Multimode Plate Reader (Molecular Devices, San Jose, CA, USA).

2.5. Glucose Uptake Assay

Glucose uptake was measured in mature 3T3-L1 adipocyte cultures (±LPS and 1 mM ACE, PRO, or BUT) for 24 h (n = 6–8/treatment group) in both basal (i.e., non-insulin stimulated) and insulin-stimulated cultures (described below) using a colorimetric Glucose Uptake Assay Kit (Abcam, Waltham, MA, USA) according to the manufacturer's instructions and as described previously [69]. Basal stimulated 3T3-L1 cell cultures were not treated with insulin but instead treated with phosphate-buffered saline for 20 min, followed by the addition of 10 mM of the glucose analog, 2-deoxy glucose, for 20 min at 37 °C. Insulin-stimulated 3T3-L1 adipocyte cultures were treated with 1 μM insulin (Millipore-Sigma) for 20 min, followed by the addition of 10 mM of 2-deoxy glucose for 20 min at 37 °C. At the end of the kit protocol, optical density was measured at 412 nm using a spectrophotometer (Molecular Devices, San Jose, CA, USA). Glucose-starved cell cultures (n = 4/treatment group) that did not receive either 2-deoxy glucose or insulin served as background negative control cultures, and there was no difference in optical density between the control and SCFA-negative control cultures.

2.6. Statistical Analysis

All data are expressed as means ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test for post hoc analysis between experimental treatment groups ($p \leq 0.05$). The Shapiro–Wilk test was used to test for normality. All analyses were conducted using GraphPad Prism, version 10 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. SCFAs Dose-Dependently Attenuate Inflammatory Adipokine Secretion in Response to LPS Stimulation

In response to LPS stimulation, all three SCFAs (ACE, PRO, and BUT) reduced leptin secretion compared to CON at the highest concentration tested (1 mM); however, at the lower SCFA concentrations assessed, there was no difference in leptin secretion between experimental groups (Figure 1A). There was no difference in the secretion of resistin (Figure 1B) or adiponectin (Figure 1C) between experimental groups at any SCFA concentration.

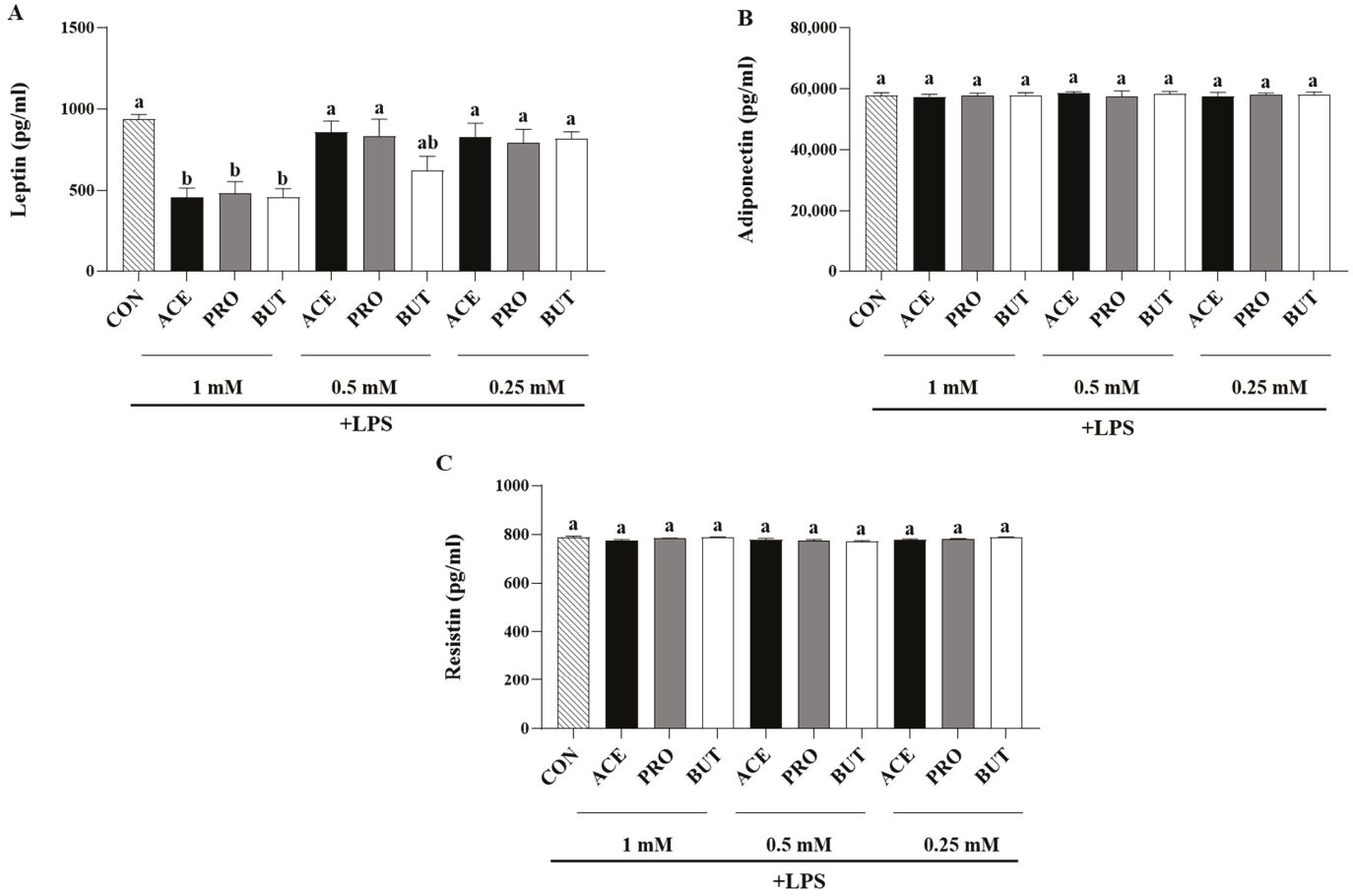


Figure 1. Individual SCFAs' dose-dependent (1 mM, 0.5 mM, and 0.25 mM) effects on LPS-stimulated 3T3-L1 adipocyte secreted protein levels [(**A**): leptin, (**B**): adiponectin, and (**C**): resistin]. Bars represent mean values ± SEM (n = 9/experimental group). Data were analyzed via one-way ANOVA followed by Tukey's multiple comparison test. Bars not sharing a lower-case letter differ ($p < 0.05$).

Inflammatory cytokine secretion in response to LPS stimulation was differentially affected by each SCFA in a dose-dependent manner (Figure 2). ACE, PRO, and BUT all reduced IL-6 secretion at the highest concentration assessed (1 mM) compared to CON ($p < 0.05$; Figure 2A), wherein IL-6 secretion was the lowest from the PRO-treated adipocytes. At the middle SCFA dose (0.5 mM), only PRO and BUT reduced IL-6 secretion in comparison to CON ($p < 0.05$), and there was no difference in IL-6 secretion between any treatment groups at the 0.25 mM SCFA concentration (Figure 2A).

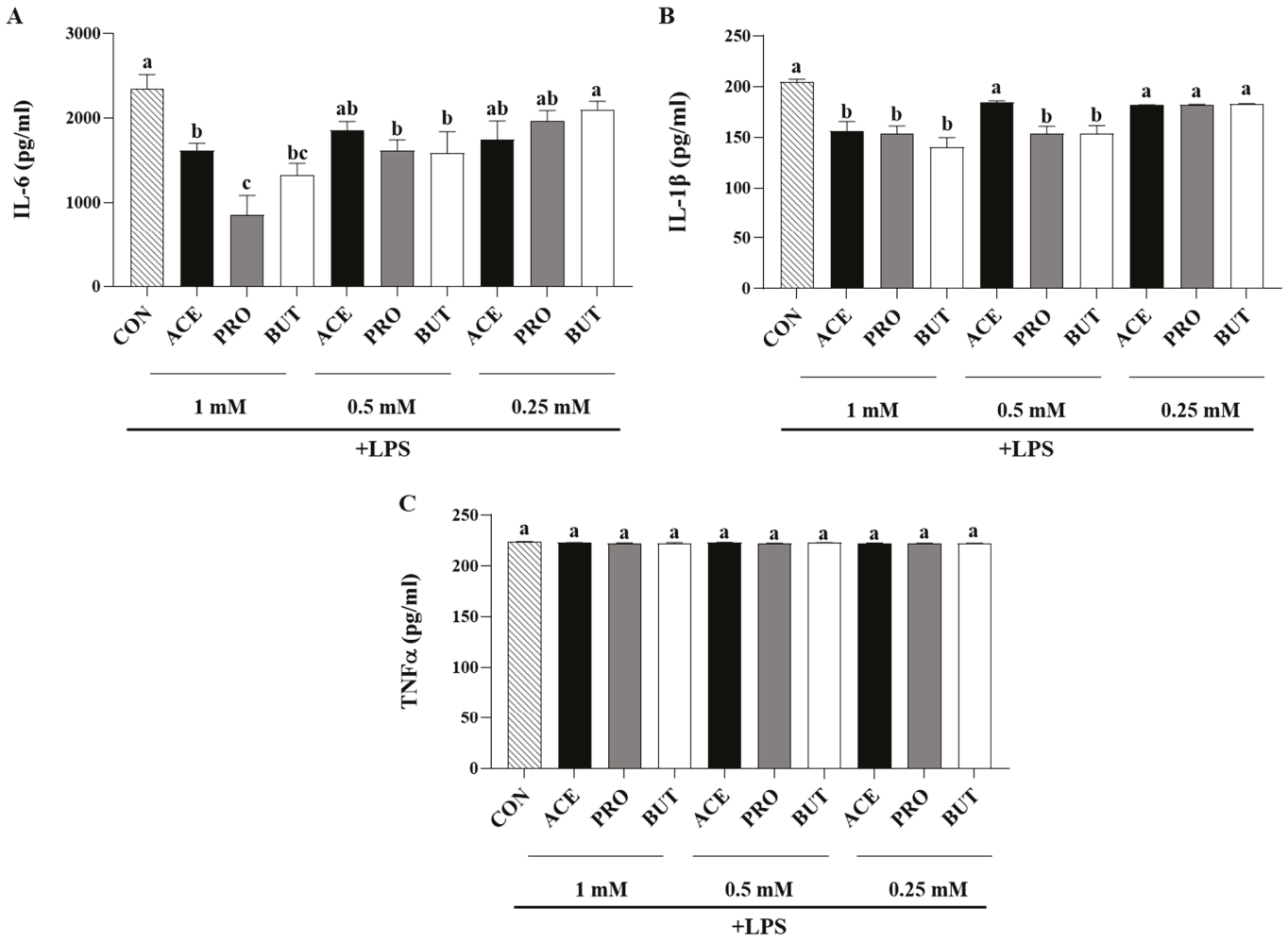


Figure 2. Individual SCFAs' dose-dependent (1 mM, 0.5 mM and 0.25 mM) effects on LPS-stimulated 3T3-L1 adipocyte secreted protein levels [(**A**): IL-6, (**B**): IL-1β, and (**C**): TNFα]. Bars represent mean values ± SEM (n = 9/experimental group). Data were analyzed via one-way ANOVA followed by Tukey's multiple comparison test. Bars not sharing a lower-case letter differ ($p < 0.05$).

All three SCFAs reduced IL-1β secretion at the 1 mM dose compared to CON ($p < 0.05$; Figure 2B), while only PRO and BUT reduced IL-1β secretion at the middle dose (0.5 mM) compared to CON ($p < 0.05$), and there were no differences between treatment groups at the lowest dose (0.25 mM) ($p > 0.05$). There was no difference between treatment groups in the secretion of TNFα at any SCFA concentration assessed ($p > 0.05$; Figure 2C).

Inflammatory chemokine secretion by adipocytes in response to LPS and SCFA is shown in Figure 3. MCP-1/CCL2 secretion was reduced by both PRO and BUT at the 1 mM concentration compared to CON ($p < 0.05$; Figure 3A), while ACE did not differ from CON ($p > 0.05$). There was no difference in MCP-1/CCL2 secretion between treatment groups at either the 0.5 mM or 0.25 mM SCFA treatment concentrations ($p > 0.05$). Only PRO reduced MCP-3/CCL7 secretion at the 1 mM concentration as compared to CON ($p < 0.05$; Figure 3B), and there were no differences between CON and PRO at the other lower concentrations assessed ($p > 0.05$). ACE and BUT did not differ from CON at any concentration tested compared to CON ($p > 0.05$). RANTES/CCL5 secretion was increased by ACE at the 1 mM concentration compared to CON ($p < 0.05$), while both PRO and BUT reduced RANTES/CCL7 secretion in comparison to CON ($p < 0.05$; Figure 3C).

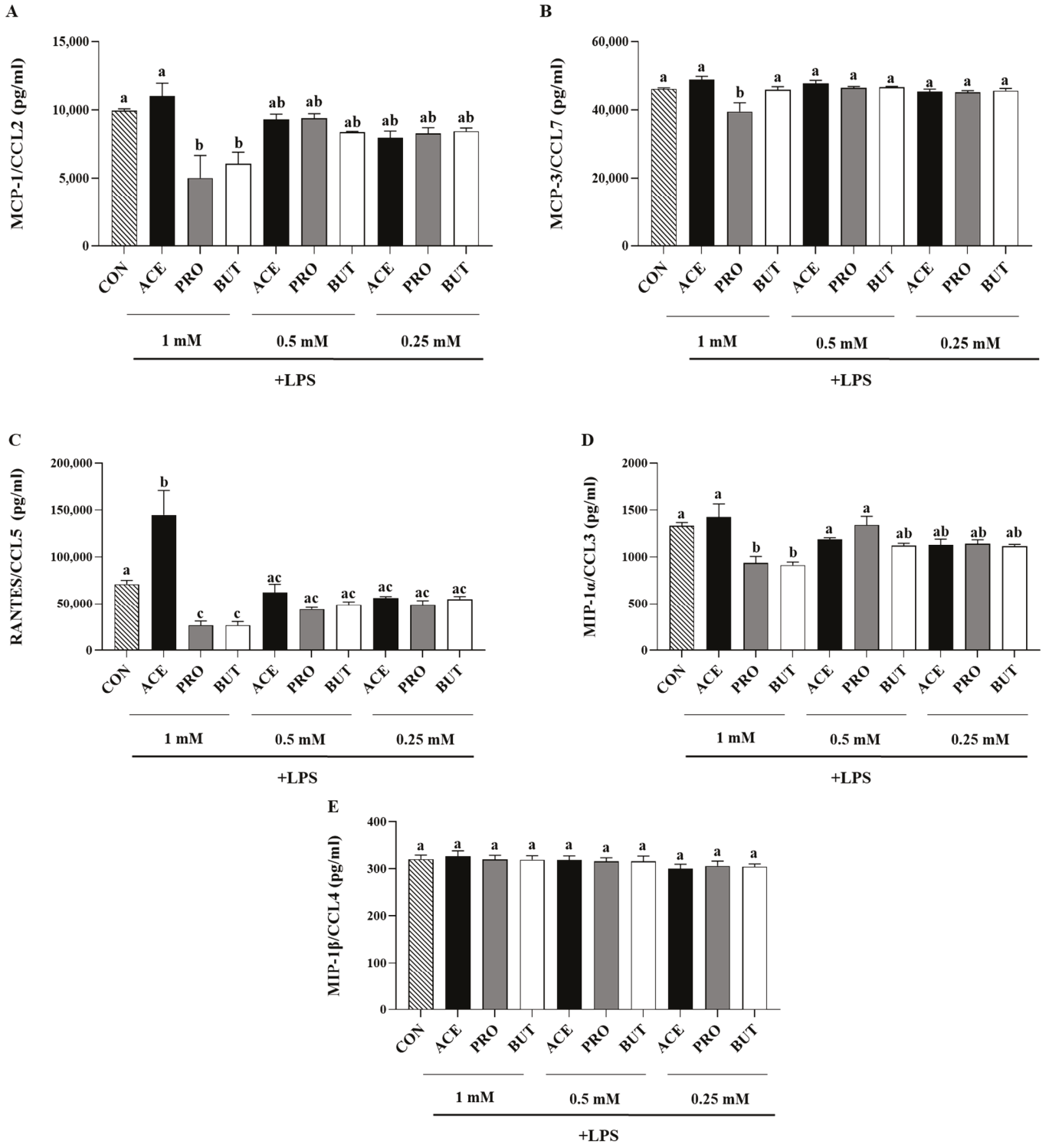


Figure 3. Individual SCFAs' dose-dependent (1 mM, 0.5 mM, and 0.25 mM) effects on LPS-stimulated 3T3-L1 adipocyte secreted protein levels [(**A**): MCP-1/CCL2, (**B**): MCP-3/CCL7, (**C**): RANTES/CCL5, (**D**): MIP-1α/CCL3, (**E**): MIP-1β/CCL4]. Bars represent mean values ± SEM (n = 9/experimental group). Data were analyzed via one-way ANOVA followed by Tukey's multiple comparison test. Bars not sharing a lower-case letter differ ($p < 0.05$).

MIP-1α/CCL3 secretion was reduced by both PRO and BUT at the 1 mM concentration compared to CON ($p < 0.05$; Figure 3D); however, there was no difference between CON and PRO or BUT at the lower concentrations, and ACE had no effect on LPS-stimulated

secretion of MIP-1α/CCL3 at any concentration tested ($p > 0.05$). MIP-1β/CCL4 secretion did not differ between any treatment groups ($p > 0.05$; Figure 3E).

3.2. Transcription Factor Activation (Ratio of Phosphorylated-to-Total) Is Dose-Dependently Reduced by SCFA in Response to LPS Stimulation

Adipocyte intracellular protein expression of transcription factors, expressed as the ratio of phosphorylated (i.e., activated)-to-total, in response to LPS stimulation, is shown in Figure 4. All three individual SCFAs reduced intracellular protein expression of STAT3 at the 1 mM concentration compared to CON ($p < 0.05$; Figure 4A). At the 0.5 mM concentration, only PRO and BUT reduced STAT3 expression compared to CON ($p < 0.05$), whereas ACE had no effect at the 0.5 mM concentration. At the lowest concentration (0.25 mM) assessed, there was no difference in STAT3 expression between treatment groups ($p > 0.05$).

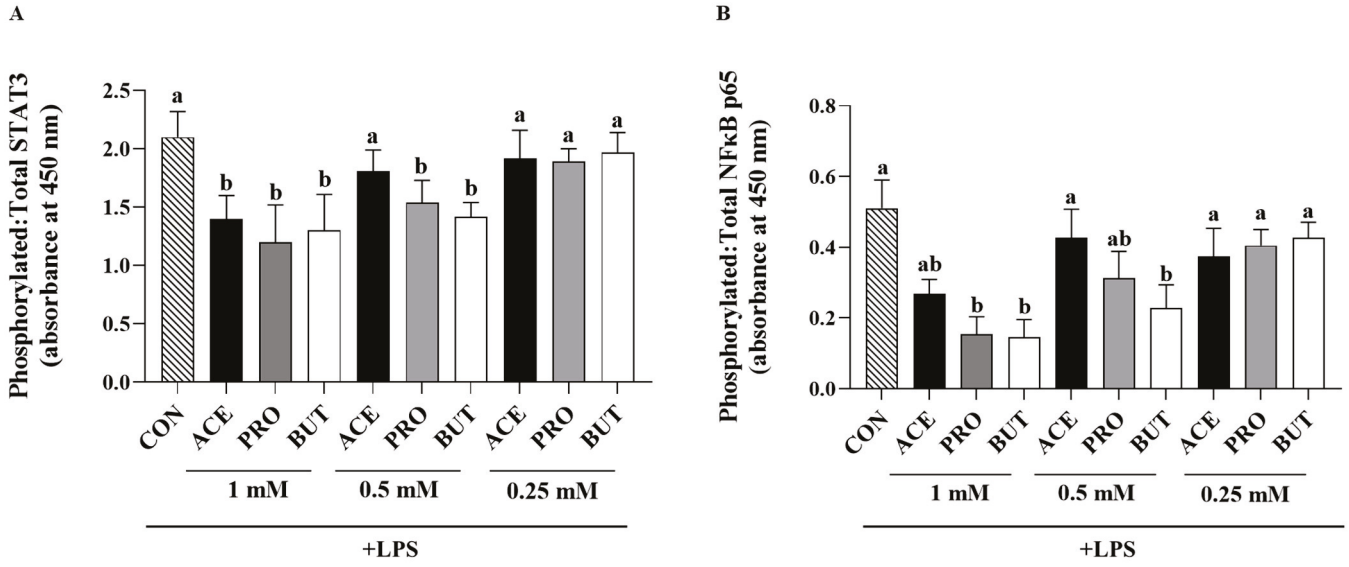


Figure 4. Individual SCFAs' dose-dependent (1 mM, 0.5 mM, and 0.25 mM) effects on LPS-stimulated 3T3-L1 adipocyte intracellular protein levels ((**A**): phosphorylated–to–total STAT3, and (**B**): phosphorylated–to–total NFκB p65). Bars represent mean values ± SEM ($n = 9$/experimental group). Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Bars not sharing a lower-case letter differ ($p < 0.05$).

In comparison to CON LPS-stimulated adipocyte cultures, NFκB p65 intracellular protein expression was reduced by both PRO and BUT at the 1 mM dose ($p < 0.05$; Figure 4B); however, ACE did not differ from CON ($p > 0.05$). At the 0.5 mM dose, only BUT reduced intracellular expression of NFκB p65 compared to both CON and the other SCFAs ($p < 0.05$). There was no difference between CON and any SCFA at the 0.25 mM dose ($p < 0.05$).

3.3. Insulin-Stimulated Glucose Uptake in LPS-Stimulated Adipocytes Is Not Affected by SCFA

Glucose uptake in basal (i.e., non-insulin-stimulated) and insulin-stimulated adipocyte cultures was assessed in CON and only 1 mM SCFA-treated cultures in the presence of LPS, as shown in Figure 5. There was no difference in glucose uptake between any treatment groups (CON and individual SCFAs) in both the basal (i.e., non-insulin simulated) and insulin-stimulated conditions ($p > 0.05$).

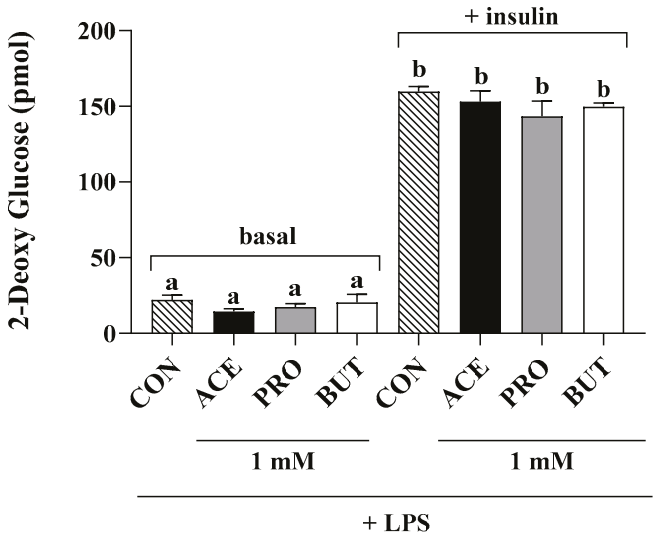


Figure 5. Effect of individual SCFAs (1 mM) on LPS-stimulated 3T3-L1 adipocyte glucose uptake under basal (i.e., non-insulin stimulated, phosphate-buffered saline for 20 min followed by 10 mM 2-deoxy glucose for 20 min at 37 °C) and insulin-stimulated conditions (1 μM insulin for 20 min followed by 10 mM 2-deoxy glucose for 20 min at 37 °C). Bars represent mean values ± SEM (n = 6–8/experimental group under basal and insulin-stimulated conditions). Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Bars not sharing a lower-case letter differ ($p < 0.05$).

4. Discussion

The results from this study demonstrate the dose-dependent effects of individual SCFAs on LPS-stimulated adipocyte adipokine secretion, activation status (i.e., ratio of phosphorylated–to–total) of transcription factors NFκB p65 and STAT3 that affect inflammatory mediator production, and glucose uptake (under both basal and insulin-stimulated conditions). The experimental approach utilized aimed to create an adipocyte cell culture microenvironment that more accurately recapitulated the conditions that obese adipocytes would experience in vivo by utilizing an LPS dose that mimics circulating LPS levels observed in obese humans [85] and rodents [86,87]. Furthermore, the doses of individual SCFAs used in this study (0.25–1 mM) more accurately reflect circulating SCFA levels in humans, which can be variable depending upon the underlying physiological condition [11–16,88,89]. Investigations into the metabolic effects of SCFA in adipose have primarily focused on select individual SCFAs, such as only acetate [49,50,53], only butyrate [48], or studies assessing differing outcomes between acetate, propionate, and butyrate [47,55–57], or consider the effects of SCFAs with a longer carbon chain length, such as valerate [54,70]. Importantly, the concentration of SCFA used in this study is within the range of SCFA concentrations observed in human blood [88,89], which highlights the importance of assessing SCFA-mediated effects at lower concentrations. Importantly, the 1 mM SCFA concentration used in this study has been shown previously to not affect adipocyte viability [54,70,71], which we also confirmed herein. In contrast, other studies assessing the metabolic effects of SCFA used higher concentrations that do not necessarily reflect circulating levels in vivo that adipose tissue would be more likely to encounter [49,56,57,74,84,90].

The cellular composition of obese adipose tissue within the stromal vascular compartment changes based on the infiltration of additional immune cells and their subsequent change in polarization and functional status [91,92], which is directly connected to tissue inflammation and metabolic dysfunction. It is equally important to identify the intact tissue adipokine secretory profile in response to individual SCFAs, and to identify the cellular source(s) of each adipokine secreted in response to SCFA. The current study investigated

the effects of individual SCFAs on the adipocyte response to LPS-mediated stimulation, as adipocytes have been identified as a critical immunomodulatory cell type in adipose tissue that is responsible for not only adipokine production (hormones, cytokines, and chemokines to recruit additional immune cells to the adipose tissue), but also the release of proinflammatory lipids, extracellular matrix proteins, and extracellular vesicles that contribute to local and systemic inflammation and metabolic function [93]. The results from the current study highlight the effect of individual SCFAs on adipocyte-mediated adipokine secretion.

The spectrum of secreted inflammatory adipokines assessed in the current study included hormones, cytokines, and chemokines, which have been shown to contribute to obese adipose tissue low-grade chronic inflammation, increased immune cell recruitment, and subsequent metabolic dysfunction [94,95], while concomitantly also assessing the effect of SCFA on the secretion of anti-inflammatory and/or insulin-sensitizing mediators such as IL-10 and adiponectin [94,96]. The majority of studies investigating the role of SCFA on adipocyte or adipose tissue adipokine gene expression and/or protein secretion utilize different model systems, such as primary tissue explants or adipocytes alone, utilize a range of SCFA concentrations, and tend to be more focused on the adipokines leptin and adiponectin [72,97,98]. A higher concentration of propionate (3 mM) has been shown to reduce the secretion of some inflammatory mediators (cytokines and chemokines, namely TNFα, RANTES/CCL5, MIP-1α/CCL3, and MIP-aβ/CCL4) from primary adipose tissue cultures [77], whereas a lower 1 mM concentration of butyrate was shown to reduce the secretion of a spectrum of inflammatory cytokines and chemokines from LPS-stimulated adipocytes under normoxic conditions (namely IL-6, IL-1β, MCP-1/CCL2, MCP-3/CCL7, MIP-1α/CCL3, and RANTES/CCL4) and prevent the rise in inflammatory mediator secretion from hypoxic adipocytes [70]; however, in both studies, the effects of other individual SCFAs were not evaluated. In the current study, the majority of the effects of SCFA on adipokine secretion were observed at the 1 mM concentration, indicating that lower SCFA concentrations (0.25 mM) are insufficient to induce a change in adipocyte adipokine secretion. PRO and BUT (at 1 mM) exerted similar effects by reducing adipocyte secretion of leptin, IL-6, IL-1β, MCP-1/CCL2, RANTES/CCL5, and MIP-1α/CCL3 (Figures 1–3), wherein a lower concentration (0.5 mM) of PRO and BUT also reduced IL-6 and IL-1β secretion (Figure 2). The reduced secretion of cytokines and chemokines in response to butyrate confirmed previous findings that highlighted functional differences between butyrate and valerate [70], and the current study highlights the functional similarities between BUT and PRO, with both SCFAs exerting an anti-inflammatory and anti-chemotactic effect in LPS-stimulated adipocytes. Importantly, BUT and PRO did not differ in the secretion of MCP-3/CCL7 (Figure 2), which demonstrates that not all SCFAs exert similar effects. In this connection, ACE was shown to reduce only leptin, IL-6, and IL-1β secretion; however, ACE significantly increased RANTES/CCL5 secretion (Figures 1–3). Higher circulating levels of RANTES are associated with impaired glucose tolerance [81]. Obese adipose tissue expression of RANTES is positively correlated with the expression of the macrophage marker CD68 [99], as well as increased local T cell accumulation [100]. This indicates that ACE may contribute to immune cell chemotaxis to inflamed adipose tissue, whereas PRO and BUT exhibited an anti-chemotactic effect that could reduce immune cell recruitment into obese adipose tissue. Upon macrophage and T cell infiltration and polarization into inflammatory phenotypes in obese adipose tissue, these cells contribute to inflammatory mediator production, increased reactive oxygen species production, endothelial dysfunction, and metabolic dysfunction, in particular, insulin resistance [93,101,102]. Moreover, leptin, which is elevated in obese adipose tissue [103], has been shown to play a key role in directing chemokine production [101]; however, all SCFAs assessed were shown to

reduce leptin secretion (Figure 1). Conflicting findings have been reported in primary adipose tissue using higher SCFA concentrations that increased leptin gene expression and secretion [72,97], or had no effect [98]. Conversely, adiponectin functions as an insulin-sensitizing and anti-inflammatory adipokine [104], whose secretion decreases in obese adipose tissue [105,106]. In the current study, adiponectin secretion did not differ between SCFA treatment groups (Figure 1); however, a high dose of acetate (10 mM) has been shown to stimulate adiponectin secretion from primary brown adipose tissue and white adipose tissue [98], and adiponectin gene expression has been shown to increase in primary adipose tissue from obese type 2 diabetic donors but had no effect on non-diabetic adipose tissue [72], which highlights that SCFAs may exert different effects based on the inflammation and metabolic status of the tissue and warrants further investigation. The addition of individual SCFAs at 5% *w*/*w* to a high-fat diet was shown to increase adiponectin gene expression [55], and the same study also observed an increase in resistin gene expression compared to high-fat diet controls. Resistin levels are increased in obesity [107,108]; however, in the current study, there was no difference in resistin secretion between treatment groups (Figure 1). Collectively, the results demonstrate dose-dependent anti-inflammatory and anti-chemotactic effects of SCFAs on the adipocyte secretory profile, with the strongest effects attributable to propionate and butyrate.

Activation of NFκB occurs downstream of LPS signaling via Toll-Like Receptor (TLR)-2 and TLR4 in adipocytes and serves as a critical transcription factor controlling the gene expression of many inflammatory cytokines and chemokines (e.g., TNFα, IL-6, MCP-1/CCL2, etc.) that contribute to obesity-associated inflammation and metabolic dysfunction [109,110]. All SCFAs reduced NFκB p65 activation (ratio of phosphorylated-to-total cellular protein) at the 1 mM dose, wherein the secretion of key inflammatory adipokines that contribute to adipocyte metabolic dysfunction, such as IL-6 and MCP-1/CCL2 [94,95], was concomitantly also reduced (Figures 1–4). Furthermore, only BUT reduced NFκB p65 activation at the 0.5 mM concentration, highlighting a dose-dependent effect of SCFAs on transcription factor activation. Similarly, STAT3 is another transcription factor that influences inflammatory adipokine gene expression and immune and metabolic functions in adipose tissue [111,112] and was dose-dependently reduced by each individual SCFA. At the 1 mM concentration, all three SCFAs reduced phosphorylated–to–total STAT3 intracellular protein levels, whereas both PRO and BUT reduced STAT3 activation at the 0.5 mM concentration, and there was no effect at the lowest concentration (0.25 mM) assessed (Figure 4). Collectively, these findings mirrored the adipokine secretion profile attributable to each SCFA, with butyrate and propionate having the strongest effects, particularly at lower concentrations compared to acetate.

Despite the connection between increased obese adipose tissue inflammatory adipokine production and metabolic dysfunction [94,95], and the anti-inflammatory and anti-chemotactic effects of predominantly PRO and BUT (and to a lesser degree ACE), insulin-stimulated glucose uptake did not differ between control and SCFA treatments in LPS-stimulated adipocytes (Figure 5). Supplementation of a high-fat diet with 5% *w*/*w* sodium butyrate was shown to reduce fasting insulin levels, the response to an insulin tolerance test, and insulin sensitivity assessed by HOMA-IR [113]. Previously, propionate (at concentrations ranging from 1 to 10 mM) was shown to increase insulin-stimulated glucose uptake, whereas the effects of butyrate to improve insulin sensitivity were only observed at a 10 mM concentration [114]. Moreover, in the same study, propionate and butyrate were shown to dose-dependently inhibit lipolysis and de novo lipogenesis, with the strongest effects observed at supraphysiological concentrations (10 mM) [114], indicating that SCFAs may also impact other metabolic functions beyond glucose uptake. In this connection, high concentrations of acetate (6.2 mM) and propionate (3.2 mM), but lower concentrations of

butyrate (0.8 mM), that are closer to those used in the current study, have been shown to promote lipid accumulation in adipocytes [115]. Further studies are required to dose-dependently investigate the breadth of potential metabolic effects attributable to individual SCFAs, and to determine both the kinetics of those effects, and if those effects are a direct consequence of SCFA signaling (i.e., through GCPR) or through altering adipokine levels within the adipose tissue microenvironment. The translational potential of this work can be increased by future studies that utilize primary adipose tissue, which can more accurately reflect in vivo conditions and comprises diverse cell types that are present in adipose tissue, in contrast to the limited scope of the current study that utilized only adipocytes from an immortalized cell line.

In order to accurately provide recommendations for dietary intakes of SCFA precursors, it is necessary to identify the SCFA concentration that is required to exert beneficial effects within the extra-intestinal tissues, such as adipose. The ability of individual SCFAs to beneficially modulate adipocyte function via anti-inflammatory and anti-chemotactic mechanisms identifies the therapeutic potential of SCFAs to improve obese adipose tissue function. Investigating strategies to increase SCFA levels, either through nutritional interventions to increase NDC dietary intakes and/or directly providing SCFA via supplementation approaches hold potential for attenuating adipose tissue dysfunction associated with obesity, although further study is required. Using physiologically relevant cell culture models can identify the mechanisms and relevant concentrations that are required to beneficially affect adipocyte function, which can be further tested within intact adipose tissue in future studies in a directed manner. Given the variables that can affect SCFA production in vivo, including the amount, type, and fermentability of each NDC consumed, the relative abundance of SCFA-producing microbial species found within the microbiota, and the absorptive capacity of the intestinal epithelium [10,62–67], comprehensive assessments of adipocyte functions using physiologically relevant SCFA concentrations [11–16,88,89] can help to address this knowledge gap and can demonstrate the potential for individual SCFAs to beneficially affect adipocyte function.

5. Conclusions

Individual SCFAs exert differential effects on LPS-stimulated adipocyte function, wherein propionate and butyrate exert a strong dose-dependent anti-inflammatory and anti-chemotactic effect on transcription factor activation and the adipokine secretory profile compared to acetate.

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Resveratrol Butyrate Esters Reduce Hypertension in a Juvenile Rat Model of Chronic Kidney Disease Exacerbated by Microplastics

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Abstract: Background: Resveratrol is recognized as a promising nutraceutical with antihypertensive and prebiotic properties; however, its bioavailability in vivo is limited. To enhance its bioactivity, we developed resveratrol butyrate esters (RBEs). This study investigates whether RBEs can mitigate hypertension induced by chronic kidney disease (CKD) and exacerbated by microplastics (MPs) exposure in juvenile rats. Methods: Three-week-old male Sprague Dawley rats were fed either regular chow or 0.5% adenine chow for three weeks. The adenine-fed CKD rats (N = 8 per group) received either 5 μM MPs (10 mg/L) or MPs combined with RBE (25 mg/L) in their drinking water from weeks 3 to 9. Results: Our results indicate that MP exposure worsened CKD-induced hypertension, while RBE treatment resulted in a reduction in systolic BP by 15 mmHg (155 ± 2 mmHg vs. 140 ± 1 mmHg, $p < 0.05$). The combined exposure to adenine and MPs was associated with nitric oxide (NO) deficiency, which RBE treatment alleviated. Additionally, our findings revealed that RBE modulated both the classical and nonclassical renin–angiotensin system (RAS), contributing to its protective effects. We also observed changes in gut microbiota composition, increased butyric acid levels, and elevated renal GPR41 expression associated with RBE treatment. Conclusions: In conclusion, in this juvenile rat model of combined CKD and MP exposure, RBE demonstrates antihypertensive effects by modulating NO levels, the RAS, gut microbiota, and their metabolites.

Keywords: resveratrol; hypertension; gut microbiota; kidney disease; microplastics; asymmetric dimethylarginine; butyrate renin–angiotensin system

1. Introduction

Phenolic compounds are abundantly found in plants and exhibit a variety of biological effects, including antioxidant, anti-inflammatory, anti-atherosclerotic, and anti-aging properties [1,2]. Resveratrol, a natural stilbene phenolic compound, is commonly utilized as a nutraceutical for the prevention of cardiovascular disease (CVD), type 2 diabetes mellitus, chronic kidney disease (CKD), and various other health conditions [3–5]. Recent research underscores the importance of understanding resveratrol's bioefficacy and bioavailability to elucidate its role in human health [6]. Like many phenolic compounds, resveratrol has low bioavailability [7], which can be influenced by several factors [8]. For example,

interactions between resveratrol and gut microbiota may affect its biological effects [9]. Notably, the limited in vivo bioactivity of resveratrol poses a significant challenge for its clinical applications. Recent evidence suggests that the esterification of resveratrol could enhance its bioactivity [10,11].

Previously, we developed esterified resveratrol-producing short-chain fatty acid (SCFA) products through Steglich reactions to enhance the bioactivity of resveratrol [12]. These resveratrol–SCFA esters exhibited antioxidant properties, with resveratrol butyrate esters (RBEs) demonstrating biological activity comparable to that of resveratrol but with improved bioavailability [13]. SCFAs, essential microbial metabolites, function as postbiotics and have been shown to positively influence human health [14,15].

In children, hypertension is often secondary to CKD, unlike in adults, where it is typically due to primary causes [16]. Despite treatment with antihypertensive medications, many children with CKD remain uncontrolled and may progress to end-stage kidney disease. Furthermore, CKD-related morbidity can be worsened by environmental pollutants, such as microplastics (MPs) [17]. Although research on the impact of MPs on kidney function is still in its early stages, there is growing concern that CKD patients may be especially vulnerable to the accumulation and damaging effects of these pollutants [18].

Resveratrol has demonstrated antihypertensive and renoprotective effects in several animal models [19], including those involving prenatal hypoxia combined with high-fat intake [20], spontaneously hypertensive rats [21], and a renovascular hypertensive two-kidney one-clip model [22]. However, these benefits have not consistently translated into positive outcomes in human populations. A meta-analysis of 10 randomized controlled trials found no significant effect of resveratrol supplementation on cardiovascular risk factors [23]. Another meta-analysis, which included 32 studies, suggested a mild renal protective effect of resveratrol in adults, though it was based on low-certainty evidence [24].

In our previous study using an adenine-induced juvenile rat model of CKD, we found that RBE supplementation improved kidney function and reduced hypertension by modulating the gut microbiota [25]. The beneficial effects of RBE on blood pressure (BP) are thought to stem from its ability to regulate key factors such as vasodilators, including nitric oxide (NO), and vasoconstrictors within the renin–angiotensin system (RAS). Building on this, the goal of our current study is to investigate whether exposure to MPs exacerbates hypertension in juvenile rats with adenine-induced CKD. Additionally, we aim to determine whether RBE treatment can mitigate these effects by influencing the gut microbiota, the NO signaling pathway, and the RAS.

2. Materials and Methods

2.1. Synthesis of Resveratrol Butyrate Esters

The RBEs were synthesized following our established protocol [11]. Initially, butyric acid (ACROS, Morris Plains, NJ, USA) was combined with resveratrol (TCI Development Co., Ltd., Shanghai, China) in tetrahydrofuran (Echo Chemical Co., Ltd., Miaoli County, Taiwan). Subsequently, 4-dimethylaminopyridine (Sigma-Aldrich, Saint Louis, MO, USA) and N-ethyl-N-(3-dimethylaminopropyl) carbodiimide (Sigma-Aldrich) were added to the mixture. The esterification reaction was conducted in the absence of light for 48 h. After the reaction was complete, the mixture was diluted with distilled water and filtered to isolate the precipitated RBEs. The final RBEs were then stored in a freezer at −20 °C for preservation.

2.2. Animals

The experimental protocol received approval from the Institutional Animal Ethics Committee (permit number: 2024022301, approval date: 27 March 2024). Sprague Dawley rats were allowed to acclimate in a temperature-controlled room (22 ± 1 °C), with 55 ± 5% humidity and a 12:12 light–dark cycle, in an AAALAC-International accredited animal facility. Pregnant rats were provided with unrestricted access to food and water until delivery, and the offspring were raised alongside their dams during the lactation period.

After weaning, two rats were housed per cage. Male rats aged three weeks were divided into two dietary groups: one group received regular chow, while the other received chow containing 0.5% adenine for three weeks to induce CKD. Only male juvenile rats were used due to their heightened susceptibility to developing hypertension at an earlier age compared to females [26]. The adenine dosage was selected based on our previously established pediatric CKD model [25]. Some of the rats were given MPs in their drinking water from week three to nine. The polystyrene MPs were procured from Magsphere (Pasadena, CA, USA), and the dosages of MPs and resveratrol were determined based on prior studies involving rats [27].

Figure 1 demonstrates the experimental protocol. The male rats were randomly assigned to five groups (n = 8 per group): a control group (CN), a CKD group receiving adenine chow (CK), an MP group receiving 5 μM microparticles (MPs) (10 mg/L), a CKD + MP group (CKMP) receiving both adenine and MPs, and a CKD + MP + RBE group (CKMPRBE) receiving adenine, MPs, and RBE at a dose of 25 mg/L in drinking water from weeks three to nine, as detailed in our previous work [25].

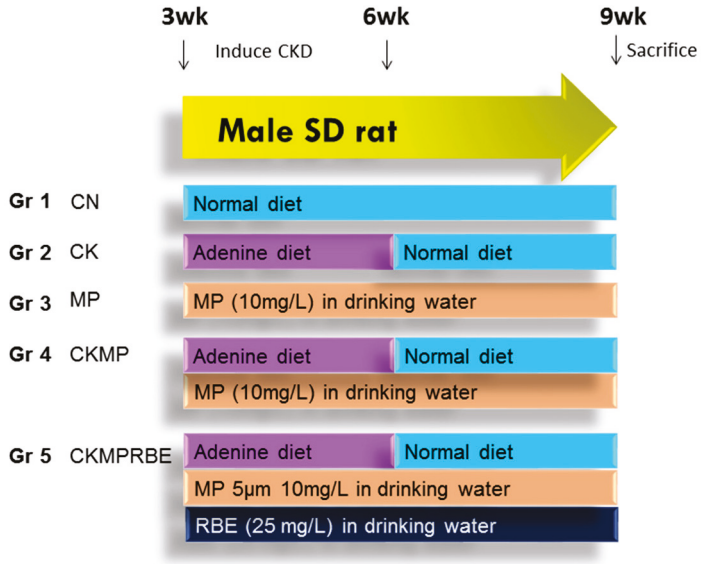


Figure 1. Experimental protocol employed in the current study.

Non-invasive BP measurements were conducted on conscious, trained rats using the tail-cuff method (CODA, Kent Scientific Corp., Torrington, CT, USA). The rats were acclimatized to the restraint holders for one week prior to the recording sessions. Three consistent BP measurements were taken, and the average value was calculated. All rats were euthanized at nine weeks of age. Fecal samples were collected in the morning, before sacrifice, by gently lifting the tail and twisting it toward the back to induce defecation. The fecal samples were then stored at −80 °C until further extraction. The rats were anesthetized by an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg) and euthanized with an intraperitoneal injection of a pentobarbital (150 mg/kg) overdose. Both kidneys were excised and weighed, and plasma samples were collected, aliquoted, and stored at −80 °C.

2.3. Gut Microbiota Metagenomics

Microbial DNA was extracted from stool samples, and metagenomic analysis was performed using 16S ribosomal RNA genes [25]. The barcode primers designed for SMRTbell library preparation (Menlo Park, CA, USA) were employed to amplify the full-length 16S rRNA gene from the genomic DNA. We conducted downstream analyses of the sequences using the QIIME2 software package [28]. From the amplicon sequence variants (ASVs), we constructed a phylogenetic tree with FastTree version 2.1.10, and each cluster

was taxonomically assigned. For α-diversity analysis, we assessed observed features and calculated the Shannon index. β-diversity was evaluated using the analysis of similarities (ANOSIM) in conjunction with Partial Least Squares Discriminant Analysis (PLSDA). We also utilized the linear discriminant analysis (LDA) effect size to identify bacterial taxa with significantly different abundances between the groups [29]. To assess statistical differences between the groups, we conducted a two-sided Welch's *t*-test using the Statistical Analysis of Metagenomic Profiles (STAMP).

2.4. Determination of Plasma SCFAs

We assessed SCFA concentrations in plasma using a Gas Chromatograph–Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA), following previously established methods [25]. The SCFAs analyzed included acetic acid, propionic acid, butyric acid, isobutyric acid, isovaleric acid, and valeric acid. Chromatographic separation was achieved using a DB-FFAP column (30 cm × 0.25 mm, 0.25 μm; Agilent Technologies), with 2-ethylbutyric acid serving as the internal standard. A 1 μL injection volume was applied with a split ratio of 5:1 at an injection temperature of 240 °C.

2.5. Quantitative PCR

Total RNA was extracted from kidney cortex samples, followed by real-time quantitative PCR (qPCR), as previously described in our methods [25]. We analyzed the expression of SCFA receptors [30] and components of the RAS [31]. All samples were analyzed in duplicate, with 18S ribosomal RNA (18S rRNA) serving as the reference gene for normalizing the qPCR data. The comparative threshold cycle (Ct) method was employed for the relative quantification of gene expression. The primer sequences are listed in Table 1.

Table 1. Primers for PCR amplification of each gene studied.

Gene	Sense	Anti-Sense
Olfr78	GAGGAAGCTCACTTTTGGTTTGG	CAGCTTCAATGTCCTTGTCACAG
GPR41	TCTTCACCACCGTCTATCTCAC	CACAAGTCCTGCCACCCTC
GPR43	CTGCCTGGGATCGTCTGTG	CATACCCTCGGCCTTCTGG
GPR109A	CGGTGGTCTACTATTTCTCC	CCCCTGGAATACTTCTGATT
Renin	AACATTACCAGGGCAACTTTCACT	ACCCCCTTCATGGTGATCTG
PRR	GAGGCAGTGACCCTCAACAT	CCCTCCTCACACAACAAGGT
AGT	GCCCAGGTCGCGATGAT	TGTACAAGATGCTGAGTGAGGCAA
ACE1	CACCGGCAAGGTCTGCTT	CTTGGCATAGTTTCGTGAGGAA
AT1R	GCTGGGCAACGAGTTTGTCT	CAGTCCTTCAGCTGGATCTTCA
ACE2	ACCCTTCTTACATCAGCCCTACTG	TGTCCAAAACCTACCCCACATAT
MAS	CATCTCTCCTCTCGGCTTTGTG	CCTCATCCGGAAGCAAAGG
AT2R	CAATCTGGCTGTGGCTGACTT	TGCACATCACAGGTCCAAAGA
R18S	GCCGCGGTAATTCCAGCTCCA	CCCGCCCGCTCCCAAGATC

2.6. Measurement of NO-Related Parameters

Plasma levels of L-arginine and asymmetric dimethylarginine (ADMA), an inhibitor of nitric oxide synthase, were measured using high-performance liquid chromatography (HP Series 1100, Agilent Technologies, Inc., Santa Clara, CA, USA) with o-phthalaldehyde and 3-mercaptopropionic acid as the derivatization reagents [25]. L-homoarginine (Fluka, Neu Ulm, Germany) was used as the internal standard.

2.7. Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM). The data were analyzed using a one-way analysis of variance (ANOVA) followed by multiple comparisons with Tukey's post hoc test. A *p*-value of less than 0.05 was considered statistically significant for all tests. The analysis was conducted using SPSS version 17.0 software.

3. Results

3.1. Anthropometrics and Blood Pressure

No mortality was observed among the five groups at nine weeks of age. The CKMPRBE group exhibited the lowest body weight (BW) (Figure 2A). The kidney weight (KW)-to-BW ratio was higher in the CK group compared to the CN and MP groups (Figure 2B). After seven weeks, adenine-exposed and MP-exposed juvenile rats demonstrated elevated systolic BP compared to the CN group (CK group: 134 ± 1 mmHg, MP group: 131 ± 1 mmHg, vs. CN group: 120 ± 1 mmHg, both $p < 0.05$), with a synergistic effect observed from their combined exposures (CKMP group: 145 ± 1 mmHg, $p < 0.05$) (Figure 2C). Importantly, the increase in systolic BP in the CKMP group was mitigated by RBE treatment (CKMPRBE group: 133 ± 1 mmHg, $p < 0.05$). At week 9, MP exposure exacerbated CKD-induced hypertension (CKMP group vs. CK group: 155 ± 2 mmHg vs. 140 ± 1 mmHg, $p < 0.05$), while RBE treatment reduced systolic BP by 15 mmHg (CKMPRBE group: 140 ± 1 mmHg, $p < 0.05$). Diastolic BP (Figure 2D) and mean arterial pressure (Figure 2E) showed similar trends as systolic BP.

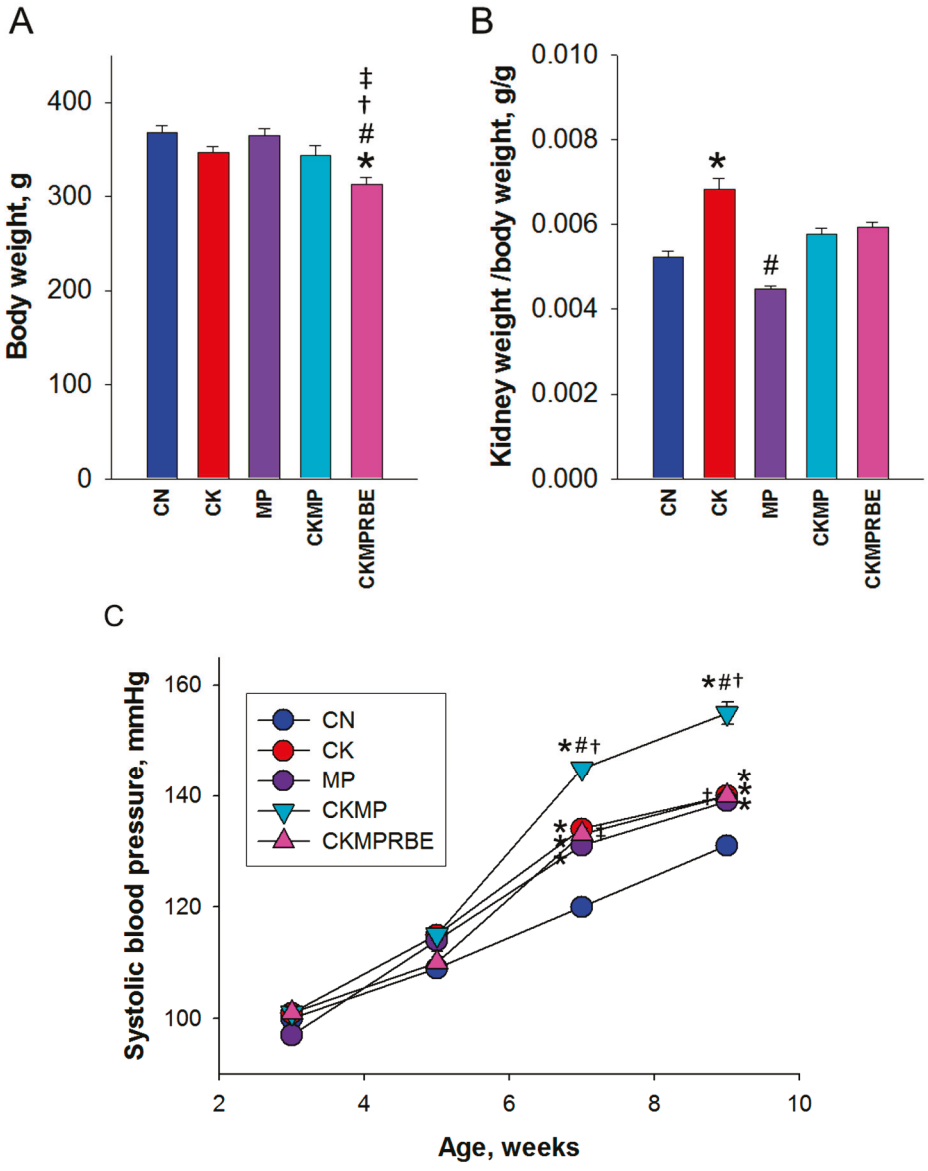


Figure 2. *Cont.*

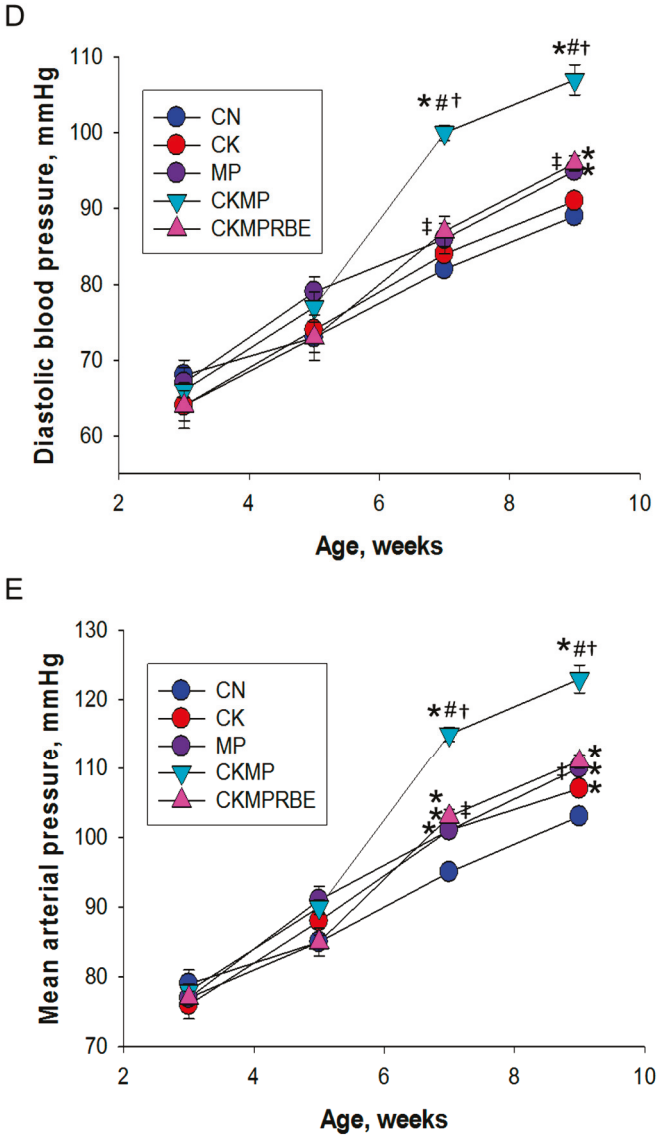


Figure 2. (**A**) Body weight, (**B**) kidney weight-to-body weight ratio, (**C**) systolic blood pressure, (**D**) diastolic blood pressure, and (**E**) mean arterial pressure in rats receiving adenine chow (CK), microplastics (MP), or resveratrol butyrate esters (RBE). N = 8/group; * $p < 0.05$, compared with rats fed with regular chow (CN), # $p < 0.05$, compared with rats fed with adenine chow (CK); † $p < 0.05$, compared with rats exposed to microplastics (MP); and ‡ $p < 0.05$, compared with rats fed adenine chow and exposed to MP (CKMP).

3.2. Plasma SCFAs and SCFA Receptors

Adenine and MP exposure differentially affected plasma concentrations of SCFAs. Adenine exposure resulted in lower plasma levels of isobutyric acid ($p = 0.042$) and valeric acid ($p = 0.02$) in the CK group, while MP exposure led to reduced propionic acid levels in the MP group ($p = 0.004$) compared to the CN group (Table 2). Notably, combined adenine and MP exposures did not impact plasma SCFA concentrations. In contrast, RBE significantly increased butyric acid levels in the CKMPRBE group compared to the CKMP group ($p = 0.002$). Plasma concentrations of isovaleric acid were consistent across all five groups, showing no significant differences.

Table 2. Plasma SCFA levels in rats receiving adenine chow (CK), microplastics (MPs), or resveratrol butyrate esters (RBEs).

Group	CN	CK	MP	CKMP	CKMPRBE
Acetic acid (μM)	1163 ± 53.9	1148.6 ± 55.1	1080.8 ± 26.1	1053.3 ± 47.5	966.2 ± 36.0 *#†
Propionic acid (μM)	6.0 ± 0.7	5.3 ± 0.5	3.4 ± 0.4 *#	4.8 ± 0.4 †	6.0 ± 0.5 †
Isobutyric acid (μM)	3.1 ± 0.3	2.4 ± 0.1 *	3.3 ± 0.3 #	2.6 ± 0.3	3.0 ± 0.4
Butyric acid (μM)	16.1 ± 0.9	18.4 ± 0.8	15.8 ± 0.7 #	17.6 ± 0.8	26.9 ± 2.3 *#†‡
Isovaleric acid (μM)	4.8 ± 0.4	5.4 ± 0.4	4.5 ± 0.2	5.2 ± 0.5	5.3 ± 0.3
Valeric acid (μM)	6.5 ± 0.2	4.9 ± 0.5 *	6.3 ± 0.2 #	5.7 ± 0.5	6.6 ± 0.3 #

N = 8/group. * $p < 0.05$, compared with rats fed with regular chow (CN); # $p < 0.05$, compared with rats fed with adenine chow (CK); † $p < 0.05$, compared with rats exposed to microplastics (MPs); ‡ $p < 0.05$, compared with rats fed adenine chow and exposed to MP (CKMP).

SCFAs can influence BP by interacting with SCFA receptors [30]. We analyzed the expression of four SCFA receptors, containing olfactory receptor 78 (OlfR78), G protein-coupled receptors 41 (GPR41), GPR43, and GPR109A. Figure 3 shows that renal Olfr78 expression was significantly higher in the CK and MP groups compared to the CN group. Additionally, combined adenine and MP exposures resulted in the lowest GPR41 expression in the CKMP group, but this reduction was restored with RBE treatment. No significant differences were observed in GPR43 and GPR109A expression between the CN and CK groups; however, both were induced by MP exposure.

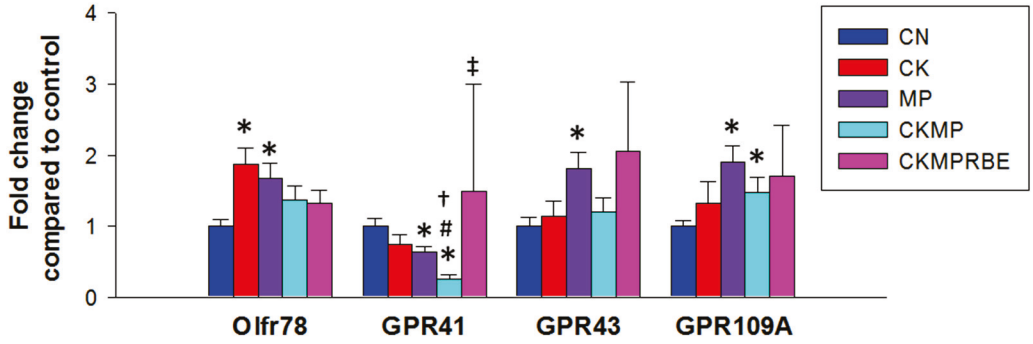


Figure 3. Renal mRNA expression of olfactory receptor 78 (OlfR78); G protein-coupled receptor 41 (GPR41), GPR43, and GPR109A in rats receiving adenine chow (CK), microplastics (MPs), or resveratrol butyrate esters (RBEs). N = 8/group. * $p < 0.05$, compared with rats fed with regular chow (CN); # $p < 0.05$, compared with rats fed with adenine chow (CK); † $p < 0.05$, compared with rats exposed to microplastics (MPs); and ‡ $p < 0.05$, compared with rats fed adenine chow and exposed to MP (CKMP).

3.3. Gut Microbiota Composition

Both α-diversity metrics—the Faith's PD index and the Shannon index—assessing microbiome richness and evenness showed no significant differences among the five groups (Figure 4A,B). Additionally, β-diversity was visualized through a PLSDA plot, which revealed five distinct clusters (Figure 4C). ANOSIM analysis confirmed significant differences among nearly all the groups (all $p < 0.05$), except for the comparison between the CN and MP groups, which did not reach statistical significance.

The LEfSe analysis revealed significant differences in taxa abundance between the CKMP and CKMPRBE groups, as illustrated in Figure 5. Notably, *Turicibacter sanguinis*, along with its associated genus and family, was more abundant in the CKMP group (LDA > 3). Additionally, the genera *Ruminococcus*, *Murimonas*, and *Robinsoniella* were also enriched in the CKMP group. In contrast, the CKMPRBE group exhibited an increase in *Ligilactobacillus murinus*, along with its corresponding genus, order, and class, compared to the CKMP group.

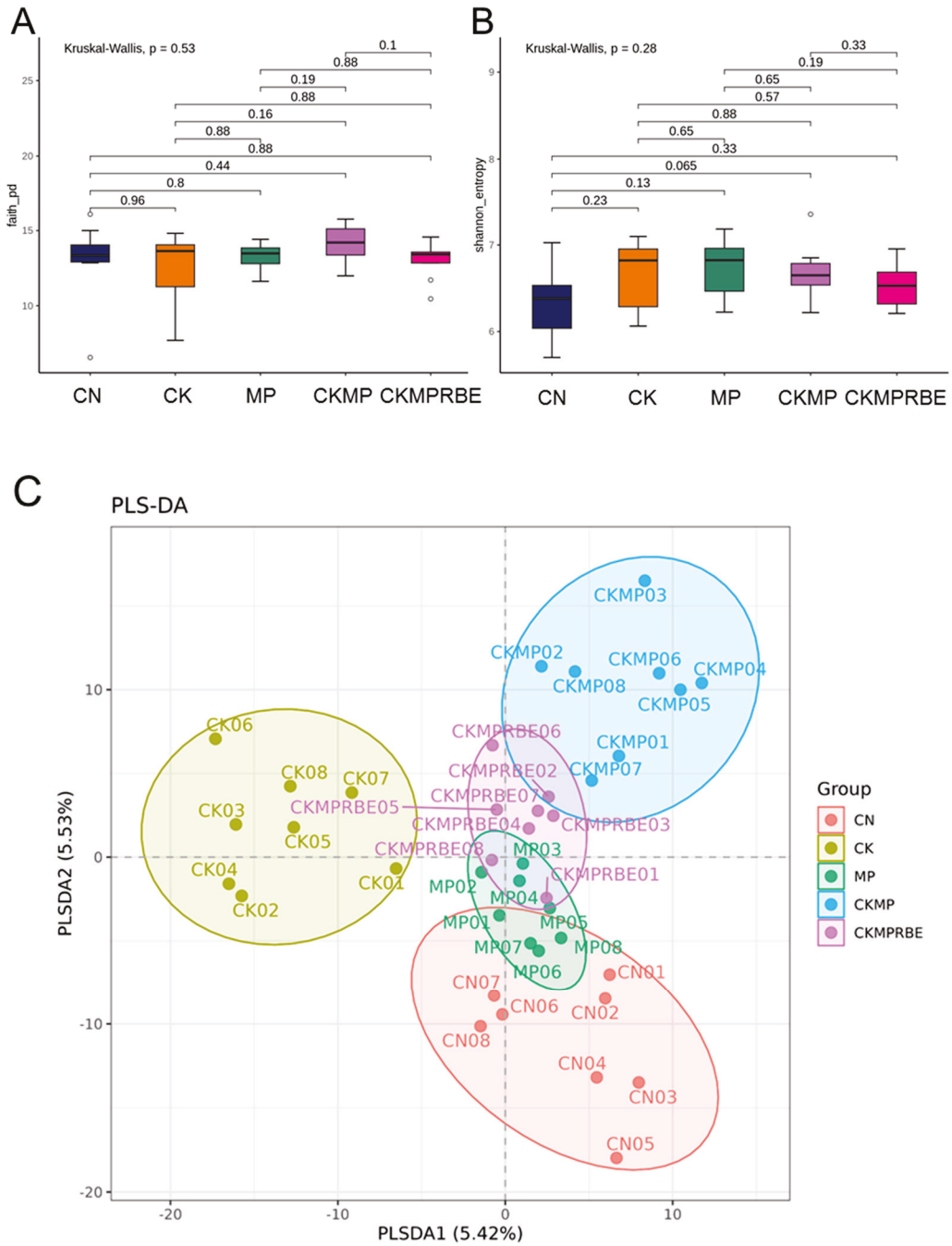


Figure 4. For α-diversity analysis among the groups, we utilized (**A**) the Faith's Phylogenetic Diversity (PD) index and (**B**) the Shannon index. For the β-diversity assessment, we performed (**C**) Partial Least Squares Discriminant Analysis (PLSDA). Each point represents the microbiota of an individual sample, with colors indicating the corresponding group. N = 8 per group. CN, rats fed with regular chow; CK, rats fed with adenine chow; MP, rats exposed to microplastics; CKMP, rats fed adenine chow and exposed to MP; and CKMPRBE, rats fed adenine chow and exposed to both MP and resveratrol butyrate esters.

To identify the differentially altered taxa at the genus level due to RBE treatment, we applied the two-sided Welch's *t*-test in STAMP to establish statistical differences. Compared to the CKMP group, RBE treatment resulted in significant changes in microbial genera, including notable increases in *Ruminococcus*, *Murimonas*, *Robinsoniella*, *Phocea*, *Ihubacter*, *Breznakia*, and *Flavonifractor*, as well as decreases in *Odoribacter*, *Emergencia*, and *Massilioclostridium* (Figure 6).

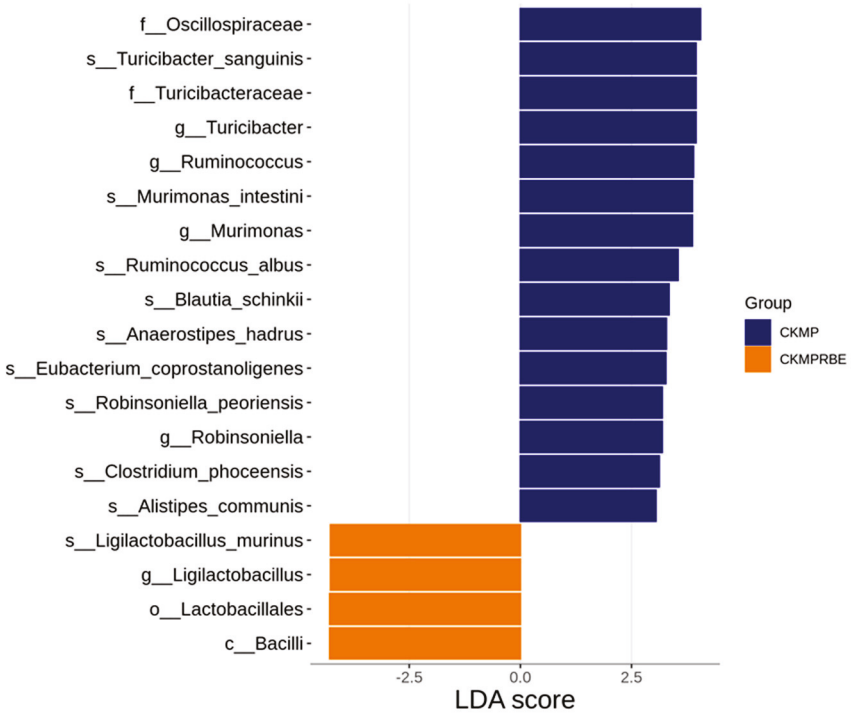


Figure 5. Linear discriminant effect size (LEfSe) analysis revealed significant differences in bacterial taxa between the CKMP (blue) and CKMPRBE (orange) groups, with LDA scores exceeding 3.0. CKMP, rats fed adenine chow and exposed to MP; CKMPRBE, rats fed adenine chow and exposed to both MP and resveratrol butyrate esters.

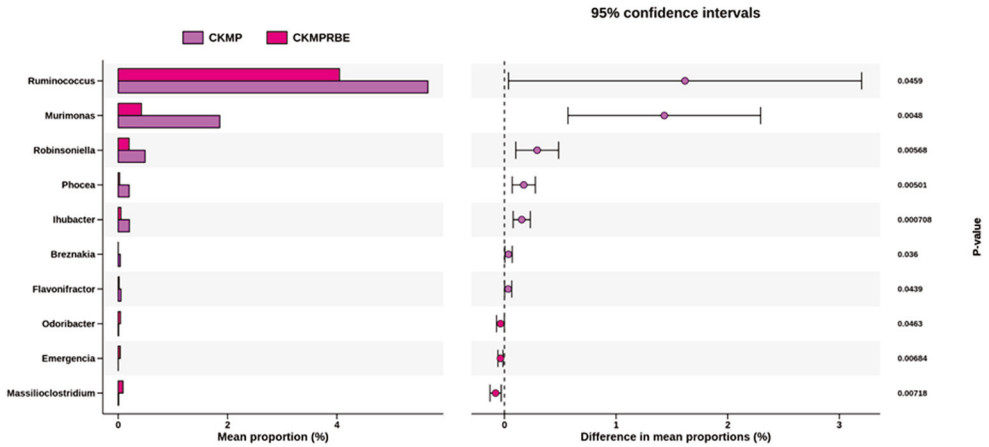


Figure 6. Differentially abundant taxa between the CKMP and CKMPRBE groups as identified by STAMP analysis with 95% confidence intervals. N = 8/group. CKMP, rats fed adenine chow and exposed to MP; CKMPRBE, rats fed adenine chow and exposed to both MP and resveratrol butyrate esters.

3.4. NO and RAS

We next assessed NO and RAS as they play crucial roles in regulating BP. NO is a potent vasodilator, with L-arginine serving as the substrate for NO synthase to produce NO, while ADMA inhibits NO production. Our results showed that plasma concentrations of L-arginine were similar across all five groups (Figure 7A). Exposure to either adenine or MP resulted in increased plasma concentrations of ADMA (Figure 7B), but there was no synergistic effect between the two. The ratio of L-arginine to ADMA, which reflects NO bioavailability [32], was diminished by adenine, MP, or their combined exposures; however, this reduction was partially reversal by the RBE treatment (Figure 7C).

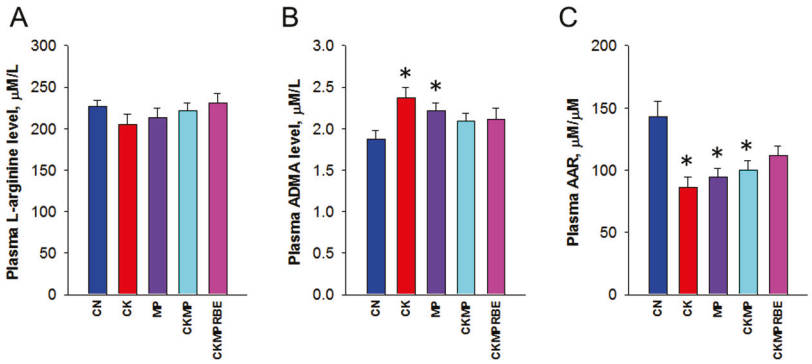


Figure 7. Plasma concentrations of (**A**) L-arginine, (**B**) asymmetric dimethylarginine (ADMA), and (**C**) their combined ratio (AAR) in rats receiving adenine chow (CK), microplastics (MPs), or resveratrol butyrate esters (RBEs). N = 8/group; * $p < 0.05$, compared with rats fed with regular chow (CN).

The RAS primarily raises BP through the action of angiotensin II (Ang II) as a potent vasoconstrictor. The binding of renin to the (pro)renin receptor (PRR) activates Ang-II-dependent and independent pathways to raise BP [31]. The classical RAS is characterized by the angiotensin-converting enzyme 1 (ACE1)-Angiotensin II type 1 receptor (AT1R) axis, which promotes vasoconstriction. In contrast, the nonclassical RAS, consisting of the ACE2-Ang-(1-7)-MAS axis and the Ang II/Ang III-AT2R pathway, counteracts the effects of the aberrant activation of the classical axis. We used qPCR to quantify changes in key RAS-related genes in rat kidneys. Figure 8 illustrates that exposure to adenine and MP tends to increase the expression of genes in the classical RAS axis, including renin and AT1R, while these increases were normalized by RBE treatment (Figure 8A). Compared to the CKMP group, RBE also reduced other components of the classical axis, such as PRR, AGT, and ACE1. Conversely, resveratrol treatment resulted in an increase in ACE2, MAS, and AT2R within the nonclassical RAS axis, which was inhibited by the combined exposure to adenine and MP (Figure 8B).

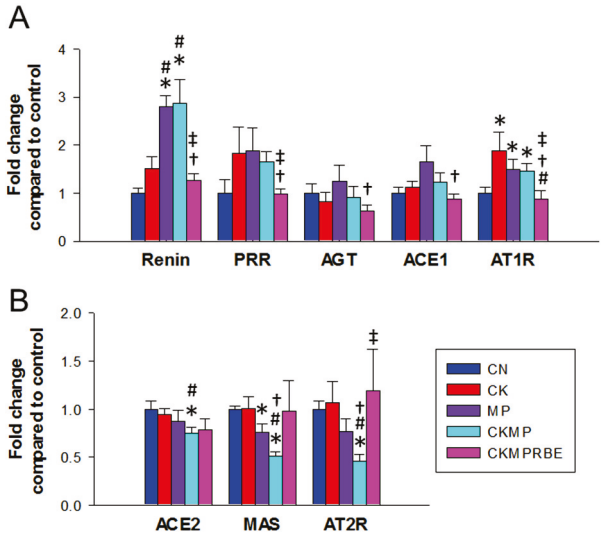


Figure 8. Renal mRNA expression of RAS-related genes in the (**A**) classical axis, which includes renin, pro(renin) receptor (PRR), angiotensinogen (AGT), ACE1, and AT1R; the (**B**) nonclassical axis, which includes ACE2, MAS, and AT2R in rats receiving adenine chow (CK), microplastics (MP), or resveratrol butyrate esters (RBE). N = 8/group. * $p < 0.05$, compared with rats fed with regular chow (CN); # $p < 0.05$, compared with rats fed with adenine chow (CK); † $p < 0.05$, compared with rats exposed to microplastics (MP); and ‡ $p < 0.05$, compared with rats fed adenine chow and exposed to MP (CKMP).

4. Discussion

In this study, we found that MP exposure exacerbated CKD-induced hypertension in young rats at nine weeks of age. Notably, we report for the first time that RBE can protect juvenile rats with CKD from hypertension exacerbated by MP exposure, resulting in a reduction in systolic BP by approximately 15 mmHg. Although the antihypertensive effect did not fully normalize BP, the 15 mm Hg reduction in systolic BP is considered clinically significant.

The key findings from our data are as follows: (1) juvenile rats exhibited heightened susceptibility to the detrimental effects of MPs, which exacerbated hypertension; (2) RBE treatments alleviated hypertension induced by the combined exposure to adenine and MP; (3) the beneficial effects of RBE were associated with increased levels of butyric acid and the elevated expression of renal GPR41; (4) combined adenine/MP-induced hypertension was linked to NO deficiency, which improved with RBE treatment; (5) RBE's protective effect against hypertension in juvenile rats was correlated with a reduction in the renal expression of renin and AT1R in the classical RAS axis, along with an increase in ACE2, MAS, and AT2R in the nonclassical RAS axis; (6) combined exposure to adenine and MPs, as well as RBE treatment, differentially influenced distinct gut microbiota profiles; and (7) the protective effect of RBE was accompanied by alterations in several bacterial populations, including an increase in *Ligilactobacillus murinus* and a decrease in *Turicibacter sanguinis*.

In accordance with previous research on the antihypertensive effects of resveratrol [33,34], the treatment of young rats with RBE, a derivative of resveratrol, resulted in an approximate 15 mmHg reduction in SBP, effectively mitigating hypertension. The antihypertensive effects of RBE in the context of pediatric CKD align with our earlier report [25]. We expanded our investigation to explore its protective role in a juvenile rat model subjected to both adenine and MP exposure. Our study demonstrates that RBE protects young rats with CKD from hypertension exacerbated by MP exposure.

One beneficial effect of RBE on hypertension may be associated with improved NO bioavailability. Previous studies have indicated that NO deficiency is implicated in CKD and hypertension [35,36]. Conversely, the antihypertensive effects of resveratrol have been attributed to its ability to enhance NO levels [33]. The data support this notion, showing that RBE's protective effect against hypertension aligns with an increased L-arginine to ADMA ratio, indicating enhanced NO bioavailability in the plasma.

Another protective effect of RBE is its regulation of the RAS. In aging mice, resveratrol has been shown to protect against arterial fibrosis by inhibiting the classical axis and stimulating the nonclassical axis [37]. In our young rat model of CKD-induced hypertension, RBE treatment decreased renin and AT1R levels in the classical RAS axis while increasing ACE2, MAS, and AT2R levels in the nonclassical axis. These findings suggest that RBE helps restore balance between the two RAS axes, promoting vasodilation and lowering BP.

Butyric acid is a crucial component of RBE and serves as a ligand for the SCFA receptors GPR41, GPR43, and GPR109A. It can influence BP by stimulating these receptors; GPR43 tends to elevate BP, while GPR41 and GPR109A can induce vasodilation. In our study, RBE treatment was found to increase plasma concentrations of butyric acid and the renal expression of GPR41 in the CKMPRBE group. These findings suggest that hypertension resulting from adenine and MP exposure may be mitigated by RBE's modulation of SCFA receptors, shifting the balance from vasoconstriction toward vasodilation.

The protective mechanisms through which RBE treatment alleviates hypertension complicated by adenine and MP exposure are also associated with alterations in gut microbiota. Previous research indicates that the beneficial effects of resveratrol are closely linked to its prebiotic properties [38]. Although RBE treatment resulted in a distinct microbiome composition compared to the other groups, there was no significant difference in α-diversity among the five groups.

So far, data on the impact of MPs on the gut microbiota remain scarce [39]. A recent study revealed that MP exposure induces size-dependent multi-organ damage, including kidney injury in mice, accompanied by gut microbiota dysbiosis [40]. Although the gut-kidney axis plays a key role in CKD-induced hypertension [41], it remains unclear whether MPs contribute to CKD-induced hypertension through gut microbiota dysbiosis. Our findings indicate that MP exposure exacerbates CKD-induced hypertension, which is associated with an increase in *Turicibacter sanguinis* and its related genus and family. This aligns with previous research demonstrating that MPs can affect soil microbial communities, leading to an increase in the abundance of the genus *Turicibacter* [42]. *Turicibacter sanguinis* is the most predominant and well-studied species within the genus *Turicibacter*. This species has been identified in animal models with irritable bowel syndrome [43]. However, its potential role in the pathogenesis of hypertension has not yet been thoroughly investigated. We found that the species *Ligilactobacillus murinus*, along with its associated genus, order, and class, were enriched in the CKMPRBE group, correlating with the antihypertensive effects of RBE. This finding is consistent with previous research indicating that *Ligilactobacillus murinus* can have lower BP in spontaneously hypertensive rats [44].

In addition, STAMP analysis identified several bacterial taxa associated with hypertension, specifically the hypertension-depleted taxa *Ruminococcus*, *Robinsoniella*, and *Flavonifractor* [45]. Our findings indicated that the BP-lowering effect of RBE is correlated with an increase in the abundance of these taxa. Furthermore, RBE was found to decrease the proportion of the genus *Emergencia*, which is known to convert carnitine into trimethylamine N-oxide (TMAO) [46]. TMAO has been linked to the exacerbation of hypertension and kidney disease [47]. Further investigation is needed to determine if RBE's protective effects against hypertension are linked to these specific microbial changes.

Our study has several limitations. First, using a single dose of RBE may not fully capture its effectiveness, and thus, further research with multiple dosing regimens is needed. Although we previously found that low-dose RBE (25 mg/L) and resveratrol (50 mg/L) similarly protected adenine-fed rats against hypertension and kidney damage [25], it remains unclear whether these protective effects are dose-dependent or may arise in a dose-independent manner, which warrants further investigation. Second, our focus on juvenile male rats highlights the need to explore potential sex differences in response to MPs and RBE. We concentrated RBE treatment on the CKMP group based on previous findings, but more research is required to assess RBE's impact on BP in MP-exposed young rats. Lastly, RBE's antihypertensive effects may involve other organs that regulate BP, necessitating further studies.

5. Conclusions

In conclusion, this study offers the first evidence that RBE may serve as a potential therapy targeting NO, the RAS, and gut microbiota to protect against hypertension induced by combined exposure to adenine and MP. Our findings provide novel mechanistic insights into the role of RBE in modulating these pathways and offer promising prospects for its application in managing hypertension. While our findings reveal novel mechanistic insights related to RBE, further investigation and clinical translation are essential to fully understand its therapeutic potential.

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Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

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

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Review

Honey as a Neuroprotective Agent: Molecular Perspectives on Its Role in Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is the most prevalent form of dementia and a major global health challenge, characterized by progressive cognitive decline and neurodegeneration. Despite decades of research, there is currently no cure, and available treatments provide only limited symptomatic relief without halting disease progression. In this context, natural compounds with multi-targeted biological activities are being explored as potential complementary therapeutic strategies. Honey, a complex natural substance rich in bioactive phytochemicals, has emerged as a promising candidate due to its antioxidant, anti-inflammatory, anti-apoptotic, and enzyme-inhibitory properties. This review summarizes the molecular mechanisms underlying the neuroprotective effects of honey in the context of AD, with a particular focus on its ability to modulate oxidative stress, mitochondrial dysfunction, inflammation, apoptosis, β-amyloid accumulation, tau hyperphosphorylation, and neurotransmission-related enzymes. Notably, the botanical origin of honey significantly influences its composition and biological activity, as evidenced by studies on avocado, manuka, acacia, kelulut, chestnut, coffee, or tualang honeys. While preclinical findings are encouraging, especially in vitro and in invertebrate and rodent models, clinical validation is still lacking. Therefore, further research, including well-designed in vivo and human studies, is needed to clarify the therapeutic relevance of honey in AD. Overall, honey may represent a promising natural adjunct in the prevention or management of AD, but current evidence remains preliminary.

Keywords: manuka; tualang; antioxidant; polyphenols; amyloid; tau protein; acetylcholinesterase; neuroinflammation; oxidative stress

1. Introduction

Alzheimer's disease (AD) is a progressive and lethal neurodegenerative disorder that represents the most prevalent form of dementia [1]. Dementias in general are the seventh cause of death globally and the second highest in high-income countries. According to the World Health Organization, more than 55 million people live with dementia worldwide

and there are nearly 10 million new cases every year. Hence, the total cost of dementia worldwide was USD 1.3 trillion in 2019, and is expected to increase to USD 2.8 trillion by 2030 as the incidence is increasing [2]. Common AD early symptoms are memory problems like difficulty remembering recent conversations, names, or events. Other frequent manifestations are alterations in word finding, vision/spatial issues, and impaired reasoning or judgment, as well as apathy and depression. In advanced cases, the behavioral, cognitive, and motor declinations, including difficulty speaking, swallowing, and walking, interfere with the daily life of the patients, even incapacitating them. This leads to a high degree of disability and morbidity, which is converted into a severe challenge for society and the healthcare system. Therefore, all the problems associated with this pathology result in an important impact in physical, psychological, social, emotional, and economic terms [3].

AD is considered a complex and multifactorial disease in which the main molecular features are the synaptic dysfunction, neurotransmission alterations, protein misfolding, and other general aging-associated features such as oxidative stress, mitochondrial dysfunction, and inflammation [4]. Two of the most accepted hypotheses of the AD etiopathogenesis are the amyloid beta (Aβ) and the tau cascade, which are proteins with different locations reported as the main causes of the disease [4–6]. The Aβ plaques and the neurofibrillary tangles of hyperphosphorylated tau are deteriorative for cells and could lead to cell death or apoptosis, as well as to neuroinflammation and oxidative stress [4,5].

Although the number of dementia patients and the aged population are expected to grow, the disease incidence appears to have declined in recent years due to the improvement in risk factors such as the cardiovascular or educational level [7,8]. That fact suggests that the identification of and reduction in the risk factors may be an effective strategy. Currently, there is no preventive or curative treatment for AD [1]. The available treatments, which mainly consist of inhibitors of the acetylcholinesterase (AChE) enzyme, only provide a temporary and slight improvement of the symptoms in mid- to moderate-AD patients [3,9]. Thus, there is an overriding need to find or develop new tools for the prevention or management of this pathology. In this sense, one of the main modifiable risk factor is the diet [3], so bioactive compounds from foods could be an excellent approach. Foods present a wide and diverse composition of chemical compounds that may act on the hallmarks of the disease [9]. In that sense, apiculture products like honey are highlighted for their wealth in phenolic compounds, mainly flavonoids, that have been demonstrated to exert health benefits partly derived from their antioxidant and anti-inflammatory potential and, therefore, could be considered as a promising tool in the onset and development of neurodegenerative diseases like AD.

Based on all the above, the aim of this article is to summarize the knowledge about the impact of honey in AD in terms of the molecular aspects of the disease. A literature search was conducted in three major databases: PubMed, Scopus, and Web of Science (WoS) (Core Collection). The goal was to identify studies investigating the effects of honey or honey-derived extracts on AD or its core pathological mechanisms. To this end, the following search equation was applied: Honey AND (Alzheimer OR amyloid OR "tau protein" OR neuroinflammation OR apoptosis OR neurotransmission OR "oxidative stress" OR "mitochondrial dysfunction" OR "ion disbalance"). This combination of terms allowed for the inclusion of studies investigating at least one of the hallmark features of AD in the context of honey administration. The combined searches yielded the following results: PubMed: 646 records, Scopus: 1013 records, and WoS: 1712 records. After title and abstract screening, studies were excluded based on the following criteria: (i) review articles, (ii) studies unrelated to the nervous system or neurodegeneration, (iii) studies involving bees or bee venom, (iv) studies on apicultural products other than honey (e.g., royal jelly, bee pollen), (v) studies using honey mixed with additives without a honey-only control,

(vi) non-English articles, and (vii) studies without accessible full text. After applying these criteria and removing duplicates, 27 unique articles were retained.

2. Phytochemical Characterization of Honeys

Honey is a natural product made by worker honeybees from honeydew or flower nectar [10–12]. It is collected by bees and transported into the hive, where concentration processes and enzymatic reactions take place to produce the honey. The composition and features of honey in terms of taste and color are linked to the type of flower source, climate, and geographical area, as well as processing methods and storage conditions [13]. This food contains a large variety of compounds, including water, sugars, proteins, minerals, vitamins, free amino acids, and phytochemicals [10,11]. Concerning the phytochemicals, the main classes of polyphenols present in this matrix are phenolic acids and flavonoids, mainly found as aglycones [13]. As mentioned, the phenolic content of honey varies notably depending on its botanical origin. Generally, lighter honeys, such as those produced from acacia, willow, ribwort, goldenrod, clover, rapeseed, sunflower, lime, thyme, and citrus flowers, tend to exhibit a lower concentration of total phenolic compounds when compared to darker varieties like chestnut, heather, forest honeydew, and buckwheat honey [14]. Among the phenolic acids most frequently identified in honey are p-coumaric, ferulic, caffeic, cinnamic, syringic, gallic, and chlorogenic acids. Regarding flavonoids, the dominant classes present include flavones, flavanols, and flavonols. Specific compounds commonly detected in honeys include quercetin, luteolin, apigenin, kaempferol, pinobanksin, chrysin, and pinocembrin. Information about the presence of phenolic compounds in different honeys is collected in Table 1 [11,14–28].

Table 1. Main phenolic compounds identified in different types of honey.

Compound/Honey Type	AH	AVH	CH	COH	EH	KH	LH	MH	MFH	RH	THH	TH
Flavonoids												
Apigenin	X		X	X	X		X	X	X		X	X
Catechin	X			X			X	X	X			X
Chrysin	X	X	X					X	X	X	X	X
Epicatechin							X	X				
Galangin	X				X		X	X			X	
Genistein	X								X			
Isorhamnetin	X		X					X	X		X	
Kaempferol	X		X	X	X	X		X	X	X	X	X
Luteolin	X		X	X	X	X	X	X	X	X	X	X
Myricetin	X			X	X						X	
Naringenin	X			X		X		X				
Pinobanksin	X	X	X		X		X	X	X	X	X	
Pinocembrin	X		X		X		X	X	X	X	X	
Quercetin	X		X	X	X	X	X	X	X	X	X	X
Rutin	X			X		X	X		X			
Phenolic acids												
2-Hydroxycinnamic acid					X		X					X
Caffeic acid	X	X	X	X		X	X	X	X		X	X
Chlorogenic acid	X			X			X	X	X		X	X
Cinnamic acid			X	X	X	X	X	X	X		X	X
Ellagic acid		X	X						X			
Ferulic acid	X	X	X	X	X	X		X	X		X	X
Gallic acid	X		X	X		X		X			X	X
p-Coumaric acid	X	X	X	X		X	X	X	X	X	X	X
p-Hydroxybenzoic acid	X		X				X	X	X			
Rosmarinic acid		X								X		
Syringic acid	X	X			X	X	X	X	X		X	X
Vanillic acid	X		X									

Abbreviations and references for each honey type: Acacia Honey (AH): [11,14,15,22,24,26,28]; AVH (Avocado Honey): [18,27]; CH (Chestnut Honey): [20,27,28]; COH (Coffee Honey): [21–23]; EH (Eucalyptus Honey): [20,27]; Kelulut Honey (KH): [24,25]; Lavender Honey (LH): [20,28]; Manuka Honey (MH): [11,16,17,22]; MFH (Multifloral Honey): [19,26–28]; Rosemary Honey (RH): [11,20,27]; Thyme Honey (THH): [11,20,27]; Tualang Honey (TH): [11,15,16,24,25]. 'X' indicates the presence of the compound in that honey variety.

These compounds are considered key contributors to honey's antioxidant properties, primarily due to their capacity to neutralize free radicals by generating more stable and less harmful molecular forms [11]. Antimicrobial, antifungal, antiviral, anti-inflammatory, anticancer, antidiabetic, antilipidemic, and protective effects on cardiovascular, nervous, respiratory, and gastrointestinal systems are some of the health benefits demonstrated by honey, supporting its use in the treatment of various disorders [10,11]. Even more, the benefits of honey in terms of oxidative stress, mitochondrial dysfunction, and inflammation, among others, mean it is considered as a promising tool in the management of healthy aging [29].

3. Effects of Honey on AD Features

As mentioned above, honey exerts beneficial effects against the main pathological mechanisms of AD. The evidence regarding the effects of different types of honey on AD-related features is summarized below and presented in Tables 2 and 3. Table 2 compiles the outcomes of studies using honey as a whole food, whereas Table 3 gathers data from studies employing honey-derived extracts. Furthermore, a schematic overview of the proposed mechanisms of action is provided in Figure 1, integrating current evidence on how honey may interact with key pathological processes in AD.

Table 2. Honey's effects on AD features.

Model	Treatment	Dosage and Duration	Effects	Reference
		Oxidative stress		
In vitro. Astrocyte cell culture injured with H_2O_2	Honey	1% (*v*/*v*) for 24 h followed by 100 µM H_2O_2 for 3 h	↑ Cell viability ↓ ROS content	[10]
In vitro. RAW 264.7 macrophages injured with LPS	Manuka honey	8 mg/mL honey with 1 µg/mL for 24 h	↓ TBARS and carbonyl protein levels ↓ Protein expression of OGG1 (DNA damage) ↑ GSH levels ↑ GR and GST activity ↑ Protein expression of Nrf-2, Keap-1, CAT and SOD	[30]
In vitro. RAW 264.7 macrophages injured with LPS	Chestnut and Eucalyptus honey	1 mg/mL Honey for 24 h followed by 1 µg/mL LPS for 24 h	= ROS content ↓ NO levels ↑ GSH levels ↑ GPx activity = GR and GST activity = Nrf-2 gene expression	[31]
In vivo. *C. elegans* worms injured with AAPH	Manuka honey	100 mg/mL for 48 h followed by 2.5 mM AAPH for 15 min	↓ ROS content	[17]
In vivo. *C. elegans* worms injured with AAPH	Avocado honey	100 mg/mL for 48 h followed by 2.5 mM AAPH for 15 min	↓ ROS content	[18]
In vivo. *C. elegans* transgenic strains	Manuka honey	100 mg/mL for 48 h	↓ SOD3 and HSP-16.2 expression = SKN-1/NRF2, DAF-16/FOXO, GST-4, and HSF-1 expression	[17]
In vivo. *C. elegans* transgenic strains	Avocado honey	100 mg/mL for 48 h	↑ DAF-16/FOXO expression ↓ SKN-1/NRF2, HSF-1, SOD-3 and HSP-16.2 expression = GST-4 expression	[18]
In vivo. *Drosophila melanogaster* dUbqn knockdown	Coffee honey	1% (*v*/*v*)	↓ Brain ROS content	[32]
In vivo. Balb/c mice injured with $AlCl_3$ (model of AD)	Honey syrup	40 mg/kg/d (i.p.) $AlCl_3$ followed by 500 mg/kg honey syrup (i.p.) for 45 days	↑ TAC ↓ TBARS = SOD activity ↑ CAT and GSH-Px activity	[33]
In vivo. C57BL/6 mice injured with $AlCl_3$ (model of AD)	Honey syrup	40 mg/kg/d (i.p.) $AlCl_3$ followed by 500 mg/kg honey syrup (i.p.) for 45 days	↑ TAC ↓ TBARS ↑ SOD, CAT and GSH-Px activity	[33]
In vivo. Male C57BL/6 mice injured with HFD	Chestnut honey	45 mg/d (orally) for 10 weeks	↓ ROS levels in brain	[13]
In vivo. Male Sprague Dawley rats injured with KA	Tualang Honey	1.0 g/kg (i.g.) five times every 12 h and 15 mg/kg KA (s.c.) 30 min after last dose of treatment	↑ Viable cells in cortex ↑ TAC in cortex ↓ TBARS levels in cortex	[34]

Table 2. *Cont.*

Model	Treatment	Dosage and Duration	Effects	Reference
		Oxidative stress		
In vivo. Male Sprague Dawley rats injured with KA	Tualang Honey	10 or 50 mg/kg honey (p.o.) and 15 mg/kg KA (s.c.) 30 min after last dose of treatment	↑ TAC ↓ MDA and NOx levels in brain = Protein carbonyl levels in brain ↑ CAT activity ↑ GSH levels	[35]
In vivo. Male Wistar rats injured with LPS	Honey	250 μg/kg LPS (i.p.) for 7 days followed by 0.31 and 0.36 g/kg honey for other 7 days	↓ MDA levels in brain ↑ GSH levels in brain	[36]
In vivo. Male Wistar rats injured with lead acetate	Honey	1.5 mL/kg of honey concomitantly with 0.2% lead for 28 days (orally)	↑ Memory function = MDA levels ↑ SOD levels ↑ GST activity = CAT levels ↑ GSH levels	[37]
In vivo. Male Sprague Dawley rats injured with LPS	Tualang honey	200 mg/kg tualang honey (i.p.) for 14 days, 5 mg/kg LPS (i.p.) applied on day 4	↑ CAT and GPx levels ↑ SOD and GR levels	[38]
In vivo. Aged male Sprague Dawley rats injured with noise stress	Tualang honey	200 mg/kg bw/day (i.g.) 14 days prior to the stress procedure and continued for an additional 14 days during the stress exposure	↑ Short- and long-term memory ↓ MDA levels in brain ↑ SOD activity in brain = GPx, GR and CAT activity in brain	[39]
In vivo. Male Sprague Dawley rats subjected to stresses	Tualang honey	1 g/kg bw/twice daily (i.g.) and exposed to stress for 28 consecutive days	↑ TAC in brain ↓ TBARS levels in brain ↑ GSH:GSSG ratio in brain ↓ GSSG levels in brain	[40]
In vivo. Male Sprague Dawley rats injured with $A\beta_{1-42}$ (AD model)	Kelulut honey	2.5 μg/μL $A\beta_{1-42}$ (icv) 1 week before 1 g/kg bw/d honey (i.g.) for 28 days	= SOD1 and MDA levels in hippocampus	[41]
		Neuroinflammation		
In vitro	Algerian honeys	IC_{50} = 1.72–7.43 mg/mL	↓ Activity of BSA denaturation induced by heat ↓ Nitrite levels	[42]
In vitro. RAW 264.7 macrophages injured with LPS	Manuka honey	8 mg/mL honey with 1 μg/mL LPS for 24 h	↓ NF-κB, iNOS, TNF-α and IL-1β gene expression ↓ TLR4, NF-κB, p-Iκ-Bα, iNOS protein expression ↓ TNF-α, IL-1β, IL-6 protein expression ↑ IL-10 protein expression	[30]
In vitro. RAW 264.7 macrophages injured with LPS	Chestnut and Eucalyptus honey	1 mg/mL honey for 24 h followed by 1 μg/mL LPS for 24 h	↓ Nitrite levels	[31]
In vitro. RAW 264.7 cell line injured with LPS	Kelulut honey	0.5 and 1% (*v*/*v*) honey for 2 h followed by the addition of 1 μg/mL LPS for another 22 h	↓ iNOS expression ↓ NO levels = COX-2 expression	[43]
In vivo. Male C57BL/6 mice injured with HFD	Chestnut honey	45 mg/d (orally) for 10 weeks	↓ Nitrite levels in brain ↓ TNF-α gene expression in brain ↓ COX-2 and iNOS protein levels in brain	[13]
In vivo. Male Wistar rats injured with HFD	Stingless Bee Honey	HFD for 8 weeks, followed by HFD with 10 mg/kg bw/d honey (i.g.) for another 8 weeks	↓ TNF-α levels in brain	[44]
In vivo. Male Sprague Dawley rats injured with KA	Tualang Honey	1.0 g/kg (i.g.) five times every 12 h and 15 mg/kg KA (s.c.) 30 min after the last dose of treatment)	↓ TNF-α and IL-1β levels ↓ GFAP and AIF-1 levels ↓ COX-2 and 5-LOX levels	[45]
In vivo. Male Wistar rats injured with LPS	Honey	250 μg/kg LPS (i.p) for 7 days followed by 0.31 and 0.36 g/kg honey for other 7 days	↓ Memory and motor impairment ↓ Nitrite levels in brain ↓ TNF-α and IL-6 levels	[36]
In vivo. Male Sprague Dawley rats injured with $A\beta_{1-42}$ (AD model)	Kelulut honey	2.5 μg/μL $A\beta_{1-42}$ (icv) 1 week before 1 g/kg bw/d honey (i.g.) for 28 days	= p-NF-κB levels in hippocampus	[41]
		Apoptosis		
In vivo. Male C57BL/6 mice injured with HFD	Chestnut honey	45 mg/d (orally) for 10 weeks	↓ Number of apoptotic cells in cortex ↓ FAS-L, P27, and BIM gene expression ↑ BCL2 gene expression	[13]
In vivo. Male Wistar rats injured with HFD	Stingless Bee Honey	HFD for 8 weeks, followed by HFD with 10 mg/kg bw/d honey (i.g.) for another 8 weeks	= Number of apoptotic cells	[44]
In vivo. Male Sprague Dawley rats injured with $A\beta_{1-42}$ (AD model)	Kelulut honey	2.5 μg/μL $A\beta_{1-42}$ (icv) 1 week before 1 g/kg bw/d honey (i.g.) for 28 days	↓ Number of apoptotic cells in dentate gyrus, CA1 and CA3 areas	[41]

Table 2. *Cont.*

Model	Treatment	Dosage and Duration	Effects	Reference
Aβ plaque damage and APP processing				
In vivo. *C. elegans* transgenic strain (CL4176, Aβ model)	Manuka honey	100 mg/mL for 72 h	↓ Aβ-induced paralysis phenotype ↓ Aβ deposits	[17]
In vivo. *C. elegans* transgenic strain (CL4176, Aβ model)	Avocado honey	100 mg/mL for 72 h	↓ Aβ-induced paralysis phenotype	[18]
In vivo. Male C57BL/6 mice injured with HFD	Chestnut honey	45 mg/d (orally) for 10 weeks	↓ Expression of genes related to APP generation and processing	[13]
In vivo. Male Sprague Dawley rats injured with LPS	Tualang honey	200 mg/kg tualang honey (i.p.) for 14 days, 5 mg/kg LPS (i.p.) applied on day 4	↓ $A\beta_{1-40}$ levels ↑ $A\beta_{1-42}$ levels	[38]
In vivo. Male Sprague Dawley rats injured with $A\beta_{1\text{-}42}$ (AD model)	Kelulut honey	2.5 μg/μL $A\beta_{1\text{-}42}$ (icv) 1 week before 1 g/kg bw/d honey (i.g.) for 28 days	↓ $A\beta_{1-42}$ depositions in dentate gyrus area = $A\beta_{1-42}$ depositions in CA1 and CA3 areas	[41]
Hyperphosphorylated tau protein damage				
In vivo. *C. elegans* transgenic strain (BR5706, tauopathy model)	Manuka honey	100 mg/mL for 72 h	↓ Mobility parameters	[17]
In vivo. *C. elegans* transgenic strain (BR5706, tauopathy model)	Avocado honey	100 mg/mL for 72 h	↓ Mobility parameters	[18]
In vivo. Male C57BL/6 mice injured with HFD	Chestnut honey	45 mg/d (orally) for 10 weeks	↓ Expression of genes related to tau protein	[13]
In vivo. Male Sprague Dawley rats injured with $A\beta_{1\text{-}42}$ (AD model)	Kelulut honey	2.5 μg/μL $A\beta_{1\text{-}42}$ (icv) 1 week before 1 g/kg bw/d honey (i.g.) for 28 days	↓ p-Tau levels in hippocampus	[41]
Imbalance of neurotransmitters and elimination-related enzymes				
In vitro	Polish honeys	Buckwheat honey (AChE inhibition 39.51%) Multi-floral honey (BChE inhibition 39.76%)	↓ AChE and BChE activity	[46]
In vitro	Acacia, raspberry, lavender, bean, buckwheat, aloe, heather, linden, eucalyptus, sunflower, goldenrod, linden, thyme, rape, rosemary, hawthorn, orange blossom honeys		↓ AChE and BChE activity	[47]
In vitro	Acacia honey	IC_{50} = 0.26% (*v*/*v*)	↓ AChE activity	[48]
In vitro: Enzyme isolated from rat liver microsomes	Chestnut honey	IC_{50} = 41.60 μg/mL	↓ MAO activity	[49]
In vivo. Male Wistar rats	Acacia honey	20% *v*/*v* (orally) for 1 week	↓ AChE activity in brain and in serum	[48]
In vivo. Male Wistar rats injured with LPS	Honey	250 μg/kg LPS (i.p) for 7 days followed by 0.31 and 0.36 g/kg honey for other 7 days	↓ AChE activity	[36]
In vivo. Aged male Sprague Dawley rats	Tualang honey	200 mg/kg bw/day (i.g.) 28 days	↓ AChE activity	[39]

Abbreviations: 5-LOX = 5-Lipoxygenase; AAPH = 2,2′-Azobis(2-amidinopropane) dihydrochloride; Aβ = amyloid β; AChE = acetylcholinesterase; AD = Alzheimer's disease; AIF-1 = allograft inflammatory factor 1; APP = amyloid precursor protein; BChE = butylcholinesterase; BSA = bovine serum albumin; *C. elegans* = *Caenorhabditis elegans*; CAT = catalase; COX-2 = cyclooxygenase 2; DAF-16 = dauer formation 16; FOXO = Forkhead box class O; GFAP = glial fibrillary acidic protein; GPx = glutathione peroxidase; GR = glutathione reductase; GSH = reduced glutathione; GST = glutathione S transferase; HFD = high-fat diet; HSF = heat shock factor; HSP = heat shock protein; IC_{50} = half-maximal inhibitory concentration; icv = intracerebroventricular; i.g. = intragastric; IL = interleukin; i.p. = intraperitoneal; iNOS = inducible nitric oxide synthase; KA = kainic acid; LPS = lipopolysaccharide; MAO = monoamine oxidase; MDA = malondialdehyde; NF-κB = nuclear factor kappa B; Nrf-2 = nuclear factor erythroid 2-related factor 2; NO = nitric oxide; OGG1 = 8-oxoguanine DNA glycosylase 1; ROS = reactive oxygen species; s.c. = subcutaneous; SKN-1 = skinhead-1; SOD = superoxide dismutase; TAC = total antioxidant capacity; TBARS = thiobarbituric acid reactive substances; TLR = toll-like receptor; TNF-α = tumor necrosis factor alpha. Symbols: ↑ increase; ↓ decrease; = no significant change.

Table 3. Effects of honey's extracts on AD features.

Model	Treatment	Dosage and Duration	Effects	Reference
Oxidative stress				
In vitro. N13 microglial cells injured with LPS	Flavonoid extract of multifloral honey	0.5 and 1 μg/mL for 30 min before 2.5 ng/mL LPS for 1 h	↓ ROS content	[19]
In vitro. HT22 cells injured with glutamate	Ethyl acetate Fraction of chestnut honey	250, 500, or 750 μg/mL for 24 h followed by 5 mM glutamate for 6 h	↓ ROS content ↑ HO-1 and GCLC protein expression	[50]
In vivo. Male Sprague Dawley rats injured with LPS	Phenolic extract of tualang honey	150 mg/kg extract (i.p.) for 14 days, 5 mg/kg LPS (i.p.) applied on day 4	↑ CAT and GPx levels	[38]
Mitochondrial dysfunction				
In vitro. HT22 cells injured with glutamate	Ethyl acetate Fraction of chestnut honey	500, or 750 μg/mL for 24 h followed by 5 mM glutamate for 6 h	↑ Mitochondrial membrane potential	[50]
Neuroinflammation				
In vitro. N13 cell culture injured with LPS	Flavonoid extract of multifloral honey	0.5 and 1 μg/mL, 30 min/6 h	↓ TNF-α, IL-1β, iNOS mRNA levels ↓ iNOS protein levels	[19]
In vitro. RAW 264.7 macrophages injured with LPS	Methanolic extract of saffron honey	3.4 or 6.8 μg/mL for 2 h, followed by 1 μg/mL LPS for 22 h	↓ NO production ↓ NF-κB, IL-6 and TNFSF9 gene expression	[51]
In vitro. RAW 264.7 macrophages injured with LPS	Methanolic extract of eryngium honeys	5.5 or 11 μg/mL for 2 h, followed by 1 μg/mL LPS for 22 h	↓ NO production ↓ NF-κB and TNFSF9 gene expression ↑ Nrf-2 gene expression	[51]
Apoptosis				
In vitro. HT22 cells injured with glutamate	Ethyl acetate Fraction of chestnut honey	500, or 750 μg/mL for 24 h followed by 5 mM glutamate for 24 h	↓ Cell death ↓ AIF protein expression ↑ Bcl-2 protein expression	[50]
In vitro. RAW 264.7 macrophages injured with LPS	Methanolic extract of saffron and eryngium honeys	3.4 or 6.8 μg/mL (saffron) or 5.5 or 11 μg/mL (eryngium) for 2 h, followed by 1 μg/mL LPS for 22 h	↓ Bax gene expression ↑ Bcl-2 gene expression	[51]
Aβ plaque damage and APP processing				
In vivo. Male Sprague Dawley rats injured with LPS	Phenolic extract of tualang honey	150 mg/kg extract (i.p.) for 14 days, 5 mg/kg LPS (i.p.) applied on day 4	↓ $A\beta_{1-40}$ levels ↑ $A\beta_{1-42}$ levels	[38]
Imbalance of neurotransmitters and elimination-related enzymes				
In vitro	Phenolic extract of Algerian honeys	IC_{50} = 0.367–0.629 mg/mL	↓ AChE activity	[42]

Abbreviations: Aβ = amyloid β; AChE = acetylcholinesterase; AD = Alzheimer's disease; AIF = allograft inflammatory factor; APP = amyloid precursor protein; CAT = catalase; GCLC = glutamate–cysteine ligase, catalytic subunit; GPx = glutathione peroxidase; HO-1 = heme oxygenase-1; IC_{50} = half-maximal inhibitory concentration; IL = interleukin; i.p.= intraperitoneal; iNOS = inducible nitric oxide synthase; LPS = lipopolysaccharide; NF-κB = nuclear factor kappa B; Nrf-2 = nuclear factor erythroid 2-related factor 2; NO = nitric oxide; ROS = reactive oxygen species; TNF-α = tumor necrosis factor alpha. Symbols: ↑ increase; ↓ decrease.

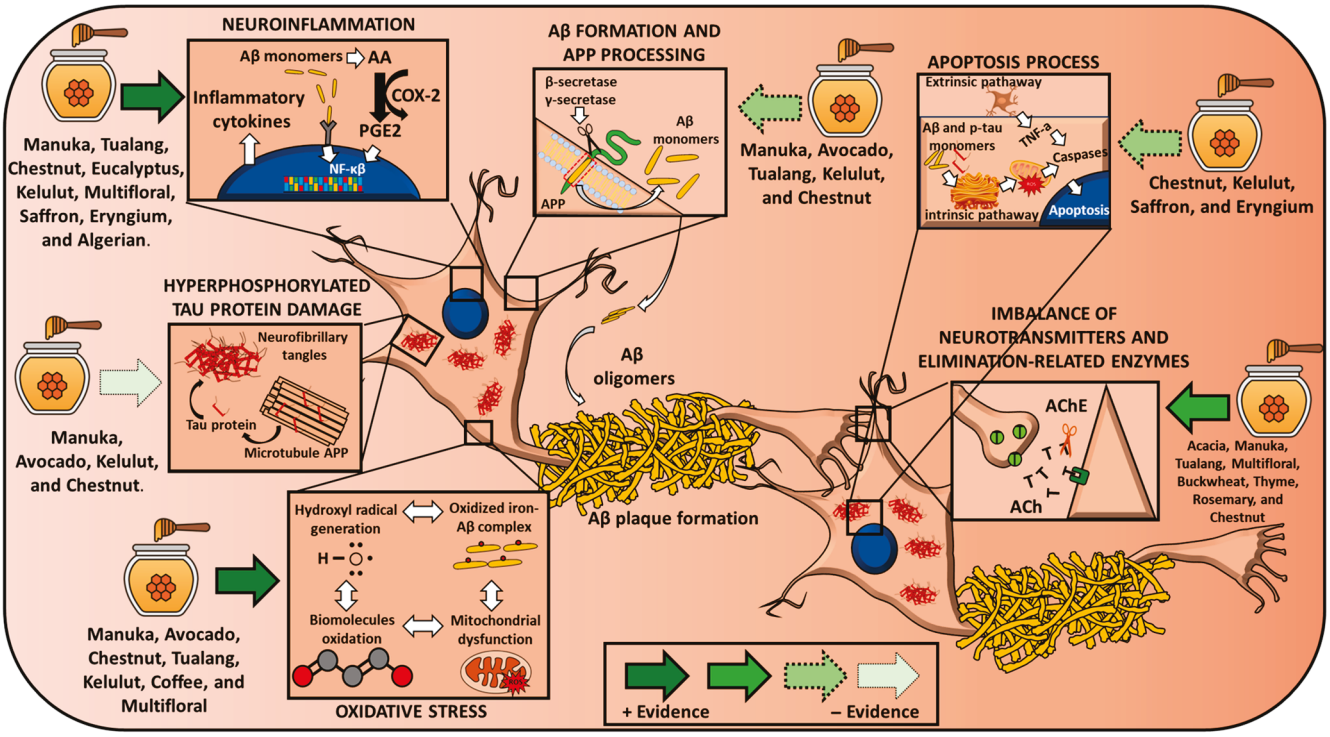


Figure 1. Effects of different honey types on the main molecular features of Alzheimer disease. Arrows' design reflects the strength of the evidence. Abbreviations: AA = arachidonic acid; Aβ= amyloid beta; APP= amyloid precursor protein; AChE = acetylcholinesterase; COX-2 = cyclooxygenase-2; PGE_2 = prostaglandin E_2.

3.1. Oxidative Stress

Oxidative stress has been widely implicated in the pathogenesis of age-related diseases like AD, contributing to neuronal damage through the accumulation of reactive oxygen species (ROS) and impaired antioxidant defenses. The beneficial activity of different types of honey or their extracts against oxidative stress in the context of the nervous system has been evaluated using both in vitro and in vivo models. In terms of extracts, those obtained from multifloral and chestnut honeys have been evaluated in cell culture models. In N13 microglial cells, lipopolysaccharide (LPS)-induced ROS production was significantly attenuated by pretreatment with 0.5 and 1 µg/mL of a flavonoid-rich extract of multifloral honey [19]. Similarly, the ethyl acetate fraction of chestnut honey extract reduced intracellular ROS levels in HT22 neuronal cells exposed to glutamate in a dose-dependent manner [50]. With respect to whole honey, both multifloral and manuka varieties have been assessed in cellular models. An attenuation of oxidative stress was found in an astrocyte cell culture damaged with H_2O_2, where honey treatment provided dose-dependent protection, with the 1% (*v*/*v*) dose showing the most significant effect on cell survival [10]. manuka honey has also demonstrated ROS-reducing effects, as observed in LPS-injured RAW 264.7 macrophages. In addition, this treatment reduced oxidative damage to lipids, proteins, and DNA, as evidenced by decreased thiobarbituric acid reactive substances (TBARS) and protein carbonyl levels, as well as reduced expression of the DNA repair enzyme 8-oxoguanine DNA glycosylase (OGG)1 [30].

In *Caenorhabditis elegans*, the increase in the ROS content caused by 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) was significantly decreased by 100 mg/mL of manuka honey [17] and the same concentration of avocado honey [18], with the effect of the avocado honey being greater. Results in the same sense were described for coffee honey (*Apis cerana*) treatment at 1% (*v*/*v*) applied to transgenic fly larvae (*Drosophila melanogaster*).

The analysis of brain homogenates revealed a lower ROS content in treated flies comparing to the control group, which is indicative of an attenuation of oxidative stress in the central nervous system [32]. *C. elegans* [52] and *D. melanogaster* [53] are widely used in in vivo models for the screening and evaluation of potential health benefits of natural or synthetic compounds. Regarding the nematode model, *C. elegans* shares many conserved biochemical pathways with mammals. Genes associated with AD in humans have orthologs in *C. elegans*, including those involved in amyloid protein precursor (APP) processing and tau protein regulation. Moreover, since the complete neuronal connectivity of the worms has been fully mapped, it provides a valuable model for studying learning and memory impairments associated with AD [52]. Similarly, *D. melanogaster* has proven useful for modeling AD, as the expression of disease-related human gene products, such as tau and $A\beta_{42}$, leads to observable and quantifiable phenotypes in flies. This makes *D. melanogaster* a practical model for investigating specific pathological features of AD. Additionally, more complex behavioral and cognitive assays can also be conducted in this model [53]. However, despite the numerous advantages of these non-vertebrate models, such as short lifespan, genetic manipulability, and cost-effectiveness, their findings cannot be directly extrapolated to humans. Therefore, results obtained in *C. elegans* and *D. melanogaster* should be further validated in higher-order animal models, such as rodents, and ultimately in clinical trials to assess translational relevance.

Experiments carried out in in vivo models of neurological damage are consistent with the results already mentioned in cells and invertebrates. Several toxic agents, such as $A\beta_{1\text{-}42}$, LPS, kainic acid (KA), $AlCl_3$, lead (Pb), or even a high-fat diet (HFD) treatment, are used in order to induce inflammation, oxidative stress, or cognitive impairment. The total antioxidant capacity (TAC) and TBARS levels, which initially were affected by $AlCl_3$ [33] or KA [34,35] in animal models, were improved by the treatment with honey syrup [33] and tualang honey [34,35], respectively. Interestingly, in latter study, authors administered the treatment as honey silver nanoparticles [35]. In addition, tualang honey, a wild polyfloral honey produced by *Apis dorsata*, increased the number of viable cells in the cortex of rats injured with KA, a well-known neurotoxic agent [54]. The LPS-induced oxidative stress was ameliorated by honey treatment in rats, as manifested the lower malondialdehyde (MDA) levels in the brain [36,38]. However, the MDA levels were not affected by kelulut honey treatment in the hippocampus of Sprague Dawley rats injured with $A\beta_{1\text{-}42}$ (AD model) [41], which could be related to the type of honey. On the other hand, treatment with chestnut honey for 10 weeks decreased the ROS and nitrite content in the brain of C57BL/6 mice injured with HFD. Authors evidenced that HFD was able to induce activation of amyloidogenic pathways, neuroinflammation and neurodegeneration; therefore, it is a suitable model for the evaluation of the natural products' effects on AD [13]. Regarding lead exposure, it has been reported to cause reduced locomotor and exploratory activity, increased anxiety-like behavior, memory impairment, enhanced lipid peroxidation, and diminished antioxidant enzyme activity. In a study using male Wistar rats exposed to lead acetate, concomitant oral administration of honey for 28 days resulted in improved memory performance. However, the treatment did not significantly alter MDA levels in the brain [37]. On the other hand, aged male Sprague Dawley rats subjected to noise-induced stress were used to evaluate the effects of tualang honey. The animals received oral administration of tualang honey for 14 days prior to the stress procedure and continuously during the 14-day stress exposure period. This treatment improved both short- and long-term memory performance and significantly reduced MDA levels in the brain [39]. Along the same line, tualang honey administered to Sprague Dawley rats subjected to stresses increased the TAC and reduced-oxidized glutathione (GSH:GSSG) ratio in the brain, while reducing the TBARS and GSSG levels [40].

Regarding antioxidant defense, the activation of nuclear factor erythroid 2-related factor 2 (Nrf-2) in various neurodegenerative models promotes the expression of antioxidant enzymes, thereby reducing oxidative stress and exerting neuroprotective effects. In this context, one study evaluated the effect of a chestnut honey extract in HT22 cells exposed to glutamate-induced toxicity. The results showed that pretreatment with the extract significantly upregulated the expression of heme oxygenase-1 (HO-1) and the catalytic subunit of glutamate–cysteine ligase (GCLC). Additionally, a trend toward increased nuclear translocation of Nrf-2 was observed following treatment with the extract, although this change did not reach statistical significance [50]. Similarly, manuka honey demonstrated antioxidant effects in RAW 264.7 macrophages exposed to LPS-induced oxidative stress. Treatment with 8 mg/mL for 24 h, in the presence of 1 μg/mL LPS, significantly reduced nitrite accumulation and increased intracellular GSH levels. Furthermore, the treatment enhanced the activity of glutathione reductase (GR) and glutathione S-transferase (GST), and upregulated the protein expression of Nrf-2, Keap1, catalase (CAT), and superoxide dismutase (SOD) [30]. In line with these findings, Cianciosi et al. [31] evaluated the effects of Italian chestnut and eucalyptus honeys in RAW 264.7 macrophages injured with LPS. Pretreatment with honey followed by stimulation with LPS significantly increased intracellular GSH levels and glutathione peroxidase (GPx) activity, while GR and GST activities remained unchanged. Notably, ROS levels and Nrf-2 gene expression were not significantly affected [31]. The specific composition of the chestnut and eucalyptus honeys used, including their polyphenolic profile and antioxidant capacity, may selectively modulate certain enzymatic pathways while leaving others unaffected, which could explain the differences between manuka and those honeys in the same experimental models. Furthermore, the timing and duration, as well as the dose applied, may not have been set optimally to trigger a full Nrf-2 activation or reduce ROS accumulation.

In other experimental models, authors reported that manuka [17] and avocado [18] honey treatments led to a decrease in SOD-3 expression in a *C. elegans* transgenic strain, accompanied by a reduction in heat shock protein (HSP)-16.2 expression, while maintaining GST-4 enzyme expression [17,18]. Interestingly, manuka honey did not affect the expression of Skinhead (SKN)-1/Nrf-2 and dauer formation (DAF)-16/Forkhead box class O (FOXO) transcription factors [17]. In contrast, avocado honey treatment resulted in an upregulation of DAF-16/FOXO and a downregulation of SKN-1/Nrf-2 expression [18]. These divergent responses likely reflect differences in the phytochemical compositions of the two honeys, suggesting that each honey type activates distinct antioxidant signaling pathways to confer cellular protection. Both honeys contain substantial amounts of compounds such as o-vanillin and ellagic acid, which may underlie their shared effects on several antioxidant markers. However, unique bioactive constituents distinguish each honey: manuka honey is enriched in flavonoids and phenolics including epicatechin, naringenin, and quercetin [17], whereas avocado honey contains notable levels of ferulic acid and isoflavones like formononetin [18].

On the other hand, tualang honey and its phenolic extract increased the depleted levels of CAT and GPx in the LPS-injured rat brain [38]. In addition, the whole honey increased the levels of the antioxidant enzymes SOD and GR [38], and reduced glutathione (GSH) content [36]. Neuroprotective effects were further supported by an increased number of Nissl-positive cells in the hippocampus, indicating preserved neuronal integrity [38]. Similarly, in a lead-induced neurotoxicity model, honey administration restored SOD, GSH, and GST activity in rat brain [37]. In a noise-induced stress model, tualang honey improved SOD activity, which correlated with decreased MDA levels and better memory performance. However, no changes were observed in GPx, GR, or CAT activity under these conditions [39]. In addition, treatment with honey syrup for 45 days attenuated the

oxidative effects of $AlCl_3$, restoring SOD, CAT, and GPx activity [33]. In contrast, a 28-day treatment with kelulut honey failed to modify SOD1 levels in the hippocampus of Sprague Dawley rats subjected to $A\beta_{1–42}$-induced injury (AD model), which is consistent with its lack of effect on oxidative damage markers in the same study [41]. Further evidence from a KA-induced excitotoxicity model supports the antioxidant potential of tualang honey. In this study, oral administration of silver nanoparticles synthesized with tualang honey significantly reduced brain nitrogen oxides (Nox) levels and increased CAT activity and GSH content [35].

As summarized in Tables 1 and 2, the dosages and treatment protocols vary greatly across the experiments, making it difficult to directly compare the effects of different honeys and determine which variety is more effective in mitigating oxidative stress. The studies used doses ranging from 10 mg/kg/day over extended periods (e.g., 45 days) to 1 g/kg administered multiple times over short intervals (e.g., five times every 12 h). Furthermore, experimental models and the agents used to induce oxidative stress also differ. Nonetheless, the overall findings suggest that various types of honey may serve as potential therapeutic agents against oxidative stress and neurodegeneration. They appear to reduce ROS and markers of oxidative damage while enhancing antioxidant defense mechanisms. Thus, in this sense, honey may represent a promising dietary approach for the management of oxidative stress in the neurological context of AD.

Considering dose extrapolation from rodents to humans, the method based on body surface area is commonly used [55]. This approach calculates the human-equivalent dose (HED) by applying species-specific conversion factors. For example, a dose of 10 mg/kg administered to rats would correspond to approximately 1.6 mg/kg in humans. For a standard 60 kg adult, this results in a total daily dose of around 96 mg. Similarly, a high dose such as 1 g/kg in rats translates to 161 mg/kg in humans, which equals 9.6 g per day for a 60 kg adult, which is still within the range of typical daily honey intake. Therefore, the doses used in several animal studies appear to be physiologically and nutritionally relevant for humans.

3.2. Mitochondrial Dysfunction

The primary mitochondrial cascade hypothesis proposes that mitochondria drive the pathogenesis of AD. Mitochondrial dysfunction drives Aβ production and tau deposition, affecting the maintenance of optimal neuronal and synaptic function. Targeting the mitochondria and/or associated proteins may hold promise for new therapeutic strategies for AD, and for providing meaningful clinical benefit. One therapeutic approach is to restore mitochondrial function and reverse oxidative stress by using antioxidants [56]. Authors evaluated the effect of an extract of chestnut honey on glutamate-exposed neurotoxicity in HT22 cells. The glutamate exposure rapidly changed the mitochondrial membrane potential of neurons, leading to a significant loss. Alternatively, the treatment with the honey extract prevented glutamate-induced mitochondrial membrane potential loss at concentrations of 500 and 750 μg/mL, in a concentration-dependent manner [50].

3.3. Neuroinflammation

Neuroinflammation is increasingly recognized as a key driver in the progression of pathology, as sustained activation of glial cells and pro-inflammatory signaling contribute to neuronal dysfunction and degeneration. Nuclear factor kappa B (NF-κB) plays a central role in the inflammatory response by promoting the expression of pro-inflammatory cytokines and mediators such as tumor necrosis factor-alpha (TNF-α), interleukins (e.g., IL-1β, IL-6), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) [51]. The induction of iNOS expression during the inflammatory process catalyzes the conversion of

L-arginine into nitric oxide (NO) [43]. Conversely, Nrf-2 exerts anti-inflammatory effects not only by enhancing antioxidant defenses but also by inhibiting the NF-κB signaling pathway, thereby suppressing the production of pro-inflammatory molecules [51]. In the context of neurodegeneration, chronic activation of NF-κB contributes to sustained neuroinflammation, neuronal damage, and disease progression. Several studies have shown that honey and its phenolic compounds can inhibit NF-κB activation by reducing its nuclear translocation or interfering with upstream signaling pathways, ultimately leading to decreased expression of inflammatory mediators.

Some studies that showed reduced ROS content also reported decreased inflammatory markers. For instance, the anti-inflammatory activity of various Algerian honeys was evaluated in vitro, and the samples exhibited a significant inhibitory effect on heat-induced bovine serum albumin (BSA) denaturation, a property related to their antioxidant capacity [42]. Kelulut honey has also demonstrated anti-inflammatory properties in RAW 264.7 cells stimulated with LPS. Specifically, concentrations of 0.5% and 1% (*v*/*v*) led to a reduction in NO levels and iNOS expression, although COX-2 expression was not altered [43]. A similar experimental model was used by Gasparrini et al. [30] to evaluate the anti-inflammatory activity of manuka honey. Their results showed that treatment with manuka honey led to a downregulation of NF-κB, iNOS, TNF-α, and IL-1β gene expression, along with a decrease in toll-like receptor (TLR)-4, NF-κB, p-Iκ-Bα, and iNOS protein levels. Additionally, the treatment reduced the protein expression of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, while increasing the expression of the anti-inflammatory cytokine IL-10 [30]. Treatment with chestnut or eucalyptus honey decreased NO levels in the macrophage cell line [31].

In the same cellular model, the anti-inflammatory effects of methanolic extracts of saffron and eryngium honeys were also investigated. RAW 264.7 macrophages were pretreated with saffron or eryngium honey extracts, and both treatments significantly reduced NO production and downregulated NF-κB and TNFSF9 gene expression. Additionally, saffron honey decreased IL-6 expression, while eryngium honey upregulated Nrf2 gene expression, suggesting activation of the antioxidant response pathway [51]. Similarly, the mRNA expression of pro-inflammatory markers was reduced in a dose-dependent manner by a flavonoid extract from multifloral honey (0.5 and 1 μg/mL) in an LPS-injured N13 microglial cell line. More specifically, TNF-α, IL-1β, and iNOS mRNA levels, and iNOS protein levels, were decreased by the treatment. Therefore, the extract is a potent inhibitor of microglial activation and a potential agent for neurodegenerative diseases involving inflammation [19].

In vivo experiments confirmed the anti-inflammatory activity of honeys in the neurological context. Tualang honey ameliorated the increase in TNF-α and IL-1β levels induced by kainic acid in different cerebral areas in rats. The glial fibrillary acidic protein (GFAP) and the allograft inflammatory factor 1 (AIF-1) levels were also significantly reduced by the treatment, as well as the levels of the enzymes COX-2 and 5-lipoxygenase (5-LOX). Thus, this honey reduced neuroinflammation in different brain regions [45]. The LPS-induced impairment of memory was improved by honey in Wistar rats [36] and by the honey and its extract in Sprague Dawley rats [57]. Additionally, locomotion activity was increased and the levels of the inflammatory markers TNF-α and IL-6 were reduced by treatment, as well as the nitrite levels [36]. NO is highly unstable and rapidly converts into nitrate and nitrite; therefore, nitrite levels are commonly assessed as an indirect yet stable indicator of NO production [51]. Similarly, chestnut decreased the HFD-induced gene expression of TNF-α as well as COX-2 and iNOS protein levels in mice [13], and stingless bee honey reduced the TNF-α levels in the brain of rats injured with HFD [44]. In contrast, kelulut honey did not alter the NF-κB levels in the hippocampus of rats injured with $A\beta_{1\text{-}42}$ [41].

This absence of effect may be related to the specific properties of kelulut honey, which also failed to influence other parameters evaluated, in contrast to other honey types that showed positive outcomes. Alternatively, it may be due to the modulation of biological pathways not assessed in the study.

As previously mentioned, the assays conducted to evaluate inflammatory markers in the context of neurological disease vary considerably in terms of the type of honey used, the methodologies applied, and the experimental models employed. These differences hinder direct comparisons and limit the ability to extrapolate the findings. Notwithstanding this, in summary, honey and its phenolic compounds exhibit strong anti-inflammatory properties and may represent very interesting therapeutic agents for the treatment of neurodegenerative diseases.

3.4. Apoptosis

Dysregulated cell death pathways contribute to the gradual loss of cognitive function, so neuronal apoptosis plays a central role in AD progression. Honey and its polyphenols were found to be useful in improving memory deficits and could act at the molecular level by involving in apoptotic activities [58]. Proapoptotic proteins include Bax and Bim, whereas Bcl-2, Bcl-xL, and Bcl-w are considered anti-apoptotic. In addition, the FAS ligand (FAS-L) has been involved in neuronal death and P-27 has been reported to promote neuronal apoptosis induced by the $A\beta_{1\text{-}42}$ peptide [13].

Although the evidence on AD is very scarce, different types of honey and their extracts have shown effects on apoptosis. Extracts from chestnut honey [50], as well as from saffron and eryngium honeys [51], have been investigated in vitro. A pretreatment with an ethyl acetate fraction of chestnut honey (500 or 750 μg/mL) decreased the AIF and increased the Bcl-2 protein expressions, as well as increased the survival of HT22 cells injured with glutamate [50]. In RAW 264.7 macrophages stimulated with LPS, methanolic extracts of saffron and eryngium honeys reduced Bax gene expression and increased Bcl-2 gene expression at concentrations selected based on MTT viability results (3.4 and 6.8 μg/mL for saffron; 5.5 and 11 μg/mL for eryngium), indicating antiapoptotic potential in an inflammatory context [51].

In vivo, chestnut [13] and kelulut honey [41] have been demonstrated to exert an influence on the apoptosis process. Treatments with chestnut and kelulut honey decreased the number of apoptotic cells in the cortex of rodents injured with HFD [13] and $A\beta_{1\text{-}42}$ [41], respectively. The effect was mediated by the downregulation of pro-apoptotic genes such as FAS-L, P27, and BIM, and the upregulation of the anti-apoptotic Bcl-2 gene. Additionally, treatment increased the brain-derived neurotrophic factor (BDNF) gene expression that previously was decreased by the HFD [13]. However, not all types of honey exerted a measurable impact. In one study, treatment with stingless bee honey in HFD-fed Wistar rats did not significantly alter the number of apoptotic cells in the brain [44].

These findings suggest that certain honey types may modulate apoptosis-related pathways, although further studies are needed to confirm these effects and clarify the specific molecular mechanisms involved.

3.5. Aβ Plaque Damage and APP Processing

As already mentioned above, the accumulation of Aβ peptides into extracellular plaques is one of the hallmark features of AD, disrupting synaptic function and triggering a cascade of neurodegenerative events. Amyloid precursor protein (APP) undergoes two alternative processing routes: the non-amyloidogenic pathway, involving α- and γ-secretases, results in soluble fragments that are subsequently degraded and cleared, whereas in the amyloidogenic pathway, β- and γ-secretases generate insoluble Aβ peptides, which aggre-

gate to form extracellular amyloid plaques [5,6]. The beta-site amyloid precursor protein cleaving enzyme (BACE1, also known as β-secretase) is implicated in the amyloidogenesis of AD and considered a key drug target as its inhibition is expected to decrease the formation of amyloid aggregates. Some analyses have revealed that compounds present in honey, such as rutin, 3,4-dicaffeoylquinic acid, nemorosone, and luteolin, have high binding affinity to the BACE1 receptor, positioning them as promising therapeutic agents [59]. *C. elegans* transgenic strains were used by some authors to investigate the effect of manuka honey [17] and avocado honey [18] in a paralysis phenotype caused by $A\beta_{1-42}$ expression. Concentration of 100 mg/mL dramatically delayed the paralysis process with both treatments [17,18]. The RNAi test revealed that the effect of manuka honey was mediated by HSP-16.2 and the SKN-1/Nrf-2 pathway [17]. HSP16.2 is a chaperone involved in the correct folding of unfolded polypeptides [5] and SKN-1 is an important antioxidant pathway. Furthermore, the Thioflavin T stain showed a reduction in Aβ deposits in treated worms [17].

Although the investigation into rodent models is scarce, the results are in line with those already presented. Honey was evaluated in a mix with other functional foods such as pomegranate and dates, and the treatment with 4 mL/kg of that mixture manifested an improvement in spatial memory and learning in an AD model based on male albino Wistar rats injected with $A\beta_{42}$ [60]. Oxidative stress and inflammation have been closely associated with increased synthesis and accumulation of Aβ peptides. In rat models, administration of LPS led to elevated levels of $A\beta_{1-42}$ and reduced $A\beta_{1-40}$ in the hippocampus compared to controls, suggesting an enhancement of amyloidogenic processes. Treatment with tualang honey mitigated these alterations, resulting in a reversal of this pattern, and higher $A\beta_{1-40}$ and lower $A\beta_{1-42}$ levels relative to the LPS group, indicating its potential to counteract oxidative damage and amyloid aggregation [38]. Similarly, kelulut honey was shown to reduce $A\beta_{1-42}$ accumulation in the dentate gyrus of rats subjected to $A\beta_{1-42}$-induced neurotoxicity [41]. On the other hand, chestnut honey decreased the expression of genes related to APP generation and processing in mice having injured HFD [13]. Reducing the levels of Aβ is a prime and a promising therapeutic strategy for treating AD, and honey could be beneficial in that sense.

3.6. Hyperphosphorylated Tau Protein Damage

Tau protein, located in the cytoskeleton microtubules, is phosphorylated by the kinases that are activated in turn by the Aβ. That phosphorylated tau leaves the microtubule and forms intracellular aggregates, which are strongly associated with neuronal dysfunction and cognitive decline [4,5]. To the best of our knowledge, there are only four studies in which the relation between honey and tau protein is addressed. The *C. elegans* transgenic strain BR5706, which expresses pro-aggregant human tau protein in neurons, was treated with manuka [17] and avocado [18] honey and the mobility was evaluated. Intriguingly, the honey-treated worms exhibited worse parameters of movement than the non-treated group in both types [17,18]. The authors evaluated those parameters in the wild strain too, and the locomotion deterioration was observed again. Moreover, the honey hydrophilic extract exerted a similar effect to that of the vehicle [17]. Thus, the sugar content of manuka or avocado honey could affect the general mobility of the nematodes and not specifically the aggregation of tau protein, but more studies are needed in order to clarify this hypothesis.

Nevertheless, more promising results have been observed in animal models. Male Sprague Dawley rats subjected to $A\beta_{1\text{-}42}$ -induced injured and treated with kelulut honey showed reduced p-Tau levels in the hippocampus [41]. Similarly, Terzo et al. [13] used a HFD-induced model in mice to evaluate the effects of chestnut honey on tau protein, reporting a downregulation of genes associated with tau expression. These findings

suggest that honey may exert beneficial effects on tau pathology; however, further studies are necessary to validate and confirm these results.

3.7. Imbalance of Neurotransmitters and Elimination-Related Enzymes

In terms of neurotransmission, AD is characterized by profound impairments in the cholinergic, glutamatergic, gamma-aminobutyric acid (GABA)ergic, and monoaminergic systems, which are closely linked to cognitive and behavioral symptoms. In particular, reduced acetylcholine levels and elevated AChE activity are strongly associated with memory loss, while glutamatergic dysregulation contributes to excitotoxicity and neuronal death [4]. The enzyme inhibitory potential of various types of honey has been extensively investigated in vitro. Buckwheat honey and multi-floral honey stood out for their AChE and butyrylcholinesterase (BChE) inhibitory activity, respectively [46]. In addition, other varieties such as acacia, raspberry, thyme, and rosemary also exhibited significant inhibition of both enzymes [47]. Moreover, the AChE inhibitory activity of manuka honey has also been demonstrated [61]. The phenolic extract of some Algerian honeys [42] and acacia honey [48] exhibited a significant inhibition of AChE. Correlations were observed between those properties and the antioxidant capacity of the honeys [42]. The in vitro results of acacia honey were confirmed by in vivo experiments in Wistar rats. The treatment reduced the AChE activity in the brain and serum [48]. The AChE activity, which was increased by LPS induction, was attenuated by honey treatment, again in Wistar rats [36]. Along the same line, tualang honey treatment for 28 days (orally) decreased the AChE activity of aged rats [39]. Monoaminoxidase (MAO) is another enzyme involved in neurodegenerative diseases such as AD. Chestnut honey and other bee products were effective in inhibiting MAO activity in enzymes isolated from rat liver microsomes. That effect was related to the antioxidant capacity of the samples [49]. As manifested, honey can be a rich source of inhibitors for ChE and other enzymes, and, therefore, may play an important role in AD treatment. Thus, those results further support the potential neuroprotective properties of different honey types, and the effect should be further explored for the management of AD.

4. Limitations and Final Conclusions

This review highlights the potential neuroprotective properties of honey and honey-derived extracts against key pathological mechanisms involved in AD, including oxidative stress, inflammation, apoptosis, and Aβ or tau-related pathways. Nevertheless, the current body of evidence remains predominantly preclinical, which significantly limits the translational applicability of the findings.

One of the main limitations is the lack of clinical trials investigating the effects of honey in AD. To our knowledge, no randomized controlled human studies have been completed or are currently registered that directly evaluate honey as a therapeutic or preventive agent for this disease. Therefore, any extrapolation of the available data to human health must be interpreted with caution. Furthermore, a high degree of heterogeneity is observed across the included studies regarding the type of honey tested, animal species or cell lines used, doses administered, treatment duration, and outcome measures employed. Even when the same variety of honey is used (e.g., manuka or chestnut), its phytochemical composition can vary substantially depending on its geographic and botanical origin, harvest season, and processing method. These factors hinder the ability to draw robust, generalizable conclusions about the comparative efficacy of different honeys and make it hard to propose standardized dosage or therapeutic protocols at this stage. Additionally, many studies rely on invertebrate models (e.g., *C. elegans*) or murine organisms, which, while valuable for mechanistic exploration, limit the direct translatability of the results to humans. The doses used in animal studies are often very high (e.g., up to 1 g/kg/day) and may not be

realistically achievable or acceptable in human dietary or therapeutic contexts. No attempts were made in the cited literature to convert these doses to human-equivalent doses, nor to address pharmacokinetic aspects such as the low oral bioavailability of polyphenols, which further complicates the clinical relevance of the findings.

To address the methodological quality and potential risk of bias in the included studies, appropriate tools were applied depending on the experimental model. For in vitro studies, the QUIN tool was used as a reference framework. Most studies showed a high risk of bias in recurrent aspects, particularly due to insufficient reporting on operator involvement, lack of blinding of outcome assessors, and inadequate standardization of measurement procedures. For in vivo studies, SYRCLE's Risk of Bias tool was considered. Although many studies provided adequate details regarding animal characteristics and experimental conditions, frequent sources of bias included lack of allocation concealment, absence of blinding, and non-randomized outcome assessment.

Despite the aforementioned limitations, honey has consistently demonstrated the ability to reduce oxidative stress, enhance antioxidant defenses, decrease inflammation, and modulate enzymes involved in neurotransmission such as AChE. However, further research is needed to explore its role in other neurodegenerative mechanisms, including apoptosis, mitochondrial dysfunction, and the accumulation of pathological hallmarks such as amyloid-beta plaques and hyperphosphorylated tau protein. These processes are closely associated with neuronal damage and loss, and are fundamental drivers of AD progression, highlighting the need for further in-depth investigation. Among the types, avocado, manuka, chestnut, kelulut, and tualang honeys have been the most extensively studied, providing valuable insights into their distinct bioactive profiles and biological impacts. To conclude, honey holds promise as a functional food or as a source of phytochemicals for the development of nutraceuticals with potential use in the neurological context, although additional studies are required to draw robust conclusions.

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Review

Food Nutrients and Bioactive Compounds for Managing Metabolic Dysfunction-Associated Steatotic Liver Disease: A Comprehensive Review

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Abstract

Metabolic dysfunction-associated steatotic liver disease (MASLD) and its progressive form, metabolic dysfunction-associated steatohepatitis (MASH), are growing global health concerns. However, pharmacological therapies for MASLD/MASH have not yet been established. Dietary interventions and their bioactive components have been explored as strategies to mitigate MASLD and MASH progression. Although specific nutrients and bioactive compounds have exhibited potential therapeutic benefits, they also exacerbate adverse outcomes. In this comprehensive review, we synthesize the protective and exacerbating or sometimes dual effects of key macronutrients, including fatty acids (saturated, unsaturated, and trans fats) and carbohydrates (fructose, glucose, and sucrose), and bioactive compounds and micronutrients, in the context of MASLD management. The evidence suggests that coffee-derived compounds, such as caffeine and chlorogenic acid, may attenuate liver injury. However, the effects on MASLD severity are inconsistent. Diets high in saturated fatty acids exacerbate MASLD pathogenesis, whereas moderate intake (7–10% of total energy) may confer metabolic benefits. Other bioactive compounds and micronutrients have been explored for their diverse roles in hepatic lipid metabolism and insulin sensitivity. Although current evidence supports the therapeutic potential of specific dietary nutrients and bioactive compounds in the management of MASLD, inconsistencies in results highlight the need for more robust, well-controlled studies, including clinical trials, to clarify the preventive and therapeutic standards for balanced food interventions in MASLD management. In particular, well-designed clinical trials are necessary before clinical application. Although this is a narrative review and the literature retrieval may be biased, we covered a wide variety of substances.

Keywords: metabolic dysfunction-associated steatotic liver disease; metabolic dysfunction-associated steatohepatitis; nutrition; fatty acid; polyphenol; vitamin; zinc; liver fibrosis; dietary intervention

1. Introduction

In 2023, the terms metabolic dysfunction-associated steatotic liver disease (MASLD) and its advanced form, metabolic dysfunction-associated steatohepatitis (MASH), were introduced to replace nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. This revision underscores the growing recognition of metabolic dysfunction as a central driver

of these conditions. Several academic societies have recently published clinical practice guidelines for the management of MASLD [1,2].

Changes in dietary patterns in recent decades, notably the increased consumption of ultra-processed foods, high-fructose diets, and energy-dense meals, combined with sedentary lifestyles, have substantially contributed to the increased prevalence of MASLD and MASH worldwide. Currently, MASLD affects approximately 38% of the global adult population and approximately 7–14% of children and adolescents. By 2040, the number of adults with MASLD is expected to increase to over 55%, highlighting an increasingly important public health concern [3]. In a large-scale cross-sectional ecological study, Younossi et al. found a strong correlation between MASLD prevalence and indicators of food insecurity, particularly the reliance on ultra-processed foods in high socio-demographic index countries [4]. These observations underscore the pivotal role of dietary quality in modulating the risk and severity of MASLD.

The pathogenesis of MASLD and MASH is complex and multi-factorial, involving metabolic dysregulation, gut–liver axis dysfunction, and immune-mediated mechanisms; however, our understanding of these processes is incomplete. The only recommended treatment approaches are lifestyle modifications, particularly dietary changes and increased physical activity. This highlights the urgent need to develop nutritional strategies for modulating key metabolic and inflammatory pathways involved in the pathophysiology of MASLD. In this context, research has increasingly focused on dietary interventions and bioactive food components as potential strategies for mitigating MASLD and MASH progression. Specifically, the effects of specific nutrients, functional foods, and dietary supplements on disease development have been explored.

Ranneh et al. reviewed the therapeutic potential of polyphenols in MASLD [5], and Mullin et al. reviewed the mechanistic roles of dietary fatty acids and sugars in MASLD progression [6]. These reviews provide valuable insights into the beneficial effects of specific dietary components. Most studies and reviews have emphasized the beneficial effects of specific nutrients and bioactive compounds in the management of MASLD. However, authors are increasingly reporting conflicting or even adverse effects. These inconsistencies underscore the complex and context-dependent nature of nutrient–liver interactions and highlight the need for more comprehensive, balanced evaluations that consider both the protective and potentially harmful effects of dietary factors on MASLD pathophysiology.

In this comprehensive narrative review, we aim to synthesize the current scientific evidence on the complex and sometimes dual roles of key macronutrients (e.g., fatty acids and carbohydrates), bioactive compounds (e.g., caffeine, chlorogenic acid [CGA], ethanol, polyphenols, like curcumin, resveratrol, silymarin, and polyamines, like spermidine), and micronutrients (e.g., vitamins D, E, and C and the trace elements zinc and selenium) in the management of MASLD. By critically evaluating the available literature, we aimed to elucidate the mechanisms of action in hepatic lipid metabolism, insulin sensitivity, oxidative stress, inflammation, and fibrosis while also highlighting discrepancies and knowledge gaps. While several previous studies have discussed the role of different dietary nutrients in the management of MASLD [7–9], this review is unique as we emphasize preclinical studies with detailed mechanism descriptions. Furthermore, we describe the effects of ethanol and polyamines (including data from our studies), which have not been widely described in previous review articles. Therefore, in this paper, we focus on the most promising and well-studied nutrients, like vitamins and polyphenols, as well as nutrients that have been rarely covered in previous review articles. Our findings inform future research directions and help develop more precise, evidence-based nutritional recommendations for managing this prevalent and complex metabolic disorder.

2. Effects of Macronutrients on MASLD

The liver uses three major macronutrients for energy: carbohydrates, fats, and proteins. Macronutrient composition, specifically carbohydrates and fatty acids, significantly influences hepatic lipid metabolism, inflammation, and insulin sensitivity, thereby playing a crucial role in the development and progression of MASLD. Recent clinical and experimental studies have investigated the influence of specific macronutrients on MASLD outcomes, highlighting both beneficial and harmful effects. The underlying mechanisms and comparative findings from preclinical and clinical studies are summarized in Table 1.

Table 1. Summary of macronutrient intervention studies for MASLD.

Nutrients and Compounds	Intervention	Main Findings	Remarks	Ref.
SFA	High SFA intake	↑ Lipotoxicity, ↑ insulin resistance, ↑ endoplasmic reticulum stress	Very low (<7%) saturated fat levels can lead to metabolic imbalances	[10–17]
PUFAs (DHA, EPA)	Varied; DHA often more potent	↓ Steatosis, ↓ inflammation, ↓ fibrosis via PPARα, SREBP-1c inhibition, GPR120 activation	Dose/ratio uncertainties remain	[18–35]
Trans fatty acids	Varied; often industrial trans fatty acids	↑ DNL, ↑ inflammation, ↑ steatosis	—	[36–40]
Fructose	High intake, chronic	↑ DNL, ↑ inflammation, ↑ fibrosis, ↑ gut dysbiosis	—	[41–59]
Glucose	High-glucose/fructose diet	↑ inflammation, ↑ perisinusoidal fibrosis	—	[42,60–62]
Sucrose	Chronic high intake	↑ steatosis, ↑ oxidative stress, ↑ fibrosis	—	[41,42,63,64]

DHA, docosahexaenoic acid; DNL, de novo lipogenesis; EPA, eicosapentaenoic acid; GPR 120, G protein-coupled receptor 120; MASLD, metabolic dysfunction-associated steatotic liver disease; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; SREBP-1c, sterol regulatory element-binding protein 1c.

2.1. Fatty Acids

Fatty acids are central to the pathogenesis and progression of MASLD and influence hepatic lipid metabolism, insulin sensitivity, oxidative stress, and inflammation. The type and quantity of consumed fatty acids can have diverse effects on liver health.

2.1.1. Saturated Fatty Acids

High SFA intake, particularly palmitic acid (C16:0) and stearic acid (C18:0), is strongly implicated in MASLD progression. SFAs serve as substrates for DNL and contribute significantly to hepatic triglyceride accumulation [10]. In addition to simple accumulation, SFAs induce lipotoxicity through several mechanisms. They can activate pro-inflammatory pathways, notably TLR4 signaling, leading to the production of cytokines (e.g., TNF-α and IL-6) and immune-cell recruitment [11,12]. SFAs also promote endoplasmic reticulum stress and mitochondrial dysfunction, contributing to oxidative stress and hepatocyte apoptosis [13]. Although relevant data from humans are limited, Rosqvist et al. (2014) reported increased liver fat accumulation and visceral adiposity in humans who overate SFAs compared with those who consumed unsaturated fat [14]. Furthermore, SFAs can directly impair insulin signaling and exacerbate systemic insulin resistance [15]. Dietary

guidelines generally recommend limiting SFA intake to <10% of the total energy intake for metabolic health [16], which is particularly beneficial for MASLD management. Extremely low-fat diets, including those very low in saturated fats, can also impair the absorption of fat-soluble vitamins (A, D, E, and K) and affect hormone production or lead to metabolic imbalances, thereby impacting overall health [17].

2.1.2. Unsaturated Fatty Acids

Unsaturated fatty acids, including monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), generally exhibit more favorable metabolic profiles than saturated fats. MUFAs, predominantly oleic acid found in olive oil, are a key component of the Mediterranean diet and have been associated with reduced MASLD severity [18]. Mechanistically, MUFAs may improve insulin sensitivity and lipid profiles by displacing saturated fatty acids (SFAs) in cellular membranes and influencing signaling pathways [19,20]. PUFAs are categorized into omega-6 (n-6), primarily linoleic acid, and omega-3 (n-3), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Although n-6 PUFAs can produce pro-inflammatory eicosanoid compounds, n-3 PUFAs generally exert anti-inflammatory effects [21]. Patients with MASH have lower hepatic levels of long-chain n-3 PUFAs than those with simple steatosis [22]. PUFAs, particularly EPA and DHA, have therapeutic potential. They activate peroxisome proliferator-activated receptor alpha (PPARα) to enhance fatty acid oxidation, suppress sterol regulatory element-binding protein 1c (SREBP-1c) to reduce de novo lipogenesis (DNL), and activate G protein-coupled receptor 120 (GPR120) to mediate anti-inflammatory effects [23,24] (Figure 1). Most clinical trials have suggested that n-3 PUFA supplementation in both children and adults with MASLD/MASH can ameliorate the clinical and pathological findings of MASLD, but some studies showed no significant effects, suggesting limited and mixed evidence [25–29]. These clinical trials have some additional limitations. For example, in the clinical trial by Nobili et al., there was no control group (placebo group) and only 20 subjects [28]. In a clinical trial of omega-3-rich *Camelina sativa* by Musazadeh et al., the observational period was only 12 weeks [26]. Even in double-blind, placebo-controlled trials, the risk of bias cannot be denied because the matching of factors, such as age, sex, comorbidities, and lifestyle, may be insufficient.

A recent experimental study demonstrated that n-3 PUFAs attenuate steatosis in MASH model mice fed a high-fat diet (HFD) by stimulating the proliferation and differentiation of pre-adipocytes, thereby promoting the recruitment of new adipocytes and reducing hepatic lipid accumulation [30]. Furthermore, hepatic inflammation was notably attenuated by DHA rather than EPA in the atherogenic HFD- and Western diet-induced MASH model mice. This is because DHA lowers hepatic inflammatory markers, including interleukin (IL)-1α, IL-1β, IL-2rm, monocyte chemoattractant protein (MCP)-1, tumor necrosis factor (TNF)-α, CD68, and osteopontin [31,32]. Furthermore, in Ldlr−/− mice fed a Western diet, DHA suppressed the hepatic expression of CD14 (both messenger RNA (mRNA) and protein) and the toll-like receptors (TLR)2 and TLR4 and reduced the nuclear abundance of nuclear factor kappa B (NF-κB)-p50 and its precursor mRNA [32,33] (Figure 1).

DHA, but not EPA, has been shown to attenuate Western diet-induced hepatic fibrosis, as evidenced by the decreased gene and protein expression of collagen type I alpha 1 chain (Col1A1) and improved histological outcomes [32,34]. The antifibrotic effect of DHA is associated with its ability to attenuate hepatic transforming growth factor (TGF)-β signaling [33]. Furthermore, a recent preclinical study demonstrated that DHA-mediated inhibition of betacellulin disrupts the integrin pathway in macrophages and impairs TGF-β-driven collagen production by hepatic stellate cells, which synergistically contributes to the development of liver fibrosis [35] (Figure 1).

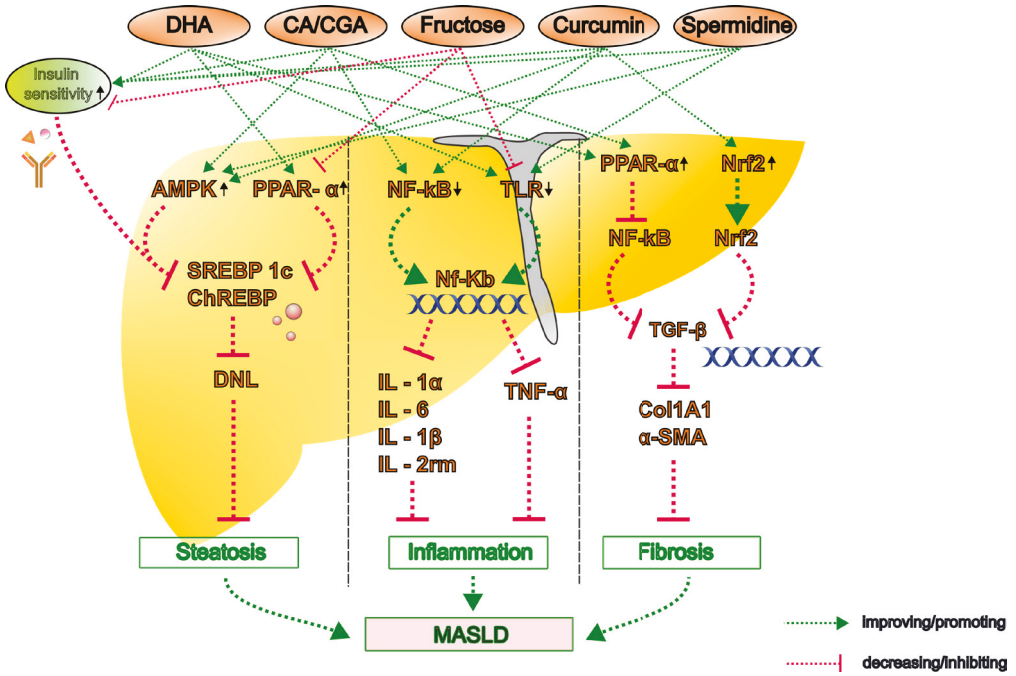


Figure 1. The proposed molecular mechanisms by which key dietary bioactive compounds influence the pathogenesis of metabolic dysfunction-associated steatotic liver disease (MASLD). Representative compounds—including caffeine, chlorogenic acid (CGA), polyphenols (curcumin, resveratrol, and silymarin), omega-3 polyunsaturated fatty acids (PUFAs), and polyamines (spermidine and putrescine)—modulate lipid metabolism, oxidative stress, inflammation, and fibrogenesis through multiple pathways. Upward arrow denotes an increase or upregulation and the downward arrow indicates decrease or downregulation in the activity or expression. α-SMA, alpha-smooth muscle actin; AMPK, AMP-activated protein kinase; CA, caffeine; CGA, chlorogenic acid; ChREBP, carbohydrate-responsive element-binding protein; Col1A1, collagen type I alpha 1; DHA, docosahexaenoic acid; DNL, de novo lipogenesis; IL, interleukin; NF-κB, nuclear factor kappa B; Nrf2, nuclear factor erythroid 2-related factor 2; PPAR, peroxisome proliferator-activated receptor; SREBP-1c, sterol regulatory element-binding protein 1c; TGF, transforming growth factor; TLR, toll-like receptor; TNF, tumor necrosis factor.

Experimental studies consistently highlight DHA as a more potent therapeutic n-3 PUFA than EPA for mitigating hepatic steatosis, inflammation, and fibrosis in MASLD. Although preclinical models have provided strong evidence for DHA's hepato-protective properties, further clinical studies are required to validate these findings and establish the optimal intake parameters, including effective dosing and appropriate n-6/n-3 PUFA ratios for managing MASLD.

2.1.3. Trans Fatty Acids

Industrially produced trans fatty acids (iTFAs), which are formed during partial hydrogenation of vegetable oils, are particularly detrimental to metabolic health. Although regulatory actions have significantly reduced the iTFA content in many food supplies, the levels of residual and naturally occurring TFAs from ruminant sources remain unclear [36]. The consumption of iTFAs is associated with an increased risk of cardiovascular disease, insulin resistance, and inflammation [37]. In the context of MASLD, iTFAs exacerbate hepatic steatosis by increasing DNL, impairing fatty acid oxidation, and promoting inflammation [38]. Animal studies have shown that high-TFA diets induce steatohepatitis features [39]. Although the human data are limited, minimizing TFA intake is a crucial dietary recommendation for preventing and managing MASLD [40].

2.2. Carbohydrates

Carbohydrates, particularly simple sugars such as fructose, glucose, and sucrose, play pivotal roles in the development and progression of MASLD. Excessive intake of these sugars, especially high-fructose corn syrup and sweetened beverages, is closely linked to hepatic steatosis, insulin resistance, and systemic inflammation.

2.2.1. Fructose

Saccharides and dietary fiber can be classified as simple or complex carbohydrates. For example, fructose is a monosaccharide classified as a simple carbohydrate found in honey, fruits, and root vegetables. The carbohydrate load describes the storage of exogenous saccharides and their influence. Although fructose has a low glycemic index, excessive consumption, primarily from sugar-sweetened beverages and processed foods containing high-fructose corn syrup, is a primary dietary driver of MASLD [41]. Unlike glucose metabolism, fructose metabolism largely bypasses the primary regulatory step of glycolysis (phosphofructokinase-1), leading to a rapid influx of DNL substrates [42]. Fructose activates the key lipogenic transcription factors SREBP-1c and carbohydrate-responsive element-binding protein (ChREBP) [41,43] (Figure 1). Fructose metabolism also generates uric acid, which induces mitochondrial oxidative stress [44]. Furthermore, fructose consumption contributes to insulin resistance, alters gut microbiota composition, increases gut permeability and endotoxemia, and promotes hepatic inflammation [45,46]. According to a meta-analysis, high fructose intake is significantly associated with increased liver fat and insulin resistance [47].

Experimental studies have demonstrated that chronic fructose intake significantly influences the expression of numerous genes involved in key metabolic pathways, including glycolysis, lipogenesis, β-oxidation/lipolysis, fructolysis, and gluconeogenesis. These effects appear to be modulated by factors such as nutritional status and sex [48–50]. High-fructose diets are also strongly associated with MASH development because they promote hepatic DNL via SREBP1c and ChREBP [51–54] (Figure 1). Fructose-induced alterations in hepatic metabolism contribute to the development of insulin resistance and gut microbiota dysbiosis [55]. As fructose metabolism predominantly occurs in the liver, it bypasses key regulatory steps in glycolysis, resulting in unregulated lipid synthesis and hepatic lipid accumulation [56].

Chronic fructose consumption has been shown to induce oxidative stress and inflammatory responses in experimental models. In C57BL/6 mice, a high-fructose diet activated TLR4 signaling in the liver, leading to increased fibrogenesis, and the severity of MASH was exacerbated [56,57]. According to Takahashi et al., fructose-fed animal models reliably exhibit pronounced hepatic steatosis and increased hepatic expression of inflammatory cytokines, such as IL-6 and TNF-α [58]. Kawasaki et al. reported that rats fed a high-fructose diet developed significantly increased macrovesicular steatosis, hepatic triglyceride accumulation, and lobular inflammation. These animals also exhibited an increased liver-to-body weight ratio and higher prevalence of lipogranulomas, closely mirroring the histopathological features of MASH in humans [59]. These findings highlight the harmful effects of a high-fructose diet on liver pathology and disease progression (Figure 1).

Recent findings from an ongoing study by our group (Osaka Metropolitan University) suggest that a high-fructose diet and decreased male hormone levels following castration synergistically exacerbated hepatic steatosis in mice. This effect appears to be mediated, at least in part, by disruptions in gut microbiota homeostasis, emphasizing the intricate interplay among diet, hormonal regulation, and gut–liver axis disturbances in MASH progression. Future research should aim to elucidate the precise molecular mechanisms

linking fructose metabolism, gut–liver axis dysfunction, and hormonal regulation to identify potential therapeutic strategies for mitigating MASLD/MASH progression.

2.2.2. Glucose

Glucose is the primary circulating sugar, and its hepatic metabolism is closely regulated by insulin. Although glucose can contribute to DNL, especially under conditions of high carbohydrate intake and hyperinsulinemia, it is generally considered less lipogenic than fructose on a gram-for-gram basis [42]. Despite being less lipogenic, excessive glucose intake, particularly from refined sources, contributes to hyperinsulinemia and lipogenesis, indirectly worsening MASLD. High-glycemic-load diets promote hepatic insulin resistance and fat accumulation. Animal studies have shown that glucose-rich diets aggravate steatosis, particularly when combined with HFDs [60]. Takahashi et al. reported that rats fed a high-fructose/high-glucose diet developed histopathological features consistent with those of humans with MASH, including macrovesicular steatosis, perisinusoidal fibrosis, and lobular inflammation, particularly localized in zone 1 of the hepatic lobule. Notably, this high-fructose/high-glucose diet increased serum alanine aminotransferase (ALT) levels and the hepatic expression of pro-inflammatory cytokines, such as IL-6, despite the animals exhibiting lower body weights than starch-fed controls. This effect was accompanied by increased epididymal adipose tissue weight, mimicking visceral obesity in metabolic syndrome [61]. Diets rich in carbohydrates with a high glycemic index, which causes rapid spikes in blood glucose and insulin levels, can promote DNL and contribute to hepatic steatosis and insulin resistance over time [62]. Considering the overall carbohydrate intake and favoring low-glycemic index sources is crucial for managing MASLD.

2.2.3. Sucrose

Sucrose (table sugar) is a disaccharide consisting of one glucose molecule and one fructose molecule. Upon ingestion, sucrose is rapidly hydrolyzed in the intestine. Consequently, high sucrose intake leads to the same detrimental metabolic effects associated with high glucose and particularly high fructose consumption, including potent stimulation of DNL and contribution to insulin resistance and hepatic fat accumulation [41,42]. Chronic sucrose intake promotes hepatic steatosis and inflammation comparable to high-fructose diets [63]. The additive effects of glucose and fructose accelerate oxidative stress and fibrotic signaling [63,64]. Limiting the intake of added sugars, including sucrose, is a key dietary strategy for managing MASLD.

3. Effects of Food Bioactive Compounds and Micronutrients on MASLD

In addition to macronutrients, various bioactive compounds and micronutrients are being investigated. Bioactive compounds derived from plant-based foods have shown considerable therapeutic potential in modulating the pathogenesis of MASLD by targeting key pathological mechanisms, including oxidative stress, inflammation, metabolic dysregulation, and fibrosis. Micronutrients, including essential vitamins and trace elements, modulate the pathophysiology of MASLD by regulating oxidative stress responses, enhancing insulin sensitivity, modulating lipid metabolism, and attenuating hepatic inflammation and fibrosis.

3.1. Bioactive Ingredients in Beverages

Bioactive compounds found in common beverages, such as coffee, tea, and plant-based drinks, have garnered significant interest for their potential roles in modulating the pathogenesis of MASLD.

3.1.1. Caffeine and Chlorogenic Acid

Caffeine and CGA are major bioactive compounds found in coffee, tea, and certain plant-based foods that have gained increasing attention, given their potential hepatoprotective effects in MASLD. Several epidemiological studies have suggested that habitual coffee consumption is inversely correlated with the progression of MASH, including reduced risk of fibrosis and hepatocellular carcinoma [65–68]. These beneficial effects appear to be mediated by both independent and combined actions of caffeine and CGA, which exert regulatory effects on lipid metabolism, oxidative stress, inflammation, and fibrogenic pathways. However, clinical and observational studies have primarily focused on whole coffee consumption, and experimental studies are required to elucidate the precise molecular mechanisms through which these compounds exert their hepatoprotective effects. A growing body of clinical and epidemiological evidence suggests that caffeine and CGA intake are associated with improvements in hepatic steatosis, inflammation, and metabolic health. These benefits are reflected in reductions in the controlled attenuation parameter and improvements in key clinical indicators, such as serum triglyceride levels and body mass index (Figure 2).

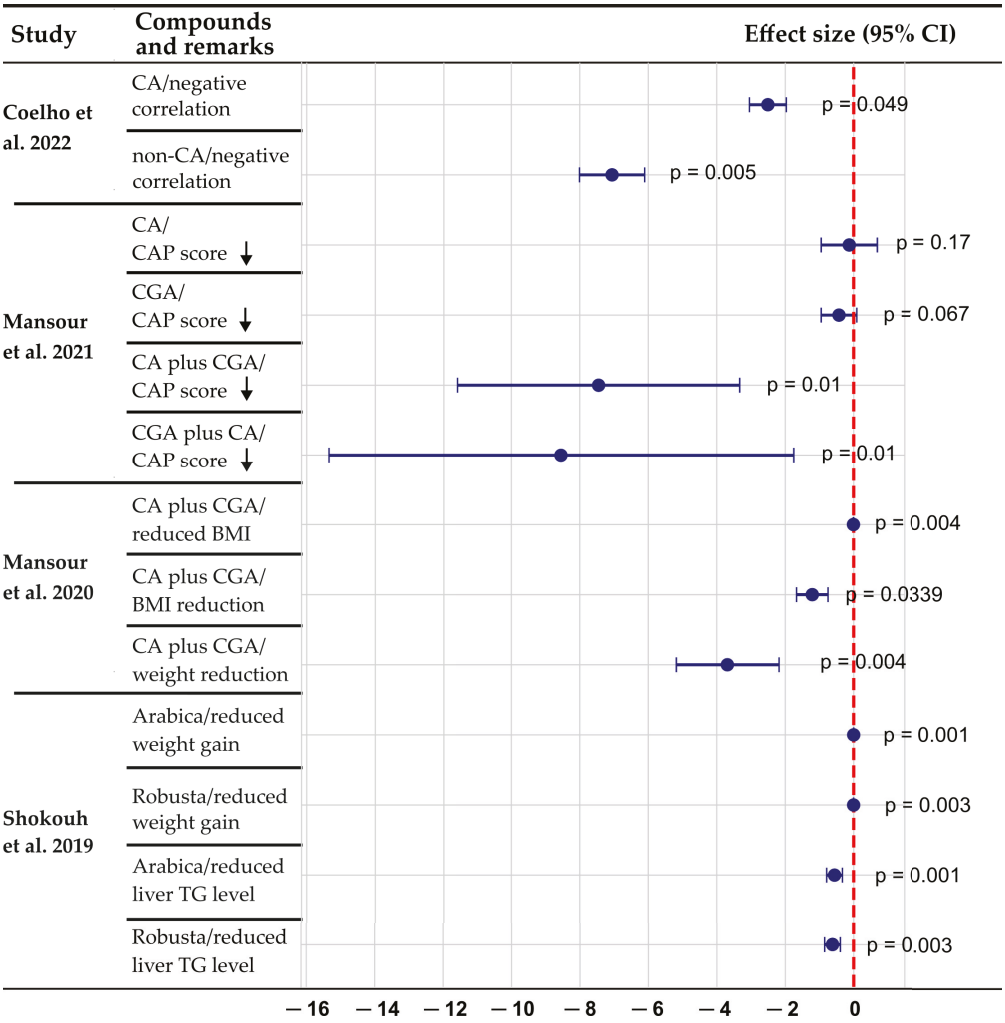


Figure 2. A forest plot of the association between coffee components and MASLD parameters. Negative values indicate the beneficial effects of coffee/caffeine and chlorogenic acid on MASLD parameters. The combination of caffeine plus chlorogenic acid appears to have stronger effects than

either component alone. Both robusta and arabica coffee varieties show significant benefits for liver triglyceride levels and weight reduction. *p*-values displayed on the right side of each data point indicate statistical significance of the observed effects, with values < 0.05 considered statistically significant. Data compiled from Coelho et al. (2022) [69], who reported negative correlations between fatty liver index and caffeine metabolites ($p = 0.049$, $p = 0.005$); Mansour et al. (2021) [70], who demonstrated effects of caffeine and chlorogenic acid on controlled attenuation parameter scores (p values ranging from 0.01 to 0.17); Mansour et al. (2020) [71], who showed significant BMI and weight reduction with caffeine plus chlorogenic acid supplementation ($p = 0.004$, $p = 0.0339$); and Shokouh et al. (2019) [72], who documented reduced liver triglyceride levels and weight gain with both arabica and robusta coffee varieties (*p* values ranging from 0.001 to 0.003). Downward arrow indicates a decrease of CAP score. BMI, body mass index; CA, caffeine; CAP, controlled attenuation parameter; CGA, chlorogenic acid; TG, triglyceride.

Most experimental studies on caffeine and CGA have reported inhibitory effects on MASLD/MASH [73–79]. Several animal studies have demonstrated that caffeine intake improves hepatic lipid metabolism by reducing DNL and enhancing fatty acid β-oxidation [73,74,76,77]. However, several studies have reported exacerbating effects [77,80–85].

Xin et al. showed that in an MASH mouse (C57BL/6J) model fed a high-fat high-carbohydrate diet, caffeine significantly reduced hepatic triglyceride accumulation by suppressing SREBP-1c and ChREBP, key regulators of lipogenesis, while upregulating PPARα and carnitine palmitoyl transferase (CPT)1, which promote lipid oxidation [76]. Velázquez et al. reported similar findings in rats fed an HFD plus 10% liquid fructose, showing that caffeine activated AMP-activated protein kinase (AMPK) signaling, leading to reduced hepatic lipid accumulation and improved insulin sensitivity [77] (Figure 1).

According to other animal studies, CGA counteracts hepatic steatosis by suppressing lipogenesis and promoting β-oxidation [75,78]. Xu et al. showed that CGA administration in HFD-fed obese mice significantly reduced hepatic triglyceride content by activating AMPK signaling and suppressing SREBP-1c and the liver X receptor (LXR)α [78].

The molecular mechanisms and pathways through which caffeine and CGA exert their antioxidant, anti-inflammatory, and antifibrotic effects have been detailed in previous reviews [86,87]. These mechanisms include the modulation of oxidative stress, the suppression of inflammation via crosstalk between IL-6 production in muscle and liver signal transducer and activator of transcription (STAT)3 activation, and the inhibition of fibrogenic pathways.

In a combined in vivo and in vitro study, Yang et al. demonstrated that CGA attenuates liver fibrosis by inhibiting the microRNA (miR)-21-regulated TGF-β1/mothers against the decapentaplegic homolog (Smad)7 signaling pathway. This inhibition resulted in the downregulation of alpha-smooth muscle actin (α-SMA) and collagen I expression in liver tissue, thereby mitigating fibrotic progression [79] (Figure 1).

Recent systematic reviews, including the one by Salvoza et al., have emphasized evidence of the effects of caffeine and CGA on MASH [80]. This review analyzed five experimental studies on the effects of caffeine and CGA; however, two of these studies reported no significant effect of caffeine or CGA administration in reducing the risk of progression to more severe stages of MASLD or MASH [77,85].

Our group previously reported that caffeine and CGA exacerbated liver inflammation and fibrosis in mice fed a choline-deficient, L-amino acid-defined HFD (CDAHFD). Both caffeine and CGA showed exacerbating effects attributed to the absence of significant changes in the expression of key lipid metabolism-related genes, including *Cpt1a*, *Srebp-1c*, and *fatty acid synthase* (*Fasn*) mRNA across experimental groups. Moreover, caffeine and CGA supplementation significantly worsened markers of liver cell injury and inflammation,

as evidenced by elevated serum ALT levels and an increased number of CD45R-positive cells in the liver. IL-6 may be associated with exacerbated effects because hepatic expression levels of *IL-6* mRNA were significantly higher in the caffeine group than in the CDAHFD group. However, the association was not definitive since *IL-6* mRNA expression levels in the caffeine group were almost the same as in the control diet group [81].

Hu et al. reported that prenatal caffeine exposure in rats increased the susceptibility of female offspring to MASLD through activation of the glucocorticoid receptor-CCAAT/enhancer binding protein-sirtuin 1 (GR-C/EBP-SIRT1) pathway [82]. However, conflicting findings have been reported in human studies. For instance, in a study involving individuals who were overweight or obese with type 2 diabetes, although caffeine metabolites were inversely correlated with the fatty liver index, direct coffee consumption was not significantly associated with MASLD parameters, including fibrosis or steatosis severity [83].

Mubarak et al. reported that CGA supplementation did not confer protective effects against metabolic syndrome features in obese mice fed an HFD. Notably, mice receiving CGA supplementation alongside the HFD exhibited increased hepatic lipid content and worsened steatosis compared to those on the HFD alone, suggesting impaired fatty acid oxidation [84].

The heterogeneity of the study conditions (e.g., experimental models, dosages, duration of treatment, and methodological approaches) likely contributed to the discrepancies observed in the research investigating the effects of caffeine and CGA on MASLD and MASH. Further investigations are required to clarify the distinct and combined effects of caffeine and CGA administration on MASH pathogenesis and progression across different species, age groups, and sexes.

3.1.2. Ethanol

Although MASLD diagnosis requires the exclusion of considerable alcohol consumption, the interaction between metabolic risk factors and alcohol intake is being increasingly recognized. Modest alcohol consumption in individuals with MASLD may accelerate disease progression compared to abstaining from alcohol [88]. This combined pathological effect is thought to involve multiple mechanisms, including enhanced oxidative stress, dysregulated lipid metabolism, and immune activation [89]. Alcohol metabolism generates reactive oxygen species and acetaldehyde; increases the nicotinamide adenine dinucleotide (NADH/NAD+) ratio, favoring steatosis; impairs mitochondrial function; increases gut permeability, leading to elevated endotoxin (lipopolysaccharide) levels; and sensitizes the liver to inflammatory stimuli [90,91].

Although MASLD/MASH is distinct from alcoholic liver disease, the emerging evidence has suggested that ethanol consumption may have paradoxical effects on disease progression. However, clinical and epidemiological studies have reported conflicting outcomes, meaning that the effect of alcohol intake on MASLD and MASH remains highly controversial. For example, studies have reported that low doses of alcohol may have protective effects, but others have demonstrated the exacerbation of liver injury at higher levels of consumption [88,92–101]. Although excessive alcohol consumption is a well-established driver of hepatic inflammation, fibrosis, and disease progression, some epidemiological studies have suggested that low-to-moderate alcohol intake, particularly red wine, may exert hepatoprotective effects [94–98]. However, other studies have contradicted these findings, indicating that even light-to-moderate alcohol intake may exacerbate fibrosis and hepatic injury [88,99–101]. Light alcohol consumption was generally defined as alcohol intake of <20 g/day, and abstinence was defined as no alcohol intake during a study.

A recent meta-analysis of cohort studies reported that moderate alcohol consumption increased the risk of advanced fibrosis (pooled odds ratio 1.56; 95% confidence interval 1.08–2.26) and hepatocellular carcinoma (hazard ratio 1.39; 95% confidence interval 1.22–1.57) in patients with MASLD. However, this association was not observed in cross-sectional studies. Notably, although alcohol use was linked to a higher risk of cirrhosis and hepatocellular carcinoma, it appeared to lower the risk of steatohepatitis [92]. Given the inconsistencies in human studies, future studies should aim to construct experimental models to delineate the direct effects of ethanol on MASH pathophysiology.

Several experimental studies have investigated the effects of ethanol in conjunction with HFDs [102–104]. The first investigation of the effects of low-dose ethanol on MASH was conducted by our group using an HFD-fed db/db mouse model. The findings demonstrated that short-term (8-week) light ethanol administration (a high-fat liquid diet supplemented with 0.1% ethanol ad libitum) significantly improved MASH parameters, including reductions in serum aspartate aminotransferase (AST) and ALT levels, and hepatic inflammation. The number of CD45R- and myeloperoxidase-positive inflammatory cells in both the hepatic lobule and portal tract was markedly lower in the low-ethanol group than in the HFD group [105].

Daniels et al. examined binge ethanol consumption in multiple MASH model mice fed an HFD, a high-fat diet with cholesterol, and a high-fat/cholesterol diet with trans fats. Their results revealed that ethanol exacerbated the inflammatory and fibrogenic properties of an atherogenic diet-induced MASH model, suggesting that alcohol acts synergistically with metabolic stressors to accelerate hepatic injury [106]. Yeh et al. demonstrated that a single ethanol binge (5 g/kg body weight) induced severe liver injury, evidenced by marked elevations in AST and ALT, in C57BL/6J mice fed a Western diet for 3 weeks [107]. However, these studies primarily examined high ethanol doses (15–31.25% ethanol), limiting their relevance to the potential short-term effects of low or moderate alcohol consumption.

Although light-to-moderate ethanol intake may exert hepatoprotective effects, the long-term effects remain uncertain. Researchers should aim to develop well-characterized MASH animal models to investigate the effects of low levels of alcohol consumption over the long and short term to determine whether it is beneficial or contributes to disease progression. Abstinence from alcohol is generally recommended for patients with MASLD [108], and the World Health Organization's global status report on alcohol and health in 2018 specifically indicates that no amount of alcohol is safe [109].

3.1.3. Tea and Its Ingredients

Green tea is a popular beverage worldwide, especially in Asia. Green tea contains various bioactive compounds, including polyphenols. Among them, catechins (a kind of polyphenol) are known for their health-promoting effects. Tea catechins include epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate (EGCG). Of these, EGCG, in particular, is highly bioactive and accounts for approximately 50% of the total polyphenols in tea leaves [110].

Green tea extract was found to prevent MASLD in mice, and the preventive effects were attributed to the activation of mitochondrial respiratory chain complexes, SIRT1, and AMPK [111–113]. EGCG improved MASLD/MASH in mice and rats, and the effects were associated with lower concentrations of profibrogenic, oxidative, and pro-inflammatory mediators and a decreased absorption of bile acids and lipids [114–116].

Several clinical trials have shown the effects of tea and its ingredients on MASLD/MASH. For example, a double-blind, placebo-controlled, randomized clinical trial found that green tea extract (GTE) supplementation (500 mg GTE tablet per day) significantly reduced ALT and AST levels after a 12-week period, whereas the placebo did not [117]. However, this

study had limitations because the number of patients was relatively small (80 patients), and the observational period was short. In a double-blind, randomized, controlled clinical trial, sour tea supplementation (450 mg capsule per day) significantly decreased serum triglyceride, ALT, and AST levels compared with the placebo [118]. However, this study also had limitations due to the small number of patients (70 patients) and short observational period (8 weeks). In a double-blind placebo-controlled study, the consumption of 700 mL of green tea with high-density catechins (1080 mg catechins) per day significantly decreased body fat and serum ALT levels and significantly improved the liver-to-spleen computed tomography attenuation ratio compared with the placebo and green tea with low-density catechins (200 mg catechins) [119]. However, clinical data on the effects of the ingredients of tea on MASLD are scarce, and further studies are required.

3.2. Polyphenols

Polyphenols are a diverse group of plant-derived compounds widely recognized for their potent antioxidant, anti-inflammatory, and metabolic regulatory properties. Notably, curcumin, resveratrol, and silymarin have been extensively studied in preclinical and clinical settings. These representative polyphenols have demonstrated potential in preventing hepatic steatosis and attenuating disease progression, making them attractive candidates for adjunctive dietary or therapeutic interventions in MASLD. A summary of their reported effects is presented in Table 2.

Table 2. Summary data from studies using polyphenol interventions for MASLD.

Nutrient and Compound	Intervention	Main Findings	Remarks	Ref.
Curcumin	1500 mg/day (clinical); varied preclinically	↓ Steatosis, ↓ inflammation, ↓ fibrosis via AMPK, Nrf2, TGF-β pathways	Bioavailability and dose variability	[120–132]
Resveratrol	Varied; often limited by bioavailability	↑ SIRT1, ↑ AMPK, ↓ lipids, ↓ inflammation	Mixed clinical results	[133–137]
Silymarin	30–80 mg/kg (preclinical)	↓ Lipids, ↓ 7-KDCA, modulates FXR	Mixed histological outcomes	[138–143]
Hesperidin	Varied; often 500–1000 mg/day for 12 weeks	Improved metabolic parameters; reduced liver enzymes (ALT, AST); decreased inflammatory markers	Limited clinical studies	[144–151]

ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; AST, aspartate aminotransferase; FXR, farnesoid X receptor; 7-KDCA, 7-ketodeoxycholic acid; MASLD, metabolic dysfunction-associated steatotic liver disease; Nrf2, nuclear factor erythroid 2-related factor 2; SIRT1, sirtuin 1; TGF, transforming growth factor.

3.2.1. Curcumin

Curcumin, the principal curcuminoid derived from turmeric (*Curcuma longa*), has garnered attention for its hepatoprotective properties in MASLD. Preclinical studies have demonstrated that curcumin can attenuate MASLD/MASH by modulating lipid metabolism, reducing oxidative stress, and suppressing inflammatory pathways. Specifically, curcumin inhibits the nucleotide-binding domain, leucine-rich-containing family, and pyrin domain-containing-3 (NLRP3) inflammasome and activates AMPK and nuclear factor erythroid 2-related factor 2 (Nrf2) pathways, leading to decreased lipid accumulation and inflammation in hepatic tissues [120] (Figure 1).

A meta-analysis of 52 preclinical studies involving 792 animals found that curcumin effectively improved key pathological markers associated with MASH, including hepatic

steatosis, inflammation, and fibrosis [121]. In one of the reviewed studies, curcumin supplementation significantly attenuated the effects of HFDs on glucose disposal, body weight/fat gain, and insulin resistance in C57BL/6J mice over 28 weeks. The study also found that curcumin reduced intrahepatic lipid content and downregulated mRNA levels of ChREBP and SREBP-1c, two key transcription factors involved in hepatic lipogenesis, as well as liver pyruvate kinase, a downstream target of ChREBP [122].

Curcumin was also found to exhibit potent anti-inflammatory properties in MASH rat models by suppressing key pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β. Curcumin reduced oxidative stress by enhancing antioxidant defenses, including increased glutathione levels and decreased malondialdehyde levels, a marker of lipid peroxidation. Curcumin enhanced the efficacy of oxidative stress factors, including superoxide dismutase, sirtuin 2 (Sir2), glutathione peroxidase, and Nrf2 [123] (Figure 1). Elkordy et al. reported that curcumin administration alleviates MASH by modulating endoplasmic reticulum stress, oxidative stress, apoptosis, and autophagy. These effects are achieved through the downregulation of protein kinase RNA-like endoplasmic reticulum kinase (PERK) and C/EBP homologous protein (CHOP) expression, the restoration of Beclin-1 levels, and the improvement of the ratio between Bcl-2-associated X protein (Bax)/B-cell lymphoma 2 protein (Bcl-2) in HFD-induced MASH model mice [124].

Curcumin has also been shown to suppress α-SMA and collagen I protein expression levels in cardiac fibroblasts treated with TGF-β1, indicating that it inhibits fibrogenesis by targeting the TGF-β/Smad3 pathway [125]. Another study found that the hepatoprotective effects of pomegranate extract and curcumin against thioacetamide-induced liver fibrosis are linked to their ability to modulate the Nrf2/heme oxygenase-1 (HO-1), NF-κB, and TGF-β/Smad3 signaling pathways [126]. Collectively, these studies indicate that curcumin attenuates fibrosis by modulating the TGF-β/Smad3 signaling pathway, leading to a significant reduction in the expression of the fibrogenesis markers α-SMA and collagen I.

Numerous clinical and epidemiological studies have investigated whether curcumin can mitigate MASLD and MASH [127–129]. For instance, a randomized, placebo-controlled trial involving 50 patients with MASLD reported that 12 weeks of curcumin supplementation (1500 mg/day) significantly reduced hepatic steatosis, serum liver enzymes, and inflammatory markers compared with a placebo [130]. Another study using a bioavailable form of curcumin, Meriva®, at a dose equivalent to 50 mg/day of pure curcumin for 8 weeks improved serum metabolite profiles associated with MASLD [131]. The results of human trials have indicated that curcumin supplementation may improve serum liver function markers, serum lipid profiles, and markers of steatosis [131].

Despite these promising findings, inconsistent outcomes regarding curcumin efficacy have also been reported. For example, although curcumin significantly reduced serum AST levels (mean difference: −3.36 U/L, 95% confidence interval: −5.35 to −1.36, $p = 0.001$), the magnitude of this effect varied across studies, and the overall heterogeneity was considerable [132]. The inconsistencies in the results may be due to challenges associated with curcumin's low bioavailability or variability in study design. Regardless, further large-scale, controlled trials are required to establish standardized dosing regimens and confirm the therapeutic efficacy of curcumin in MASLD management.

3.2.2. Resveratrol

Resveratrol, a natural polyphenol found in grapes, berries, and peanuts, has been extensively studied for its potential benefits in metabolic disorders, including MASLD [133]. Preclinical studies have shown that resveratrol can improve insulin sensitivity, reduce hepatic lipid accumulation, and exert anti-inflammatory effects by modulating pathways such as SIRT1 and AMPK. SIRT1 activation can improve insulin sensitivity, enhance mito-

chondrial biogenesis and function, promote fatty acid oxidation, and reduce DNL [134] (Figure 1).

However, the clinical evidence remains inconclusive. Several randomized controlled trials have yielded mixed results regarding the efficacy of resveratrol in improving liver function and reducing hepatic steatosis in patients with MASLD [134,135]. Factors contributing to these inconsistencies include variations in study populations, dosages, treatment durations, and the low bioavailability of the compound [136]. To address these challenges, research has focused on enhancing the bioavailability of resveratrol through novel nano-drug delivery systems [137], which may improve its therapeutic potential in MASLD. Nonetheless, further well-designed clinical trials are required to validate these approaches and determine the role of resveratrol in MASLD treatment.

3.2.3. Silymarin

Silymarin, a flavonolignan complex extracted from milk thistle (*Silybum marianum*), has been traditionally used for its hepatoprotective effects. In the context of MASLD, silymarin exhibits antioxidant, anti-inflammatory, and antifibrotic properties. A recent preclinical study revealed that silymarin, administered at a dose of 80 mg/kg, was more effective at mitigating liver steatosis and reducing lipid accumulation than a lower dose of 30 mg/kg silymarin or a combination of silybin and isosilybin A [138]. Notably, silymarin was shown to regulate bile acid metabolism by reducing the concentration of 7-ketodeoxycholic acid (7-KDCA), thereby modulating farnesoid X receptor (FXR) activity through a negative feedback mechanism [138].

Clinical studies have suggested that silymarin can modulate the gut microbiota composition and reduce liver stiffness, potentially through interactions with the FXR pathway [139,140]. However, a meta-analysis of clinical trials found that silymarin may improve liver enzymes (e.g., ALT and AST) in patients with MASLD, but its effects on liver histology (steatosis, inflammation, and fibrosis) are less consistent [141,142]. A randomized, double-blind, placebo-controlled trial demonstrated that silymarin supplementation significantly decreased liver stiffness in patients with MASLD, indicating potential benefits in reducing hepatic fibrosis [140]. However, another study found that essential phospholipids were more effective at improving liver function and reducing fibrosis than silymarin when assessed using FibroScan® [143]. These discrepancies highlight the need for further research to determine optimal dosing strategies and treatment durations and to elucidate the mechanisms underlying the effects of silymarin on MASLD.

3.2.4. Hesperidin

The polyphenol hesperidin is a flavanone glycoside found in citrus fruits that consists of aglycon hesperetin and disaccharide rutinose. Hesperidin possesses antioxidant and anti-inflammatory properties and is expected to have beneficial effects on various diseases [144,145].

Preclinical studies showed that hesperidin prevents MASLD in experimental animals. This was attributed to its inhibition of pyroptosis and endoplasmic reticulum stress-induced inflammation and the promotion of fatty acid β-oxidation via the activation of SIRT1/peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) [146–148]. A recent study showed that hesperetin also prevents MASLD in rats [149].

In a randomized, placebo-controlled, double-blind clinical trial, MASLD patients administered a 1 g hesperidin capsule for 12 weeks showed significant improvement in ALT, γ-glutamyltransferase, total cholesterol, triglyceride, high-sensitivity C-reactive protein, TNF-α, NF-κB, and hepatic steatosis compared with the placebo group [150]. However,

the study has limitations because the number of patients was small (50 patients), and the period of observation was short. A randomized controlled clinical trial with one hundred MASLD patients, which was performed by the same group and used 1 g hesperidin supplementation for 12 weeks, found a significant reduction in body mass index, glucose homeostasis parameters, and hepatic steatosis compared with the control group [151]. Although promising effects were gained in these clinical studies, studies with more patients and a longer observational period are required in the future. Histopathological evaluations via liver biopsy are also desirable.

3.3. Polyamines

Polyamines, such as putrescine, spermidine, and spermine, are small aliphatic amines naturally present in endogenous metabolism and various dietary sources, including whole grains, soy products, mushrooms, and fermented foods. Polyamines have garnered growing interest in the context of MASLD owing to their capacity to influence hepatocellular lipid accumulation, mitochondrial function, and fibrotic progression. In particular, experimental studies have suggested that polyamines confer protective effects against hepatic steatosis and liver injury, highlighting their potential as novel nutritional or pharmacological targets for managing MASLD. A summary of their reported effects is presented in Table 3.

Table 3. Summary data from polypamine intervention studies for MASLD.

Nutrient and Compound	Intervention	Main Findings	Remarks	Ref.
Putrescine	80 mg/kg in mice	↓ Inflammation, ↓ fibrosis via PPARα/γ, NF-κB inhibition	Endogenous levels may correlate with severity	[152–156]
Spermidine	Dietary supplementation (preclinical)	↑ Autophagy, ↓ inflammation, ↑ SIRT1/AMPK	Lack of clinical trials	[157–159]
Spermine	Supplemented or endogenous	↓ Apoptosis, ↑ autophagy, ↓ inflammation (via ATG5)	Limited direct MASLD evidence	[160–163]

AMPK, AMP-activated protein kinase; MASLD, metabolic dysfunction-associated steatotic liver disease; NF-κB, nuclear factor kappa B; PPAR, peroxisome proliferator-activated receptor; SIRT1, sirtuin 1.

3.3.1. Putrescine

Putrescine, the simplest polyamine, is derived from ornithine decarboxylation and serves as a precursor for spermidine and spermine biosynthesis. Clinical studies directly assessing the effects of putrescine supplementation in patients with MASLD are lacking. However, studies using animal models have revealed that the oral administration of putrescine improves gut mucosal morphology by suppressing inflammatory responses [152,153] and augmenting albumin synthesis and mitochondrial DNA copy number in the liver [154]. Our research group recently investigated the role of putrescine supplementation in a CDAHFD-induced db/db mouse model of MASH (unpublished data). Our findings demonstrated that putrescine significantly reduced the markers of inflammation, hepatocellular injury, and fibrosis. Histological evaluation revealed a significant reduction in hepatocyte ballooning, accompanied by a reduction in steatosis grade, intralobular inflammation, perisinusoidal fibrosis, and fibrosis stage in the putrescine-treated group compared with those in the CDAHFD group. The protective effects of putrescine in MASH appear to be partially mediated through the PPAR-α and PPAR-γ signaling pathways.

A recent experimental study by Dasdelen et al. demonstrated that putrescine supplementation reduced liver damage by exerting anti-inflammatory and antioxidant effects,

including NF-κB and IL-6 inhibition [155]. However, another study reported that elevated endogenous hepatic putrescine and increased ornithine decarboxylase levels correlate with greater MASLD severity [156]. Future research should focus on elucidating the regulatory pathways governing putrescine metabolism, optimizing therapeutic dosing strategies, and clarifying the long-term impact of putrescine on liver health.

3.3.2. Spermidine

Spermidine, a naturally occurring polyamine derived from putrescine, plays a pivotal role in cellular homeostasis, autophagy, and mitochondrial functions. Spermidine levels decline with age, and spermidine supplementation has protective effects against various age-related diseases. A key mechanism is the induction of autophagy, including lipophagy, which helps clear lipid droplets and damaged organelles [157]. Preclinical studies have indicated that spermidine can ameliorate hepatic steatosis and inflammation in MASLD models, potentially via autophagy induction and AMPK activation [158,159] (Figure 1). In dietary supplementation models, spermidine has been shown to enhance autophagic flux, reduce hepatic steatosis, and suppress the expression of pro-inflammatory cytokines, such as TNF-α and IL-1β, thereby mitigating liver injury and fibrosis [159] (Figure 1). Moreover, spermidine's ability to modulate lipid metabolism through SIRT1 activation and the mammalian target of rapamycin inhibition is associated with improved hepatic insulin sensitivity and reduced oxidative stress.

Although these findings collectively highlight the promising therapeutic potential of spermidine, especially as a modulator of autophagy and inflammation, robust clinical trials are lacking. Such trials will be required to elucidate the long-term safety profile and optimal dosing and identify interactions with polyamine metabolic pathways. A deeper understanding of these mechanisms is critical for translating preclinical success into viable dietary interventions for managing MASLD.

3.3.3. Spermine

Spermine, a naturally occurring polyamine synthesized from spermidine, is involved in cellular homeostasis, including the regulation of oxidative stress, apoptosis, and immune responses. Preclinical studies have demonstrated that spermine supplementation can alleviate acute liver injury by inhibiting pro-inflammatory responses in liver-resident macrophages via ATG5-dependent autophagy pathways [160]. Spermine has also been shown to enhance liver barrier function, modulate amino acid transporters, and suppress apoptosis in animal models, suggesting hepatoprotective effects [161].

Kukoamines A and B are polyamine-conjugated alkaloids structurally linked to a spermine-like backbone. While spermine modulates immune cell polarization and enhances autophagy, kukoamines A and B reduce hepatic steatosis and oxidative stress by downregulating SREBP-1c and pro-inflammatory cytokines [162,163]. Despite these promising findings, direct clinical evidence of the efficacy of spermine in managing MASLD remains limited. Further research, including well-designed clinical trials, is required to elucidate the therapeutic potential and safety profile of spermine supplementation in patients with MASLD.

3.4. Vitamins

Vitamins play essential roles in maintaining hepatic function and systemic metabolic balance, and several vitamins have emerged as promising candidates for therapeutic intervention in MASLD. Vitamins D, E, and C have been extensively investigated for their potential to mitigate oxidative stress, inflammation, and fibrogenesis associated with steatosis and steatohepatitis. Relevant findings from the reviewed preclinical and clinical studies are summarized in Table 4.

Table 4. Summary data of the reviewed studies on vitamin and essential trace element interventions for MASLD.

Nutrients and Compounds	Intervention	Main Findings	Remarks	Ref.
Vitamin D	Various oral regimens	↓ Inflammation, ↑ insulin sensitivity, modulates vitamin D receptor pathways	Inconsistent clinical outcomes	[164–174]
Vitamin E	800 IU/day (PIVENS)	↓ Oxidative stress, ↓ inflammation, ↓ ballooning	Potential risk at high doses	[175–179]
Vitamin C	Supplement or dietary	↓ Reactive oxygen species, regenerates vitamin E, synergistic antioxidant	Limited as monotherapy	[180–184]
Zinc	Dietary supplement (varied)	↓ Inflammation, ↑ metabolism, antioxidant	Results depend on duration and diet	[185–192]
Selenium	Dietary/co-supplemented	↑ Glutathione peroxidase activity, ↓ oxidative stress	Narrow therapeutic window	[193–196]
Sodium	Dietary intake long-term observational survey	Higher or suboptimal intake of magnesium, potassium, and calcium was common in MASLD patients, highlighting targets for dietary intervention	Limited experimental observation	[197–199]

PIVENS, pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease.

3.4.1. Vitamin D

Alongside its classical role in calcium homeostasis, vitamin D also influences insulin signaling, lipid metabolism, and immune modulation, with deficiencies frequently observed in patients with MASLD. Vitamin D deficiency, particularly low serum levels of 25-hydroxy vitamin D [25(OH)D] (optimal value: $\geq$30 ng/mL), is prevalent among individuals with MASLD and has been consistently associated with increased hepatic steatosis, insulin resistance, and inflammatory activity [164]. Several observational studies have reported that reduced 25(OH)D levels are correlated with greater severity of steatosis, inflammation, and fibrosis, further linking vitamin D status to the progression of liver disease and broader metabolic dysfunction [164–167]. Vitamin D receptor activation exerts anti-inflammatory effects, improves insulin sensitivity, and modulates the activation of hepatic stellate cells [168]. However, clinical trials of vitamin D supplementation in MASLD have produced inconsistent results with regard to improvements in liver histology and biochemical markers, suggesting a complex relationship influenced by factors such as dosage, baseline levels, and patient genetics [169]. Clinical trials have primarily examined the effects of vitamin D supplementation on the serum concentrations of inflammatory markers and lipid profiles in patients with MASLD [165,170,171]. In these clinical trials, subjects were orally administered 50,000 IU of vitamin D3 (cholecalciferol) weekly; 50,000 IU of vitamin D3 every 14 days; or 2000 of IU vitamin D3 daily. In a randomized controlled trial, Barchetta et al. found that oral vitamin D supplementation did not significantly improve hepatic steatosis or metabolic parameters in patients with MASLD or type 2 diabetes, indicating limited therapeutic efficacy in this subgroup [165].

Rodent studies have suggested that vitamin D deficiency or supplementation significantly influences MASH development and progression. Cordeiro et al. investigated the effects of vitamin D supplementation in Western diet-fed obese rats. Their findings revealed that vitamin D supplementation reduced visceral adiposity and normalized leptin

and circulating TNF-α levels, suggesting a protective role against diet-induced obesity and inflammation [172]. Other experimental studies have indicated that vitamin D and its receptor play crucial roles in suppressing fibrogenic signaling, thereby attenuating hepatic fibrosis [173]. In addition, vitamin D regulates miRNAs involved in liver lipid metabolism and hepatocyte turnover, including miR-122, miR-34a, and miR-21 [174].

In conclusion, vitamin D supplementation appears to confer protective effects against metabolic disturbances associated with MASH. However, the clinical findings underscore the need for further well-designed clinical trials to clarify its role as a therapeutic agent for managing MASLD.

3.4.2. Vitamin E

Vitamin E (α-tocopherol), a lipid-soluble antioxidant, improves liver histology in non-diabetic patients with MASH by reducing oxidative injury and inflammatory responses [175]. As a major lipophilic antioxidant, vitamin E protects cell membranes from lipid peroxidation because oxidative stress is a key driver of MASH progression [176]. The PIVENS trial demonstrated that, compared with a placebo, vitamin E (800 IU/day) improved liver histology (steatosis, inflammation, and ballooning) in non-diabetic adults with MASH [176]. Based on this study outcome, guidelines recommend vitamin E for this specific patient subgroup [177]. Vitamin E supplementation has also been associated with reductions in the serum markers of liver inflammation and improvements in liver histology in patients with MASLD [178]. However, concerns about potential long-term risks at high doses (e.g., increased all-cause mortality and hemorrhagic stroke risk) warrant caution and limit its widespread use [179].

3.4.3. Vitamin C

Vitamin C (ascorbic acid), a water-soluble antioxidant, has garnered attention for its potential hepatoprotective effects in MASLD because of its capacity to neutralize reactive oxygen species and regenerate vitamin E. Given the prominent role of oxidative stress in MASLD pathogenesis, the antioxidant properties of vitamin C are of particular interest.

Preclinical studies have demonstrated that vitamin C supplementation mitigates hepatic steatosis and oxidative stress. For instance, in a choline-deficient diet-induced MASLD rat model, vitamin C administration markedly reduced the histological alterations associated with fatty liver disease. Moreover, the role of vitamin C in regenerating other antioxidants, such as vitamin E, indicates that it has potential synergistic effects in combating oxidative liver damage [180]. In addition, vitamins C and E can exert antioxidative effects at different sites because of their respective hydrophilic and lipophilic qualities [181].

Clinical investigations have yielded promising results. A randomized controlled trial involving patients with NAFLD reported that daily oral vitamin C supplementation improved liver health and associated metabolic parameters, including glucose metabolism and adiponectin levels [182]. A cross-sectional study also found that higher dietary vitamin C intake was inversely associated with the risk of MASLD, suggesting a protective role for vitamin C against liver fat accumulation [182,183].

Despite these encouraging findings, the evidence remains inconclusive regarding the efficacy of high-dose vitamin C supplementation as a standalone treatment for MASLD. Some studies have suggested that vitamin C is beneficial when combined with other antioxidants, such as vitamin E or N-acetylcysteine. However, dedicated trials are required to clarify its role and optimal dosage [110,184]. Furthermore, although observational studies have indicated a potential protective association between serum vitamin C levels and MASLD risk [183], a causal relationship has not yet been established. This should be the focus of future Mendelian randomization analyses.

In summary, vitamin C exhibits potential hepatoprotective properties in the context of MASLD through its antioxidant effects and its involvement in metabolic processes. However, further well-designed clinical trials are required to determine its therapeutic efficacy, optimal dosing strategies, and long-term safety for managing MASLD.

3.5. Essential Trace Elements

Deficiencies in essential trace elements involved in antioxidant defense and metabolic regulation have been implicated in MASLD. Among them, zinc and selenium have garnered considerable interest in MASLD research. They regulate oxidative stress, inflammation, and insulin signaling, which are key disease progression mechanisms. A summary of the relevant studies is presented in Table 4.

3.5.1. Zinc

Zinc is a cofactor of numerous enzymes, including the antioxidant enzyme copper–zinc superoxide dismutase, and it plays a role in insulin signaling and immune function [185]. Zinc deficiency is common in patients with chronic liver disease and may exacerbate oxidative stress and insulin resistance in MASLD [186]. Some studies have suggested that zinc supplementation alone or combined with other antioxidants may improve liver enzymes and metabolic markers in patients with MASLD [187].

Zinc, an essential trace element, plays a critical role in various biological processes, including immune function, antioxidant defense, and lipid metabolism. The evidence from rodent studies indicates that zinc homeostasis significantly influences MASH development and progression. Preclinical research has suggested that zinc deficiency exacerbates hepatic steatosis, oxidative stress, and inflammation, which are key drivers of MASH pathology [188–191]. Zinc supplementation has potential hepatoprotective effects, including the regulation of lipid metabolism and the attenuation of inflammatory responses. The experimental models show conflicting results regarding the efficacy of zinc supplementation in mitigating MASH-related hepatic impairment. Although several studies have suggested beneficial effects on lipid and glucose metabolism, others have indicated limited or no impact on the metabolic dysregulation associated with HFD-induced liver disease [192].

Zinc can alleviate metabolic disturbances induced by an HFD, particularly lipid and glucose homeostasis. Qi et al. reported that dietary zinc supplementation in HFD-fed mice and zinc treatment in HepG2 cells effectively attenuated hepatic steatosis and improved glucose and lipid metabolism, suggesting a regulatory role for zinc in hepatic function and metabolic pathways [192]. Conversely, Bolatimi et al. found no significant differences in the expression levels of hepatic function and fatty acid transport/metabolism-related genes, such as the hepatocyte nuclear factor (Hnf)4α and PPARα, between the control and zinc-treated groups. The authors attributed these discrepancies to the shorter duration of zinc treatment (8 weeks) compared with longer-term supplementation studies, which may be necessary to observe significant metabolic improvement.

Inconsistent findings in experimental models suggest that the efficacy of zinc supplementation in MASH may depend on several factors, including treatment duration, dosage, dietary composition, and co-supplementation with other micronutrients. Further well-designed long-term studies are needed to clarify the precise role of zinc in metabolism and its therapeutic potential in managing MASH.

3.5.2. Selenium

Selenium is an essential component of selenoproteins, including glutathione peroxidase, which are critical antioxidant enzymes [193]. Selenium deficiency can impair antioxidant defenses. Although some studies have linked low selenium status to increased MASLD risk or severity [193,194], other studies have reported conflicting findings or poten-

tial toxicity with high selenium intake [195]. Co-supplementation of zinc and selenium in high-calorie diet-fed rats reduced the serum levels of ALT and AST, fasting plasma glucose and insulin, homeostatic model assessment for insulin resistance (HOMA-IR), vascular endothelial growth factor, and malondialdehyde, further supporting the hepatoprotective effects of both the essential trace elements zinc and selenium [195,196]. However, more in-depth studies are required to fully understand the role of selenium supplementation in MASLD and optimize its use.

3.5.3. Sodium

The association between dietary sodium and MASLD has garnered attention. According to a recent retrospective data search, subjects with MASLD exceeded the recommended daily allowance (RDA) for sodium (241% of RDA) [197]. A recent longitudinal cohort study also suggested that high sodium intake in patients with MASLD is associated with increased disease incidence but decreased all-cause mortality [198]. A recent bidirectional Mendelian randomization study revealed a significant association between "salt added to food" and increased MASLD risk (odds ratio: 1.5–2.1), and subsequently, "salt added to food" was considered a causal risk factor for MASLD [199]. Further experimental studies are required to evaluate the effects of dietary sodium on MASLD and identify the associated mechanisms.

4. Discussion and Conclusions

This paper comprehensively reviews the effects of various food nutrients and bioactive compounds against MASLD. However, as this was a narrative review, the method used for the literature retrieval was not systematic and may have been biased. Furthermore, while this review focuses on MASLD, it is important to note that an increasing body of research indicates that natural compounds, such as flavonoids, resveratrol, saponins, and β-carotene, exert protective effects against alcoholic liver disease (ALD), as well as MASLD, and could thus be used in targeted therapies [200]. This is probably because MASLD and ALD share numerous common mechanisms [201].

Natural compounds have limitations that must be considered: (1) The clinical efficacy of most natural compounds is limited due to their low bioavailability. (2) Defining the most appropriate drug form and dosage is difficult without standardized pharmaceutical technology. Thus, the optimal liver protection effect cannot be realized. (3) Most compounds have not been subjected to double-blind, placebo-controlled clinical trials to evaluate their efficacy, and the mechanisms of action of natural compounds are still unclear because studies are limited [202]. Consequently, there are safety concerns [203]. In addition, most peer-reviewed publications are biased toward positive results, and challenges are generally not discussed [204].

Oral administration is the most commonly used method for nutrients, but other methods must be considered, depending on the patient's condition. Optimal dosing strategies, safety profiles, potential adverse effects, drug–nutrient interactions, and long-term safety should also be considered, as acceptable daily intake is set for many nutrients, and overdose may cause various toxic effects. For example, caffeine overdose can cause dizziness, increased heart rate, insomnia, and diarrhea. Caution is especially necessary in long-term administration.

MASLD and its more progressive form, MASH, represent a global health crisis. As pharmacological therapies for MASLD have not been established, attention has increasingly shifted to dietary interventions and nutritional strategies as viable therapeutic avenues. This comprehensive review underscores the multifaceted role of macronutrients, bioac-

tive compounds, and micronutrients in mitigating or exacerbating MASLD pathogenesis and progression.

The primary causes of conflicting results in animal experiments are differences in animal models (e.g., species and strain, diet) and experimental methods (e.g., dosage, route of administration, and observational period). Researchers should use the most appropriate animal model for the study and the experimental method applicable to humans. The primary causes of conflicting results in clinical trials are the differences in the subjects (e.g., age, sex, comorbidities, and lifestyle) and study design (e.g., blinding, randomization, number of subjects, administration method, and dosage). A study design with minimal bias and the inclusion of many subjects is desirable. Researchers should specify the cause when conflicting results appear and determine the most reliable data.

The conclusions of this review are based primarily on preclinical studies. The gap between animal models and human disease is particularly relevant for MASLD research. Consequently, if researchers use animal models whose pathophysiology differs from human MASLD, translating the results to humans is difficult. Vacca et al. [205] generated an unbiased ranking of murine dietary models based on their proximity to MASLD and showed that Western diets align closely with human MASH. It is essential to use reliable animal models to translate the results of preclinical studies to humans.

The evidence consistently indicates that dietary fat, particularly the quality and type of fatty acids, significantly affects hepatic lipid metabolism and inflammation. PUFAs, especially DHA, have potent antisteatotic, anti-inflammatory, and antifibrotic properties in preclinical models, making them promising therapeutic candidates. However, further research is required to establish optimal intake parameters, including effective dosing and appropriate n-6/n-3 PUFA ratios, for managing MASLD. In contrast, excessive intake of SFAs and iTFAs exacerbates hepatic steatosis and inflammatory responses via mechanisms involving endoplasmic reticulum stress, TLR activation, and mitochondrial dysfunction. These findings support the current dietary guidelines advocating for reducing SFA and TFA intake in individuals with MASLD. However, studies have confirmed that extremely low-fat diets, including those very low in saturated fats, can impair the absorption of fat-soluble vitamins (A, D, E, and K) and affect hormone production or lead to metabolic imbalances.

Carbohydrate quality is another important factor to consider when managing MASLD. High fructose intake, particularly from sugar-sweetened beverages, is strongly associated with increased DNL, insulin resistance, and gut–liver axis disruption, thereby promoting MASH development. Although glucose and sucrose are less lipogenic than fructose alone, their combined metabolic burden contributes to hepatic fat accumulation and fibrosis. Therefore, limiting the consumption of high-glycemic foods and added sugars remains a crucial dietary goal.

In addition to macronutrients, numerous bioactive compounds exhibit context-dependent effects on liver pathology. Polyphenols such as curcumin, resveratrol, and silymarin generally exert hepatoprotective effects through anti-inflammatory, antioxidative, and antifibrotic mechanisms. However, the bioavailability limitations and conflicting clinical trial results warrant cautious interpretation and highlight the need for improved delivery systems and standardized protocols. Likewise, compounds such as caffeine and CGA exert protective and exacerbating effects, depending on the dosage, treatment duration, and experimental model. These inconsistencies highlight the necessity for rigorous and well-controlled investigations to delineate their exact role in MASLD therapy.

Polyamines, particularly putrescine, spermidine, and spermine, have emerged as novel dietary modulators of hepatic metabolism and immune regulation. Although animal studies have suggested beneficial roles in reducing hepatic inflammation and fibrosis, the

duality of endogenous versus exogenous effects and the lack of clinical trials underscore the need for mechanistic clarity and translational research.

Micronutrients, including vitamins D, E, and C, and trace elements, such as zinc and selenium, also play critical roles in the pathophysiology of MASLD. Vitamin E is the supplement most widely shown to improve liver histology among non-diabetic patients with MASH. In preclinical studies, vitamin D and zinc show promising anti-inflammatory and antifibrotic effects, but the human data are inconsistent. Although essential, selenium has a narrow therapeutic window and exhibits potential effects when co-supplemented with zinc. Therefore, individualized assessments and supplementation strategies are recommended.

In summary, the current body of evidence supports the implementation of diets rich in unsaturated fatty acids, polyphenols, and essential micronutrients while optimizing harmful fats and simple sugars for managing MASLD. However, there are considerable gaps in the literature regarding the dosage, bioavailability, long-term safety, and efficacy of the different potential components of such diets in diverse populations. Future research should focus on well-designed clinical trials, standardized nutritional protocols, and integrative approaches considering genetic, environmental, and lifestyle factors to establish evidence-based dietary guidelines for MASLD prevention and treatment. In particular, DHA and vitamin E should be further investigated using well-designed clinical trials to bring them to clinical practice.

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Abbreviations

α-SMA, alpha-smooth muscle actin; ALD, alcoholic liver disease; ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; AST, aspartate aminotransferase; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2 protein; CDAHFD, choline-deficient, L-amino acid-defined, high-fat diet; C/EBP, CCAAT-enhancer-binding protein; CGA, chlorogenic acid; CHOP, C/EBP homologous protein; ChREBP, carbohydrate-responsive element-binding protein; Col1A1, collagen type I alpha 1; CPT, carnitine palmitoyl transferase; DHA, docosahexaenoic acid; DNL, de novo lipogenesis; EGCG, epigallocatechin gallate; EPA, eicosapentaenoic acid; Fasn, fatty acid synthase; FXR, farnesoid X receptor; GPR 120, G protein-coupled receptor 120; GR, glucocorticoid receptor; GTE, green tea extract; HFD, high-fat diet; Hnf, hepatocyte nuclear factor; HO-1, heme oxygenase-1; HOMA-IR, homeostatic model assessment for insulin resistance; IL, interleukin; iTFA, industrially produced trans fatty acid; 7-KDCA, 7-ketodeoxycholic acid; LXR, liver X receptor; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; MCP, monocyte chemoattractant protein; miR, microRNA; MUFA, monounsaturated fatty acid; NADH/NAD+, nicotinamide ade-

nine dinucleotide; NF-κB, nuclear factor kappa B; NLRP3, nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3; Nrf2, nuclear factor erythroid 2-related factor 2; 25(OH)D, 25-hydroxy vitamin D; PERK, protein kinase RNA-like endoplasmic reticulum kinase; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; RDA, recommended daily allowance; SFA, saturated fatty acid; Sir2, sirtuin 2; SIRT1, sirtuin 1; Smad, mothers against the decapentaplegic homolog; SREBP-1c, sterol regulatory element-binding protein 1c; TGF, transforming growth factor; TLR, toll-like receptor; TNF, tumor necrosis factor.

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Review

Ashwagandha (*Withania somnifera*) and Its Effects on Well-Being—A Review

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Abstract

In recent decades, the mental health and sleep quality of populations have worsened globally, leading healthcare professionals and researchers to seek new, safe therapies that extend beyond traditional pharmacological treatments. *Withania somnifera*, commonly known as ashwagandha, appears to be a valuable element for improving overall quality of life due to its abundance of active substances and known multidirectional effects on the human body. This review aims to critically evaluate the available literature on ashwagandha extract and its potential roles in enhancing well-being, with a focus on reducing stress, fatigue, anxiety, and depressive disorders; improving sleep quality; and enhancing cognitive function. Furthermore, an attempt has also been made to highlight the limitations associated with the use of this plant extract in clinical practice and indicate directions for future research.

Keywords: ashwagandha; depression; anxiety; stress; sleep quality; cognitive function

1. Introduction

In recent years, the global prevalence of mental health issues and sleep disturbances has increased, leading healthcare providers and the public to seek integrative methods beyond traditional drug therapies. As worries regarding the side effects and shortcomings of usual treatments for mood disorders, anxiety, and insomnia have grown, the quest for effective, evidence-based natural remedies has intensified [1]. One plant that has garnered notable scientific and clinical attention is *Withania somnifera*, also known as ashwagandha.

Ashwagandha is a small shrub native to the Indian subcontinent, the Middle East, and parts of Africa. The name "ashwagandha" derives from Sanskrit and translates to "smell of a horse," a reference to its unique aroma and its reputed ability to impart strength and vitality akin to that of a horse. Used for over 3000 years in Ayurvedic medicine, ashwagandha is considered a rasayana—a rejuvenating herb that promotes longevity, resilience to stress, and overall health [2]. It has traditionally been employed to enhance stamina, restore energy, reduce fatigue, and combat stress-related conditions [3,4].

Phytochemically, ashwagandha contains several biologically active constituents that mediate its therapeutic effects. The most studied compounds are withanolides (Figure 1), a group of steroidal lactones that exhibit anti-inflammatory, antioxidant, immunomodulatory, and neuroprotective properties [5]. Other notable components include alkaloids (such as

somniferine and tropine), sitoindosides, and acylsterylglucosides, many of which have been suggested to modulate the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic nervous system [6]. These biochemical effects contribute to ashwagandha's ability to function as an adaptogen, helping the body to mitigate and adapt to psychological and physical stressors [7].

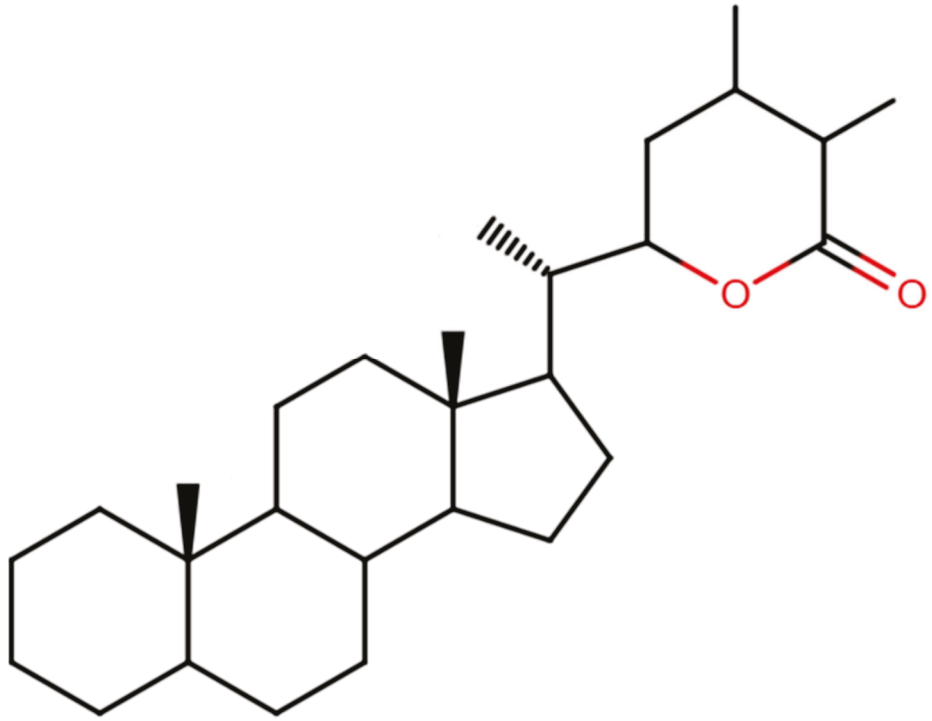


Figure 1. Basic chemical structure of withanolides.

The global burden of mental health disorders is substantial. According to the World Health Organization (WHO), depression affects over 280 million people worldwide and is a leading cause of disability. Anxiety disorders—including generalized anxiety disorder (GAD), panic disorder, and social phobia—affect an estimated 301 million individuals [8]. In parallel, insomnia and sleep disturbances have become increasingly prevalent in modern societies, often exacerbated by digital overstimulation, work-related stress, and social isolation. Epidemiological studies indicate that acute or transient insomnia affects 30% to 50% of adults, while chronic insomnia—defined as sleep difficulties occurring at least three times per week for three months or more—impacts approximately 10% to 15% of the adult population [9].

These conditions frequently coexist and can profoundly impact the quality of life, work performance, interpersonal relationships, and physical health of individuals. Chronic stress and poor sleep are associated with an increased risk of cardiovascular disease, metabolic syndrome, and immune dysfunction [10,11]. In clinical practice, pharmacotherapy remains the primary treatment modality for mood and sleep disorders. Antidepressants, such as selective serotonin reuptake inhibitors (SSRIs) and serotonin–norepinephrine reuptake inhibitors (SNRIs), alongside benzodiazepines and non-benzodiazepine hypnotics, are commonly prescribed [12]. However, these medications often induce side effects, including sedation, weight gain, sexual dysfunction, dependency, and withdrawal symptoms. Consequently, many individuals choose to discontinue treatment or explore complementary options [13].

In light of these challenges, there has been a significant increase in both public and academic interest in natural health products and herbal medicine. Consumers are increasingly

drawn to botanicals that offer therapeutic benefits alongside a safer profile. Ashwagandha has emerged as a leader in this movement, owing to its traditional usage and an expanding body of scientific research that supports its potential psychotropic effects. This review aims to critically evaluate studies on ashwagandha and its potential benefits for overall well-being, specifically in terms of mood regulation, stress relief, sleep enhancement, and cognitive function. PubMed and Google Scholar databases were searched using a combination of keywords: ashwagandha, *Withania somnifera*, stress, depression, anxiety, insomnia, sleep, fatigue, cognitive function, and memory. The search mainly included studies published between 2009 and 2025. The review primarily included in vivo studies, particularly clinical trials.

Through synthesizing the available evidence, we aim to determine whether ashwagandha can serve as a viable adjunct or alternative to standard treatments, while also identifying areas where further research is needed.

2. The Effects of Ashwagandha on Stress, Anxiety, and Depression

Stress is currently a widespread symptom that causes deterioration of quality of life. In an evolving world, there has been a rise in psychiatric disorders as well as an escalation in social issues, including substance abuse and related concerns and addictions. An increasing number of individuals are directing their attention towards lifestyle choices and the equilibrium between work and personal life to mitigate stress, anxiety, and depression [14]. We are seeking remedies, psychological support, and pharmacological treatment. Observations of human physiology suggest that the experience of stress is linked to hormonal disorders, primarily within the HPA axis, leading to elevated cortisol levels in the bloodstream [15].

In the study by Lopresti et al. [16], it was shown that the use of ashwagandha at a dosage of 240 mg/day for 60 days led to a reduction in the feeling of stress and fatigue assessed using the Hamilton Anxiety Rating Scale (HAM-A) and the Depression, Anxiety, and Stress Scale-21 (DASS-21). It has also been shown to reduce morning cortisol levels and increase testosterone levels in men, but not in women [16]. Another study evaluated 54 adults with generalized depression [17]. It was shown that administering 500 mg of ashwagandha root extract caused significant improvements in the Perceived Stress Scale (PSS) and GAD-7 scale, a reduction in morning cortisol in saliva, an increase in serotonin in urine, and no effect on nitric oxide (NO), glutathione (GSH), and malondialdehyde (MDA) concentrations [17]. In the study by Baker et al. [18], the experience of stress and coding abilities of students exposed to stress during study were assessed. The study results indicate that a daily dose of 700 mg of ashwagandha enhances sleep, boosts energy, and improves mental clarity, thereby helping to lower stress levels [18]. The researchers observed that ashwagandha in the dose mentioned above had no effect on stress over a 30-day follow-up period. It appears that a more prolonged period of use is necessary to relieve stress [19].

In a study of overweight and obese men, no reduction in stress levels on the PSS was observed compared to the placebo. Interestingly, men showed an increase in free testosterone and luteinizing hormone levels [4]. In a study examining 52 adults who were administered 300 mg of ashwagandha extract daily, it was found that this extract had a beneficial effect on weight management and cortisol reduction in individuals experiencing chronic stress [20]. Another recent study evaluated the effectiveness of various doses in 98 healthy individuals. It examined the 14-item PSS along with stress-related biochemical parameters. Changes in the HPA axis were confirmed, regardless of the dosage [21]. The findings indicate that ashwagandha positively impacts the reduction in stress and anxiety, primarily by altering hormonal levels. The results of the studies discussed above are shown in Table 1.

Table 1. Summary of research on the effects of ashwagandha on stress, anxiety, and depression.

Characteristics of the Group and Duration of the Study	Daily Doses of Ashwagandha Extract	Effect on Stress, Anxiety, and Depression	Study
60 stressed, healthy adults; 60 days	240 mg	Reducing feelings of anxiety and stress.	[16]
54 stressed, healthy adults; 60 days	500 mg	Reducing feelings of anxiety and stress.	[17]
60 healthy college students aged 18–50; 30 days	700 mg	Enhancing sleep, boosting energy, and improving mental clarity. No effect on stress over 30 days of follow-up.	[18] [19]
120 overweight or mildly obese men and women aged 40–75 years; 12 weeks	400 mg	No reduction in the level of stress.	[4]
52 adults aged 18–60; 8 weeks	300 mg	Stress reduction, weight loss, and cortisol level reduction.	[20]
98 adults; 8 weeks	125, 250, and 500 mg	Reduction in mild to moderate stress regardless of dose.	[21]

Arumugam et al. [22] conducted a meta-analysis examining the effects of ashwagandha on stress and anxiety. Their review of studies involving 558 participants revealed a significant effect of ashwagandha, compared to a placebo, on the PSS, Hamilton Anxiety Scale (HAS), and serum cortisol levels. Mild side effects were noted, and the need to evaluate the long-term safety of ashwagandha extract was emphasized [22]. Another systematic review and meta-analysis, which included five clinical trials (254 participants), revealed that ashwagandha significantly reduces HAM-A scores; however, the researchers emphasized the need for studies with larger sample sizes [23].

Given the widespread prevalence of depression, anxiety, and stress in society, it appears that the studies mentioned above were conducted in relatively limited populations. Additionally, some researchers have suggested that the study duration should be extended to adequately observe clinical changes, such as reductions in stress levels. Furthermore, comparative data are deficient regarding the efficacy of ashwagandha extract compared to that of antidepressants and anti-anxiety medications.

3. The Effects of Ashwagandha on Fatigue and Sleep Quality

As sleep is a crucial physiological process that allows the human organism to regenerate both physical and mental dimensions, its quality and duration are essential [24]. These factors make insomnia a significant challenge for patients who suffer from it, as it diminishes quality of life due to a lack of proper regeneration and rest. Insomnia is a very common sleep disorder nowadays, and, due to the adverse effects of most available medications, innovative strategies are under consideration by scientists around the world [25]. Furthermore, prevalent high stress and insufficient sleep and rest often lead to pathological fatigue, which can reduce both mental and physical efficiency throughout the day and impact overall quality of life.

Based on their animal study, Kaushik et al. [26] have suggested that ashwagandha extract may positively affect sleep not due to withanolides but rather due to triethylene glycol (TEG), which is found in ashwagandha leaves and may be the most effective component for promoting sleep.

Although the exact molecule responsible for the influence on sleep has not yet been identified, numerous scientific articles, including randomized, double-blinded, prospective studies, confirm ashwagandha's beneficial effect on physiological processes. Its influence

has been observed in individuals aged 18 to 85 [4,24,25,27–29]. This age range is significant, as all groups—young adults, adults, and senior citizens—are susceptible to insomnia stemming from various causes. Young adults and adults primarily experience insomnia due to stress, while the elderly are often affected by the aging process, age-related diseases, and pain [29]. The fact that ashwagandha is effective across all age ranges makes it a promising and relatively safe remedy, as noted by Dimpfel et al. [27], who reported some mild side effects. Specifically, one patient experienced an allergic reaction, and two patients (who were treated with hypotensive drugs) reported an increase in blood pressure. The most influential aspects of ashwagandha extract intake include improved sleep quality, waking up refreshed, and enhanced sleep efficiency. Additionally, it reduces the time it takes to fall asleep, increases mental alertness upon waking, and extends total sleep duration [4,24,25,27–30]. Authors often point out that the improvement is dose-dependent up to a certain point. Salve et al. [28] observed that a 300 mg ashwagandha dose administered twice daily appears to be more effective than a 125 mg dose administered twice daily. Pérez-Piñero et al. [24] compared the effects of different ashwagandha doses with a combination of ashwagandha and tryptophan. The results showed that the 600 mg ashwagandha extract dose had the most significant positive influence on sleep, followed by the 250 mg ashwagandha dose group, which had the second-best effect. In contrast, the combination of 250 mg ashwagandha and tryptophan exhibited the weakest effect, although it still demonstrated improvement compared to the placebo group [24].

Murthy et al. [31] performed a study on animals and cell cultures that presented the molecular influence of ashwagandha—the extract positively affects the gene expression of the ρ1 subfamily of GABAA receptors and histamine type 3 receptors, both in a dose-dependent manner (the gene expression increase was higher in 30 μg/mL than in 15 μg/mL ashwagandha extract arm of the study). The authors also emphasized the dose-dependent reduction in time to fall asleep and the increase in sleep duration in animals [31]. Another study conducted on animals compared the influence of water (WA) and enzyme-treated (EA) ashwagandha root extracts on sleep parameters in mice and rats. Park et al. [32] observed that the enzyme-treated extract—likely due to the removal of starch—resulted in increased sleep duration even at higher doses. In contrast, the water extract demonstrated its peak impact at a 60 mg/kg dose, with no positive change and even a decrease in sleep duration at higher doses. Both extracts reduced sleep latency in a group of pentobarbital-treated mice; however, this effect did not reach statistical significance. In this model, both treatments notably extended sleep duration, with the EA extract proving more effective. On the other hand, in a model of caffeine-induced insomnia, the EA extract significantly reduced sleep latency and increased sleep duration at higher doses (100 and 150 mg/kg) [32].

Fatigue is a problematic condition that reduces human efficiency and involves cognitive and mental impairment. It can result from inadequate sleep, poor sleep quality, or occur independently of these factors. Unfortunately, it is quite common in modern society [33]. Ashwagandha is also regarded as an anti-fatigue agent. This effect is linked to its relaxing, stress-relieving, and sleep-inducing properties [4,33–35]. An interesting study in this field was performed by Cooley et al. [36]. They compared ashwagandha extract at a dosage of 300 mg twice daily for 12 weeks with the effects of standard psychotherapy in a group of women and men experiencing moderate to severe anxiety. In this double-blind, randomized trial, ashwagandha showed greater reductions in subjective fatigue, physical fatigue, motivation, and concentration levels compared to conventional psychotherapy [34]. Several studies also emphasize that *W. somnifera* extract has greater anti-fatigue effects than those of the placebo [4,34,35]. However, Lopresti et al. tested ashwagandha extract at a dose of 600 mg in overweight male patients aged 40 to 70, and reported a decrease

in fatigue in both the ashwagandha and placebo groups [33]. The results of the studies discussed above are shown in Table 2.

Table 2. Summary of research on the effects of ashwagandha on fatigue and sleep quality.

Characteristics of the Group and Duration of the Study	Daily Doses of Ashwagandha Extract	Effect on Sleep	Effect on Fatigue	Study
120 overweight or mildly obese men and women aged 40–75 years; 12 weeks	400 mg	Quality increased in both groups.	A larger decrease in the ashwagandha group and a smaller decrease in the placebo group.	[4]
59 men and women aged 18–49 years; 30 days	225 mg	N/A	There was a small decrease in the ashwagandha group.	[34]
57 overweight men aged 40–70 years; 16 weeks	600 mg	N/A	Improvement in both groups with no statistically significant difference.	[33]
13 women and men aged 18–59 years; 4–7 days	400 mg	N/A	Less mental fatigue in tests in the ashwagandha group.	[35]
16 women and men aged 60–75 years; 4 weeks	300 mg	Increase in sleep quality and refreshment after sleep.	N/A	[27]
41 employees with moderate to severe anxiety; 12 weeks	600 mg	N/A	A decrease in physique and subjective fatigue in the ashwagandha group compared to psychotherapy.	[36]
60 women and men aged 18–55 years; 8 weeks	250 mg or 600 mg	Increase in sleep quality in ashwagandha groups, with more effectiveness in the ashwagandha 600 mg/day group.	N/A	[28]
Mice and rats; 4 weeks	Ashwagandha root water extract (AW) vs. enzyme-treated ashwagandha root extract (EA)	Decrease in sleep latency—statistically insignificant for AW and EA. Sleep duration increases with peak performance in the 60 mg/kg dose for AW, while sleep duration increases for EA even in higher doses (60, 80, 100, and 150 mg/kg). Decreased sleep latency and increased sleep duration in caffeine-induced insomnia mice for EA.	N/A	[32]
130 women and men aged 20–55 years; 90 days	300 mg	Increase in sleep quality.	N/A	[30]
C6 rat glioma-established cell line and Swiss albino male mice	15 mg/ml or 30 mg/ml	Increase in $GABA_A\rho 1$ and H3 receptor gene expression in a dose-dependent manner. Sleep latency decreases and sleep time increases in a dose-dependent manner.	N/A	[31]

Table 2. *Cont.*

Characteristics of the Group and Duration of the Study	Daily Doses of Ashwagandha Extract	Effect on Sleep	Effect on Fatigue	Study
60 women and men aged 18–60 years suffering from insomnia; 10 weeks	600 mg	Decrease in sleep latency and wake after sleep onset. Increase sleep efficiency, total time in bed, total sleep time, and subjective sleep quality.	N/A	[25]
52 young adults of both genders; 90 days	250 mg or 250 mg + tryptophan or 600 mg	Sleep quality improvement from the highest to the lowest: ashwagandha 600 mg group, ashwagandha 250 mg group, ashwagandha 250 mg + tryptophan 175 mg group, and placebo group.	N/A	[24]
39 women and men aged 60–85 years; 12 weeks	600 mg	An increase in sleep quality and mental alertness in the ashwagandha group.	N/A	[29]
Male C57BL/6 mice with EEG monitoring	Alcohol-based ashwagandha leaf extract vs. water-based ashwagandha leaf extract vs. cyclodextrin-assisted aqueous ashwagandha leaf extract	No influence on REM and NREM sleep phases in alcohol extract; NREM duration increases in water and cyclodextrin extracts.	N/A	[26]

N/A—not available.

Cheah et al. [37] confirm, in their meta-analysis, the beneficial effect of ashwagandha extract on sleep-related regeneration. Nevertheless, the aforementioned meta-analysis highlights that data on severe adverse effects of ashwagandha extract are limited, and further studies should focus on safety issues related to its long-term use [37].

4. The Effects of Ashwagandha on Cognitive Function and Memory

Cognitive functions and memory are fundamental to daily functioning, learning, and decision-making processes. Impairments in cognitive functions and memory can significantly impact a patient's quality of life and adversely affect overall mental well-being. The pathogenesis of cognitive impairment is multifaceted; however, the primary mechanism involves damage to neuronal tissue. This damage varies according to the underlying cause, which may include aging, psychological stress, brain injury, neurological disorders, and chronic diseases such as hypertension and diabetes [38].

Recent research has highlighted intriguing findings regarding the impact of ashwagandha on cognitive function. Table 3 summarizes these studies.

Remenapp et al. [39] studied 58 healthy subjects aged 18 to 54 who received 225 or 400 mg of ashwagandha extract daily for 30 days in a randomized, double-blind, placebo-controlled clinical trial. In contrast to the placebo group, both ashwagandha groups demonstrated significant improvements in flexibility, complex attention, executive functioning, processing time, and reaction time [39]. Likewise, in a double-blind, randomized, placebo-controlled clinical study, Gopukumar et al. [30] studied 130 healthy, cognitively sound adults aged 20–55 who received 300 mg/day of ashwagandha root extract for 90 days. The

results showed a significant improvement in recall memory and a reduction in the total error rate when recalling patterns [30].

In line with the abovementioned studies, Leonard et al. [34] examined 59 healthy participants aged 18 to 60, administering 225 mg of ashwagandha extract daily for 90 days. They have observed improved word recall, choice reaction time, picture recognition, digit vigilance, and the Stroop Color–Word Test [34]. The Stroop Color–Word Test serves as a diagnostic tool for cognitive impairment, requiring the participant to identify the colors printed in a contrasting font. This test checks mainly attention, vigilance, and executive functions [40].

Table 3. Summary of research on the effects of ashwagandha on cognitive function and memory.

Characteristics of the Group and Duration of the Study	Daily Doses of Ashwagandha Extract	Effects on Cognitive Function and Memory	Study
130 participants 20–55 years old; 90 days	300 mg	Improvement in recall memory. A decrease in the total error rate in recalling patterns.	[30]
59 participants 18–60 years old; 30 days	225 mg	Improvement in word recall, choice reaction time, picture recognition, digit vigilance, and Stroop Color and Word test.	[34]
58 participants 18–54 years old; 30 days	225 or 400 mg	Improvement in flexibility, complex attention, executive functioning, processing speed, and reaction time.	[39]
120 participants 30–75 years old; 8 weeks	600 mg	Improvement in episodic memory, working memory, and accuracy of attention.	[41]
50 adults; 8 weeks	600 mg	Significant improvements in immediate and general memory, executive function, sustained attention, and information processing speed.	[42]
40 participants with MCI; 60 days	225 mg	Improvement in immediate memory, general memory, working memory, and visuospatial processing. Increase in MMSE scale.	[43]

Kale et al. [41] reported similar effects in a prospective, randomized, placebo-controlled study in which 120 healthy subjects took 600 mg of ashwagandha root extract daily for 8 weeks. The extract was well tolerated and reported no clinically significant side effects [41].

Another study highlighting the improvement of cognitive functions was conducted by Choudhary et al. [42], who provided 50 participants with mild cognitive impairment (MCI) with 300 mg of ashwagandha root extract twice daily for 8 weeks. Significant improvements were observed in immediate and general memory, executive function, sustained attention, and information processing speed [42].

Rai et al.'s [43] study reported very interesting outcomes. They studied 40 patients with MCI who received 225 mg/day of ashwagandha extract for 60 days. They observed improvements in immediate memory, general memory, working memory, and visuospatial processing, as well as an increase in the Mini-Mental State Examination (MMSE) scale [43]. This scale is a screening test for cognitive impairment, primarily used in elderly individuals. It assesses the main cognitive domains, including orientation, registration, attention, recall, language, and visuospatial skills [44].

Several attempts have been made to elucidate the potential mechanism of action of ashwagandha extracts in human cells. Kuboyama et al. investigated in vitro that withanolide A isolated from ashwagandha root extract regenerates neurites and recovers synapses in heavily damaged mouse neurons [45]. Another study by Kuboyama et al. shows that withanolide A, IV, and VI are responsible for enhancing the outgrowth of human

neuroblastoma SH-SY5Y dendrites and axons [46]. These studies indicate the regenerative effect of ashwagandha extract on nerve cells. Secondarily, it may improve the neural transmission between synapses, which is beneficial for patients with cognitive impairment. Unfortunately, the exact mechanism by which ashwagandha improves cognitive function and memory is still unknown.

According to the aforementioned studies, supplementation with ashwagandha extract appears beneficial for individuals in good health and for patients suffering from cognitive impairment. It improves daily functioning, memory, and visuospatial functioning, reduces stress, and improves sleep quality. The recommended daily dosage ranges from 225 to 600 mg. The daily dosage and length of therapy do not notably influence the outcomes achieved. Importantly, no significant adverse effects were observed during the trials in any of the study groups. On the other hand, the studies mentioned above are characterized by the limited size of the participant groups and the brief duration of the investigations. Furthermore, all studies evaluated the efficacy of ashwagandha extract in comparison to a placebo, thereby rendering it infeasible to conclude its effectiveness relative to standard pharmacotherapy for cognitive impairment.

5. Limitations

The studies discussed in the chapters above indicate that ashwagandha can be a valuable supplement for improving mood and sleep quality, reducing fatigue and stress, and improving memory and cognitive function. Nevertheless, discussing its potential contraindications as a stand-alone therapeutic agent and an adjunct to standard treatment seems necessary. Regarding the aforementioned studies, it is essential to note the small number of participants involved. Moreover, many studies were conducted over a limited duration (ranging from 30 days to a maximum of 16 weeks), which may have failed to demonstrate the complete clinical effect. This relatively brief duration of research may also inadequately reflect the impact of prolonged use of ashwagandha extract, particularly in instances of self-medication. Interestingly, in most studies, ashwagandha's efficacy has been compared to a placebo, so data comparing its effects with standard pharmacotherapy (e.g., SSRIs, hypnotics) are lacking. It appears to be a valid approach to closely examine the potential dangers associated with the unregulated use of this plant material.

Most studies suggest that the recommended dose of ashwagandha is 250–600 mg/day [28]. The most common and popular form of ashwagandha is powder, which contains the herb's dried root, leaf, or combination [47]. The preparations with ashwagandha extract available on the pharmaceutical market are mainly dietary supplements, which simplifies registration procedures and may impact the quality of the available products [48]. Some studies have used sustained-release capsules containing ashwagandha extract, which may be associated with increased bioavailability and, consequently, improved utilization of the active ingredients contained in this plant [30]. Moreover, due to the wide range of phytochemicals found in ashwagandha, it is crucial to develop a suitable analytical method to measure the various withanolides in ashwagandha, allowing its extracts to be standardized [49].

The clinical trial on the safety of ashwagandha showed that supplementation with ashwagandha root extract at 300 mg/day for 8 weeks demonstrated no toxic effects in healthy individuals [50]. In contrast, there are increasing reports of potential liver-damaging effects, which are even referred to as ashwagandha-induced cholestatic hepatitis. This is particularly dangerous for patients with pre-diagnosed liver disease and can even lead to acute and chronic liver failure syndrome [51–53]. Björnsson et al. [52] detailed a case involving five patients who experienced jaundice, nausea, pruritus, and abdominal discomfort after using ashwagandha preparations for 2 to 12 weeks. The average age of the patients was

43 years, with three being men. The findings indicated that the liver damage was cholestatic or mixed, although none of the patients experienced liver failure. Notably, in one patient who was also taking *Rhodiola rosea*, a potential herb–herb interaction was suspected to have contributed to the liver damage [52]. This case highlights the possible dangers of concomitant use with other adaptogens. Another reported case involved a 39-year-old woman who presented with a week-long history of jaundice and nausea after taking a supplement containing ashwagandha root extract. The patient displayed abnormalities in liver enzymes linked to jaundice, and a liver biopsy showed acute cholestatic hepatitis with subepithelial necrosis; however, no features indicating chronicity were observed [52]. Additional cases of herb-induced liver injury (HILI) involve a man and a woman who purchased ashwagandha supplements online to boost fertility [54]. A 36-year-old male took ashwagandha extract at a dosage of 450 mg three times a day for six months. His symptoms included nausea, itching, and dark urine. In contrast, a 30-year-old female took 450 mg of ashwagandha daily and experienced itching after 45 days. In both cases, there were elevated levels of serum bilirubin and liver enzymes, specifically aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP). After stopping ashwagandha supplements, both individuals showed improvements in their clinical condition and liver function [54].

Not all patients are suitable candidates for ashwagandha. Those with hyperthyroidism who present symptoms like irritability, restlessness, anxiety, hand tremors, palpitations, psychomotor agitation, muscle weakness, fatigue, and reduced libido fall into this category. While products containing ashwagandha root extract have demonstrated effectiveness in alleviating these symptoms, their use is contraindicated for individuals with hyperthyroidism, as they can worsen the condition. Research indicates that this raw material can raise the levels of 3, 3′, 5-triiodothyronine (T3) and tetraiodothyronine (T4), which is detrimental for those suffering from hyperthyroidism [55]. An illustrative example of a perilous trend related to self-medication with ashwagandha is the case of a 73-year-old woman who experienced supraventricular tachycardia, hyperthyroid symptoms, and markedly low thyroid-stimulating hormone (TSH) levels following two years of utilizing ashwagandha extract as a self-treatment for hypothyroidism. Discontinuing the supplement resulted in complete symptom resolution and enhanced biochemical markers [55]. Ashwagandha root extract is known for treating male infertility, but it is not recommended for men with hormone-sensitive prostate cancer since it may boost testosterone production, accelerating disease progression. Additionally, ashwagandha is contraindicated for those who are pregnant or planning to conceive, as high doses of ashwagandha root extract could result in miscarriage [56,57].

Interactions with other drugs also remain an important issue. Due to its synergistic properties, ashwagandha root extract may enhance the effects of anti-anxiety, sleep, muscle-relaxant, and sedative medications. This ingredient shows additive effects with anticonvulsants, barbiturates, and benzodiazepines, potentially leading to heightened adverse reactions, including impaired motor coordination, muscle weakness, headaches, reduced libido, muscle tremors, and drowsiness [58]. Analysis of herb–drug interactions has shown that the concomitant use of ashwagandha with antidepressants can lead to several dangerous side effects. Interaction with reboxetine may result in testicle pain and ejaculatory dysfunction, while interaction with sertraline can cause severe diarrhea. Additionally, combining ashwagandha with escitalopram can lead to myalgia, epigastric pain, nausea, vomiting, restless legs syndrome, and a severe cough. In contrast, the combination with paroxetine may result in generalized myalgia, ophthalmalgia, and ocular hypertension. From a pharmacokinetics perspective, the inhibition of CYP3A4 and CYP2D6

by ashwagandha increases the concentration and side effects of antidepressants that are metabolized by these cytochromes [59].

It seems that ashwagandha may not be suitable for everyone, and potential side effects should be taken into account when selecting such a supplement, especially for elderly patients and those taking other medications. Additionally, there is a lack of substantial data regarding the long-term effects of ashwagandha extract use and its safety. Moreover, researchers face the challenge of standardizing ashwagandha formulations, which is inherently linked to the quality of the preparations used.

6. Conclusions

At present, due to stress and overwork, mood and sleep disorders are affecting an increasing number of people. Hence, natural remedies with proven effects are being actively searched for. Ashwagandha is a plant that covers many needs, not only related to mental health but also somatic health. Studies have proven its positive effects in terms of reducing stress and fatigue using objective scales (HAM-A, DASS-21) and in laboratory tests, such as lowering morning cortisol levels and increasing testosterone levels in men. Modulation of the HPA axis and the amelioration of neuronal transmission between synapses contribute to the reduction in anxiety and insomnia, thereby improving cognitive ability and overall mental health.

Most studies have used ashwagandha root extract, while others have used leaves or both. Depending on the expected effect, it is essential to systematize the composition to maximize the result. This will confirm the positive impact of a specific ingredient in an exact dose for a particular ailment (e.g., TEG contained in leaves and its effect on insomnia). Approving ashwagandha as a drug or medical device would enable better control over the preparations available for use by researchers and patients. The high quality of preparations containing ashwagandha extract, coupled with an in-depth examination of side effects and the specific patient groups that may experience adverse reactions, is a significant concern that requires further investigation. Moreover, when recommending ashwagandha extract, it is essential to consider the patient's medical condition and any potential interactions with other medications they may be taking.

Future research on ashwagandha extract should focus on standardizing formulation protocols, long-term safety trials, and testing on vulnerable populations, especially the poly-treated elderly and those with endocrine disorders. Additionally, mechanistic studies using omics and neuroimaging tools may also provide valuable scientific insights.

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Review

Molecular Mechanisms and Therapeutic Potential of Mulberry Fruit Extract in High-Fat Diet-Induced Male Reproductive Dysfunction: A Comprehensive Review

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Abstract: High-fat diet (HFD)-induced obesity represents a significant challenge to male reproductive health, affecting approximately 13% of the global adult population. This comprehensive review synthesizes current evidence regarding mulberry (*Morus alba* L.) fruit extract's therapeutic potential for HFD-induced male reproductive dysfunction. Through comprehensive analysis of the peer-reviewed literature from multiple databases (PubMed, Web of Science, Scopus, and Google Scholar; 2005–2024), we evaluated mulberry extract's effects on testicular morphology, spermatogenesis, sperm parameters, and the underlying molecular mechanisms. Mechanistic studies reveal that standardized mulberry extract mediates protective effects through multiple pathways: enhanced antioxidant enzyme activities (SOD: +45%, Catalase: +38%, GPx: +35%), reduced inflammatory markers (TNF-α: −64%, IL-6: −58%), and modulated NF-κB signaling (−42.3%). These effects are facilitated by mulberry's rich phytochemical profile, particularly anthocyanins (2.92–5.35 mg/g dry weight) and polyphenols (4.23–6.38 mg/g). The extract demonstrates particular efficacy in preserving seminiferous tubule integrity and maintaining blood-testis barrier function, with treated groups maintaining up to 85% of normal tubular architecture compared to HFD controls. Key molecular mechanisms include AMPK/SIRT1 pathway activation (2.3-fold increase), enhanced mitochondrial function (67% increase in mtDNA copy number), and epigenetic regulation of metabolic pathways. Temporal analysis indicates optimal therapeutic effects after 28 days of treatment, with initial improvements observable within 14 days. While current evidence is promising, limitations include predominant reliance on rodent models and lack of standardized extraction protocols. Future research priorities include well-designed human clinical trials, standardization of preparation methods, and investigation of potential synergistic effects with other therapeutic agents. This comprehensive review indicates that mulberry extract is a promising therapeutic candidate for obesity-related male infertility, warranting further clinical investigation.

Keywords: mulberry fruit; high-fat diet; obesity; male reproduction system; sperm quality

1. Introduction

The global obesity epidemic has reached unprecedented levels, with obesity rates nearly tripling since 1975 and affecting over 650 million adults worldwide, representing 13% of the global adult population [1,2], and is a major risk factor for male infertility. Indeed, male infertility has now emerged as a significant public health and economic concern, affecting approximately 15% of couples worldwide with estimated annual healthcare costs exceeding $9 billion in developed nations [3,4]. Therefore, identifying an effective remedy to mitigate obesity-related male infertility is a now pressing priority, and this review aims to explore the therapeutic potentials of mulberry and its natural fruit compounds.

Recent epidemiological studies have revealed a concerning correlation between obesity and male infertility [5–8]. Meta-analyses of 32 studies involving 115,158 participants have revealed that obese men (BMI > 30 kg/m^2) have significantly reduced sperm concentration (mean difference: -2.04×10^6/mL, 95% CI: -2.95 to -1.13) and total sperm count (mean difference: -7.81×10^6/mL, 95% CI: -10.31 to -5.31) compared to men with normal BMI. Specifically, obese men have a significantly higher risk of infertility (OR: 1.28, 95% CI: 1.06–1.55, $p < 0.001$) compared to normal-weight men, with a dose-dependent relationship between BMI increase and fertility decline [6].

A comprehensive analysis by Skoracka et al. (2020) demonstrates that nutritional factors are critically underestimated in male reproductive health, with direct correlations between dietary patterns and sperm parameters. Their systematic review of clinical and experimental studies reveals that diet quality significantly impacts reproductive outcomes through multiple pathways, including sperm? membrane composition, energy metabolism, and hormonal regulation [9].

High-fat diet (HFD) consumption, a primary driver of obesity, disrupts male reproductive function through distinct pathophysiological mechanisms [10]. Molecular and proteomic analyses have revealed that HFD induces significant alterations in testicular morphology and function, characterized by reduced seminiferous tubule diameter (24.8 $\pm$ 3.2% reduction), disrupted blood–testis barrier integrity, and compromised Sertoli-germ cell junctions [11]. These structural modifications are accompanied by metabolic perturbations in Sertoli cells, with studies showing disrupted energy metabolism and mitochondrial function [12]. HFD-induced oxidative stress in testicular tissue leads to increased production of reactive oxygen species (ROS), triggering lipid peroxidation and DNA damage in developing sperm cells [13,14].

These metabolic and oxidative changes trigger complex cascades of reproductive dysfunction through multiple interrelated pathways. The disruption extends to the hypothalamic–pituitary–gonadal axis, resulting in reduced testosterone production and impaired spermatogenesis [15]. HFD consumption induces significant alterations in the testicular antioxidant defense system, characterized by marked reductions in key antioxidant enzymes: superoxide dismutase (42.3 $\pm$ 3.2% decrease), catalase (38.4 $\pm$ 2.9% decrease), and glutathione peroxidase (35.7 $\pm$ 3.1% decrease). Concurrently, lipid peroxidation increases 2.4-fold, indicating substantial oxidative damage to cellular membranes [14]. The inflammatory response encompasses the activation of multiple signaling cascades, with NF-κB pathway activation showing a 3.2-fold increase. This activation leads to elevated pro-inflammatory cytokine expression, including TNF-α (2.8-fold increase), IL-6 (2.4-fold increase), and IL-1β (2.1-fold increase). Oxidative stress markers demonstrate parallel increases, with 8-OHdG and malondialdehyde levels elevated 2.7-fold and 2.3-fold, respectively [11]. Notably, these diet-induced reproductive impairments exhibit transgenerational effects. First-generation (F1) offspring demonstrate a 23% reduction in fertility rates, accompanied by altered DNA methylation patterns in 47 reproductive genes and significant decreases in sperm parameters (count reduced by 32%, motility decreased by 24%). These

effects persist into the second generation (F2), albeit at a reduced magnitude, with a 16% reduction in fertility rates and sustained alterations in metabolic gene expression and glucose homeostasis [16].

Current therapeutic approaches for obesity-related male infertility demonstrate limited efficacy [17], with conventional treatments achieving success rates below 30% and failing to address underlying molecular mechanisms [18]. This therapeutic limitation has stimulated investigation into natural compounds with pleiotropic properties, among which mulberry (*Morus alba* L.) fruit extract has emerged as a potential therapeutic agent, as the mulberry fruit extract is rich in bioactive compounds like flavonoids, phenolics, and anthocyanins [19–21]. Comprehensive phytochemical analyses have elucidated the extract's bioactive profile, with significant concentrations of anthocyanins (2.92–5.35 mg/g dry weight), predominantly cyanidin-3-glucoside and cyanidin-3-rutinoside, flavonoids (1.02–2.06 mg/g), and polyphenols (4.23–6.38 mg/g) [22]. These phytochemicals exhibit diverse pharmacological activities, including antioxidant, anti-inflammatory, antidiabetic, neuroprotective, and anticancer properties [19,21,23]. The biosynthesis of these compounds involves pathways such as phenylpropanoid and flavonoid biosynthesis, with key enzymes like phenylalanine ammonia-lyase and chalcone synthase [19]. Mulberry extracts and isolated compounds have shown potential in treating various health conditions, including cardiovascular diseases and gastrointestinal problems [23]. Additionally, mulberry supplementation in food products enhances stability, sensory properties, and antimicrobial activity [23]. Despite its promising therapeutic potential, more systematic research is needed to fully understand and utilize mulberry's health benefits [20].

While independent investigations have examined HFD's impact on male fertility and the general health benefits of mulberry consumption, a comprehensive analysis of mulberry fruit extract as an intervention for HFD-induced reproductive dysfunction remains unexplored. Critical questions persist regarding optimal therapeutic protocols, long-term efficacy, and precise mechanisms of action in reproductive tissues. This review critically evaluates current evidence supporting the therapeutic potential of mulberry fruit extract in HFD-induced male reproductive dysfunction, with particular emphasis on testicular morphology, spermatogenesis, sperm parameters, and underlying molecular mechanisms. Understanding these multifaceted interactions is crucial for developing targeted interventions for obesity-related male infertility, a condition affecting millions globally. The insights gained from this analysis may fundamentally reshape therapeutic strategies for male reproductive dysfunction, offering hope for improved fertility outcomes in an increasingly obesity-challenged world. Moreover, this review establishes a framework for future investigations into natural compounds as interventions for complex metabolic–reproductive disorders, potentially opening new avenues in reproductive medicine.

2. Methods

2.1. Search Strategy

A comprehensive literature search was conducted across four major electronic databases: PubMed, Web of Science, Scopus, and Google Scholar [24,25]. The search strategy employed a combination of relevant keywords and phrases into three core categories: (i) Intervention terms: ("mulberry" OR "*Morus alba*" OR "white mulberry"); (ii) Condition terms: ("high-fat diet" OR "obesity" OR "hyperlipidemia" OR "dyslipidemia"); and (iii) Outcome terms: ("male fertility" OR "male infertility" OR "testicular morphology" OR "spermatogenesis" OR "sperm quality" OR "sperm parameter" OR "oxidative stress" OR "inflammation" OR "hormonal regulation"). Articles published in peer-reviewed journals were included if they met the following criteria: (i) written in English, (ii) published between 2005 and 2024, and (iii) directly addressed the effects of

mulberry or its bioactive components on male reproductive outcomes associated with HFD-induced conditions. Seminal articles published before 2005 were also reviewed when deemed pivotal for providing foundational knowledge. Studies were identified using a two-phase approach: an initial screening of titles and abstracts to exclude irrelevant articles, followed by a full-text review to ensure relevance and methodological quality. Reference lists of included studies were also manually searched to identify additional relevant articles. The literature search and analysis process was conducted in accordance with best practices outlined by [25], ensuring robust coverage of the biomedical literature.

2.2. Inclusion and Exclusion Criteria

In our comprehensive review, we carefully selected studies based on specified criteria. We included research that examined how mulberry fruit extract affects male reproductive health. These studies used either animal models fed HFDs, laboratory experiments with cells or tissues, or clinical trials involving human participants. It was important that the research provided measurable data on reproductive outcomes or offered insights into how mulberry extract works in the body. We only considered peer-reviewed original research articles or high-quality systematic reviews published in reputable scientific journals.

On the other hand, we excluded certain types of studies from our review. We did not include research studies that focused solely on female reproductive health, as our interest was specifically in male reproductive parameters. Studies that used mulberry extracts from parts of the plant other than the fruit, such as the leaves or bark, were not included unless they directly compared these to the fruit extract. We also chose not to include conference abstracts, non-peer-reviewed articles, or non-English publications. Lastly, we also excluded any research that did not provide sufficient details about their methods or lacked quantitative results.

By adhering to these inclusion and exclusion criteria, we ensured that our review focused on the most relevant, reliable, and comprehensive information available about the effects of mulberry fruit extract on male reproductive health in the context of HFDs.

2.3. Data Extraction and Synthesis

Data from the included studies were extracted independently by four reviewers, with each study evaluated for key parameters, including study design, sample size, intervention details (dosage, duration, administration), human participants or animal model characteristics, reproductive outcomes (e.g., testicular morphology, sperm parameters, hormonal levels), proposed mechanisms of action, and quantitative results and statistical analyses. Discrepancies in data extraction were resolved through discussion with the other two reviewers to ensure accuracy and consistency. We synthesized this information (from the extracted data) to provide a comprehensive overview of the current state of knowledge, using both narrative synthesis and, where possible, quantitative analysis of key outcomes. The quality of evidence was assessed by considering factors such as study design, methodological rigor, consistency of findings across studies, directness of evidence, and applicability of results.

This comprehensive approach enabled us to provide a robust and comprehensive overview of the current knowledge regarding the potential of mulberry fruit extract as an intervention for HFD-induced male reproductive dysfunction while identifying areas requiring further investigation. The methodology emphasizes thorough literature coverage and careful evidence synthesis, providing a foundation for understanding both the current state of knowledge and future research directions in this field.

3. Results and Discussion

Our analysis reveals multiple levels of interaction between HFD, reproductive dysfunction, and therapeutic intervention with mulberry extract. The following sections present our findings, beginning with the fundamental effects of HFD on reproductive parameters (Table 1), progressing through the therapeutic agent's composition (Table 2), to its effects (Table 3) and underlying mechanisms (Table 4).

3.1. Effects of High-Fat Diet on Male Reproductive Health

HFD consumption has been consistently associated with detrimental effects on male reproductive health, demonstrated through both animal studies and human observational research [1,2]. Research has revealed that HFDs have wide-ranging negative effects on male reproductive health. At the structural level, HFDs significantly alter testicular structure, reducing the size of sperm-producing tubes (seminiferous tubules) and the thickness of sperm-generating tissue, while increasing non-functional space between these structures [11,26]. These structural changes are often accompanied by disruptions in important cellular connections (Sertoli cell-germ cell junctions) and diminished Leydig cell populations, which are essential for testosterone production [27].

The molecular impact of HFD extends to spermatogenesis regulation, leading to decreased sperm quantity and quality. Studies have reported decreased expression of key spermatogenesis markers (e.g., BOULE, DAZ, and DAZL) and disrupted meiotic progression [28]. Notably, these molecular perturbations manifest as quantifiable changes in sperm parameters. Clinical studies have revealed that men who consume HFDs and obese individuals often show lower sperm counts, reduced sperm motility, and more sperm with abnormal morphology [29,30]. The testicular environment under HFD conditions is characterized by increased oxidative stress and altered cellular function. Research has demonstrated that HFD leads to elevated production of reactive oxygen species and inflammatory mediators, potentially causing DNA damage in spermatozoa [31,32]. Recent evidence has further emphasized the significance of dietary factors in male fertility, highlighting how nutritional choices directly influence reproductive parameters [9].

The pathophysiological effects manifest through multiple mechanisms (Table 1). These alterations span structural, functional, and molecular levels, creating a complex pattern of reproductive dysfunction. Furthermore, HFD consumption alters the hypothalamic–pituitary–gonadal (HPG) axis, leading to reduced testosterone levels, increased estradiol, and potential leptin resistance, a key metabolic hormone [15,33]. These hormonal changes further contribute to impaired spermatogenesis and testicular function. The testicular environment in HFD conditions is characterized by chronic inflammation and oxidative stress, with increased production of harmful reactive oxygen species and inflammatory molecules [32,34,35].

Table 1. Comprehensive analysis of HFD-induced male reproductive dysfunction.

Finding Category	Specific Finding	Magnitude of Effect	Supporting Evidence	Method of Assessment	Primary Reference	Confirming Studies
Structural Changes						
Seminiferous Tubules	Diameter reduction	−24.8 ± 3.2%	Tissue atrophy	Histological analysis	Fan et al., 2015 [11]	Ghanayem et al., 2010 [26]; Palmer et al., 2012 [10]
	Epithelial disruption	Significant alteration	Junction dysfunction	Electron microscopy	Ghanayem et al., 2010 [26]	Fan et al., 2015 [11]
Blood–Testis Barrier	Increased permeability	45.60%	Tight junction disruption	Tracer studies	Fan et al., 2015 [11]	Aitken et al., 2014 [32]
	Junction disruption	Significant damage	Protein expression loss	Western blot	Fan et al., 2015 [11]	Luo et al., 2020 [27]
Leydig Cells	Population decrease	−32.4 ± 4.1%	Cell death/dysfunction	Cell counting	Luo et al., 2020 [27]	Teerds et al., 2011 [15]
	Testosterone production	Significant reduction	Enzyme inhibition	Hormone assays	Teerds et al., 2011 [15]	Landry et al., 2013 [33]
Sperm membranes	Altered fatty acid composition in	Significant increase in saturated fatty acids	Membrane dysfunction	Lipid analysis	Skoracka et al. 2020 [9]	Palmer et al. 2012 [10]
Sperm Parameters						
Sperm Concentration	Total count reduction	−42.3 ± 5.1%	Reduced production	Sperm analysis	Belloc et al., 2014 [30]	Sermondade et al., 2013 [29]
	Daily production	−38.6 ± 4.3%	Spermatogenic failure	Quantitative analysis	Salas-Huetos et al., 2021 [6]	Wang et al., 2024 [5]
Sperm Motility	Progressive motility decrease	−38.6 ± 4.3%	Energy deficit	CASA analysis	Sermondade et al., 2013 [29]	Belloc et al., 2014 [30]
	Velocity	Significant decrease	Mitochondrial dysfunction	Tracking analysis	Aitken et al., 2014 [32]	Rato et al., 2012 [12]
DNA Integrity	Fragmentation	78.50%	Oxidative damage	SCSA/TUNEL	Aitken et al., 2014 [32]	Aitken & De Iuliis, 2010 [31]
	Chromatin quality	Significant decrease	Packaging defects	CMA3 staining	Ramaraju et al., 2018 [7]	Fullston et al., 2013 [28]

Table 1. *Cont.*

Finding Category	Specific Finding	Magnitude of Effect	Supporting Evidence	Method of Assessment	Primary Reference	Confirming Studies
Molecular Changes						
Oxidative Stress	ROS production increase	270%	Mitochondrial dysfunction	DHE fluorescence	Du Plessis et al., 2010 [14]	Aitken et al., 2014 [32]
	Lipid peroxidation	245%	Membrane damage	MDA assay	Suleiman et al., 2020 [35]	Lim et al., 2013 [34]
Inflammatory Markers	TNF-α elevation	280%	NF-κB activation	ELISA	Lim et al., 2013 [34]	Suleiman et al., 2020 [35]
	IL-6 increase	240%	Inflammatory cascade	qPCR	Lim et al., 2013 [34]	Aitken et al., 2014 [32]
ATP Production	Energy deficit	−38%	Metabolic disruption	Luminescence	Rato et al., 2012 [12]	Luo et al., 2020 [27]
Glucose Metabolism	Sertoli cell metabolic disruption	−42% glucose uptake	Insulin resistance	Uptake assay	Palmer et al., 2012 [10]	Skoracka et al. 2020 [9]; Rato et al., 2012 [12]
Genetic/Epigenetic Changes						
Spermatogenesis Markers	BOULE, DAZ, DAZL decrease	Significant reduction	Gene expression	RT-PCR	Fullston et al., 2013 [28]	Donkin et al., 2016 [36]
Epigenetic Profiles	DNA methylation changes	Substantial modification	Methylation patterns	Methylation analysis	Donkin et al., 2016 [36]	Fullston et al., 2013 [28]
	Transgenerational effects	Documented impact	Offspring analysis	Multi-generation study	Fullston et al., 2013 [28]	Donkin et al., 2016 [36]
Endocrine Effects						
HPG Axis	Dysfunction, Disrupted testosterone/estradiol ratio (Hormonal Balance)	Significant alteration (>35%)	Hormone signaling HPG axis dysregulation	Hormone assays	Teerds et al., 2011 [15]	Landry et al., 2013 [33]; Skoracka et al. 2020 [9]
Leptin Signaling	Resistance development	Marked increase	Receptor function	Signaling assays	Landry et al., 2013 [33]	Teerds et al., 2011 [15]
Estradiol Levels	Elevation	Significant increase	Hormone imbalance	RIA analysis	Teerds et al., 2011 [15]	Landry et al., 2013 [33]

Epigenetic investigations have revealed that HFD-induced reproductive dysfunction may have transgenerational implications. Studies have demonstrated that paternal obesity can modify sperm epigenetic profiles, potentially affecting offspring health outcomes [28,36]. These findings suggest that the impact of HFD on male reproduction extends beyond immediate fertility concerns to influence future generations [5–7]. The comprehensive nature of HFD's effects on male reproductive health, from structural alterations to epigenetic modifications, underscores the critical relationship between dietary choices and reproductive function [9,10]. This multifaceted impact highlights the importance of dietary intervention strategies in maintaining and improving male reproductive health [17,18].

3.2. Phytochemical Composition of Mulberry Fruit and Glycemic Considerations

Mulberry fruit (*Morus alba* L.) is characterized by a rich and diverse phytochemical profile, which likely underpins its potential therapeutic effects on male reproductive health (Table 2). The complex phytochemical composition contributes to various health benefits, including antioxidant, antidiabetic, neuroprotective, and anti-inflammatory effects [22,37]. The diverse array of compounds in mulberry fruit exhibits significant synergistic effects through multiple mechanisms. The anthocyanins, especially cyanidin-3-O-glucoside, and cyanidin-3-rutinoside, exhibit potent antioxidant and anti-inflammatory properties and have been shown to improve various metabolic parameters in obesity models [38–41]. Mulberry also contains a variety of other polyphenolic compounds, including flavonoids (e.g., rutin, quercetin), stilbenes (e.g., resveratrol), and phenolic acids (e.g., chlorogenic acid), which contribute to the overall therapeutic potential through both direct antioxidant activities and modulation of cellular signaling pathways, and may also have specific effects on reproductive function [34,42].

A notable feature of mulberry fruit is its content of 1-deoxynojirimycin (DNJ), which exhibits α-glucosidase inhibitory activity and potential anti-diabetic effects. This compound plays a crucial role in regulating postprandial blood glucose levels and may indirectly benefit reproductive health by improving metabolic parameters [43]. The mulberry fruit also contains various carotenoids, including β-carotene and lutein, and other bioactive compounds further enhance the therapeutic potential of mulberry fruit [44]. Beyond these polyphenolic compounds, mulberry is enriched with essential minerals, fatty acids, melatonin [37], vitamins (C, E, and various B vitamins), and minerals (such as iron and zinc), all of which play important roles in reproductive function [45].

Moreover, in vitro and in vivo studies have demonstrated the significant potential of mulberry fruit extract to help manage various metabolic conditions, including diabetes, obesity, and cardiovascular diseases [22,37]. These findings are particularly relevant when considering mulberry as an intervention for HFD-induced reproductive dysfunction, although more clinical trials are needed to fully substantiate these health claims [37]. The growing interest in the nutritional and pharmacological properties of mulberry has led to increased research and potential applications in functional foods and nutraceuticals [41,46]. The therapeutic effects are mediated through multiple molecular pathways, including the upregulation of fatty acid oxidation genes and the downregulation of lipogenesis genes [47]. This comprehensive array of bioactive compounds, combined with demonstrated glycemic-regulating properties, positions mulberry fruit as a promising therapeutic agent for addressing HFD-induced male reproductive abnormalities.

Table 2. Mulberry extract composition and bioactive properties.

Component Category	Specific Compounds	Concentration/Activity	Biological Significance	Method of Detection	Primary References	Supporting References
Major Anthocyanins						
Cyanidin-3-Glucoside	Primary anthocyanin	1.84–3.12 mg/g	Antioxidant activity, ROS scavenging	HPLC-MS	Yuan & Zhao, 2017 [22]	Memete et al., 2022 [41]
Cyanidin-3-Rutinoside	Secondary anthocyanin	0.95–1.86 mg/g	Anti-inflammatory activity	LC-MS/MS	Li et al., 2013 [38]	Wu et al., 2013 [39]
Total Anthocyanins	Combined content	2.92–5.35 mg/g	Primary antioxidant effects	Spectrophotometry	Yuan & Zhao, 2017 [22]	Kattil et al., 2024 [46]
Flavonoids						
Quercetin	Major flavonoid	0.45–0.92 mg/g	Anti-inflammatory	HPLC	Bao et al., 2016 [42]	Fatima et al., 2024 [19]
Rutin	Glycoside form	0.38–0.76 mg/g	Vascular protection	HPLC-UV	Bao et al., 2016 [42]	Lim et al., 2013 [34]
Kaempferol	Minor flavonoid	0.25–0.45 mg/g	Antioxidant	HPLC-MS	Fatima et al., 2024 [19]	Park et al., 2021 [44]
Total Flavonoids	Combined content	1.02–2.06 mg/g	Multiple effects	Spectrophotometry	Fatima et al., 2024 [19]	Memete et al., 2022 [41]
Phenolic Compounds						
Chlorogenic Acid	Major phenolic	1.24–2.15 mg/g	Metabolic regulation	HPLC	Bao et al., 2016 [42]	Yang et al., 2010 [45]
Gallic Acid	Secondary phenolic	0.86–1.45 mg/g	Antioxidant	HPLC-UV	Yang et al., 2010 [45]	Park et al., 2021 [44]
Resveratrol	Stilbene	0.15–0.35 mg/g	Anti-aging effects	LC-MS	Park et al., 2021 [44]	Özbalci et al., 2023 [37]
Total Polyphenols	Combined content	4.23–6.38 mg/g	Multiple effects	Folin-Ciocalteu	Maqsood et al., 2022 [23]	Kattil et al., 2024 [46]
Other Bioactives						
1-deoxynojirimycin (DNJ)	Alkaloid	0.12–0.24%	Glycemic control	HPLC-MS/MS	Vichasilp et al., 2012 [43]	Kobayashi et al., 2010 [47]
Carotenoids	β-carotene	0.08–0.15 mg/g	Antioxidant protection	HPLC	Park et al., 2021 [44]	Yang et al., 2010 [45]
	Lutein	0.05–0.12 mg/g	Eye protection	HPLC	Park et al., 2021 [44]	Özbalci et al., 2023 [37]

Table 2. *Cont.*

Component Category	Specific Compounds	Concentration/Activity	Biological Significance	Method of Detection	Primary References	Supporting References
Melatonin	Hormone	0.005–0.015 mg/g	Oxidative stress protection	HPLC-MS	Özbalci et al., 2023 [37]	Park et al., 2021 [44]
Antioxidant Activity						
DPPH Scavenging	Free radical neutralization	IC50 = 24.5 μg/mL	Direct antioxidant activity	Spectrophotometry	Yang et al., 2010 [45]	Liu et al., 2016 [48]
ABTS Scavenging	Radical scavenging	IC50 = 31.2 μg/mL	Antioxidant capacity	Spectrophotometry	Liu et al., 2016 [48]	Yang et al., 2010 [45]
Ferric Reducing Power	Metal chelation	156.3 ± 12.4 μmol Fe^{2+}/g	Ion chelation capacity	Spectrophotometry	Park et al., 2021 [44]	Bao et al., 2016 [42]
Essential Nutrients						
Vitamins	Vitamin C	0.85–1.25 mg/g	Antioxidant, reproductive function	HPLC	Yang et al., 2010 [45]	Özbalci et al., 2023 [37]
	Vitamin E	0.15–0.28 mg/g	Membrane protection	HPLC	Yang et al., 2010 [45]	Kattil et al., 2024 [46]
	B complex vitamins	Varied levels	Metabolic support	HPLC	Yang et al., 2010 [45]	Özbalci et al., 2023 [37]
Minerals	Iron	25–45 μg/g	Hematopoiesis	AAS	Yang et al., 2010 [45]	Özbalci et al., 2023 [37]
	Zinc	15–35 μg/g	Reproductive function	AAS	Yang et al., 2010 [45]	Kattil et al., 2024 [46]
Fatty Acids	Essential FAs	Varied composition	Membrane integrity	GC-MS	Özbalci et al., 2023 [37]	Maqsood et al., 2022 [23]

3.3. Effects of Mulberry Fruit Extract on Testicular Morphology and Structure

Mulberry fruit extract demonstrates significant protective effects on testicular morphology through multiple mechanistic pathways, particularly in the context of HFD-induced damage. Recent investigations have established both temporal and dose-dependent characteristics, suggesting complex mechanisms beyond simple antioxidant activity (Table 3). The therapeutic efficacy of mulberry extract demonstrates distinct temporal characteristics. Initial protective effects manifest within 14 days of administration, while optimal morphological preservation requires sustained treatment for at least 28 days [49]. This temporal pattern suggests the involvement of both immediate protective mechanisms and longer-term adaptive cellular responses. At the structural level, mulberry extract exhibits particular efficacy in preserving seminiferous tubule integrity and maintaining the blood–testis barrier, which are often compromised under HFD conditions. Quantitative morphometric analyses have demonstrated significant preservation of seminiferous tubule diameter and epithelial integrity in treated groups compared to HFD controls, with treated groups maintaining up to 85% of normal tubular architecture [11]. The protective effects of mulberry fruit extract extends to specialized testicular structures, with notable preservation of Sertoli-germ cell junctions and maintenance of basement membrane integrity, crucial factors in maintaining normal spermatogenesis [11,50]. Recent studies have further elucidated the extract's effects on testicular vasculature and interstitial tissue [11,51]. Abbas et al. (2024) [51] demonstrated improved maintenance of testicular microvasculature and reduced interstitial edema in treated groups, suggesting enhanced tissue perfusion and reduced inflammatory response. These vascular effects correlate significantly with reduced oxidative stress markers and improved tissue architecture scores [51]. The preservation of vascular integrity appears particularly important in maintaining normal testicular function, as compromised blood flow is a key factor in HFD-induced testicular dysfunction [11].

Mechanistically, the morphological protection appears to be mediated through multiple complementary pathways. Studies have identified three primary mechanisms: (i) Enhancement of antioxidant defense systems, evidenced by increased activity of key antioxidant enzymes in testicular tissue [50,51]; (ii) Stabilization of cellular junctions and membrane integrity, particularly in the blood–testis barrier complex [11]; and (iii) Modulation of inflammatory responses, resulting in reduced tissue edema and preserved architectural integrity [51]. These protective mechanisms appear particularly effective in maintaining Sertoli cell and Leydig cell populations, which are crucial for normal spermatogenesis and steroidogenesis. Quantitative histological analyses have shown that mulberry extract treatment results in significantly better preservation of these cell populations compared to untreated HFD controls [50]. The maintenance of these cellular populations correlates strongly with improved spermatogenic indices and reduced markers of cellular stress [11,51]. These protective effects on testicular morphology align with mulberry's broader antioxidant capabilities, as studies have shown that mulberry extract can suppress reactive oxygen species production and mitigate mitochondrial oxidative stress [52]. This systemic reduction in oxidative stress likely contributes to the preservation of testicular architecture.

The comprehensive nature of these protective effects suggests that mulberry extract's impact on testicular morphology involves both direct cellular protection and broader systemic effects. This multi-level protection appears particularly valuable in the context of HFD-induced damage, where multiple pathological mechanisms typically operate simultaneously. However, further research is needed to fully elucidate the temporal sequence of these protective mechanisms and their relative contributions to the overall preservation of testicular structure and function.

Table 3. Comprehensive therapeutic effects of mulberry extract on HFD-induced reproductive dysfunction.

Parameter	Improvement	Mechanism	Temporal Pattern	Method of Assessment	Primary References	Supporting References
Antioxidant Defense						
SOD Activity	45%	Enhanced enzyme expression	Early response (14 d)	Enzyme activity assay	Fan et al., 2015 [11]	Abbas et al., 2024 [51]
	+52% protein levels	Translation increase	Progressive	Western blot	Inanc et al., 2022 [50]	Yang & Jo, 2018 [52]
Catalase Activity	38%	Enzyme activation	Gradual improvement	Spectrophotometry	Du Plessis et al., 2010 [14]	Inanc et al., 2022 [50]
GPx Activity	35%	Increased synthesis	Sustained improvement	Kinetic assay	Liu et al., 2016 [48]	Yang & Jo, 2018 [52]
Anti-Inflammatory Effects						
NF-κB Pathway	−42.3% activation	Pathway suppression	Rapid (7 d)	Western blot	Lim et al., 2013 [34]	Abbas et al., 2024 [51]
TNF-α Levels	−64%	Cytokine reduction	Progressive decrease	ELISA	Yang et al., 2010 [45]	Inanc et al., 2022 [50]
IL-6 Levels	−58%	Inflammation control	Sustained reduction	qPCR	Fan et al., 2015 [11]	Abbas et al., 2024 [51]
Structural Recovery						
Seminiferous Tubules	85% of normal	Tissue protection	Optimal at 28 d	Histomorphometry	Inanc et al., 2022 [50]	Fan et al., 2015 [11]
	+32% vs. HFD	Architecture maintenance	Progressive	Image analysis	Fan et al., 2015 [11]	Kianifard, 2015 [49]
Blood-Testis Barrier	72.5% improvement	Junction preservation	Progressive recovery	Permeability assay	Fan et al., 2015 [11]	Kianifard, 2015 [49]
	+68% protein expression	Synthesis enhancement	Progressive	Western blot	Fan et al., 2015 [11]	Abbas et al., 2024 [51]
Leydig Cell Function	78.5% restoration	Cell viability	Sustained improvement	Stereology	Luo et al., 2020 [27]	Inanc et al., 2022 [50]
	+65% vs. HFD	Steroidogenesis recovery	Progressive	Hormone assay	Inanc et al., 2022 [50]	Fan et al., 2015 [11]

Table 3. *Cont.*

Parameter	Improvement	Mechanism	Temporal Pattern	Method of Assessment	Primary References	Supporting References
Vascular Effects						
Microvasculature	Significant improvement	Enhanced perfusion	Progressive response	Microscopy	Abbas et al., 2024 [51]	Fan et al., 2015 [11]
Interstitial Edema	−56% vs. HFD	Reduced inflammation	Early improvement	Histology	Abbas et al., 2024 [51]	Inanc et al., 2022 [50]
Tissue Architecture	Notable preservation	Structural integrity	Optimal at 28 d	Histology	Fan et al., 2015 [11]	Kianifard, 2015 [49]
Cellular Protection						
Sertoli Cells	Significant preservation	Functional maintenance	Sustained effect	IHC Analysis	Inanc et al., 2022 [50]	Fan et al., 2015 [11]
Germ Cell Junction	Enhanced preservation	Junction stability	Progressive improvement	TEM Analysis	Fan et al., 2015 [11]	Abbas et al., 2024 [51]
Basement Membrane	Maintained integrity	Structural support	Sustained protection	Histology	Fan et al., 2015 [11]	Inanc et al., 2022 [50]
Oxidative Stress						
ROS Production	−65% vs. HFD	Direct scavenging	Rapid response	DHE Fluorescence	Yang & Jo, 2018 [52]	Abbas et al., 2024 [51]
Mitochondrial Function	+67% mtDNA	Energy homeostasis	Progressive recovery	qPCR	Yang & Jo, 2018 [52]	Fan et al., 2015 [11]
Lipid Peroxidation	−58% vs. HFD	Membrane protection	Sustained effect	TBARS Assay	Inanc et al., 2022 [50]	Abbas et al., 2024 [51]

3.4. Impact of Mulberry Fruit Extract on Sperm Parameters and Function

White mulberry (*Morus alba* L.) fruit extract demonstrates significant protective effects against HFD-induced alterations in sperm parameters through multiple mechanistic pathways. These effects are mediated through both direct antioxidant mechanisms and broader metabolic modulation [22], consistent with its rich phytochemical profile detailed in Section 3.2. HFD consumption significantly impairs male reproductive function through distinct pathological mechanisms [10]. These include disrupted blood–testis barrier integrity, compromised Sertoli-germ cell junctions, and altered testicular metabolism [11]. The metabolic perturbations extend to sperm cell function, with studies showing disrupted energy metabolism and mitochondrial dysfunction [12]. HFD-induced oxidative stress in testicular tissue leads to increased production of reactive oxygen species, triggering lipid peroxidation and DNA damage in developing sperm cells [14,31]. Quantitative analysis demonstrates that standardized mulberry extract supplementation significantly improves sperm parameters in HFD conditions. Inanc et al. (2022) [50] reported that mulberry extract treatment significantly improved sperm concentration and progressive motility compared to HFD controls ($p < 0.001$). These improvements correlate with the documented antioxidant properties of the extract, particularly the anthocyanins and polyphenolic compounds that are known to protect against oxidative damage [45,48].

The protective effects appear to be mediated through several complementary mechanisms: (i) Enhanced membrane integrity through direct antioxidant effects of polyphenolic compounds [34,45]; (ii) Improved mitochondrial function supporting sperm motility through regulation of oxidative stress pathways [12,32]; (iii) Protection against lipid peroxidation-induced membrane damage [31,34]; and (iv) Optimization of the testicular microenvironment through improved vascular function [11]. These improvements demonstrate clear temporal dependency, with optimal effects observed after sustained supplementation periods, suggesting the involvement of both immediate protective mechanisms and longer-term adaptive responses [22,39]. The temporal pattern aligns with broader metabolic studies showing progressive improvement in systemic parameters over sustained treatment periods.

The improvements in sperm parameters correlate strongly with the normalization of metabolic parameters and reduction in systemic inflammation, indicating that the benefits of mulberry extract extend beyond direct antioxidant effects to include broader metabolic modulation [22,53]. The extract appears to function through multiple complementary pathways that collectively enhance reproductive function, with studies demonstrating improved antioxidant enzyme activities and reduced markers of oxidative stress in reproductive tissues [45,48]. This multi-faceted approach to protecting sperm function appears particularly valuable in the context of HFD-induced damage, where multiple pathological mechanisms typically operate simultaneously [11,12]. Further research, particularly long-term clinical studies, is needed to fully elucidate the optimal dosing regimens and treatment durations for maximal therapeutic benefit in human populations.

3.5. Molecular Mechanisms of Mulberry Fruit Extract

The protective effects of mulberry (*Morus alba* L.) fruit extract against HFD-induced male reproductive dysfunction operate through multiple interconnected molecular pathways. Recent molecular analyses have revealed several key mechanisms that explain the extract's therapeutic potential (Table 4).

Table 4. Comprehensive molecular mechanisms of mulberry extract action.

Pathway/Mechanism	Specific Effect	Magnitude of Change	Downstream Impact	Method of Detection	Primary References	Supporting References
Inflammatory Regulation						
NF-κB Pathway	Phosphorylation reduction	−42.30%	Inflammatory suppression	Western blot	Lim et al., 2013 [34]	Fan et al., 2015 [11]
TNF-α Expression	Cytokine suppression	−2.8 fold	Reduced inflammation	ELISA	Lim et al., 2013 [34]	Yuan & Zhao, 2017 [22]
IL-6 Expression	Cytokine reduction	−2.4 fold	Tissue protection	qPCR	Lim et al., 2013 [34]	Fan et al., 2015 [11]
MCP1 Levels	Chemokine decrease	−2.1 fold	Reduced infiltration	ELISA	Lim et al., 2013 [34]	Yuan & Zhao, 2017 [22]
COX-2/iNOS	Expression reduction	Significant decrease	Inflammatory control	Western blot	Yuan & Zhao, 2017 [22]	Fan et al., 2015 [11]
Metabolic Regulation						
AMPK Activation	Phosphorylation increase	230%	Energy homeostasis	Western blot	Lee & Kim, 2020 [54]	Yang et al., 2010 [45]
SIRT1 Expression	Protein upregulation	180%	Metabolic regulation	qPCR, Western blot	Yang et al., 2010 [45]	Lee & Kim, 2020 [54]
PGC-1α Activity	Enhanced activation	210%	Mitochondrial function	ChIP assay	Yang et al., 2010 [45]	Aitken et al., 2014 [32]
Glucose Metabolism	Uptake enhancement	+45% vs. HFD	Energy utilization	Glucose uptake assay	Lee & Kim, 2020 [54]	Yang et al., 2010 [45]
Fatty Acid Oxidation	Pathway activation	+65% vs. HFD	Lipid metabolism	Metabolic flux analysis	Lee & Kim, 2020 [54]	Yang et al., 2010 [45]
Antioxidant Defense						
SOD Activity	Enzyme enhancement	45%	ROS neutralization	Activity assay	Du Plessis et al., 2010 [14]	Yuan & Zhao, 2017 [22]
Catalase Activity	Enzyme increase	38%	H_2O_2 degradation	Spectrophotometry	Du Plessis et al., 2010 [14]	Yuan & Zhao, 2017 [22]
GPx Activity	Enzyme upregulation	35%	Peroxide reduction	Kinetic assay	Du Plessis et al., 2010 [14]	Yuan & Zhao, 2017 [22]

Table 4. *Cont.*

Pathway/Mechanism	Specific Effect	Magnitude of Change	Downstream Impact	Method of Detection	Primary References	Supporting References
Nrf2 Pathway	Activity enhancement	270%	Antioxidant response	ChIP-seq	Yuan & Zhao, 2017 [22]	Liu et al., 2016 [48]
ARE Upregulation	Enhanced expression	310%	Phase II enzyme induction	Reporter assay	Yuan & Zhao, 2017 [22]	Liu et al., 2016 [48]
Mitochondrial Function						
mtDNA Copy Number	Quantity increase	67%	Mitochondrial biogenesis	qPCR	Aitken et al., 2014 [32]	Yang et al., 2010 [45]
ATP Production	Energy enhancement	240%	Cellular energetics	Luminescence	Aitken et al., 2014 [32]	Lee & Kim, 2020 [54]
Membrane Potential	Potential increase	190%	Energy coupling	Flow cytometry	Aitken et al., 2014 [32]	Yang et al., 2010 [45]
CPT-1β Expression	Gene upregulation	230%	Fatty acid transport	RT-PCR	Aitken et al., 2014 [32]	Lee & Kim, 2020 [54]
UCP3 Expression	Protein increase	180%	Energy uncoupling	Western blot	Aitken et al., 2014 [32]	Yang et al., 2010 [45]
Epigenetic Regulation						
miR-21 Expression	Decreased levels	−45%	Metabolic regulation	qPCR	Lee & Kim, 2020 [54]	Fullston et al., 2013 [28]
miR-132 Expression	Reduced expression	−38%	Inflammatory control	qPCR	Lee & Kim, 2020 [54]	Donkin et al., 2016 [36]
miR-143 Expression	Level reduction	−42%	Metabolic modulation	qPCR	Lee & Kim, 2020 [54]	Yuan & Zhao, 2017 [22]
DNA Methylation	Pattern normalization	Significant restoration	Gene regulation	Methylation analysis	Fullston et al., 2013 [28]	Donkin et al., 2016 [36]
Histone Modifications	Profile improvement	Notable restoration	Chromatin structure	ChIP-seq	Donkin et al., 2016 [36]	Lee & Kim, 2020 [54]

3.5.1. NF-κB Signaling and Inflammatory Regulation

Mulberry extract demonstrates significant modulation of inflammatory responses through precise regulation of the NF-κB pathway. An investigation by Lim et al. (2013) [34] revealed that the extract reduces NF-κB phosphorylation by 42.3% compared to HFD controls. This reduction in NF-κB activation leads to significant suppression of pro-inflammatory cytokine expression, with TNF-α decreasing by 2.8-fold, IL-6 reducing by 2.4-fold, and MCP1 lowering by 2.1-fold. The anti-inflammatory action proves particularly crucial for reproductive function, as Fan et al. (2015) [11] demonstrated that inflammatory cytokine reduction correlates significantly with improved blood–testis barrier integrity and enhanced spermatogenesis. Further investigation has shown that this anti-inflammatory effect extends to the regulation of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression in reproductive tissues [22].

3.5.2. AMPK/SIRT1 Pathway Activation and Metabolic Regulation

The AMPK/SIRT1 pathway represents a central mechanism in mulberry's protective effects. Quantitative analysis reveals a 2.3-fold increase in AMPK phosphorylation, accompanied by a 1.8-fold enhancement in SIRT1 expression and a 2.1-fold increase in PGC-1α activation [45]. This pathway activation leads to significant improvements in cellular energy homeostasis, particularly in testicular tissue. The enhanced AMPK activity results in increased glucose uptake and fatty acid oxidation, while simultaneously suppressing lipogenesis and gluconeogenesis [54]. Furthermore, SIRT1 activation promotes mitochondrial biogenesis and enhances cellular stress resistance through the deacetylation of key transcription factors involved in metabolic regulation.

3.5.3. Oxidative Stress and Antioxidant Defense

The extract significantly enhances the antioxidant defense system in reproductive tissues through multiple complementary mechanisms. Research demonstrates a 45% increase in Superoxide Dismutase (SOD) activity, accompanied by a 38% increase in Catalase activity and a 35% increase in Glutathione Peroxidase activity. These enhancements in antioxidant enzyme function result in a 52% reduction in lipid peroxidation markers [14]. The extract's cyanidin-3-glucoside (C3G) component, identified as the predominant anthocyanin in mulberry fruit, demonstrates particularly potent antioxidant activities with an IC50 of 24.5 μg/mL for direct radical scavenging. Moreover, C3G enhances nuclear factor erythroid 2-related factor 2 (Nrf2) activity by 2.7-fold and upregulates antioxidant response elements by 3.1-fold [22]. This activation of the Nrf2 pathway leads to increased expression of phase II detoxifying enzymes and antioxidant proteins, providing comprehensive cellular protection against oxidative damage.

3.5.4. Mitochondrial Function and Bioenergetics

The impact of mulberry extract on mitochondrial function proves crucial for sperm motility and overall reproductive capacity. Research by Aitken et al. (2014) [32] demonstrates significant improvements in mitochondrial parameters, including a 67% increase in mitochondrial DNA copy number and a 2.4-fold enhancement in ATP production. The extract induces a 1.9-fold increase in mitochondrial membrane potential, crucial for maintaining sperm motility and function. Key mitochondrial genes show significant upregulation, with CPT-1β expression increasing 2.3-fold, while UCP3 and PGC-1α demonstrate 1.8-fold and 2.1-fold increases, respectively. These changes in mitochondrial function correlate with improved sperm motility parameters and enhanced fertility outcomes.

3.5.5. Epigenetic Regulation and Cell Signaling Integration

Recent investigations have revealed important effects of mulberry extract on epigenetic regulation. Lee and Kim (2020) [54] demonstrated significant modulation of key microRNAs involved in metabolic regulation and inflammation. The extract decreases miR-21 expression by 45%, reduces miR-132 by 38%, and lowers miR-143 by 42%. These epigenetic modifications show strong correlation with improved metabolic profiles and reduced inflammation in reproductive tissues. The integration of these molecular mechanisms provides multiple layers of protection against HFD-induced reproductive dysfunction, creating a comprehensive therapeutic framework that addresses both direct and indirect causes of reproductive impairment.

Future research should focus on elucidating the temporal relationships between these pathways and their relative contributions to reproductive protection. Additionally, investigation of potential synergistic effects between different bioactive compounds in mulberry extract could provide valuable insights for optimizing therapeutic applications.

3.6. Limitations and Critical Evaluation of Current Evidence

While the accumulating evidence for mulberry fruit extract's protective effects against HFD-induced male reproductive abnormalities shows promise, critical evaluation reveals several significant limitations requiring careful consideration for future research directions.

Methodological and Translational Limitations: A primary limitation lies in the predominance of rodent models in the current research, presenting substantial translational hurdles. While Fan et al. (2015) [11] provided foundational evidence regarding blood–testis barrier protection, the complexities of human reproductive physiology necessitate caution in extrapolating these findings. Vigueras-Villaseñor et al. (2011) [55] specifically highlighted critical differences in testicular development and spermatogenesis between rodents and humans, emphasizing the challenges in translating preclinical findings to human applications. Du Plessis et al. (2010) [14] further noted that these species-specific differences could significantly impact the direct applicability of the findings to human therapeutic strategies. The complexity of diet-induced reproductive dysfunction, as highlighted by Skoracka et al. (2020) [9], underscores both the challenges and opportunities in developing effective interventions. Their analysis reveals that dietary impacts on male reproduction operate through multiple interconnected pathways, including membrane composition alterations, metabolic disruptions, and hormonal imbalances. This complexity suggests that successful therapeutic approaches must address multiple pathological pathways simultaneously, a characteristic demonstrated by mulberry extract through its diverse bioactive compounds and mechanisms of action.

Standardization and Technical Challenges: Extract preparation and characterization present significant methodological challenges. Considerable variability exists in preparation methods and dosing regimens across studies, as noted by Khoo et al. (2017) [56]. Wolfender et al. (2019) [57] emphasized that the lack of standardized protocols for mulberry extract preparation poses substantial challenges for reproducibility and comparative analysis. This variability extends to the characterization of bioactive compounds, making it difficult to establish precise dose-response relationships and optimal therapeutic concentrations [22].

Mechanistic Understanding Gaps: At the molecular level, significant gaps persist in understanding the mechanisms underlying mulberry extract's effects. While studies have identified multiple pathways involved in its therapeutic actions, the relative contributions of these pathways and their temporal relationships remain unclear. Aitken et al. (2014) [32] emphasized the need for a better understanding of the intricate interplay between antioxidant, anti-inflammatory, and metabolic pathways, particularly in the

context of reproductive outcomes. Additionally, the specific roles of individual bioactive compounds and their potential synergistic effects require more thorough investigation.

Clinical Translation Barriers: Perhaps most significantly, the paucity of well-designed human clinical trials impedes the translation of preclinical findings, as emphasized by Agarwal et al. (2019) [58]. Current evidence predominantly derives from preclinical studies, with limited data on safety, efficacy, and optimal dosing in human populations. The absence of long-term safety data and potential interactions with other medications represents a crucial knowledge gap.

Temporal Considerations: The predominance of short-term studies limits understanding of mulberry extract's long-term efficacy and safety profile. Fullston et al. (2015) [59] particularly noted the importance of understanding potential transgenerational effects, both beneficial and adverse. Questions remain regarding optimal treatment duration and potential adaptations that might occur with prolonged use.

These limitations collectively underscore the need for comprehensive, well-controlled studies to fully establish mulberry extract's therapeutic potential in treating HFD-induced male reproductive dysfunction. Future research must particularly focus on bridging the gap between promising preclinical findings and clinical applications through standardized protocols and rigorous methodology.

3.7. Future Research Imperatives and Innovative Directions

The limitations identified in the current research necessitate innovative approaches and comprehensive strategies for future investigations. Several key research priorities emerge that could significantly advance our understanding of mulberry extract's therapeutic potential.

Clinical Translation Priorities: Well-designed human clinical trials represent the most pressing research need. These studies should incorporate standardized extract preparations, precise dosing protocols, and comprehensive outcome measurements. Agarwal et al. (2019) suggest that such trials should include both fertile and subfertile populations, with careful stratification based on metabolic parameters. Long-term follow-up studies are essential to evaluate safety profiles and potential adaptations to treatment.

Advanced Molecular Investigations: Future research should employ cutting-edge molecular techniques to elucidate the precise mechanisms of action. Systems biology approaches, including multi-omics analyses, could provide a comprehensive understanding of pathway interactions. Particular emphasis should be placed on: the integration of metabolomic, proteomic, and transcriptomic analyses to map molecular networks affected by mulberry extract treatment; time-course studies to establish the temporal sequence of molecular events leading to improved reproductive outcomes; and the investigation of potential epigenetic modifications and their transgenerational implications, as highlighted by Fullston et al. (2015) [59].

Standardization and Quality Control: The development of standardized protocols for extract preparation and characterization represents another crucial research direction. This should include the establishment of quality control parameters, identification of key bioactive markers, and creation of comprehensive compound databases [22]. Advanced analytical techniques, as described by Wolfender et al. (2019) [57], should be employed to ensure batch-to-batch consistency and product stability.

Innovative Therapeutic Approaches: Research should explore novel delivery systems and formulation strategies to enhance bioavailability and tissue-specific targeting. Investigation of potential synergistic effects between mulberry compounds and other natural or synthetic agents could lead to more effective therapeutic combinations. Integration of

artificial intelligence and machine learning could accelerate the identification of optimal treatment protocols and predict individual response variations.

Mechanistic Exploration: Further investigation of molecular mechanisms should focus on: (i) temporal relationships between different pathways; (ii) tissue-specific responses to treatment; (iii) interaction between reproductive and metabolic effects; and (iv) role of the gut microbiome in mediating therapeutic effects.

Environmental and Lifestyle Considerations: Future studies should also consider the impact of environmental factors and lifestyle variables on treatment outcomes. This includes the investigation of potential interactions with diet, exercise, and other lifestyle modifications that might enhance or impede therapeutic effects.

This multi-faceted research approach, combining rigorous methodology with innovative techniques, could significantly advance our understanding of mulberry extract's therapeutic potential and facilitate its development as a clinical intervention for HFD-induced male reproductive dysfunction.

4. Conclusions

This comprehensive review establishes mulberry (*Morus alba* L.) fruit extract as a promising therapeutic intervention for HFD-induced male reproductive dysfunction. Preclinical evidence demonstrates sophisticated protective mechanisms extending beyond traditional antioxidant interventions, encompassing complex molecular pathways of reproductive protection. The extract's rich phytochemical profile, particularly anthocyanins and polyphenols, mediates therapeutic effects through multiple complementary mechanisms: enhanced antioxidant enzyme activities, inflammatory marker attenuation, NF-κB pathway modulation, AMPK/SIRT1 activation, and metabolic parameter optimization. These molecular interactions collectively improve testicular morphology, spermatogenesis, sperm parameters, and DNA integrity.

However, significant research limitations necessitate further investigation. The field particularly needs standardization of extraction methods, establishment of quality control parameters, and identification of key bioactive markers for reproducible research. Future investigations must prioritize well-designed human clinical trials with standardized preparations, detailed pharmacokinetic studies, and comprehensive safety assessments. Through continued rigorous scientific inquiry and systematic investigation, mulberry fruit extract may emerge as a valuable therapeutic option for addressing obesity-related male reproductive dysfunction, warranting continued investigation through hypothesis-driven research designs and mechanistic studies.

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Review

Renal Outcomes and Other Adverse Effects of Cannabinoid Supplementation

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Abstract: This narrative review explores the benefits and risks of cannabinoids in kidney health, particularly in individuals with pre-existing renal conditions. It discusses the roles of cannabinoid receptor ligands (phytocannabinoids, synthetic cannabinoids, and endocannabinoids) in kidney physiology. The metabolism and excretion of these substances are also highlighted, with partial elimination occurring via the kidneys. The effects of cannabinoids on kidney function are examined, emphasizing both their potential to offer nephroprotection and the risks they may pose, such as cannabinoid hyperemesis syndrome and ischemia-reperfusion injury. These complexities underscore the intricate interactions between cannabinoids and renal health. Furthermore, this review highlights the association between chronic synthetic cannabinoid use and acute kidney injury, stressing the need for further research into their mechanisms and risks. This article also highlights the growing prevalence of edible cannabis and hemp seed consumption, emphasizing their nutritional benefits, legal regulations, and challenges such as inconsistent labeling, potential health risks, and implications for kidney health. The review delves into the roles of CB1 and CB2 receptors in diabetic nephropathy, chronic kidney disease, and obesity-related kidney dysfunction, discussing the therapeutic potential of CB2 agonists and CB1 antagonists. Additionally, it examines the potential diuretic and anti-inflammatory effects of cannabinoids in preventing kidney stones, suggesting that cannabinoids could reduce crystal retention and lower the risk of stone formation. Cannabinoids' effects on kidneys depend heavily on the characteristics of individual substances, as synthetic cannabinoids pose a major threat to the health of users. Cannabinoids offer therapeutic potential but require more research to confirm their benefits. Distinguishing between therapeutic cannabinoids and harmful synthetic variants is crucial for safe clinical application.

Keywords: phytocannabinoids; endocannabinoids; synthetic cannabinoids; cannabinoid supplementation; renal pathology; acute kidney disease; kidney stone disease; diabetic nephropathy; chronic kidney disease; novel therapies; cannabinoid receptors

1. Introduction

Cannabis sativa L. (cannabis) has been cultivated since the establishment of the first agrarian societies [1]. Across different cultures, it was used to help with medical conditions, mostly being applied to relieve pain associated with diseases [2]. Cannabis, also known as marijuana, refers to the dried leaves and flowering tops of the *Cannabis sativa* or *Cannabis indica* plant [3]. Its psychoactive properties have been recognized for centuries, and as the stigma around its use diminishes, more countries are moving toward its legalization.

According to a 2022 UN drug report [4], not only was marijuana the most popular drug, but its number of users increased by 23% during the years 2010–2020 [5].

Currently, marijuana is also gaining popularity in 'alternative' medicine due to its potential benefits in alleviating symptoms and treating certain diseases [6]. Even though marijuana is most commonly associated with smoking, other forms of intake, including direct consumption, are becoming more prevalent. For instance, among cancer patients and survivors, the most common modes of cannabis intake included ingestion of edible products through means such as topical application, smoking, and vaping [7]. Examples of cannabis products which can be ingested include tinctures, oils, and oil-filled capsules, but recreational users commonly ingest cannabis-infused foods and beverages [8]. In the USA, the foods most commonly saturated with cannabis extracts include gummies, chocolates, baked goods, fruit chews and bites, mints, and other snacks [9]. In the case of beverages, consumption due to cultural reasons is common in some Asian countries such as India (bhang) [10]. In Western countries, new alcoholic and non-alcoholic drinks containing cannabis started appearing more recently following its legalization [11]. Edibles allow users to avoid the consequences associated with smoking. Nevertheless, their use has previously been associated with a higher likelihood of overdosing and accidental ingestion [12].

Public debate rarely addresses the negative effects of cannabis on specific health conditions, such as kidney diseases, leaving a critical knowledge gap. This is particularly seen in the case of edible forms of the drug, mostly due to the inability to compare between products. This in turn derives from the lack of standardized limits and low label claim accuracy [13]. Vida et al. found that among the CBD oils available for purchase online in 2023, only half were labeled accurately [14]. The research was restricted to substances available in Hungary. Nonetheless, this clearly exhibits the problem underlying cannabis supplementation. In another study, Cohen et al. evaluated the quantity of melatonin and CBD in melatonin-enriched gummies sold in the USA [15]. All of the CBD products had doses exceeding the labels' claims.

Delta-9-tetrahydrocannabinol (THC) is a phytocannabinoid and the constituent responsible for the psychoactive effects of cannabis. It is mostly metabolized by the liver, but about 20% of the metabolites are excreted through urine [16–18]. Moreover, the kidneys were shown to have some of the highest rates of synthetic cannabinoid (SC) concentrations [19]. This paper explores the mechanisms of cannabinoid metabolism and excretion via the kidneys, highlighting their potential implications for renal function.

This suggests the potential for renal complications, particularly in vulnerable populations or in individuals with pre-existing kidney conditions. This review summarizes both the positive and (particularly) the negative impacts of cannabinoids in major kidney diseases, summarizing the recent findings. Additionally, it provides a categorization of cannabinoid types and their receptors, discussing the potential clinical usefulness of cannabis-derived substances for kidney health. Furthermore, it examines the connections between cannabis and major kidney diseases, such as chronic kidney disease and acute kidney injury, presenting findings from new studies which investigated these associations. Cannabis supplementation poses significant challenges to renal health which demand further investigation.

The effects of cannabinoids on renal health are complex and influenced by factors such as their dosage, mode of administration, and individual variability. This review discusses the role of cannabinoid receptors in kidney tissues, analyzes the effects of different classes of cannabinoids on kidney health, and synthesizes findings from clinical trials and preclinical studies to evaluate the dual impacts of cannabinoid intake on kidney health. By bridging this gap in understanding, this work aims to provide an in-depth discussion on how cannabis influences kidney disorders and renal physiology, offering healthcare

professionals a clearer picture of the risks and potential clinical applications of cannabinoids in renal medicine.

This research was performed with the use of the PubMed, Google Scholar and Scopus databases. The inclusion criteria included works referencing cannabinoids, kidneys or kidney disease-related health conditions which were published before October 2024 and summarized in the form of a narrative review.

2. Cannabinoid Molecules

2.1. Natural Cannabinoids

Cannabis contains many chemical compounds which exert diverse effects on cannabinoid (CB) receptors [20–22]. More than 100 substances which belong to the phytocannabinoids are believed to constitute the pharmacological effect of the extract from the cannabis plant. The two most investigated substances are THC and cannabidiol (CBD). There are other phytocannabinoids which are divided into groups, with the main representants being cannabichromene (CBC), cannabielsoin (CBE), cannabigerol (CBG), cannabicyclol (CBL), cannabinol (CBN), cannabinodiol (CBND), cannabitriol (CBT) and Δ8-trans-tetrahydrocannabinol (Δ8-THC) [20].

THC acts as a partial agonist of CB1, binds to CB2 and partially activates it [21].

THC is known for its psychotropic effect and may influence one's mental state, exacerbating psychotic disorders and leading to depression. Its psychotropic effect can be explained by both its CB1 agonism and lipophilic structure, which enables THC to cross the blood–brain barrier. In the kidneys, THC is believed to stimulate prostaglandin production [22,23].

On the contrary, CBD is believed to be an inverse agonist or allosteric modulator of CB1 and CB2 [24,25]. CBD possibly mitigates some harmful effects of THC (e.g., the cataleptic effect [26]), although there is still no strong evidence that CBD alleviates THC-induced psychotic symptoms [27]. Nevertheless, the fact that there is interaction with CB1 implicates that CBD can also modulate inflammatory processes, as demonstrated in studies using in vitro and in vivo models [28–31]. The nephroprotective effect of CBD was shown in an experiment with a gentamicin-induced nephrotoxicity model. CBD administration enhanced the activity of farnesoid X receptors (FXR) [32]. An FXR is a nuclear receptor activated by bile acid, and its activation counteracts an inflammatory response. Another anti-inflammatory mechanism of CBD is crosstalk with Toll-like receptor (TLR) downstream pathways [33]. TLRs are receptors which can recognize particles of a pathogenic origin (e.g., bacterial DNA, lipopolysaccharides (LPSs), and viral RNA). It was demonstrated that hemp extracts reduce LPS-induced inflammatory cytokine expression (namely TNF- α and IL-6) in the canine cell line [34], and cannabidiol effectively reduced the LPS-induced inflammatory response in Wistar albino female rats. The reduction was reflected by lowering the level of TNF- α and caspase 3 [35]. These findings suggest that cannabinoids might also be useful in alleviating kidney pathologies in humans.

The plant has been used as a material for smoking (leaves, stems and flower buds) and producing cloth and tools. The seeds are an oil source used in food preparation as a substitute for other cooking oils. They were used for medical and nutritional purposes, as they were believed to alleviate joint pain and cognitive impairment [36]. Nowadays, they are also added to various food products such as cakes, pastries, bread, dairy and meat to make them more palatable and provide additional micronutrients. The seeds consist of fat (~30%), fiber (~25%) and protein (~20%) [37]. Indeed, studies indicate that supplementation of hemp seeds increases essential amino acids (phenylalanine, histidine and lysine) and crucial cations such as iron (Fe) and zinc (Zn) [38]. Apart from these constituents, cannabis-derived dietary products may contain a certain number of cannabinoids. Other edible parts

of cannabis such as stems and leaves possess relatively high amounts of THC and CBD (even reaching 0.2% and 2%, respectively) [37]. Therefore, only its seeds are allowed for consumption [39]. The concentration of the cannabinoids varies depending on a plethora of factors, including the age, harvest time, condition of culturing (humidity, fertilizers and light amount) and storage conditions [40]. The seeds abound with neither THC nor CBD (they contain only traces of these compounds) [39]. CBG, CBN and CBC can also be detected [41]. Although THC concentrations in food are tightly regulated by law in many countries, CBD is sometimes added to hemp oil, and this combination may exert a relevant pharmacological effect.

2.2. Synthetic Cannabinoids

Synthetic cannabinoids (SCs) are chemicals which interact with targets in the endocannabinoid system. The illicit market abounds with SCs, which are characterized by greater agonistic effects on CB receptors than THC. Hence, these drugs demonstrate both more devastating adverse effects and more severe psychotic symptoms. Usually, solutions containing SCs consist of organic solvents (methanol, ethanol and acetone) and are sprinkled onto plant mixtures (e.g., mint, thyme leaves, flowers and barks) [42].

Cannabinoids are usually absorbed through the respiratory tract and oral mucosa when smoked, although some products are intended for oral use in tablet, capsule or powder form.

While translation of SCs' properties into nutritional or medical practice appears to be ill-founded, the substances antagonizing CB1 are feasible options for obesity treatment and modifying dietary habits. It is known that CB1 signaling is involved in the regulation of voluntary food intake and lipogenesis [43]. Caution should be practiced since CB1 antagonists (e.g., rimonabant) may affect mental health, contributing to depression development [44]. However, in evaluating how common diagnoses of obesity are, a molecule safely suppressing CB1 signaling would be a potent tool as a supplement to functional food. Paying more attention to the role of cannabinoid receptors in homeostasis maintenance offers the prospect of manipulating these elements via food ingredients.

Recently, plenty of reports suggested an association between SC ingestion and health complications concerning not only the central nervous system but also the heart, liver and kidneys [45]. Some clinical records described acute tubular necrosis and acute interstitial nephritis upon histological examination in SC consumers [46]. Nephrotoxicity of SCs is likely due to strong CB1 agonism. Various synthetic substances exhibiting CB1 antagonism or CB2 agonism have been examined in preclinical models (e.g., rimonabant, AM251, AM1241 and AM630) [47–50]. They demonstrated the nephroprotective effect, which could be used in the future in the therapy of toxic or inflammatory renal injury.

2.3. Endocannabinoids

Human organisms produce their own CB ligands, and these substances belong to a network referred to as the endocannabinoid system. The best-characterized endogenously produced CB agonists are anandamide (AEA) and 2-arachidonoylglycerol (2-AG). They are created by enzymatic reactions involving lipids of the cell membrane (phosphatidylinositol 4,5-bisphosphate (PIP2), arachidonic acid (ARA) and phosphatidylethanolamine (PE)) [51]. One of the pathways for 2-AG synthesis begins with the production of phosphatidylinositol 4,5-bisphosphate (PIP2), which is cleaved by phospholipase C (PLC) [52]. This cleavage results in diacylglycerol (DAG) formation. The second step is the conversion of DAG to 2-AG via diacyloglycerol lipase. AEA is produced using PE, which merges with ARA, and this reaction is catalyzed by N-acyltransferase [53]. As a result, a product called N-arachidonoyl phosphatidylethanolamine (NAPE) is synthetized. NAPE serves as the

precursor of anandamide (AEA), which is formed by NAPE-specific phospholipase D. The 2-AG is mainly hydrolyzed by monoacylglycerol lipase into ARA and glycerol, whilst AEA is degraded by the enzyme fatty acid amide hydrolase (FAAH) into ARA and ethanoloamide. These routes of production are not the only ones [52,53], but the others will not be discussed in this paper.

We note that the described pathways of the synthesis share common elements with the production of other eicosanoids. Hence, cyclooxygenase (COX) inhibitors, such as non-steroid anti-inflammatory drugs, may interfere with the synthesis–degradation rate of these endocannabinoids. On one hand, the inhibition of COX leads to a higher concentration of ARA, which is also a substrate for prostaglandin synthesis [54]. On the other hand, some COX inhibitors exhibit the ability to reduce FAAH activity, which directly elevates the level of AEA [55]. Therefore, certain effects of the endocannabinoids, which are described further, can be modulated by easily available substances.

AEA acts as a CB1 partial agonist. It also exhibits activity for CB2, although less compared with CB1. In turn, 2-AG exerts an agonistic effect on CB1 and CB2 to a greater degree than AEA [56,57]. Both bind to the transient receptor potential cation channel vanilloid 1 (TRPV1) receptor, but AEA also binds and activates some subtypes of the peroxisome proliferator-activated receptor (PPAR) family [58]. There are also less-investigated endocannabinoids which interact with CB receptors, and this group includes N-arachidonoyl dopamine (NADA) [59] and 2-arachidonyl glyceryl ether [51,60]. Some others are presented in Table 1. More studies are required to assess their roles in healthy kidneys.

Table 1. Names of selected cannabinoids and their effects on CB receptors.

Substance	Agonist or Antagonist	Source
ENDOCANNABINOIDS		
AEA	CB1/CB2 partial agonist	Justinová et al., Hanus et al. [56,60]
2-AG		
N-arachidonoyl dopamine	CB1/CB2 agonist	Bisogno et al. [61]
2-arachidonyl glyceryl ether	CB1 agonist	Hanus et al. [60]
Oleamide		Leggett et al. [62]
Virodhamine	CB1 antagonist (partial agonist in vitro) CB2 agonist	Fezza et al. [63]
SYNTHETIC CANNABINOIDS		
AKB48	CB1/CB2 agonist	Canazza et al. [64]
AM-251	CB1 antagonist	Barutta et al. [48]
AM-356	CB1/CB2 agonist	Nuria et al. [65]
AM-1220		Andersen et al. [66]
AM-694	CB1 agonist	Bertol et al. [67]
AM-2201	CB1/CB2 agonist	Corli et al. [68]
CP-47,497		Hruba et al. [69]
CP-55,940		Tomiyama et al. [70]
CRA-13		Gardin et al. [71]

Table 1. *Cont.*

Substance	Agonist or Antagonist	Source
Drinabant	CB1 antagonist	Zuurman et al. [72]
Hemopresine		Heimann et al. [73]
Ibipinabant		Lange et al. [74]
Hu-210	CB1/CB2 agonist	Martín-Calderón et. al. [75]
JWH-015	CB2 agonist	Fechtner et al. [76]
JWH-018	CB1/CB2 agonist	Tirri et al. [77]
JWH-081		Basavarajappa et al. [78]
JWH-122		Cannaert et al. [79]
JWH-250	CB1/CB2 agonist	Ossato et al. [80]
Otenabant	CB1 antagonist	Hadcock et al. [81]
RCS-4	CB1/CB2 agonist	Banister et al. [82]
RCS-8		Schubert et al. [83]
STS-135		Canazza et al. [84]
Rimonabant	CB1 antagonist	Huang et al. [85]
Surinabant		Rinaldi-Carmona et al. [86]
PHYTOCANNABINOIDS		
Cannabichromene (CBC)	CB/1CB2 partial agonist	Zagzoog et. al. [87]
Cannabidiol (CBD)	CB1/CB2 antagonist (negative allosteric modulator)	Laprairie et al. [24]
Cannabigerol (CBG)	CB1/CB2 partial agonist	Zagzoog et al. [87]
Cannabinol (CBN)		Rhee [88]
Tetrahydrocannabinol (THC)		Zagzoog et al. [87]

AEA = anandamide; CB = cannabinoid receptor; 2-AG = 2-arachidonoylglycerol; CBC = cannabichromene; CBD = cannabidiol; CBG = cannabigerol; CBN = cannabinol; THC = tetrahydrocannabinol.

As noted, CBs are also expressed in the kidneys. Renal CB1 expression occurs in podocytes, mesangium, intercalated cells of the collecting duct, proximal convoluted and distal tubules, and afferent and efferent arterioles [89,90]. As mentioned earlier, CB2 expression in the kidneys remains controversial, but some studies indicate that podocytes [91] and proximal tubule cells [92] exhibit CB2 expression.

3. Cannabinoid Interaction with Renal Physiology

3.1. Cannabinoid Receptors

Cannabinoid receptors, both CB1 and CB2, are widely expressed in the human body. They are G protein-coupled receptors (GPCRs). GPCRs are transmembrane receptors usually containing seven lipophilic domains and a heterotrimeric complex of three units: G_α, G_β, and G_γ. The G_α unit may occur in various forms (Gs, Gi, or Gq) and influences intracellular cAMP levels (Gs and Gi) or acts through IP3 and DAG elevation (Gq) as a second mediator in cellular downstream pathways. Both CB1 and CB2 bind Gi, and hence their activation leads to a decrease in the intracellular cAMP level. However, CB1 may sometimes bind Gs or Gq proteins [93]. The Gβ-Gγ complex of CB plays an important role in ion channel blockage and opening and the mitogen-activated protein kinase (MAPK) cascade. CB1 activation is reported to negatively regulate some types of calcium channels (N type, P/Q type, and R type) and positively regulate inwardly rectifying potassium

channels [94,95]. CB regulates the activity of MAPK, which includes extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 mitogen-activated protein kinases (p38s) [96]. MAPK is crucial for the survival cascade of the cell and its metabolism.

CB1 is expressed predominantly in the central nervous system (CNS), although evidence of CB1 transcription can be detected in many other organs (e.g., the kidneys, adrenal gland, heart, lungs, reproductive organs, liver, and gastrointestinal tract) [90,94,97,98] and certain immune cells [94,99]. In turn, CB2 was thought to be expressed mainly in immune cells (macrophages, NK cells, and lymphocytes) [94]. To date, CB2 has been discovered in many other sites such as the pancreas, lungs, and reproductive organs [58,91,92,100,101]. The presence of CB2 in the kidneys is debatable [102], but many authors reported its detection [91,92]. It is claimed that CB1 exerts a rather pro-inflammatory and pro-fibrogenic effect (which does not apply to CNS, however), whereas CB2 exerts an anti-inflammatory effect [89].

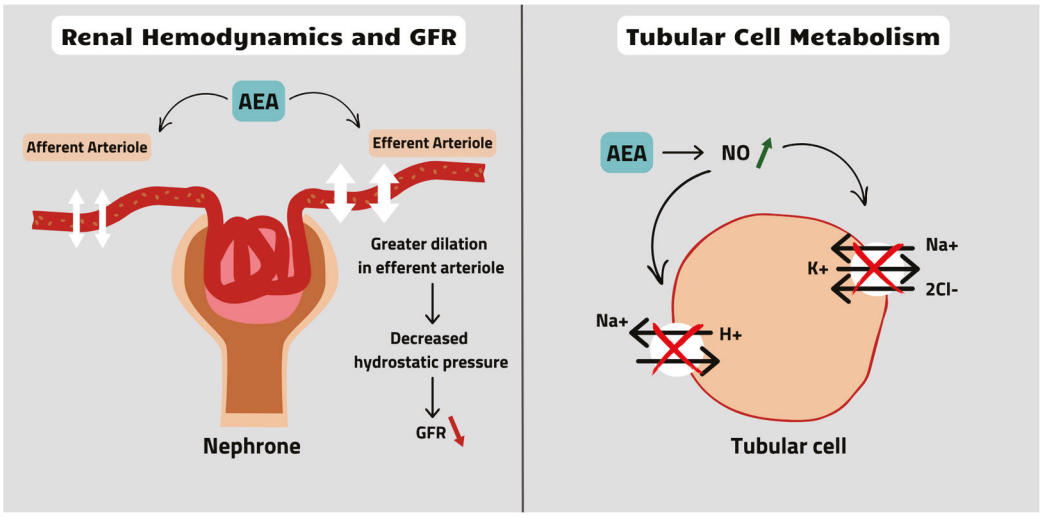
3.2. The Endocannabinoid System

The endocannabinoid system is responsible for regulating renal hemodynamics. We note that the relative levels of endocannabinoid (namely the AEA/2-AG ratio) may differ in some regions in the kidneys. For instance, the AEA concentration is higher in the renal medulla than in the cortex, whereas the concentration of 2-AG in the medulla and the cortex is comparable and equal to the cortical concentration of AEA [103–105]. AEA plays a significant role in kidney functions, influencing the glomerular filtration rate (GFR). This effect is believed to relate to CB1 agonism of AEA, which at the temporary local administration causes vasodilation in the kidneys [104,105]. Mesangial and endothelial cells are involved in the response due to their ability to synthesize and metabolize AEA [104,105]. The coexistence of a decrease in GFR and vasodilation of arterioles may seem paradoxical. This can be explained by the fact that AEA dilates efferent arterioles to a greater degree than afferent arterioles. Overall, this results in decreased hydrostatic pressure in the glomerular capillaries [106], which affects the flow of blood in the kidneys and reduces the GFR (Figure 1a).

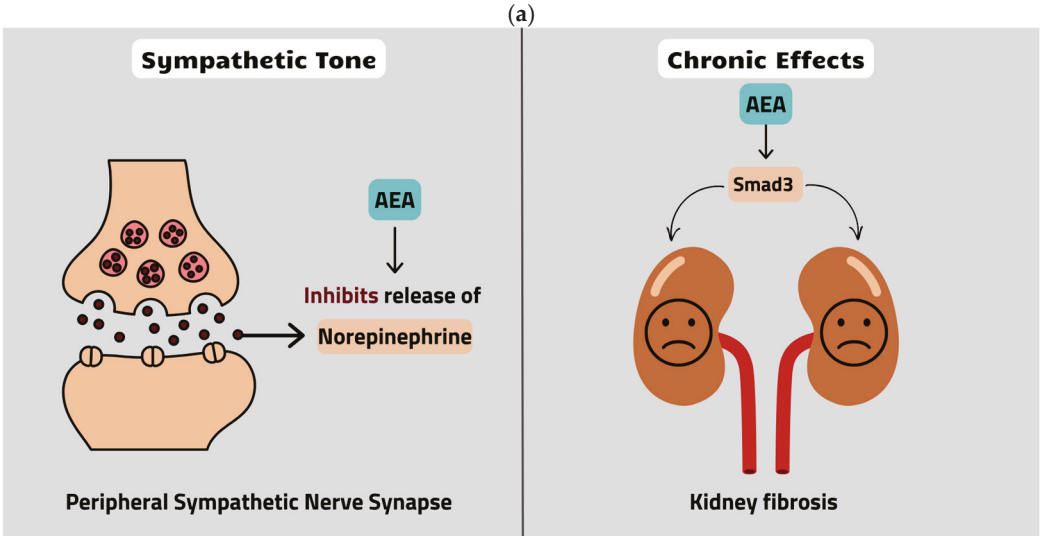
There are a few possible mechanisms which lead to this vasodilatory effect. Firstly, AEA increases nitric oxide production, which is a potent vasodilating agent [107]. This effect is attributed to TRPV1 rather than CB1 activation [108]. Secondly, AEA exhibits an inhibitory effect on norepinephrine release in the peripheral sympathetic nerves [109] (Figure 1b). Since the sympathetic nervous system is responsible for enhancing GFR via adrenergic stimulation, another mechanism of action is the TPRV1-dependent release of calcitonin gene-related peptide (CGRP) [110], which is also released in the kidneys and acts as a vasorelaxant [111].

AEA's influence on diuresis remains unclear. A decrease in GFR reduces urine production. Nonetheless, some studies imply that AEA may enhance diuresis. AEA modifies tubular cell metabolism by reducing oxygen consumption, which was observed in a rat experiment. Decreased oxygen consumption is probably linked to a nitric oxide increase due to AEA. Additionally, nitric oxide leads to Na^+/H^+ transporter and $Na^+/K^+/2Cl^-$ co-transporter inhibition [112], as shown in Figure 1a. This effect is a key point in the activity of loop diuretics, with highly potent agents increasing urine production. Another study conducted by Ritter et al. also indicated that sodium reabsorption is controlled by AEA [103], but not all studies support this [105,113]. The role of 2-AG in healthy kidneys is still not fully known, although some studies indicate its importance in renal pathologies.

Endocannabinoid disbalance may lead to an inflammatory response and damage to organs.



(**a**)



(**b**)

Figure 1. Schematic summary of effects of AEA on kidney function and structure. (**a**) Renal hemodynamics and GFR and tubular cell metabolism. (**b**) Sympathetic tone and chronic effects. AEA = anandamide; GFR = glomerular filtration rate; NO = nitric oxide; AEA = anandamide. Red arrows signify decrease, green arrows increase in certain substances and red cross a blockage.

It is worth mentioning that chronic AEA administration may induce fibrosis, and this phenomenon was shown in an experiment conducted by Golosova et al. [113], who observed increased activity in the Smad3 signaling pathway (Figure 1b). Another important observation was that chronic AEA intravenous administration exacerbated hypertension.

It is known that elevated 2-AG levels in the kidneys accompany renal injury [114,115]. In an experiment conducted by Zhou et al. [116], 2-AG elevation accelerated fibrosis and lipotoxicity. This molecule's precise role has yet to be elucidated, although it seems that the duration of exposure to 2-AG plays a crucial role here.

3.3. Excretion and Metabolism

As previously mentioned, about 20% of THC metabolites are excreted in urine (20–35%) [117]. This is due to the high lipophilicity of THC, which leads to significant renal tubular reabsorption and limited excretion of the unchanged drug [117]. Similar pharmacokinetic properties were observed for other cannabinoids, with studies suggesting that they are primarily excreted via feces [118].

It is impossible to discuss the excretion of cannabinoids without addressing their metabolism. Cannabinoids are mainly metabolized in the liver. THC is metabolized by microsomal hydroxylation and oxidation [119]. The process is catalyzed by enzymes of the cytochrome P450 (CYP) complex. The specific enzymes responsible for the hepatic metabolism of THC are CYP2C and CYP3A. These enzymes turn THC into 11-Hydroxy-Δ9-tetrahydrocannabinol (11-OH-THC), excreted mainly through feces, and 11-Nor-9-carboxy-Δ9-tetrahydrocannabinol (11-COOH-THC), excreted mainly via urine [118].

The metabolism of CBD is also associated with the CYP complex. CBD is metabolized to the active metabolite 7-Hydroxy-CBD (7-OH-CBD) by the enzymes CYP2C19 and CYP3A4. Studies suggest that CYP2C9 may also play a significant role in metabolizing CBD to the active 7-OH-CBD [120].

The involvement of the kidneys in the excretion and metabolism of CBD and THC may be significant to optimizing the planning of therapies for kidney diseases using these substances.

3.4. Effect on Kidney Function

As was mentioned, THC and CBD interact with two distinct receptors in the kidneys: CB1 and CB2 [121]. Studies on animals indicate that blocking CB1 may decrease albuminuria and renal fibrosis. CB2 activation may potentially be protective, as described earlier [122].

Vomiting is sometimes listed as one of the negative effects of synthetic cannabinoids [123]. While they are generally used to prevent vomiting, there are reported cases where they have the opposite effect [124].

Chronic use of Indian hemp was connected to pre-renal kidney injury associated with cannabinoid hyperemesis syndrome. One of the symptoms of this syndrome is the previously mentioned vomiting [124]. It can be assumed that intractable vomiting may affect renal blood flow [117].

CBD may be significant in the ischemia-reperfusion mechanism [124]. It has been demonstrated by Fouad et al. that CBD administered just before reperfusion has a protective effect on renal tissue. The research was conducted on rats. The renal parameters and kidney histopathology were examined in rats treated with CBD and in a control group [125]. There was a reduction in inflammation and oxidative damage. The rats treated with CBD by Soares et al. showed lower myeloperoxidase activity and decreased levels of TNF-alpha and IL-1beta cytokines. Additionally, malondialdehyde, a marker of lipid peroxidation and protein carbonylation, was also lower compared with animals subjected to ischemia and reperfusion without CBD administration [126]. This suggests that CBD may play an important role in protecting the kidneys from the adverse effects of reperfusion. Its anti-inflammatory and antioxidative actions are significant.

THC has been linked to a dose-dependent temporary increase in heart rate and an elevation in blood pressure [127]. Murray et al., in a case crossover study, noted that compared with non-users, marijuana users were less likely to have a history of hypertension (30% versus 44%; $p = 0.002$) [128]. Nevertheless, a definitive association with hypertension has not been established. Orthostatic hypotension was reported with self-reported acute cannabis [129]. This may limit the scope of cannabinoid usage in kidney diseases. Nonetheless, tolerance develops after 1–2 days of repeated exposure, resulting in reversal of the previously described negative effects [117]. Some studies suggest that CBD may have a blood pressure-lowering effect [130–132]. The available studies do not provide clear information regarding the impact of cannabinoids on kidney perfusion or overall function over longer observation periods. Thus, further research is needed.

4. Adverse Renal Effects Associated with Cannabinoid Use

4.1. Diabetic Nephropathy

Diabetes mellitus (DM) is one of the most common chronic diseases, globally rising to a rate of 10.5% among people 20–79 years old [133]. Its progression can lead to multiple and severe complications, one of which is diabetic nephropathy (DN). This can be caused by both types of DM and is present in as many as 40% of people suffering from this disease [134]. Multiple mediators and complex pathways have been proven to have roles in its development [135]. Until recently, little attention has been paid to cannabinoids' involvement in both the origin as well as the management of DN.

Previously, researchers have examined elevated CB1 expression in the podocytes of patients with DN. As noted, CB1 agonists may promote an inflammatory response in tissues, and this statement may be supported by studies conducted by Barutta et al., who with the use of the CB1 antagonist AM251 established that such a blockade prevents the loss of nephrin, podocin, and zonula occludens-1 (Figure 2a) [48].

This in turn may explain why the use of AM251 also improved albuminuria, which was significantly increased in diabetic mice, and reduced apoptosis. Further studies proved that CB1 blockading can protect db/db mice by diminishing the upregulation of collagen and plasminogen activator inhibitor-1 synthesis, which are caused by high glucose levels [136]. On the contrary, CB2 was shown to be downregulated in kidney biopsies of patients with advanced DM [91].

CB2 activation demonstrated a nephroprotective effect in renal disorders in mouse models, decreasing proinflammatory cytokines and reducing apoptosis [137,138]. Moreover, some studies showed improvement in albuminuria in renal dysfunction.

Therapy with the CB2 agonist was established to limit albuminuria in obese animal models [139] and in a mouse model of diabetic nephropathy [91], which opened new perspectives to the treatment of patients with diabetes. Most recent studies showed the CB2 antagonist's potential and came to the conclusion that it may ameliorate DN in rats [140].

Multiple genetic analyses have been published as part of a search for explanations for these phenomena [141–144]. One of them [143] established a genome-wide association study, which identified a variant in the gene encoding of CB2 related to DN in the United Arab Emirates and Japanese cohort. Nonetheless, a similar association failed to be demonstrated in European populations. Conversely, a study performed on a Polish cohort found that rs10493353 (G1359A) polymorphism in the CB1 gene is associated with diabetic nephropathy and diabetic retinopathy in patients with type 2 DM (T2DM) [142]. A similar study was conducted on a northern Chinese population [144], confirming that an allele of the CB1 gene rs10493353 may potentially be protective of DN in type 2 DM patients. Nevertheless, further research and clinical trials need to be performed to draw any conclusions from these data.

4.2. Chronic Kidney Disease

Chronic kidney disease is an irreversible, progressive clinical syndrome [145] characterized by extracellular matrix substituting a functional renal tissue [146]. It can result from various chronic pathologies. An urgent need for new medications to help with its progression has led researchers to target the endocannabinoid system.

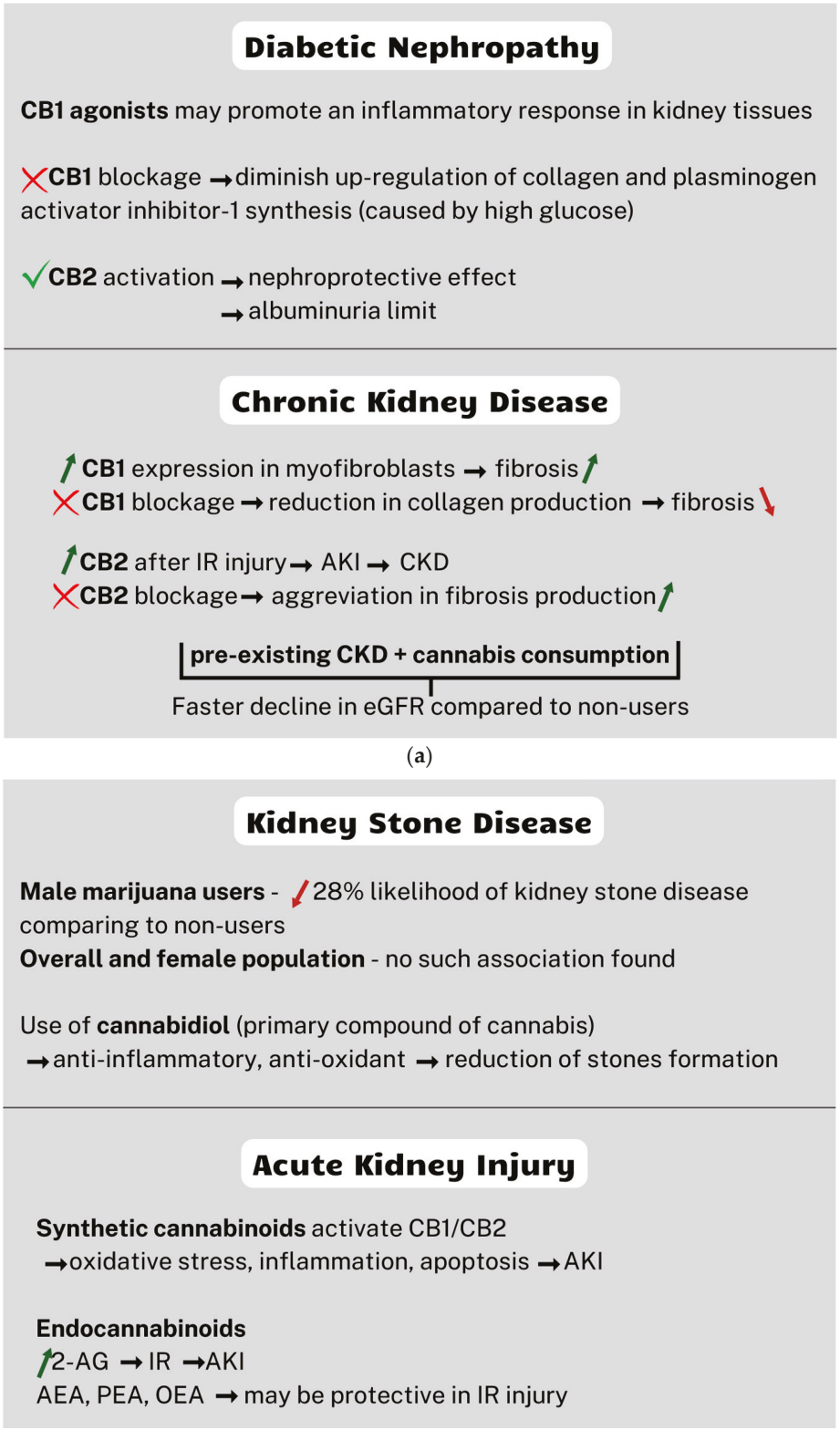


Figure 2. Summary of key mechanisms of cannabinoids in renal pathologies: (**a**) diabetic nephropathy and chronic kidney disease and (**b**) kidney stone disease and acute kidney injury. 2-AG = 2-arachidonoylglycerol; AEA = anandamide; AKI = acute kidney injury; CB1 = cannabinoid receptor 1; CB2 = cannabinoid receptor 2; CKD = chronic kidney disease; eGFR = estimated glomerular filtration rate; IR = ischemia and reperfusion; OEA = N-oleoylethanolamine; PAE = N-palmitoylethanolamine; CBD = cannabidiol. Red arrows signify decrease, green arrows increase in certain substances and red cross a blockage.

Analogous to DN, CB1 was shown to be overexpressed in patients with other non-metabolic CKDs [147]. Most importantly, increased expression was shown in myofibroblasts, the cells most crucial to the process of fibrosis. The latter study additionally established that the blockade of CB1 led to a reduction in collagen expression in vitro. Similar results were obtained by Dao et al. in patients with chronic allograft dysfunction, a leading cause of transplant failure [148], suggesting yet another therapeutic potential for CB1 antagonists. The relation between CB2 and renal fibrogenesis seems to be more complex. Its role in CKD has been considered controversial for some time. The latest studies have shown that CB2 is increased time-dependently after a 3 day period of ischemia-reperfusion injury [50]. This fact is crucial, considering that at this point, acute injury can progress into CKD. In contrast to this, Lecru et al. reported that CB2 blockading using synthetic antagonists has aggravated fibrosis progression [147].

The study conducted a post hoc analysis using data from the ASSESS-AKI study to assess the impact of cannabis consumption on kidney health outcomes over an average follow-up period of 4.5 years. Among the participants without chronic kidney disease, cannabis use was not associated with significant declines in the estimated glomerular filtration rate (eGFR) or an increased risk of CKD. However, in individuals with pre-existing CKD, cannabis consumption was linked to a faster annual decline in the eGFR compared with non-users, although it did not independently increase the risk of progressive CKD (Figure 2a). Further research is necessary to understand cannabis consumption's impact, particularly within the context of existing kidney disease and potential long-term consequences [149].

Obesity is a growing public health problem which is correlated with other diseases. The disease has been described as a proposed target for CKD reduction in the global population. It has also been established that obese patients have a higher likelihood of CKD progression [150]. Moreover, studies have reported alterations in the kidney endocannabinoid system in obese mice models [151]. Obesity has previously been linked to lipid accumulation in animal models [152]. Despite it not being a well-described phenomenon in humans, it is an important factor to note when referencing the subject. The accumulation of lipids may lead to renal lipotoxicity and, in turn, fibrosis. Udi et al. established an association between obesity-related renal toxicity and the activation of CB1 [151]. Even though CB1 depletion in the renal proximal tubule was not shown to lead to any beneficial effects regarding obesity itself, it was associated with the lessening of lipid accumulation. Nevertheless, there is a need for further studies to assess the exact mechanisms of the described phenomena.

4.3. Kidney Stone Disease

Kidney stone disease, also called nephrolithiasis or urolithiasis, is a condition characterized by the formation of solid crystal aggregates in the urinary tract. The risk factors of this condition are genetic predisposition, metabolic factors, and the environment [153]. Importantly, kidney stones are not solely isolated issues; they are often indicators of broader systemic health problems [153].

A cross-sectional study based on data obtained from the National Health and Nutrition Examination Survey was conducted to determine if marijuana use is related to kidney stones. Marijuana use was assessed through questionnaires, categorizing participants as frequent users or non-users based on frequency and duration. Kidney stone history was determined via self-reporting. Covariates, including demographics, lifestyle, and medical factors, were considered to adjust for potential confounders in the analysis.

The NHANES survey found that the men who used marijuana had a 28% lower likelihood of reporting kidney stones compared with non-users (Figure 2b). The diuretic

effects of cannabinoids shorten the time crystals stay in the kidneys, which decreases the risk of developing kidney stones [154]. However, this association was not found in the overall or female populations.

This study examined the relationship between marijuana use and kidney stones using a nationally representative NHANES dataset. It assessed both the history and frequency of use. Comprehensive adjustment for confounders and robust sensitivity analyses enhanced its reliability. A large sample size ensured generalizability, and gender-specific analyses revealed population differences.

It should be mentioned that this was a cross-sectional study, and there was a possibility of selection bias. In addition, there was no association between more frequent ($\geq$7 times/week) marijuana use and kidney stones. These results do not provide strong evidence but can be taken into consideration.

Previous research has demonstrated that cannabinoids can increase urine output in mice without affecting the excretion of Cl^- and K^+ [155]. The urine output was measured over 6 h with no access to food or water, using weigh boats to collect and weigh voided urine in 2 h intervals to minimize evaporation. Drug and vehicle doses were randomized and administered subcutaneously, with combination treatments delivered in split volumes to reduce time-dependent biases.

This study demonstrates that cannabinoid agonists induce diuresis in mice, with a biphasic effect where lower doses increase urine output via the central CB1 receptors and higher doses reduce output, possibly involving peripheral CB1 receptors. The diuretic effect at lower doses was weakly natriuretic without altering chloride or potassium excretion. These findings suggest a complex role of cannabinoid receptors in regulating water balance.

The effect shown above may shorten the duration of crystal retention in the kidneys, which can potentially lower the risk of kidney stone development, as was said in an update on the current concepts of kidney stone disease in Adv Urol. 2018 [156]. In Adv Nutr. 2023, it was also underlined that low urine output is one of the causes of nephrolithiasis [157].

What is more, cannabidiol, a primary component of cannabis, has anti-inflammatory and antioxidant abilities [136]. Inflammation and oxidative stress have crucial roles in the formation of kidney stones [158,159]. Therefore, it is possible that cannabidiol mitigates inflammatory responses and reduces oxidative stress, potentially contributing to a lower risk of kidney stone development (Figure 2b) [154].

Given the potential diuretic and anti-inflammatory effects of cannabinoids, additional research is necessary to investigate their role in kidney stone formation and prevention.

4.4. Acute Kidney Injury Induced by Synthetic Cannabinoids

To highlight the importance of thorough research into cannabinoid treatment, we aim to illustrate acute kidney injury (AKI). It is an example of a serious condition caused by synthetic cannabinoids. It is essential to emphasize the adverse impact of these substances on our body's function. We have to keep in mind the importance of proper training and expertise in cannabinoid treatment.

AKI is described as a sudden loss of the kidneys' ability to excrete. This condition can lead to irreversible loss of kidney cells and nephrons, which can potentially cause CKD. It can manifest as a mild and temporary or severe and persistent [160]. In AKI, acute tubular necrosis (ATN), resulting from ischemia or exposure to nephrotoxic substances, is a frequently encountered occurrence [161].

Ischemia and reperfusion (IR) injury is a major cause of AKI. It has been shown that kidney injury associated with reperfusion is correlated with increased levels of 2-AG in the kidneys, which may have significant clinical and therapeutic implications (Figure 2b) [114].

IR injury reduces endocannabinoid signaling and Na^+/K^+ ATPase expression and activity in LLC-PK1 cells (immortalized epithelial cell line derived from pig renal proximal tubules) and rat kidney cortexes. However, treatment with a CB1 or CB2 agonist reverses these effects in a CB1-dependent manner, indicating that CB1 activation may represent a novel pro-homeostatic mechanism, restoring proper Na^+/K^+ ATPase function following IR damage in renal proximal tubule cells [162].

New studies have demonstrated that AEA has renoprotective effects against mercuric chloride-induced kidney injury. It possesses anti-inflammatory, antioxidant, and anti-apoptotic properties, involving modulation of various molecular pathways including WNT-5A/BCL-2, IP3/NFATc1, HMGB-1/RAGE/NF-κB, and caspase cascades (Abdallah DM, Scientific Reports 2023) [163]. Furthermore, a study revealed that N-acylethanolamines such as AEA, N-oleoylethanolamine (OEA), and N-palmitoylethanolamine (PEA) may have a protective function against renal ischemia-induced injury [164]. These studies suggest potential therapeutic implications of the endocannabinoid system in addressing ischemia-related renal disorders.

One of the causes of AKI is synthetic cannabinoids, which are a type of psychoactive substance. They interact with the endocannabinoid system within many organs in the body. They also have the ability to react with CB1 and CB2. Activation of these receptors leads to the initiation of signaling pathways such as those for oxidative stress, inflammation, and apoptosis, which have the potential to cause detrimental changes in organs such as the liver, kidneys, heart, or brain. This can result in negative health consequences, including liver injury and damage, AKI, arrhythmias, myocardial infarction, and cognitive and motor impairment (Figure 2b) [165].

AKI associated with synthetic cannabinoids (SCs), such as K2, the brand name of one synthetic herbal mixture containing synthetic cannabinoids, often manifests as a sudden and severe condition. The majority of patients are young males who do not have any medical history of kidney disease. Typically, symptoms develop shortly after K2 intake. The most frequent manifestations of K2 ingestion are gastrointestinal problems such as nausea and vomiting. General constitutional symptoms occur as well. This condition, which ahs been increasingly observed in the United States, typically resolves rapidly with fluid resuscitation. SCs may accumulate in the kidneys due to the presence of CB1 and CB2 on podocytes in the human glomeruli [165].

The definitive pathophysiology of SC-associated AKI is still unspecified [166]. However, clinical evidence from renal biopsies and medical reports shows that ATN continues to be the most common pathology in AKI caused by K2 [165,167]. Specifically, SC XLR-11 has been identified as a nephrotoxic substance related to adverse effects on the mitochondrial function of proximal tubules [168].

A systematic review based on 55 young SC abusers who experienced AKI showed that renal damage was evidenced by elevated serum creatinine levels in 49 individuals, representing 89% of the cases [167]. Renal biopsy, conducted in 33% of the cases, unveiled acute tubular damage, acute tubulointerstitial nephritis, and acute interstitial nephritis, in descending order of occurrence. Nevertheless, diagnosing AKI related to SCs is a significant challenge in both the clinical and forensic fields. Considering these complexities, it is crucial to maintain a high level of suspicion for SC-related AKI, especially after ruling out other potential causes and when serum creatinine levels are elevated [167]. Due to SCs' absence of standard blood and urine toxicology tests, nephrologists need to keep this in mind when diagnosing cases of unexplained AKI [168].

Additionally, a systematic review and meta-analysis conducted by Zihao Zhao found that targeting cannabinoid receptors can significantly improve kidney function in cases of renal dysfunction. Specifically, the blockade or knockout of CB1 significantly reduced

blood urea nitrogen (SMD: −1.67), serum creatinine (SMD: −1.88), and albuminuria levels (SMD: −1.60). The activation of CB2 significantly reduced serum creatinine (SMD: −0.97) and albuminuria levels (SMD: −2.43) compared with the control group. These findings suggest that CB1 antagonists and CB2 agonists may reduce inflammatory responses and provide renal protective effects, maintaining therapeutic potential in various types of kidney disease [169].

In the United States, researchers analyzed data from 9,930,007 hospitalized patients with acute myocardial infarction (AMI) from the Nationwide Inpatient Sample from 1 January 2001 to 31 December 2020. Patients <18 and >80 years old were excluded. To differentiate the groups, cannabis users were compared with non-users. Those categorized as "drug abuse" based on ICD-9 and ICD-10 were excluded. Outcome differences were analyzed using multivariate regression models, which were adjusted for patient characteristics (e.g., sex, age, race, insurance, and weekend admission) and comorbidities such as hypertension, diabetes, smoking, dyslipidemia, chronic kidney disease, obesity, and prior cardiac interventions.

A total of 117,641 (1.2%) of these patients reported cannabis use. After conducting statistical tests and applying propensity score matching, they found that the cannabis users had a higher risk of developing acute kidney injury (aOR = 1.103, $p < 0.01$) [170].

The study was limited by the use of a modest CBG dose, early timing of assessments (20–60 min post dosing), and the remote Zoom-based methodology, which precluded physical exams, vital sign measurements, and open-ended adverse event reporting. Additionally, the sample consisted of experienced cannabis users rather than a clinical population, limiting generalizability, and multiple statistical comparisons were not corrected, increasing the potential for type I errors.

However, this study used a large, nationally representative sample spanning 20 years, which enhanced the generalizability and robustness of the findings. Additionally, it used rigorous statistical methods, including multivariate regression and propensity score matching, to adjust for confounding variables, ensuring more accurate comparisons between cannabis users and non-users, which makes this study valuable [170].

The abovementioned information highlights the critical need for recognizing the risks cannabinoids pose to kidney health. Ongoing research into cannabinoid receptor interactions offers promising therapeutic approaches, but careful attention and expertise are essential to safely manage these treatments.

5. Conclusions

This narrative review discussed the impact of various cannabinoids on kidney function. It covers new perspectives which are to be addressed in the future as well as the state of the art of molecular and animal model-based research.

Cannabis and its derivatives, including edibles and hemp seeds, are increasingly consumed for recreational, nutritional, and medicinal purposes, offering potential benefits but also posing risks such as inconsistent labeling, health implications, and overdosing. The growing prevalence of these products underscores the need for standardized regulations, accurate labeling, and further research to address health concerns, particularly for vulnerable conditions like kidney diseases.

Cannabinoid receptors CB1 and CB2 are present throughout the human body, including in the kidneys. More precisely, they are expressed in arterioles, proximal and distal tubules as well as podocytes and the mesangium, where their main role is controlling blood flow. Cannabinoids, both endogenous and exogenous, interact with CB1 and CB2 in the kidneys, affecting tubular function and renal parameters and thus acting similar to nutrients. Blocking CB1 has been shown to limit albuminuria, renal fibrosis, blood urea

nitrogen, and serum creatinine, among other substances, which potentially could be useful in the treatment of various pathologies. These data seem even more promising with the acknowledgement of CB1 overexpression in DN and non-metabolic CKD. Activation of CB2 seems to act analogously to a CB1 blockade, which may explain its downregulation in kidney biopsies obtained from patients with advanced DM.

In contrast, the use of SCs is a major threat to renal health. Not only can they cause AKI, but their interaction with CBs can also have detrimental effects on other organs, as they were present in multiple cases among users. This underlines the importance of specifying what cannabinoid one is referring to when discussing the matter. Furthermore, there is a lack of evidence regarding both the long-term effects of these substances and the genetic factors' role in the response to them in the context of kidney function.

Insufficient data regarding the subject is hampering repurposing of already-registered drugs and the submission for approval of others, as most of the presented effects have low grades of evidence. This state in turn impedes proper care of patients. Further studies should also focus on patients who choose to self-medicate with cannabinoids, as there remains a research gap regarding this group.

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Abbreviations

2-AG	2-arachidonoylglycerol
AEA	Anandamide
AKI	Acute kidney injury
AMI	Acute myocardial infarction
ARA	Arachidonic acid
ATN	Acute tubular necrosis
CB	Cannabinoid receptor
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CBC	Cannabichromene
CBD	Cannabidiol
CBE	Cannablesoin,
CBG	Cannabigerol
CBL	Cannabicyclol
CBM	Cannabinoid-based medication
CBN	Cannabinol
CBND	Cannabinodiol
CBT	Cannabitriol
CGRP	Calcitonin gene-related peptide
CKD	Chronic kidney disease
CNS	Central nervous system
COX	Cyclooxygenase
CUD	Cannabis use disorder
CYP	Cytochrome P450
DAG	DIacyloglycerol
DM	Diabetes mellitus

DN	Diabetic nephropathy
ERKs	Extracellular signal-regulated kinases
eGFR	Estimated glomerular filtration rate
FAAH	Fatty acid amide hydrolase
FXR	Farnesoid X receptor
GFR	Glomerular filtration rate
GPCR	G protein coupled receptor
IR	Ischemia and reperfusion
JNKs	C-Jun N-terminal kinases
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
NADA	N-arachidonoyl dopamine
NAPE	N-arachidonoyl phosphatidylethanolamine
PIP2	4,5-bisphosphate
PE	Phosphatidylethanolamine
PPAR	Peroxisome proliferator-activated receptor
PLC	Phospholipase C
SCs	Synthetic cannabinoids
THC	Delta-9-tetrahydrocannabinol
TLR	Toll-like receptor
TRPV1	Transient receptor potential cation channel vanilloid 1
Δ8-THC	Δ8 -trans-tetrahydrocannabinol
11-OH-THC	11-Hydroxy-Δ9-tetrahydrocannabinol
11-COOH-THC	11-Nor-9-carboxy-Δ9-tetrahydrocannabinol
7-OH-CBD	7-Hydroxy-CBD

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Review

Polyphenol Intervention Ameliorates Non-Alcoholic Fatty Liver Disease: An Updated Comprehensive Systematic Review

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Abstract: Non-alcoholic fatty liver disease (NAFLD) has recently emerged as a challenging metabolic disorder with a strong emphasis on its prevention and management. Polyphenols, a group of naturally occurring plant compounds, have been associated with a decreased risk of various metabolic disorders related to NAFLD. The current systematic review aims to critically assess evidence about the ameliorative effect of polyphenol supplementation on NAFLD patients. A PRISMA systematic search appraisal was conducted in PubMed, Scopus, Web of Science Core Collection, and all relevant studies published prior to April 2024 and met the inclusion criteria were included. Twenty-nine randomized clinical trials (RCTs) comprised 1840 NAFLD patients. The studies primarily examined eleven phenolic compounds, including turmeric, curcumin, resveratrol, genistein, catechin, green tea extract, hesperidin, and silymarin. Turmeric and curcumin decreased liver enzymes, inflammatory cytokines, lipid profile, insulin resistance, and NAFLD score, while resveratrol did not present consistent results across all the studies. Most studies on silymarin showed a reduction in liver enzymes and lipid profile; however, no changes were observed in inflammatory cytokine levels. The dietary supplementation of hesperidin and naringenin or green tea extract caused improvements in liver enzyme, lipid profile, and inflammatory cytokine, while genistein supplementation did not modulate blood lipid profile. In conclusion, dietary supplementation of polyphenols could potentially prevent and ameliorate NAFLD. Still, the inconsistent results across the included RCTs require further clinical research to establish optimal dosage and duration.

Keywords: fatty liver; inflammation; non-alcoholic; polyphenols; systematic review

1. Introduction

Concomitant with the worldwide surge in metabolic syndrome, diabetes, and obesity, an abrupt surge has been witnessed in the ubiquity of non-alcoholic fatty liver disease (NAFLD) [1]. NAFLD exhibits a worldwide prevalence of 30.05%, with the most elevated rates occurring in Latin America (44.37%), followed by Middle East and North Africa (36.53%), South Asia (33.83%), Southeast Asia (33.07%), North America (31.20%) and Western Europe (25.10%) [2]. It is noteworthy that an estimated 22.5% of individuals diagnosed with this non-alcoholic steatohepatitis (NASH) progress to hepatocellular carcinoma, while approximately 20% experience the development of cirrhosis [3].

NAFLD includes a spectrum of clinical and pathological liver conditions, ranging from isolated fat accumulation and fibrosis to cirrhosis, with a potential link to the development of hepatocellular carcinoma. It is characterized by the presence of steatosis in more than 5% of hepatocytes, as detected through histological or radiological examination;

NAFLD diagnosis requires the exclusion of secondary factors, such as substantial alcohol consumption (less than 30 g/day for men and less than 20 g/day for women), hereditary liver disorders, or viral hepatitis [4–6]. The primary theory explaining the development of NAFLD is known as the "two-hit" hypothesis. According to this model, the first event, or "hit", results in steatosis, while the second "hit" leads to necro-inflammation and fibrosis. The initial "hit" is often linked to insulin resistance, and various factors such as oxidative stress, cytokine effects, and fatty acid toxicity are considered potential contributors to subsequent hepatocellular injury [5]. Tumor necrosis factor-alpha (TNF-α) in the liver is associated with oxidative stress and may play a role in insulin resistance. Moreover, TNF-α is implicated in fibrosis through the direct activation of hepatic stellate cells and by stimulating the production of tumor growth factor (TGF)-beta, a potent cytokine promoting fibrosis [7]. According to the "second hit" theory, the dysfunction of peripheral adipose tissue in insulin resistance triggers lipid breakdown, elevating free fatty acid levels in the bloodstream. Unhealthy lifestyle choices exacerbate this elevation, causing an overflow of fatty acids to the liver beyond its transport capacity, leading to liver deposition and subsequent steatosis, which is named "the first blow". Prolonged excessive fat accumulation induces endoplasmic reticulum stress, mitochondrial dysfunction, and oxidative stress. This cascade results in the release of inflammatory factors, intensifying liver cell damage and propelling the shift from simple fatty liver to NASH, rendering it "the second blow". Peripheral adipose tissue exhibiting insulin resistance diminishes adiponectin secretion while increasing pro-inflammatory factors, amplifying the body's inflammatory response and exacerbating insulin resistance, establishing a detrimental cycle [8].

In the past 20 years, there has been an increase in NAFLD and NASH not only in Western countries, where a sedentary lifestyle and obesity are prevalent along with high-calorie diets, but also in urban areas of developing countries [9]. Despite several therapeutic options proposed for NAFLD and NASH in recent years, none have proven sufficient in improving liver function and addressing hepatocyte stress and inflammation. Currently, there are no approved treatments for NAFLD, and available options mainly focus on advising lifestyle changes, such as adopting a healthy diet low in fats and carbohydrates and engaging in regular physical activity [10]. One of the most common types of healthy diet is the Mediterranean diet, where polyphenols are abundantly available [10].

Plant-derived foods exhibit a diverse array of secondary metabolites, with polyphenols emerging as a particularly abundant and nutritionally pivotal class of phytochemicals [11]. The collection of phenolic structures exceeds 8000 compounds, with more than 500 identified in plant foods, constituting the dietary polyphenols. In recent decades, many investigations have investigated the health-promoting attributes inherent to polyphenols. The consistent inclusion of polyphenol-rich foods in dietary regimens has demonstrated an association with a potential decrement in the prevalence of conditions such as cardiovascular diseases, colon cancer, liver disorders, obesity, and diabetes [12]. Evidence supports that polyphenols can alleviate oxidative stress and inflammatory cascades, modulate immune responses, and elicit various pharmacological effects [13]. Different in vitro and in vivo studies have found that polyphenols could have a direct impact in alleviating or preventing the onset of NAFLD and NASH by regulating the Nrf-2 and AMPK [11].

Numerous randomized controlled trials (RCTs) investigating the effect of dietary polyphenols on the mitigation of NAFLD or NASH have been documented. Nevertheless, these RCTs' outcomes and therapeutic interventions exhibit heterogeneity in study populations, polyphenol type, dosages and duration, which makes cross-study comparisons challenging and provides inadequate foundational framework for clinicians in planning treatment protocols for NAFLD or NASH. In addition, a lack of standardized dosage recommendations and consensus on specific phenolic compounds further contributes to inconsistent outcomes. Thus, the current systematic review aims to synthesize data from divers RCTs to clarify common findings, identify trends associated with specific polyphenols, and examine dosage ranges and treatment durations to highlight emerging therapeutic standards that can improve clinical management of NAFLD.

2. Materials and Methods

2.1. Search Strategy

To ensure transparency and methodological accuracy in the review process, this systematic review aimed to provide a comprehensive and unbiased evaluation of the available evidence by adhering to the PRISMA guidelines and checklist. To identify relevant studies, an electronic literature search was performed in three scientific databases (PubMed, Scopus, and Web of Science Core Collection), and all relevant studies published prior to April 2024 that met the inclusion criteria were included. The search method employed a mix of appropriate keywords and Medical Subject Headings (MeSH) terms related to non-alcoholic fatty liver (NAFLD), non-alcoholic steatohepatitis (NASH), dietary supplement, polyphenols, and intervention (Table S1). Restrictions were applied based on language and publication type. Furthermore, the reference lists of relevant articles and review papers were screened to identify any supplementary eligible studies (Figure 1).

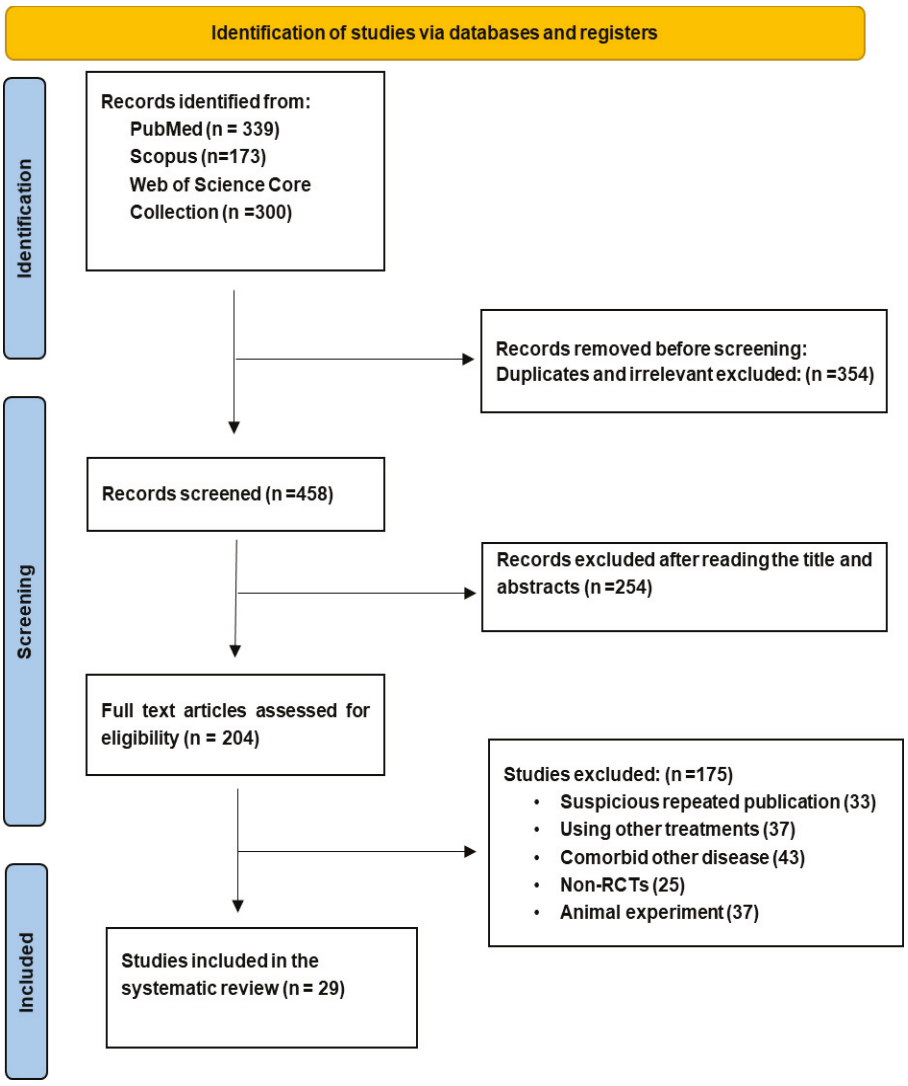


Figure 1. PRISMA flowchart of the included studies in the systematic review.

2.2. Eligibility Criteria and Study Selection

The following inclusion criteria were established to ensure the selection of suitable studies. The study design included randomized controlled trials (RCTs), case–control studies, cross-sectional studies, and prospective cohort studies. Reviews, conference abstracts, animal studies, and in vitro studies were excluded. The included participants were aged 18 years old or older and diagnosed with NAFLD. Studies examining the effect of

polyphenols as a dietary intervention in managing NAFLD were considered for inclusion. The outcomes of interest were critical indicators of NAFLD, including body mass index (BMI), Homeostasis Model Assessment-Insulin Resistance (HOMA-IR), liver enzymes (GGT, ALT, and AST), blood lipids [total cholesterol (TC), TG, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C)], and inflammatory biomarkers. Our systematic review exclusively included articles published in the English language. Initial screening of all identified studies based on titles and abstracts was conducted by two independent reviewers, S.L.P and I.A. Subsequently, full-text articles of included studies were obtained for a more thorough assessment. Any disagreement was addressed through discussion and, when required, with the engagement of a third reviewer to reach a consensus.

2.3. Data Extraction and Risk of Bias Assessment

Two independent reviewers conducted data extraction utilizing a standardized form to ensure consistency. Extracted information included the study characteristics such as authorship, publication year, geographical location, study design, sample size, participant demographics, specifics of the dietary intervention, study duration, primary and secondary outcomes, and significant findings. The methodological quality of the included RCTs was evaluated by employing the Cochrane Collaboration's risk of bias assessment tool. Two reviewers autonomously assessed bias risk, with any disparities resolved through consensus discussions.

3. Results

3.1. Study Selection and Characteristics

The PRISMA guidelines were followed in reporting the selection process of the included studies, as shown in Figure 1. After performing an initial search, 812 papers were collected, followed by an exclusion of 354 studies due to being duplicates and being irrelevant to the topic. Then, an extensive reading of the titles and abstracts was performed to exclude 254 studies unrelated to polyphenol supplementation and NAFLD. A total of 204 studies were assessed entirely in accordance with the eligibility criteria. Of these, 29 studies were deemed suitable for inclusion in the systematic review [14–42].

The main characteristics of the 29 included studies are exhibited in Table 1. Among those, an RCT protocol was presented [29]. In 13 studies, a randomized controlled trial design was employed, while 15 studies utilized a double-blind placebo-controlled trial design where participants and researchers were unaware of the treatment allocation. However, two studies incorporated more than two groups to assess different doses or combinations of treatments [34,39]. The most significant number of trials was executed in Iran, with 20 studies. Following closely behind were studies conducted in Italy, with three trials, and Denmark, with two trials. Additionally, one trial was conducted in Malaysia, China, Australia, and Pakistan. The studies primarily investigated eleven phenolic compounds, including turmeric, curcumin, resveratrol, anthocyanins, naringenin, genistein, catechin, green tea extract, hesperidin, silybin, and silymarin.

Table 1. Characteristics of the included studies in the systematic review.

First Author's Last Name, Year, Country	Sample Size I/C	Mean Age (yrs)	Gender (M/F)	Study Design	Intervention and Dose	Control	Duration	Main Results
Turmeric and Curcumin								
(Kalhori et al. 2022) Iran [14]	I: 21 C: 21	42.5 ± 8.3	I: 10/11 C: 8/13	Randomized, double-blind, placebo-controlled trial	Turmeric 3000 mg	Placebo 3000 mg	12 weeks	↓ BMI ↓ AST ↓ ALT ↓ lipid profile ↓ HOMA-IR ↓ sirtuin-1

Table 1. *Cont.*

First Author's Last Name, Year, Country	Sample Size I/C	Mean Age (yrs)	Gender (M/F)	Study Design	Intervention and Dose	Control	Duration	Main Results
(jarhahzadeh et al. 2021), Iran [15]	I: 32 C: 32	42.2 ± 10.5	I: 19/13 C: 19/13	Randomized double-blind clinical trial	Turmeric 2000 mg	Placebo 2000 mg	12 weeks	↓ ALT ↓ AST ↓ lipid profile ↓ GGT
(Mirhafez et al. 2021) Iran [16]	I: 35 C: 37	42.5 ± 5.6	I: 17/11 C: 11/17	Double-blind, randomized, placebo-controlled Trial	Curcumin 250 mg	Placebo 250 mg	8 weeks	↓ BMI ↓ ALT ↓ AST ↓ lipid profile ↓ adverse events
(Saberi-Karimian et al. 2020) Iran [17]	I: 27 C: 28	18–70	NA	Randomized controlled trial	Curcuminoids 500 mg + piperine 5 mg	Placebo	8 weeks	↓ NAFLD severity score ↓ TNF-α ↓ MCP-1 ↓ EGF ↓ ALT ↓ AST
(Cicero et al. 2020) Iran [18]	I: 40 C: 40	55 ± 5.6	NA	Double-blind, placebo-controlled clinical trial	Curcumin 200 mg + phosphatidylserine 120 mg + phosphatidylcholine 480 mg + piperine 8 mg	Placebo	8 weeks	↓ Fasting plasma insulin (FPI), ↓ HOMA index ↓ waist circumference, ↓ blood pressure, ↓ TG ↑ HDL-C, ↓ GGT ↓ fatty liver index ↓ cortisol
(Moradi et al. 2020) Iran [19]	I_1: 11 I_2: 11 C: 23	65.8 ± 4.8	I: 0/22 C: 0/22	Randomized double-blind clinical trial	I_1: Curcumin 80 mg I_2: resistance training + curcumin 80 mg	Resistance training without curcumin or placebo	12 weeks	↓ ALT ↓ AST ↔ BMI ↔ weight
(Saadati et al. 2019) Iran [20]	I: 27 C: 23	46.8 ± 3.6	I: 13/14 C: 14/9	Randomized, placebo-controlled, clinical trial	Curcumin 1500 mg	Placebo	12 weeks	↓ weight ↓ BMI ↓ waist circumference ↓ Hip circumference ↓ blood lipid profile ↓ HOMA-IR ↓ hepatic steatosis ↓ Hepatic fibrosis ↓ALT ↓ AST ↓ GGT
(Panahi et al. 2019) Iran [21]	I: 35 C: 35	46.4 ± 11.2	I: 20/15 C: 19/16	Randomized controlled parallel-group trial	curcuminoids 500 mg coadministered with 5 mg of piperine	Placebo	12 weeks	↓ ESR ↓ AST ↓ ALT ↓ ALP ↔ TG ↔ HDL-C ↓ LDL ↓ NAFLD severity

Table 1. *Cont.*

First Author's Last Name, Year, Country	Sample Size I/C	Mean Age (yrs)	Gender (M/F)	Study Design	Intervention and Dose	Control	Duration	Main Results
(Rahmani et al. 2016) Iran [22]	I: 37 C: 40	47.89 ± 2.5	I: 19/21 C: 19/21	Randomized double-blind placebo-controlled trial	curcumin 70 mg	Placebo	8 weeks	↓ BMI, ↓ TC ↓ LDL-C ↓ TG ↓ ALT ↓ AST ↓ glucose
				Resveratrol				
(Heebøll et al. 2016) Denmark [23]	I: 13 C: 13	18–70	I: 9/4 C: 8/5	Placebo-controlled, randomized clinical trial	Resveratrol 1500 mg	Placebo	26 weeks	↔ ALP ↓ AST ↓ ALT ↔ GGT ↔ BMI ↔ Weight ↔ TNF-α ↔ Lipid profile ↔ HOMA-IR
(Chen et al. 2015) China [24]	I: 30 C: 30	45.5 ± 3.8	I: 8/22 C: 20/10	Randomized controlled trial	Resveratrol 300 mg	Placebo	12 weeks	↓ AST ↓ ALT ↔ GGT ↓ HOMA-IR ↓ TC ↓ LDL ↔ TG ↓ TNF-α ↔ BMI ↔ Weight
(Chachay et al. 2014) Australia [25]	I: 10 C: 10	46.8 ± 12.5	I: 10/0 C: 10/0	Randomized, double-blind, placebo-controlled trial	Resveratrol 3000 mg	Placebo	8 weeks	↔ Weight ↔ BMI ↑ HOMA-IR ↑ ALT ↑ AST ↔ TNF-α ↔ blood lipid profile ↔ CRP ↓ IL-6
(Farzin et al. 2020) Iran [26]	I: 25 C: 25	39.5 ± 11.2	NA	Randomized, double-blind, placebo-controlled clinical trial	Resveratrol 600 mg	Placebo	12 weeks	↓ waist circumference ↓ BMI ↓ weight ↔ lipid profile ↔ liver enzymes (AST, ALT, ALP and GGT).
(Faghihzadeh et al. 2014) Iran [27]	I: 25 C: 25	45.4 ± 12.5	I: 18/7 C: 17/8	Randomized, double-blind, controlled clinical trial	Resveratrol 500 mg	Placebo	12 weeks	↔ weight ↔ BMI ↑ GGT ↓ ALT ↓ AST ↔ CRP ↑ IL-6 ↔ TNFα ↓ Fibrosis

Table 1. *Cont.*

First Author's Last Name, Year, Country	Sample Size I/C	Mean Age (yrs)	Gender (M/F)	Study Design	Intervention and Dose	Control	Duration	Main Results
(Kantartzis et al. 2018) Denmark [28]	I: 53 C: 52	18–70	I: 22/32 C: 30/24	Randomized, double-blind, placebo-controlled clinical trial	Resveratrol 150 mg	Placebo	12 weeks	↔ NAFLD score ↔ HOMA-IR ↔ AST ↔ ALT ↔ GGT ↔ Hs-CRP ↔ IL-6
Anthocyanins								
(Sangsefidi et al. 2021) Iran [29]	I: 25 C: 25	42.6 ± 10.7	NA	Double-blind randomized clinical trial	Anthocyanins 320 mg (cornelian cherry fruit extract)	caramel color, allura red color, and natural flavorings with a color, appearance, taste, and texture similar to the cornelian cherry extract, but without any anthocyanins.	12 weeks	-
Naringenin								
(Namkhah et al. 2021) Iran [30]	I: 22 C: 22	45.7 ± 10.7	I: 12/10 C: 13/9	Randomized, double-blind, placebo-controlled, clinical trial	Naringenin 200 mg	Placebo	4 weeks	↓ NAFLD score ↓ Weight ↓ BMI ↔ AST ↔ ALT ↓ TG ↓ TC ↓ LDL
Genistein								
(Amanat et al. 2018) Iran [31]	I: 41 C: 41	45.55 ± 6.1	I: 30/11 C: 31/10	Randomized, controlled trial	Genistein 250 mg	Placebo	8 weeks	↓ Weight ↓ BMI ↓ waist circumference ↓ TG ↔ TC ↔ LDL ↔ HDL ↔ AST ↔ ALT ↓ HOMA-IR ↓ TNF-α ↓ IL-6
Catechin								
(Sakata et al. 2013) Iran [32]	I_1: 5 I_2: 7 C: 5	30–65	NA	Double-blind placebo-controlled study	I_1: 200 mg catechin I_2:1080 mg catechin	green tea-flavored beverage with 0 mg catechin	12 weeks	↔ weight ↔ BMI ↓ ALT

Table 1. *Cont.*

First Author's Last Name, Year, Country	Sample Size I/C	Mean Age (yrs)	Gender (M/F)	Study Design	Intervention and Dose	Control	Duration	Main Results
				Green tea extract				
(Hussain, 2017) Pakistan [33]	I: 40 C: 40	20–55	I: 26/14 C: 28/12	Randomized placebo-controlled trial	Green tea extract 500 mg	Placebo 500 mg of cellulose	12 weeks	↓ body weight ↓ BMI ↓ HOMA-IR ↓ Lipid profile ↓ AST ↓ ALT ↓ Hs-CRP
				Hesperidin				
(Yari et al. 2021) Iran [34]	I_1: 22 I_2: 24 I_3: 22 C_1: 21	44–47	I_1:11/11 I_2: 13.9/10.1 I_3: 13/12 C:10.5/11.5	Open-labeled randomized controlled trial	I_1: Hesperidin 1000 mg I_2: Flaxseed 30,000 mg I_3: Hesperidin 1000 mg + Flaxseed 30,000 mg	30,000 mg flaxseed or Placebo	12 weeks	↓ BMI ↓ waist-circumference ↓ ALT ↔ AST ↓ GGT ↓ HOMA-IR ↓ Lipid profile ↓ Hs-CRP ↓ TNF-α ↓ NF-κB ↓ Fibrosis score ↓ Steatosis score
(Cheraghpour et al. 2019) Iran [35]	I: 24 C: 25	47.30 ± 12.73	I: 10/13 C: 12/12	Randomized, double-blind, controlled clinical trial	Hesperidin 1000 mg	Placebo	12 weeks	↓ ALT ↔ AST ↓ GGT ↔ HOMA-IR ↓ TG ↓ TC ↔ LDL-C ↓ Hs-CRP ↓ TNF-α ↓ NF-κB ↔ Fibrosis score
				Silymarin and Silybin				
(Federico et al. 2019) Italy [36]	I: 60 C: 30	47–54	I: 29/31 C: 19/11	Randomized placebo-controlled trial	Silybin-phospholipid 606 mg, + vitamin D 20 mg + vitamin E 30 mg	Placebo	24 weeks	↓ ALT ↓ GGT ↓ HOMA-IR ↓ Steatosis ↔ lipid profile ↔ CRP ↔ TNF-α ↓ IL-18 ↓ TGF-β
(Loguercio et al. 2012) Italy [37]	I: 69 C: 69	40.8 ± 10.3	NA	Randomized controlled trial	Silybin 94 mg + phosphatidyl-choline 194 mg + vitamin E	Placebo	48 weeks	↔ BMI ↓ ALT ↓ AST ↓ GGT ↓ HOMA-IR ↓ NAFLD score

Table 1. *Cont.*

First Author's Last Name, Year, Country	Sample Size I/C	Mean Age (yrs)	Gender (M/F)	Study Design	Intervention and Dose	Control	Duration	Main Results
(Wah Kheong, 2017) Malaysia [38]	I: 49 C: 50	49.6 ± 12.7	I: 24/25 C: 22/28	Randomized, double-blind, placebo-controlled trial	silymarin 2100 mg	Placebo	48 weeks	↔ body weight ↔ HOMA-IR ↓ AST ↔ ALT ↔ GGT ↔ TC ↔ HDL ↔ LDL ↓ TG ↓ Fibrosis ↓ NAFLD score
(Navarro et al. 2019) Italy [39]	I_1: 26 I_2: 27 C: 25	47–50 ± 11.7	I_1: 18/9 I_2: 13/13 C: 14/11	Randomized, double-blind, placebo-controlled trial	I_1: Silymarin 420 mg I_2: Silyamarin 700 mg	Placebo	48 weeks	↔ AST ↔ ALT ↔ HOMA-IR ↔ Fibrosis ↔ NAFLD score
(Hashemi, 2009) Iran [40]	I: 50 C: 50	39.5 ± 10.3	I:28/22 C: 29/21	Placebo-controlled trial	Silymarin 280 mg	Placebo	24 weeks	↔ BMI ↔ lipid profile ↓ AST ↓ ALT
(Solhi et al. 2014) Iran [41]	I: 33 C: 31	39.36–43.6	I: 19/14 C: 15.5/15.5	Randomized clinical trial	Silymarin 210 mg	Placebo	8 weeks	↓ AST ↓ ALT
(Anushiravani et al. 2019) Iran [42]	I: 30 C: 30	47.0 ± 9.1	I + C: 77/73	Double-blind randomized placebo-controlled trial	Silymarin 140 mg	Placebo	12 weeks	↓ BMI ↓ waist-circumference ↓ Lipid profile ↓ AST ↓ ALT

I: Intervention group, C: Control group, M: Male, F: Female, NM: Not Mentioned. ↑: significantly increased, ↓: significantly decreased, ↔: no significant change.

As shown in Table 1, 1840 patients with NAFLD were included in the study, and their ages ranged from 18 to 70 years. The sample sizes across the studies varied from a low of 10 subjects per group [25] to a high of 69 subjects per group [37]. Although certain studies did not provide specific gender data, the overall gender distribution across the included studies was approximately 491 males and 429 females. The included trials examined a broad range of phenolic compounds, administered in a single form or combined with other compounds such as vitamin D, E, piperine, phosphatidylcholine, or flaxseed. All phenolic supplements were provided orally in capsule form, ranging from 8 to 48 weeks. Turmeric and its by-product, namely curcumin, was the most examined phenolic compound in 11 studies, while resveratrol and silymarin were studied in six trials for each one.

3.2. Liver Enzymes

The primary outcomes of the included studies were liver enzymes (AST, ALP, and ALT), lipid profile, inflammatory cytokines, insulin resistance, and anthropometric changes (Table 1). A total of 28 studies measured liver enzymes, including ALT, AST, and GGT. Curcumin and its derivatives significantly reduced liver enzymes across the included trials [14–22]. While resveratrol showed a significant reduction in AST and ALT in three trials [23,24,27], one trial revealed an increase in liver enzymes [25], and two trials failed to show a statistically significant impact [26,28]. Naringenin, catechine, and catechine-rich green tea extract were also found to stimulate a reduction in liver enzymes [30–33]. Hesperidin supplementation demonstrated a considerable decrease in ALT and GGT but

not AST [34,35]. Out of seven trials that used silybin and silymarin supplementation, five trials only showed a significant reduction over AST, ALT, and GGT [36,37,40–42].

3.3. Lipid Profile

A total of 14 trials reported blood lipid profiles, including TG, LDL, TC, and HDL, while six studies reported specific lipid parameters. Of these 14 trials, seven studies showed significant improvement in blood lipid profile due to turmeric, curcumin, green tea extract, hesperidin, and silymarin [14–16,20,33,34,42]. The remaining seven studies involving the consumption of resveratrol [23,25,26], genistein [31], silybin [36], and silymarin [38,40] discovered non-significant amelioration for the whole lipid profile.

3.4. Inflammatory Markers and NAFLD Score

Inflammatory markers were assessed in 11 studies with controversial outcomes. In particular, five studies highlighted a significant reduction in TNF-α levels after supplementation with curcumin [17], resveratrol [24], genistein [31], and hesperidin [34,35]. In contrast, three studies on resveratrol [23,25,27] and one study on silybin [36] did not discover any significant improvement in TNF-α levels. Similarly, resveratrol and silybin failed to improve the serum levels of CRP [25,27,36], while green tea extract and hesperidin induced a significant amelioration, as shown in Table 1 [34,35,40]. IL-6 levels were reported to be controversial post-supplementation with resveratrol [25,27,28], but genistein supplementation significantly reduced IL-6 [31].

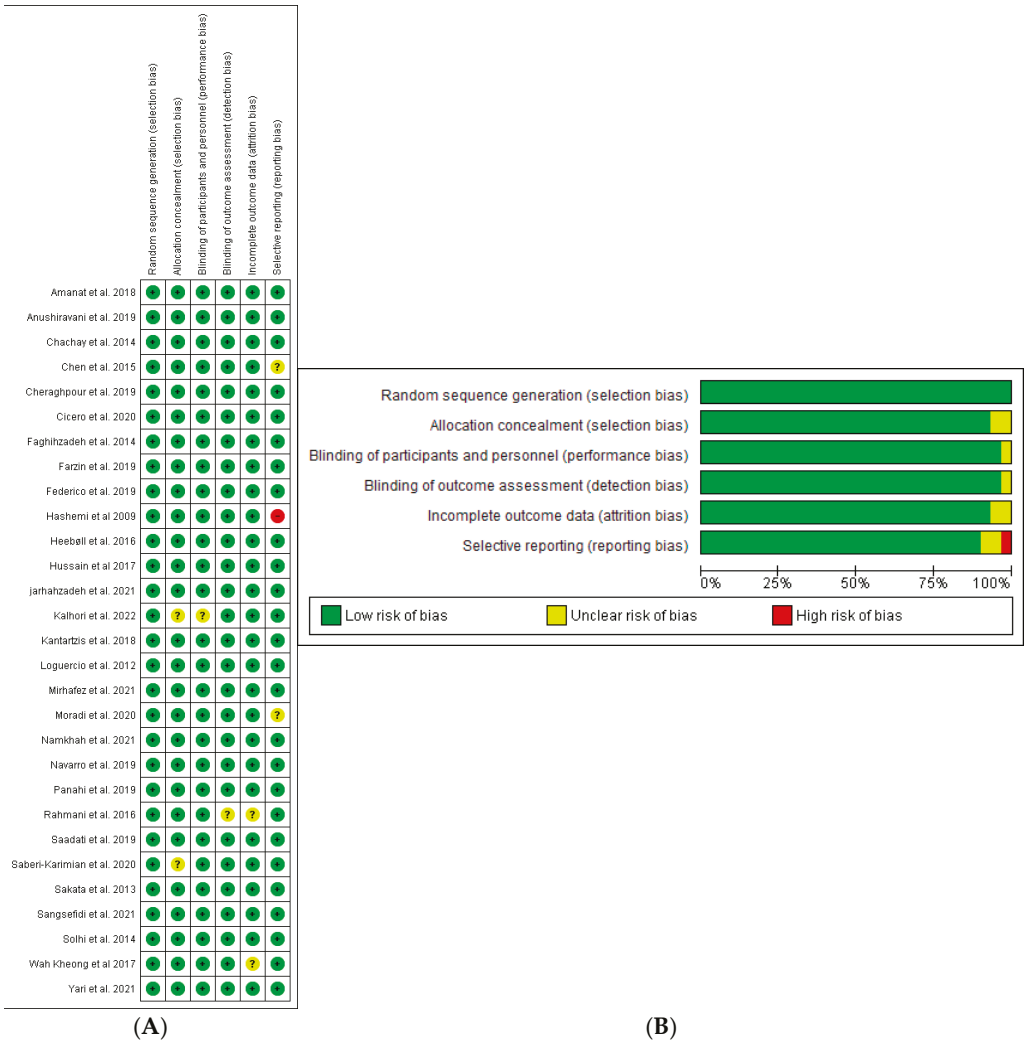
Seven studies reported NAFLD scores among all the included trials. Five trials found a significant improvement in NAFLD scores due to intervention with silymarin [38], silybin [37], naringenin [30], and curcumin [17,21]. In addition, hepatic fibrosis was noticed to improve after supplementation with curcumin [20], hesperidin [34,35], and silymarin [38].

3.5. HOMA-IR and Body Mass Index

A total of nine trials found a significant improvement in HOMA-IR values. This significant modulation was achieved following intervention with turmeric [14], curcumin [18,20], resveratrol [24], genistein [31], green tea extract [33], hesperidin [34], and silybin [36,37]. Also, BMI was highlighted in 16 studies, but in 9 trials, BMI was significantly decreased following intervention with curcumin and turmeric [14,16,20], resveratrol [26], naringenin [30], genistein [31], green tea extract [33], hesperidin [34], and silymarin [42].

3.6. Risk of Bias Assessment

As shown in Figure 2, the risk of bias assessment for the included studies indicated a generally low level of bias across all the domains. In all the included studies, randomization processes were consistently rated as low-risk, indicating cohesive methodologies for participant allocation. The majority of included trials received a low-risk rating in terms of deviation. However, a moderate risk of bias was notable in two studies [14,17]. A total of 28 studies received a low risk of bias in missing outcomes, except for [22]. The domain of outcome measurement was predominantly rated as low-risk, but Kheong et al. (2017) [38] was rated with a moderate risk, raising concern about the consistency of data collection. Finally, the assessment of reported results demonstrated a low risk of bias; however, four studies indicated a moderate risk.



(**A**) (**B**)

Figure 2. Risk of bias assessment for the included studies. (**A**) summary of risk of bias, (**B**) risk of bias graph. Symbols: (+) low risk, (?) unclear risk of bias, (−) high risk of bias [14–42].

4. Discussion

The findings of the current systematic review highlight the potential therapeutic benefits of dietary polyphenols in managing NAFLD. Among the 29 included RCTs involving 1840 participants, curcumin, silymarin, and hesperidin consistently demonstrated significant improvements in liver enzymes (AST, ALT, and GGT), lipid profile (LDL, HDL, TG and TC), and reductions in inflammatory cytokines (TNF-α, IL-6 and CRP). However, other polyphenols including resveratrol showed inconsistent results across trials while naringenin and genistein were supported by a limited number of studies.

The precise prevalence of NAFLD is not known, but it is increasing and in parallel with diseases such as diabetes, dyslipidemia, and obesity. It is believed that the prevalence is 75% among obese patients and can rise to 90% in severely obese patients [43]. NAFLD affects 20% to 30% of the whole world population, and less than 25% are diagnosed with NAFLD in Asian countries. Obesity is a significant factor in the development of NAFLD through genetic predisposition as well as dietary behavior [44]. There is no pharmacological intervention for NAFLD except lifestyle modification with a specific diet and increasing physical activity [11]. Therefore, discovering potential therapeutic agents is still required.

Medicinal plants and their extracted phytochemicals, including polyphenols, have been proposed to ameliorate various diseases for centuries. Polyphenols are a wide range of plant-derived compounds with water-soluble characteristics [45]. However, bioavailability of polyphenols varies widely depending on their chemical structure and forms of deliv-

ery [12]. Encapsulation technologies such as liposomal or nanoparticle systems enhance polyphenol absorption and increase systemic availability. For example, nano-formulations including liposomes and polymeric nanoparticles enhance significantly the bioavailability of curcumin by increasing its stability, solubility and resistance to gastrointestinal degradation [46]. Three independent reviews have highlighted that nano-curcumin is effective in reducing liver enzymes, hepatic steatosis, and inflammation [47]. However, a clinical trial investigating the supplementation effect of raw turmeric on liver enzymes and fatty liver degree in NAFLD patients reported no significant changes [48]. Consuming raw dietary polyphenols often exhibited lower bioavailability due to their glycosylated forms, which subsequently reduce the therapeutic effect [46].

In the human diet, polyphenols, like vitamins, are the most abundant antioxidant compounds with many biological activities [49]. The ability of polyphenols to prevent oxidative stress, increase fatty acid beta-oxidation, and regulate insulin resistance has been documented in experimental studies [50]. Furthermore, fruit-rich polyphenols can facilitate the export of hepatic TG and reduce lipogenesis through the brain vagus–liver axis [51]. The hepato-protective properties of certain pure polyphenols, including turmeric, resveratrol, silybin, and catechin, against steatosis have been observed in HegG2 cells challenged with oleic acid [52]. Several RCTs have recently examined phenolic compounds' efficacy in NAFLD patients. Therefore, the current systematic review aimed to summarize and provide up-to-date clinical evidence by which scientists and clinicians could learn the potential therapeutic possibilities of phenolic isolated compounds. The analysis of 29 RCTs with 1840 patients revealed a promising role of phenolic compounds in ameliorating various biomarkers and clinical outcomes associated with NAFLD.

Liver enzymes were a consistent finding across the included studies, particularly ALT, AST, and GGT. These enzymes mainly involve hepatocellular biological functions, including amino acid metabolism and glutathione synthesis [53]. At the preclinical level, polyphenol supplementations were proven to induce hepatocellular protection, decreasing AST, ALT, and GGT [11]. Most of the included trials indicated low levels of liver enzymes post-phenolic intervention, but resveratrol supplementation demonstrated controversial results. Chachay and his colleagues [25] found an increment in AST and ALT after supplementing NAFLD patients with 3000 mg of resveratrol, while supplementing 600 mg and 150 mg of resveratrol for 12 weeks did not induce changes in AST, ALT, and GGT levels [26,28]. However, serum levels of AST, GGT, and ALT are general biomarkers for liver damage rather than liver function. Therefore, the factors contributing to their decreased response to polyphenol supplementation could be multifactorial, potentially involving reductions in inflammatory cytokines including TNF-α and IL-6. These cytokines are key mediators of hepatocellular inflammation and cell injury [54].

NAFLD is intricately associated with inflammation, which affects hepatocytes and manifests systemically by elevated levels of CRP in NAFLD patients [55]. Moreover, pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 have a crucial function in the progression of NAFLD by stimulating hepatocellular inflammation and inducing fibrosis and cell necrosis [54]. Polyphenols have been reported to ameliorate pro-inflammatory cytokines in NAFLD animal models in parallel with histopathological improvements [11]. In the current systematic review, curcumin, resveratrol, genistein, green tea extract, and hesperidin have effectively reduced pro-inflammatory cytokines. However, studies that examined silybin or silymarin did not report any significant changes in TNF-α, IL-6, CRP, and IL-1β. As mentioned earlier, the anti-inflammatory properties of the polyphenols could be due to the downregulation of pro-inflammatory cytokine expression by inhibiting the phosphorylation of NF-κB and MAPK [56]. Activating IL-1β and TNF-α requires the COX-2 enzyme, which is found to be inhibited by curcumin, resveratrol, green tea extract, and hesperidin [57–60]. Dysregulation of lipid metabolism stimulates endoplasmic reticulum stress, which further amplifies the inflammatory response in hepatic cells by upregulating NF-κB [56,61]. Resveratrol, curcumin and green tea have been found to regulate fatty acid oxidation which further leads to lower levels of inflammatory cytokines [62–64].

The accumulation of lipid metabolites in hepatocytes exceeding 5% is a histological criterion for diagnosing NAFLD, associated with the induction of oxidative stress under NAFLD pathophysiology. According to the included trials, turmeric, curcumin, green tea extract, hesperidin, and silymarin significantly decreased TG, LDL, and TC [14–16,20,33,34,42]. The primary transcription factors responsible for lipids accumulation and metabolism are PPAR-α, PPAR-γ, SREBP-1c, and C/EBP-α gene [61]. Regulating lipid metabolism via PPAR-γ and SREBP-1c pathways attenuated hepatic steatosis in an NAFLD rat model after supplementation with curcumin, turmeric, green tea extract, hesperidin, and silymarin [65]. While resveratrol downregulated the gene expression of PPAR-γ and SREBP-1c in the NAFLD rat model [66], the current reviewed RCTs did not show consistent decrease in lipid profile. However, activating AMPK, a potent inhibitor for SREBP-1c, through reducing de novo lipogenesis has become a therapeutic target for addressing NAFLD. Also, AMPK plays another role in ameliorating NAFLD by inhibiting the phosphorylation of acetyl-CoA carboxylase, preventing fatty acid synthesis, and increasing fatty acid oxidation. Turmeric, curcumin, resveratrol, green tea extracts, silybin, and genistein have activated AMPK in rats and cell models [67]. These studies have also found that AMPK activation is coupled with a decrement in insulin resistance, which is also reflected by improvements in fasting blood glucose and, ultimately, in NAFLD scores [67]. Due to polyphenol supplementation, insulin resistance showed a significant improvement, reflected by a reduction in BMI and body weight among NAFLD patients based on the RCTs. While sirtuins are a family of signaling proteins involved in cellular metabolic regulation, curcumin, turmeric, resveratrol, green tea extract, and silymarin have been found to activate sirtuin, a deacetylate of liver kinase B_1 ($LKBT_1$), followed by AMPK activation [62–64,68].

Most recently, several systematic reviews have been performed to determine the role of specific classes of polyphenols in ameliorating NAFLD [3,56]. However, our study has a strength in highlighting certain explanations related to the included studies. The inconsistent findings across all the included studies are due to the different types of polyphenols, which vary in molecular structures and properties [49]. The biological properties of polyphenols are also affected by their interaction with the food matrix, which reduces the bioavailability of polyphenols [69]. Furthermore, the dietary intake of NAFLD patients in the included studies could skew the findings, especially if the trials were carried out in different geographical locations where the polyphenol intake of the population sample could be higher or lower. For example, the Mediterranean diet has been associated with moderate to high polyphenol intake and prevents or reverses NAFLD [70]. Another strength that can be noticed in our study is the attempt to gather all the recent available RCTs across all types of polyphenols. This is crucial because it allows for additional research on the role of phenolic compounds in improving NAFLD.

However, a few limitations are present in the current systematic review. The findings of the included studies had difficulty in generalization, as the RCTs had a small sample population. Because these samples were derived from various countries, their findings might not be generalizable to the global population. The significance of this consideration is particularly apparent when evaluating data sourced from clinical trials performed in developing and developed countries, as baseline differences among the populations might exist. Furthermore, the included studies tended to examine the exact exposure measurement, but they assessed the outcomes differently. Finally, this study investigated RCTs published in English, which might not represent all available evidence and could lead to inconsistent conclusions.

5. Conclusions

The current study attempted to provide a systematically comprehensive evaluation of the effect of dietary polyphenol supplementation against NAFLD development. Our analysis of 29 studies reveals that curcumin, silymarin, and hesperidin demonstrate significant potential in ameliorating critical markers of liver dysfunction, including a reduction in liver enzymes (AST, ALT, and GGT), improvement in lipid profile (LDL, HDL, TC, and TG), and

reduction in inflammatory cytokines (TNF-α, IL-6, and CRP). However, certain polyphenols (such as resveratrol) have failed to show consistent improvement in NAFLD makers, and other polyphenols (such as naringenin and genistein) lack RCTs. Given the variability in the provided evidence, further high-quality, large-scale RCTs with standardized interventions are still required to initiate clear clinical guidelines for using polyphenol supplementation among NAFLD patients. Until such evidence is available, clinicians should consider the preliminary findings and exercise caution when recommending polyphenol supplements.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu16234150/s1, Table S1: Electronic search strategies. PRISMA 2020 Checklist [71].

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nutrients

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Review

Nutritional Strategies for Chronic Craniofacial Pain and Temporomandibular Disorders: Current Clinical and Preclinical Insights

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Abstract: This narrative review provides an overview of current knowledge on the impact of nutritional strategies on chronic craniofacial pain associated with temporomandibular disorders (TMDs). Individuals experiencing painful TMDs alter their dietary habits, avoiding certain foods, possibly due to chewing difficulties, which might lead to nutrient deficiencies. Our literature investigation revealed that the causal links between nutritional changes and craniofacial pain remain unclear. However, clinical and preclinical studies suggest that nutraceuticals, including vitamins, minerals, polyphenols, omega-3 fatty acids, isoprenoids, carotenoids, lectins, polysaccharides, glucosamines, and palmitoylethanolamides, could have beneficial effects on managing TMDs. This is described in 12 clinical and 38 preclinical articles since 2000. Clinical articles discussed the roles of vitamins, minerals, glucosamine, and palmitoylethanolamides. The other nutraceuticals were assessed solely in preclinical studies, using TMD models, mostly craniofacial inflammatory rodents, with 36 of the 38 articles published since 2013. Our investigation indicates that current evidence is insufficient to assess the efficacy of these nutraceuticals. However, the existing data suggest potential for therapeutic intervention in TMDs. Further support from longitudinal and randomized controlled studies and well-designed preclinical investigations is necessary to evaluate the efficacy of each nutraceutical intervention and understand their underlying mechanisms in TMDs.

Keywords: chronic pain; diet; nutrition; nutraceuticals; temporomandibular disorders

1. Introduction

Chronic temporomandibular disorders (TMDs) present a range of conditions affecting deep craniofacial tissues, including the temporomandibular joint (TMJ) and jaw muscles [1]. These disorders are the second most prevalent musculoskeletal chronic pain condition [2]. Traditional medical strategies often rely on pharmacological interventions for chronic TMD pain management, which vary in efficacy and carry the risk of adverse effects [3,4]. Notably, guidelines from the American College of Physicians suggest that drugs might not be the first-line treatment for chronic pain [5]. In recent decades, there has been a growing body

of evidence highlighting the significance of complementary and integrative approaches in managing chronic pain [6–11]. These approaches generally involve a diverse range of treatments categorized by their delivery modes, including mind–body therapy (e.g., massage, mindfulness), bioenergetic therapy (e.g., acupuncture) [10,12], and biologically based therapy (e.g., nutrition).

Among these modalities, dietary patterns have emerged as significant health influencers, playing critical roles in managing chronic diseases, including chronic pain [13,14]. Prioritizing a healthy diet and nutritional interventions remains crucial among modifiable behaviors. Addressing this complexity necessitates a comprehensive examination of how nutrients function within the body, particularly in the context of managing chronic pain.

Poor dietary choices, often associated with a Western diet marked by excessive consumption of processed foods, too much added salt, sugar, and unhealthy fats, have been linked to various chronic pain conditions [15]. For example, an energy-dense diet characterized by a high sugar intake has been positively correlated with chronic back pain [16]. Individuals exhibiting dietary patterns similar to those of the Western diet are more prone to developing rheumatoid arthritis [17], with high-sugar diets suspected to contribute to its onset [18]. Additionally, increased dietary salt intake has been associated with non-specific low back pain, possibly due to posterior lumbar subcutaneous edema [19]. Cross-sectional surveys indicate that red meat, fish, and legumes are frequently reported to exacerbate joint pain in rheumatic diseases [20], while excessive consumption of cured meats and sweetened beverages has been linked to increased psychological distress in fibromyalgia patients [21]. Similarly, obesity and elevated body fat content are documented to heighten the risk of fibromyalgia [22]. Furthermore, a high-sucrose diet has been found to expedite arthritis progression in rheumatoid arthritis models [23].

In contrast to the preceding discussion, dietary modifications (e.g., a plant-based diet) and specific nutritional approaches offer significant benefits in promoting health and alleviating chronic pain [24–27]. Furthermore, it has been demonstrated that a range of constituents in foods can modulate neural functions associated with chronic pain [26–29]. Hence, it is reasonable to hypothesize that alternative nutritional factors are pivotal in controlling chronic TMD pain.

Given these considerations, although evidence regarding the effects of nutritional approaches on chronic deep craniofacial pain seen in TMDs is limited, several reports suggest that such approaches could significantly alleviate painful TMDs [30–32]. This narrative review article aims to assess the current understanding of the effectiveness of dietary habits and nutritional approaches for managing painful TMDs. It seeks to explore studies from the past 25 years and provide an updated investigation into the potential impact of nutritional approaches on TMDs in both clinical and preclinical settings. Due to existing evidence, there is particular emphasis on elucidating the effects of specific nutritional compounds, as shown in Table 1. Importantly, the focus is on compounds derived from ordinary foods such as vegetables, fruits, and fish. Further, given the scarcity of clinical evidence to evaluate such hypotheses, exploring preclinical research, particularly in preclinical models, could provide valuable insights into the effects of nutritional interventions on deep craniofacial nociception [33,34].

Table 1. List of nutraceuticals. Individual nutraceuticals discussed are shown in parentheses.

Nutraceuticals	
Vitamin	Vitamin D, Vitamin B, Vitamin C
Mineral	Magnesium, Zinc, Strontium, Sulfur
Polyunsaturated fatty acids	Omega-3 fatty acids, Docosahexaenoic acid
Polyphenols	Quercetin, (-)-epigallocatechin-3-gallate, Resveratrol, Curcumin
Isoprenoids	Limonene, Citral
Carotenoid	Crocin, Lutein
Lectin	
Polysaccharide	
Glucosamine	
Palmitoylethanolamide	

2. Methods

2.1. Search Strategy

This review article adheres to the rigorous guidelines set by the Scale for the Assessment of Narrative Review Articles [35]. The exhaustive search for relevant clinical articles was meticulously conducted using mainly Med-line PubMed, targeting keywords outlined in Table 2. Only articles written in English and published between January 2000 and March 2024 were primarily considered for inclusion, with strict exclusion criteria applied to unpublished results, proceedings papers, personal communications, and conference reports. During the backward search process, we identified significant papers published before 2000 or not found in PubMed, which were deemed relevant and therefore included for analysis. This article primarily focuses on clinical and preclinical evidence highlighting the nutritional roles in chronic pain associated with TMDs, occasionally comparing these findings with other chronic pain conditions. Particularly, in preclinical literature, evidence demonstrating nutritional roles in craniofacial nociceptive responses indicated by behavioral or neural responses is included for discussion. Thus, this narrative review article focuses less on the molecular mechanisms underlying painful TMDs and craniofacial nociception. Furthermore, it does not include reports in Table 2 from animal models mimicking pain conditions related to the trigeminal nerves, such as neuropathic pain, headaches, and toothaches. The screening process was conducted with utmost precision by three reviewers (K.P., Y.I., and M.H.), while the task of forward and backward searching was expertly executed by another two reviewers (K.O. and Y.K.). The eligibility of articles was carefully assessed by two supervisors (K.Y., and N.F.) to maintain the highest standards of quality and reliability.

Table 2. List of keywords employed in this study. For searching clinical and preclinical studies, the keyword was selected from categories "a" or "b" and "c", respectively.

Category	Keywords
a. Clinical	"temporomandibular disorder" OR "TMD" OR "temporomandibular joint" OR "TMJ" OR "masseter muscle" OR "orofacial pain" OR "patient" OR "clinical"
b. Preclinical	"craniofacial tissue" OR "animal model" OR "Complete Freund's Adjuvant" OR "CFA" OR "formalin" OR "mice" OR "rat" OR "nociception" OR "preclinical model"
c. Exposure	"antioxidant" OR "diet" OR "dietary" OR "dietary supplement" OR "supplement" OR "fatty acid" OR "fiber" OR "food" OR "mineral" OR "natural product" OR "nutraceutical" OR "nutrition" OR "supplement" OR "phytochemical" OR "polyphenol" OR "vitamin" OR "fruits" OR "vegetable" OR "isoprenoid" OR "carotenoid" OR "lectin" OR "polysaccharide" OR "glucosamine"

2.2. Quality of Evidence Assessments

Risk of bias was evaluated using the Cochrane Risk of Bias Tool for randomized controlled trials in clinical articles. Each item was categorized as low, high, or unclear risk of bias. For pre-clinical articles, the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) criteria were used [36]. The assessment criteria were evaluated by three different authors (K.P., Y.H., and K.O.).

3. Results

3.1. Characteristics

3.1.1. Clinical Articles

Twelve randomized controlled trials revealed the roles of nutraceuticals categorized into four groups on painful TMDs (Table 3). Five studies employed the oral administration of each nutraceutical solely, while seven studies employed the oral administration of each

nutraceutical with additional interventions, such as hyaluronic acid injection into the TMJ [37–43]. Ten of these twelve studies focused on the effects of neutraceuticals on TMJ pain. One study examined the effects of minerals (magnesium) on myogenic types of TMDs. The remaining studies examined the effects of vitamin B12 on both muscle and TMJ pain.

Table 3. Clinical studies evaluating the effects of nutraceutical interventions in Temporomandibular Disorders.

Authors	Pain Condition	Study Duration	Interventions	Groups	Outcomes
Vitamins					
Gupta et al., 2022, India [37]	Axis I group II TMDs with vitamin D levels < 30 ng/mL.	3 months	Vitamin D tablets 60,000 IU once a week for eight weeks.	1. Splint alone 2. Splint + Vitamin D supplement	In TMD patients with vitamin D deficiency, a significant difference was seen in VAS score and maximum mouth opening between the splint with vitamin D supplementation and the splint with a placebo drug.
Reis et al., 2023, Brazil [38]	Chronic myofascial pain and arthralgia.	1 month	Methylcobalamin (B12) 1000 μg/day, orally.	1. Laser and B12 placebo 2. Effective laser + B12 placebo 3. Effective laser + B12	Vitamin B12 facilitates the inhibitory effects of laserpuncture in treating painful TMDs.
Minerals					
Refahee et al., 2022, Egypt [44]	Myofascial pain and trigger points in the masseter muscle.	6 months	Magnesium sulfate (MS, 0.41 mMol/mL) 2 mL, trigger point injection.	1. Saline 2. Magnesium sulfate	MS reduced the facial pain scores, and the maximum mouth opening distance was higher up to 3 months in the MS than in the saline group.
Kiliç, 2021, Turkey [39]	Temporomandibular joint osteoarthritis (TMJ OA).	12 months	GCM supplementation, containing 750 mg GH, 600 mg chondroitin sulfate, and 350 mg MSM at 2 × 1 dosage daily for 3 months.	1. arthrocentesis plus intraarticular hyaluronic acid (HA) injection only 2. arthrocentesis plus intraarticular HA injection followed by 3 months of GCM	GCM supplementation after arthro-centesis plus intraarticular hyaluronic acid injection produced no additional clinical benefits or improvements for patients with TMJ-OA compared with arthrocentesis plus intraarticular hyaluronic acid injection alone.
Glucosamine					
Thie et al., 2001, Canada [45]	Degenerative joint Disease of TMJ.	4 months	Glucosamine sulfate (GS) 500 mg tid for 90 days, orally.	1. Ibuprofen 2. GS	GS decreased TMJ pain compared with ibuprofen administrations.

Table 3. *Cont.*

Authors	Pain Condition	Study Duration	Interventions	Groups	Outcomes
Damlar et al., 2014, Turkey [40]	Internal derangements of TMJ	2 months	1500 mg glucosamine and 1200 mg chondroitin sulfate (CS) /day, orally.	1. Tramadol HCl 2. glucosamine and chondroitin sulfate	A combination of glucosamine and chondroitin sulfate reduced pain compared with the tramadol group.
Haghighat et al., 2013, Iran [46]	Painful TMJ, TMJ crepitation or limitation of mouth opening.	3 months	Glucosamine sulfate (GS) 1500 mg/day, orally.	1. Ibuprofen 2. GS	GS improved craniofacial pain and mandibular opening distance compared to baseline and showed more post-treatment improvement when compared with ibuprofen.
Cahlin et al., 2011, Sweden [47]	TMJ OA	6 weeks	Glucosamine sulfate (GS) 1200 mg/day, orally.	1. Placebo drug 2. GS	GS showed less beneficial effects compared with the placebo group in reducing pain associated with TMJ osteoarthritis.
Nguyen et al., 2016, USA [41]	Capsulitis, disk displacement, disk dislocation, or painful osteoarthritis of TMJ	12 weeks	1500 mg of glucosamine hydrochloride (GH) and 1200 mg of chondroitin sulfate (CS)/day, orally.	1. Placebo drug 2. GH + CS	GH-CS reduced craniofacial pain compared with the placebo group.
Cen et al., 2017 [42]	TMJ OA	1 year	Two tablets of 240 mg glucosamine hydrochloride (GH) tid for 3 months, orally	1. Placebo + HA injection 2. GH + HA injection	GH with HA injection decreased craniofacial pain at 1 month and one year follow-up compared to baseline. One year later, pain score was reduced, and IL-6 and IL-1β levels were lower in group GH + HA than in group placebo + HA.
Yang et al., 2018, China [43]	TMJ OA	1 year	Glucosamine hydrochloride (GH) 1.44 g/day for 3 months, orally.	1. Placebo + hyaluronate sodium injection 2. GH + hyaluronate sodium injection	GH and hyaluronate sodium injection improved maximal mouth opening distance and facial pain intensity compared to the placebo + hyaluronate sodium injection group in the long-term follow-up.

Table 3. *Cont.*

Authors	Pain Condition	Study Duration	Interventions	Groups	Outcomes
Palmitoylethanolamide					
Marini et al., 2012, Italy [48]	TMJ OA or arthralgia	2 weeks	Palmitoylethanolamide (PEA) 300 mg in the morning and 600 mg in the evening for 7 days and then 300 mg twice a day for 7 more days, orally.	1. Ibuprofen 2. PEA	PEA improved pain related to maximum mouth opening compared with the ibuprofen-treated group.

3.1.2. Preclinical Articles

Thirty-eight articles were assessed. Of these, thirty-three were conducted with rats, one with mice, and four with both mice and rats (Table 4). Two articles specifically employed female rats [49,50]. Effects of nutraceuticals on nociception were assessed through facial pain-like behaviors or neural activities evoked by craniofacial nociceptive stimulation. Models were primarily developed through craniofacial injections of inflammatory agents, including formalin, complete Freund's adjuvant (CFA), mustard oil, capsaicin, and zymosan. Seven studies assessed nociceptive neural activities by quantifying the number of action potentials in the trigeminal ganglion or trigeminal subnucleus caudalis evoked by craniofacial stimulation [51–57]. Behavioral and neural responses were compared between groups treated with vehicle and nutraceutical.

Table 4. Preclinical studies evaluating the effectiveness of nutraceutical interventions in TMD models.

Authors	Model	Interventions	Groups	Outcomes
Vitamins				
Erfanparast et al., 2014 [58]	Male rats, formalin test	Vitamin B12, 1, 2, and 4 mg/kg, i.p. or peripheral (2.5, 5 and 10 μg/rat) injections.	1. Saline 2. Diclofenac 3. Vitamin B12 4. Vitamin B12 + Diclofenac	Vitamin B12 reduced facial pain-like behavior. Co-treatments with vitamin B12 and diclofenac facilitated antinociceptive effects.
Erfanparast et al., 2017 [59]	Male rats, formalin test	Vitamin B12, 0.5 μL intra-hippocampal injection.	1. Saline 2. Naloxone 3. Vitamin B12 4. Vitamin B12 +Naloxone	Vitamin B12 reduced facial pain-like behaviors associated with laserpuncture intervention.
Minerals				
Cavalcante et al, 2013 [60]	Male rats, carrageenan-induced TMJ inflammation	Magnesium chloride ($MgCl_2$), 10, 30, and 90 mg kg/day, orally.	1. Naive (no carrageenan) 2. Carrageenan + Vehicle 3. Carrageenan + $MgCl_2$	$MgCl_2$ reduced facial pain-like behaviors evoked by mechanical threshold.
Srebro et al., 2018 [61]	Male rats, formalin test	Magnesium sulfate (MS), 0.005–45 mg/kg, s.c.	1. Naïve (no formalin) 2. Formalin + Vehicle 3. Formalin + MS	MS reduced facial pain-like behaviors.
Srebro et al., 2023 [62]	Male rats, formalin test	Magnesium sulfate (MS), 5, 15 mg/kg, s.c.	1. Saline 2. Cromoglycate 3. MS 4. MS + Cromoglycate	MS reduced formalin-induced facial pain-like behaviors.

Table 4. *Cont.*

Authors	Model	Interventions	Groups	Outcomes
Alves et al., 2017 [63]	Male rats, zymosan-induced TMJ inflammation	Strontium, 0.5, 5, or 50 mg/kg, orally.	1. Naïve (no zymosan) 2. Zymosan 3. Zymosan + Indomethacin 4. Zymosan + Strontium ranelate	Strontium reduced the facial pain-like behaviors evoked by mechanical threshold.
Fatty acids				
Marana et al, 2022 [49]	Female rats, type II bovine collagen (CII) +CFA-induced rheumatoid arthritis (RA)	Omega-3 FAs, 300 mg/kg/day, orally.	1. Naïve (non-RA) 2. ovariectomized rats (OVX) 3. OVX + RA 4. OVX + Omega-3 FAs	Omega-3 FAs reduced TMJ damage and increased proinflammatory cytokine levels.
Ceotto et al., 2022 [64]	Male rats, CII +CFA-induced RA	Omega-3 FAs, 85 mg/kg/day, orally.	1. Naïve (non-RA) 2. RA 3. RA + Omega-3 FAs 4. RA + Omega-3 FAs +Aspirin	Omega-3 FAs reduced TMJ damage identified and increased various cytokines.
Barbin et al., 2020 [65]	Rats, Sex: not described, CFA-induced facial inflammation	Omega-3 FAs, 300 mg/kg/day, orally.	1. Naïve (no CFA) 2. CFA + Saline 3. CFA + dexamethasone 4. CFA + Omega-3 FAs	Omega-3 FAs reduced TMJ damage identified by histo-morphometric analysis. Differences in the level of IL-1 beta, TNF-alpha, and IL-10 between control and CFA groups were found.
Nakazaki et al., 2018 [66]	Male rats, CFA-induced TMJ inflammation)	Docosahexaenoic acid (DHA), 328 mg/kg, i.p.	1. Naïve (no CFA) 2. CFA + Saline 3. CFA + DHA	DHA reduced mechanical behavioral sensitivities and neural activities in the trigeminal subnucleus caudalis.
Polyphenols				
Sashide et al., 2024 [51]	Male rats, CFA-induced facial inflammation	Quercetin, 1–10 mM, local injection.	1. Before quercetin injection 2. After quercetin injection	Quercetin reduced facial stimulation-evoked neural activities in the trigeminal ganglion (TG).
Toyota et al., 2023 [52]	Male rats, naïve	Quercetin, 1, 10 mM, local injection.	1. Before quercetin injection 2. After quercetin injection	Quercetin reduced facial stimulation-evoked nociceptive neural activities in the TG.
Liu et al., 2024 [53]	Male rats, LPS into the TG	Quercetin, 10 mM, trigeminal ganglion (TG) injection.	1. Naïve (no LPS) 2. LPS + Saline 3. LPS + Quercetin	Quercetin reduced mechanical hyperalgesia-like behaviors in rats with the intra-TG injection of LPS.
Itou et al., 2022 [54]	Male rats, CFA-induced whisker pad inflammation	Quercetin, 50 mg/kg, i.p.	1. Naïve (no CFA) 2. CFA + Saline 3. CFA + Diclofenac 4. CFA + Quercetin	Quercetin reduced mechanical hyperalgesia-like behaviors and neural activities in the trigeminal subnucleus caudalis.

Table 4. *Cont.*

Authors	Model	Interventions	Groups	Outcomes
Uchino et al., 2023 [55]	Male rats, naïve	(-)-epigallocatechin-3-gallate (EGCG), 1, 10 mM, local injection.	1. Before EGCG injection 2. After EGCG injection	EGCG reduced neural activities in the trigeminal subnucleus caudalis region evoked by mechanical stimulation to the facial skin.
Shimazu et al., 2016 [56]	Male rats, naïve	Resveratrol (RSV), 1–10 mM, local injection.	1. Before RSV injection 2. After RSV injection	Resveratrol suppresses the excitability of the trigeminal subnucleus caudalis neurons.
Ma et al., 2020 [67]	Male rats, CFA-induced facial inflammation	Resveratrol (RSV), 40 mg/kg or 80 mg/kg, i.p.	1. Naïve (no CFA) + Dimethyl sulfoxide (DMSO) 2. Naïve (no CFA) + RSV 3. CFA + DSMO 4. CFA + RSV	Resveratrol increased the CFA-decreased mechanical withdrawal threshold.
Takehana et al., 2017 [57]	Male rats, naïve	Resveratrol, 2 mg/kg, i.v.	1. Before RSV injection 2. After RSV injection	Resveratrol reduced neurotransmission of the trigeminal nucleus caudalis neurons evoked by mechanical stimulation.
Mittal et al., 2009 [50]	Male and female rats, formalin test	Curcumin, 25, 50, 100, 200, 400, and 600 mg/kg, i.p.	1. DMSO 2. Curcumin 3. Diclofenac + Curcumin	Curcumin reduced facial pain-like behaviors.
Luca et al., 2014 [68]	Male mice, formalin test	Curcumin, 120 mg/kg, orally.	1. Vehicle 2. Curcumin	Curcumin reduced facial pain-like behaviors.
Wu et al., 2017 [69]	Male rats, formalin test	Curcumin, 50 mg/kg, i.p.	1. Naïve (no formalin) 2. Formalin 3. Formalin + Amiloride 4. Formalin + Curcumin	Curcumin reduced facial pain-like behaviors.
Yeon et al., 2010 [70]	Male rats, capsaicin test	Curcumin, 5, 25, or 50 mg/kg, i.p.	1. Naïve (no capsaicin) 2. Naïve (no capsaicin) + Curcumin 3. Capsaicin + Vehicle 4. Capsaicin + Curcumin	Curcumin reduced capsaicin-induced thermal hyperalgesia-like behaviors in rats.
Isoprenoids				
Pereira et al., 2022 [71]	Male rats, formalin test	Limonene (LIM), 50 mg/kg, orally.	1. Naïve (no formalin) 2. Formalin 3. Formalin + Morphine 4. Formalin + LIM 5. Formalin + LIM/HPβCD	Limonene reduced TMJ pain-like behaviors.
Santos et al., 2023 [72]	Male rats, formalin test or CFA-induced TMJ inflammation	Citral, 100 and 300 mg/kg, orally.	1. Formalin + Tween80 2. Formalin + Citral 1. Naïve (no CFA) + Tween80 2. CFA + Tween80 3. CFA + Citral	Citral reduced formalin-induced orofacial nociceptive behavior and mechanical hyper-nociception in CFA-induced TMJ inflammation.

Table 4. *Cont.*

Authors	Model	Interventions	Groups	Outcomes
Santos et al., 2022 [73]	Male mice and rats, formalin, mustard oil, cinnamaldehyde, menthol, and capsaicin tests	Citral, 0.1, 0.3, or 1.0 mg/kg, orally.	1. Naïve (non-inflamed) 2. Inflamed + vehicle 3. Inflamed + Citral	Citral reduced TMJ- or masseter muscle-evoked pain-like behaviors via TRPV1, TRPM3, and TRPM8 mechanisms.
Carotenoids				
Tamaddonfard et al., 2015 [74]	Male rats, capsaicin test	Crocin, 10 and 40 μg/rat, Intra-fourth ventricle injection.	1. Capsaicin + Saline 2. Capsaicin + Morphine 3. Capsaicin + Crocin 4. Capsaicin + Morphine + Crocin	Intra-fourth ventricle administration of crocin reduced facial pain-like behavior independent of opioid mechanisms.
Shimazu et al., 2019 [75]	Male rats, mustard oil-evoked inflammation	Lutein, 10 mg/kg, i.p.	1. Naïve (no mustard oil) 2. Mustard oil 3. Mustard oil + Lutein	Lutein reduced facial pain-like behaviors and c-Fos responses in the trigeminal caudalis and upper cervical dorsal horn.
Syoji et al., 2018 [76]	Male rats, CFA-induced inflammation	Lutein, 10 mg/kg, i.p.	1. Naïve (no CFA) 2. CFA 3. CFA + Lutein	Lutein reduced mechanical hyperalgesia-like behaviors via the Cox-2 signaling cascade.
Lectins				
Rivanor et al., 2018 [77]	Male rats, formalin or carrageenan or capsaicin test	Lectin, 0. 1, 1, or 10 mg/kg, i.v.	1. Naïve (non-inflamed) 2. Inflamed + Saline 3. Inflamed + Lectin	Lectin from green seaweed Caulerpa cupressoides reduced orofacial pain-like behaviors.
Rivanor et al., 2014 [78]	Male rats, Zymosan	Lectin, 0.1, 1, or 10 mg/kg, i.v.	1. Naïve (no zymosan) 2. Zymosan + Saline 3. Zymosan + Indomethacin 4. Zymosan + Lectin	Lectin from the green seaweed Caulerpa cupressoides reduced mechanical hyper-nociception.
Leite et al., 2022 [79]	Mice and rats, Sex: not described, capsaicin, formalin test	Lectin, 0.25, 0.5, 1, 10 mL/kg, i.p.	1. Naïve (non-inflamed) 2. Inflamed + Saline 3. Inflamed + Lectin	Parkia platycephala lectin reduced orofacial pain-like behavior.
Alves et al., 2018 [80]	Male rats, formalin test	Lectin, 0.001–0.1 mg/kg, i.v.	1. Naïve (no formalin) 2. Formalin 3. Formalin + Morphine 4. Formalin + Lectin	Lectin from Abelmoschus esculentus reduced orofacial pain-like behavior.
Freitas et al., 2016 [81]	Male rats, zymosan-induced TMJ inflammation	Lectin, 0.01–1 mg/kg, i.v.	1. Naïve (no zymosan) 2. Zymosan + Saline 3. Zymosan + Indomethacin 4. Zymosan + Lectin	Lectin from Abelmoschus esculentus reduced mechanical hyperalgesia-like behaviors.
Damasceno et al., 2016 [82]	Mice and rats, Sex: not described, formalin, capsaicin, glutamate test	Frutalin, 0.25, 0.5, or 1 mg/kg, i.p.	1. Naïve (non-inflamed) 2. Inflamed + Saline 3. Inflamed + Frutalin	Frutalin reduced facial pain-like behaviors via nitrogenic oxide mechanisms.

Table 4. *Cont.*

Authors	Model	Interventions	Groups	Outcomes
Polysaccharides				
Araújo et al., 2017 [83]	Male rats, formalin test	Sulfated polysaccharide (SP), 0.03, 0.3 or 3.0 mg/kg, s.c.	1. Naïve (no formalin) 2. Formalin 3. Formalin + Sulfated polysaccharide	SP from the red seaweed Solieria filiformis reduced facial pain-like behaviors, inhibited the plasma extravasation and inflammatory cytokines release, and increased the β-endorphin release in the trigeminal caudalis.
Souza et al., 2019 [84]	Male mice or rats, formalin test	Sulfated polysaccharide (SP), 5, 10 mg/kg, orally	1. Naïve (no formalin) 2. Formalin 3. Formalin + Sulfated polysaccharide	SP from the marine algae Hypnea pseudomusciformis reduced orofacial pain-like behaviors.
Rodrigues et al., 2014 [85]	Male rats, zymosan-evoked TMJ inflammation	Sulfated polysaccharide (SP), 1, 3 and 9 mg/kg, s.c.	1. Naïve (no zymosan) 2. Zymosan + Saline 3. Zymosan + Indomethacin 4. Zymosan + Sulfated polysaccharide	SP from the green seaweed Caulerpa cupressoides var. lycopodium reduced mechanical hyperalgesia-like behaviors.
Palmitoylethanolamide				
Bartolucci et al., 2018 [86]	Male rats, CFA-induced facial inflammation	Palmitoylethanolamide (PEA), 10 mg/kg, i.p.	1. Naïve (no CFA) 2. Naïve (no CFA) + PEA 3. CFA + Vehicle 4. CFA + PEA	PEA reduced facial hyperalgesia-like behaviors after TMJ inflammation.

Abbreviations: i.p., intraperitoneal injection; i.v., intravenous injection; s.c., subcutaneous injection; TG, trigeminal ganglion.

3.2. Risk of Bias Assessment

3.2.1. Clinical Articles

The risk of bias assessment for each article is described in detail in Table S1. No article was identified as having a high risk of bias.

3.2.2. Preclinical Articles

The risk of bias assessment for each preclinical article is detailed descriptively in Table S2. Seven articles (18.4%) displayed a high risk of bias, while 33 articles (86.8%) had more than six out of ten items classified as "unclear". In the text, no articles described the issues related to items, including the domain of allocation concealment, random housing, and random outcome assessment.

3.3. Effect of Interventions—Outcomes

3.3.1. Clinical Articles

Ten out of twelve articles documented the beneficial effects of each nutraceutical in improving painful TMDs. However, two reported limited effects of them on osteoarthritis-type TMDs [39,47].

3.3.2. Preclinical Articles

As shown in Table 4, all articles demonstrated inhibitory effects on craniofacial nociception indicated by decreases in pain-like behaviors or neural activities in response to craniofacial stimulation. The number of articles assessing each nutraceutical varied from one (palmitoylethanolamide) to twelve (polyphenol). Methodologically, different

types of models with different pain assessments were conducted. Eighteen articles assessed the effects of nutraceuticals on acute facial pain-like behaviors evoked by formalin (twelve articles), capsaicin (two articles), or several agents (four articles). Twenty articles evaluated the effects of nutraceuticals on persistent facial pain-like behaviors in inflammatory pain models, such as CFA (ten articles), zymosan (four articles), and others. These variations in the assessment of each nutraceutical's efficacy make it challenging to precisely analyze their beneficial effects on pain, including determining appropriate doses, sample sizes, treatment durations, and differences in efficacy between nutraceuticals. However, all articles revealed the inhibitory roles of nutraceuticals on craniofacial nociception.

4. Discussion

4.1. Characteristics of Dietary Habits and Nutritional Intakes in Painful TMDs

Recent research sheds light on the promising role of nutritional strategies in managing chronic pain conditions [87–90]. These approaches not only present a potentially cost-effective alternative to conventional medical treatments but also demonstrate a safer profile [91]. Moreover, they align with a patient-centered approach to treating chronic pain [8,92]. Emerging evidence suggests that specific dietary patterns exhibit beneficial effects on health promotion. For example, with a notable emphasis on a plant-based diet [26], recognized for its preventive and therapeutic impacts on diverse chronic diseases such as diabetes [93], cardiovascular diseases [94], and depression [95], the benefits extend to chronic pain as well. Notably, Mediterranean, vegetarian, vegan, or high-protein diets have shown efficacy in alleviating chronic pain associated with fibromyalgia [88,96], rheumatoid arthritis [97], musculoskeletal pain [98,99], low back pain [16], and headaches [100,101].

On the other hand, compared to the aforementioned chronic pain, the impact of dietary habits and regimens on TMDs, particularly the alleviating effects on painful TMDs, remains less clear. Before considering dietary and nutritional approaches for patients with TMDs, evaluating their nutritional status and habits is crucial. Furthermore, neurobiologically, the concepts discussed above, except headache, are based on chronic pain related to spinal pain mechanisms, while there remains uncertainty regarding their applicability to painful TMDs. Evidence indicates that TMDs involving trigeminal pain systems regulating deep craniofacial nociception exhibit distinct neural nociceptive features compared to spinal pain systems [1,33,102]. Thus, there is a need to assess the current knowledge of the nutritional roles in managing painful TMDs. In light of our above discussion, it is crucial to emphasize the distinctive attributes of TMDs, particularly their correlation with masticatory dysfunction and chronic pain in the deep craniofacial tissues. These factors exert a more pronounced influence on eating behaviors in TMD patients experiencing chronic pain compared to individuals with chronic pain in areas outside the orofacial region [103,104]. As a result, changes in dietary patterns, coupled with potential nutrient deficiencies, could lead to notable changes in trigeminal nociceptive mechanisms, potentially affecting the management of painful TMDs. Preclinical studies provide supporting evidence for this notion. For example, trigeminal inputs associated with chewing hard foods, as opposed to soft foods, have been implicated in reducing hindpaw nociception [105]. Furthermore, masticatory function might play inhibitory roles in nociception by activating descending pain controls [106], while dysfunctions in those controls are well documented in TMD patients [1,33]. This suggests that modifications in dietary behaviors, leading to changes in trigeminal systems, could induce functional alterations in the central nervous system beyond the trigeminal systems to some extent. Furthermore, these findings underscore the unique characteristics that differentiate the underlying mechanisms of TMDs from those of other pain conditions, particularly in terms of their nutritional implications. Hence, a possible link exists between painful TMDs and dietary modifications; however, they do not definitively establish a causal relationship. In other words, these observations could elucidate how TMDs influence dietary habits and masticatory function, yet they do not address the impact of specific dietary regimens or their constituents on TMD pain.

It is possible that alterations in dietary patterns and nutritional intake, potentially leading to deficiencies of specific nutrients, could contribute to the onset or aggravation of TMDs [32]. TMD patients often adapt their diets by cutting food into smaller pieces [103], and those experiencing chronic masticatory pain consume raw whole fruit less frequently [107]. Studies using the Test of Masticating and Swallowing Solids (TOMASS) reveal that TMD patients take longer to consume certain foods [108]. Reports indicate that TMD patients might eliminate specific foods like meat, apples, and bread, favoring softer cooking methods for vegetables and meat [109,110], potentially leading to deficiencies in certain nutrients. Furthermore, myogenic TMDs could be linked to reduced dietary fiber intake, which is prominently found in plant-based foods [111]. On the other hand, a recent study demonstrated that the macro-nutritional status—such as levels of energy, protein, carbohydrate, and fat—of individuals with TMDs is similar to that of healthy individuals, although this study evaluated only the amount of macronutrients using 24 h dietary recall methods [108]. While at this stage it is difficult to draw strong statements about the differences in dietary habits and nutritional status in daily life with painful TMDs, these findings suggest that micronutrients rather than macronutrients might be essential for regulating deep craniofacial nociception. The following section elucidates the existing knowledge regarding the impact of several nutrients on painful TMDs. It also examines preclinical evidence from animal models of deep craniofacial pain [33].

4.2. Nutraceutical, Nutrition, and Painful TMDs

Nutraceutical, a term commonly used, has attracted considerable interest among individuals aiming to self-manage their chronic pain using readily accessible supplements [27,112]. Several analogous nutraceutical terms are found, such as functional foods, nutritional, and dietary supplements. Currently, the definition of nutraceutical varies between researchers and even countries. Therefore, the distinction between these terms is beyond the scope of this article. Recent reports, including meta-analyses, have emphasized various nutraceuticals emerging as viable options for managing a wide range of chronic pain conditions, such as osteoarthritis [113], neuropathy [114], fibromyalgia [115], irritable bowel syndrome [116], and headache [117]. On the other hand, our literature search reveals less comprehensive elucidation on the roles of nutraceutical interventions in TMDs. As conclusive statements to draw the efficacy of each nutraceutical are premature due to limited evidence, we discuss articles that explore the role of nutraceuticals in chronic pain conditions, including TMDs, in the following sections.

4.2.1. Vitamins

Vitamins are a group of organic compounds such as four fat-soluble vitamins (A, D, E, K) and nine water-soluble vitamins (B and C). Multiple clinical studies have revealed serum levels of specific vitamins and their supplementary impacts on chronic TMD pain. Our literature search found two clinical and two preclinical articles evaluating the direct effects of vitamins D and B12 on painful TMDs and craniofacial pain-like responses in preclinical models. Indeed, other studies indicated a significant possibility of the influence of vitamin D on TMDs. This focus is likely derived from the prevalence of articles that explore the correlation between vitamin D and bone metabolism in the TMJ but not pain, which is beyond our aims. Such findings are particularly relevant in the context of painful conditions like osteoarthritis [118–123]. Subsequently, we discussed the current knowledge of reports that assessed the roles of other vitamins, including vitamin B complex and vitamin C [124].

1. Vitamin D

Vitamin D deficiency has been implicated in the development of musculoskeletal disorders, leading to reduced bone density, muscle weakness, and chronic pain [125,126]. Several articles have suggested a potential association between vitamin D deficiency and an increased risk of developing painful TMDs [118,122]. However, research findings on its serum levels in TMD patients have been inconsistent. Some studies have demonstrated

decreased levels [37,119,120], while others have found less significant change [121,127,128]. The reasons for these discrepancies are unclear; however, decreased levels of vitamin D have been observed in various types of TMDs, including intra-articular disorders [37,121] and extra-articular disorders such as myogenic TMDs [127]. These findings could be consistent with observations of vitamin D deficiency in other conditions, such as fibromyalgia [125] and osteoarthritis [129]. Furthermore, polymorphisms in the vitamin D receptor have been linked to disc degeneration-related pathologies, such as osteoarthritis [130]. However, their susceptibility to TMJ internal derangement, TMJ osteoarthritis, and pain remains uncertain [131]. These findings suggest that the development of painful TMDs associated with vitamin D is primarily attributed to the level of vitamin D itself rather than the processing of its ligand reception at least. Emerging reports have demonstrated the beneficial effects of vitamin D supplementation on various types of chronic pain. However, the findings have been inconsistent. For example, a previous cohort study and a recent systematic review have demonstrated that vitamin D supplementation significantly relieves chronic pain in conditions such as rheumatoid arthritis and fibromyalgia [132–134]. Conversely, other randomized controlled trials have found minimal effects of vitamin D supplementation on musculoskeletal pain [135]. Despite such inconsistencies, Lombardo et al. suggested that the potential benefits of vitamin D supplementation in alleviating chronic musculoskeletal pain and fibromyalgia might be observed in individuals deficient in vitamin D [134]. Consistent with the reports above, Kui et al. [122] indicated that patients suffering from TMDs with deficient levels of vitamin D are likely to benefit from vitamin D supplementation. A randomized controlled trial demonstrated that the beneficial effects of splint therapy combined with vitamin D supplementation on painful TMDs were evident in patients with lower serum levels of vitamin D [37].

2. Vitamin B complex and C

Our literature search revealed that one clinical and two preclinical articles investigated the influences of vitamin B on painful TMD and craniofacial nociception. No clinical and preclinical evidence evaluating the effects of Vitamin C on painful TMDs is found. Vitamin B complex (Bs) encompasses a group of eight essential water-soluble vitamins: B1 (thiamine), B2 (riboflavin), B3 (niacin), B5 (pantothenic acid), B6 (pyridoxine), B7 (biotin), B9 (folate), and B12 (cobalamin). We found the reports elucidating on the role of vitamin Bs, except vitamins B1, 3, 5, and 7, in nociception in clinical and preclinical settings of TMDs because those of other vitamin B have not been identified in our literature search. In the context of TMDs, clinical studies have shown variations in serum levels of these vitamins among patients. One study [123] found that TMD patients commonly exhibit deficiency in vitamin B12, while vitamin B9 levels remain unaffected. Conversely, another study [124] observed that TMD patients with a history of implant surgery showed deficiencies in vitamins B1, B6, and B12, as well as in vitamin C. However, those studies did not determine the association of chronic pain with the levels of each vitamin. Regarding other chronic pain conditions, a study reported a significant association between myofascial pain syndrome and reduced serum levels of vitamin B12, while levels of vitamin B9 remained unaffected [136]. Notably, the study also revealed a negative correlation between vitamin B12 levels and psychological distress, which plays a crucial role in exacerbating chronic pain [136]. These findings indicate the potential impact of vitamin B12 on myofascial pain syndrome and psychological distress. Additionally, in the cases of trigeminal pain systems, riboflavin (vitamin B2) and vitamin B6 have shown efficacy in preventing migraine headaches [137–139], and vitamins B6, B9 and B12 have been closely linked to secondary burning mouth syndrome [140]. Further, the web-based dietary search revealed that low levels of vitamin B12 intake might be associated with non-specific chronic pain [141]. Interestingly, while individuals are aware that vitamin B12 deficiency correlates with lower consumption, relying solely on meat is insufficient to prevent such deficiency [141]. It is well known that vitamins B6 and B12 are primarily obtained naturally through the consumption of various animal products, including meat, poultry, fish, and eggs, while

vitamin B9 is found in dark green leafy vegetables (such as spinach and kale), legumes (like lentils and beans), citrus fruits and juices, avocado, and nuts.

Clinical research highlights the association between deficiencies in vitamin Bs and the development or alleviation of chronic pain conditions, particularly neuropathic pain [114,142–145]. Karaganis et al. commented that while most beneficial outcomes were reported against baseline measures, few positive comparisons were made against the placebo group [144]. Further, there is a report suggesting that vitamin B12 might not have beneficial effects on neuropathic pain [146], while methylcobalamin, the activated form of vitamin B12, could alleviate diabetic neuropathy, low back pain, and neuralgia [147]. The existing clinical literature concerning vitamin B supplementation's efficacy in managing painful TMDs is somewhat scarce. However, one study has indicated that the use of vitamin B12 supplementation might enhance the inhibitory effects of photobiomodulation techniques, such as laser therapy and acupuncture, despite the study's small sample size [38]. Consequently, preclinical studies could provide valuable insights into the potential contributions of vitamin B. One research group has delved into the effects of vitamin B12 on orofacial nociception through the orofacial formalin test in two separate reports [58,59]. In the first study, systemic administration of vitamin B12 demonstrated inhibition of orofacial formalin-induced nocifensive behaviors [58]. Notably, vitamin B12 exhibited enhanced antinociceptive effects when administered systemically alongside diclofenac. In the second study, intra-hippocampal administration of vitamin B12 also reduced orofacial nocifensive behaviors, potentially mediated by its involvement in opioid mechanisms [59]. Furthermore, despite the use of different orofacial pain models, consistent results have been observed regarding the beneficial effects of vitamin B, containing B1, B6, and B12, in alleviating the impacts on orofacial nocifensive behaviors in rats with chronic constriction injury of the infraorbital nerve [148].

Vitamin C, also known as ascorbic acid, is also a water-soluble vitamin that cannot be synthesized in the human body and is found prominently in fruits and vegetables. Clinical studies have demonstrated the association between vitamin C deficiency and chronic spinal pain conditions, including musculoskeletal pain, cancer-related pain, and orthopedic post-surgical pain [149]. In the case of TMDs, our literature search has not yielded reports demonstrating the impact of altered vitamin C levels on painful TMDs. This absence of findings could be interpreted in light of several observations. Firstly, vitamin C deficiency has been noted in elderly hospitalized patients, with trauma and surgery known to cause a significant reduction in serum vitamin C levels [150]. It is widely acknowledged that in many cases of TMDs, extensive surgery or obvious trauma is not apparent. Mehra et al. [124] reported vitamin C deficiency in TMD patients; however, these patients were not typical cases of TMDs, as they underwent implant surgery. Secondly, a retrospective study has revealed that a significant deficiency of serum vitamin C level was more likely to be seen in males than females [151], whereas TMD patients are well documented to be predominantly female [152]. Thirdly, although deficiency of vitamin C level (<11 μmol/L) is relatively rare in developed countries like the United States (6%) [153,154], it is unclear if this is the case for painful TMDs. As aforementioned, specific features of TMDs, like masticatory dysfunction, might hinder the intake of a vitamin C-rich diet, such as fruits and vegetables [107,109,110]. At present, the effects of vitamin C supplementation on TMDs remain unknown. However, there is substantial evidence suggesting that vitamin C effectively inhibits the response of various proinflammatory biomarkers, including interleukins and tumor necrosis factor, as well as counteracting oxidative stress conditions [29,149,155,156]. These factors are thought to be potentially associated with TMD pathology. Furthermore, vitamins C and D are also documented to have antioxidant and anti-inflammatory properties, capable of reducing reactive oxygen species production and inflammation [29]. Additionally, in cases of pain related to the trigeminal nervous system, supplementation with vitamin C has demonstrated efficacy in alleviating other forms of orofacial pain, such as pain induced by the third molar extraction [157].

4.2.2. Minerals

The relationship between minerals and chronic pain varies depending on the specific minerals and types of chronic pain conditions involved. Several studies have examined these complexities within TMDs [119,123,124,128]. These investigations have primarily focused on serum levels of minerals such as magnesium (Mg), zinc (Zn), and calcium (Ca), yet their findings have shown inconsistencies. For instance, TMDs characterized by TMJ disk displacement with reduction exhibited similar levels of Mg compared to healthy controls [119,128], while TMDs following implant surgery displayed lower Mg levels than controls [124]. Similarly, while some studies reported lower levels of Ca in certain TMD cases [119], others found them comparable to healthy controls [128]. However, these studies overlooked myogenic types of TMDs, potentially limiting the scope of their conclusions. Additionally, they did not evaluate the correlation between these minerals and painful TMDs, further restricting the comprehensiveness of their findings. Furthermore, a cross-sectional study revealed that patients with TMDs displayed significantly lower serum levels of potassium despite being within the normal range compared to healthy controls. The association of potassium levels with painful TMDs remains unclear [123]. Potassium, primarily sourced from fruits and vegetables, might be challenging for TMD patients to consume, potentially correlating with TMD development. Despite limited evidence, several reports suggest that changes in mineral levels, possibly due to dietary customs and behaviors, might play a role in TMDs. These studies further highlight the potential roles of mineral supplementation in the treatment of painful TMDs. Our literature search based on one clinical and four preclinical articles demonstrated the inhibitory impacts of painful TMDs and craniofacial nociception; however, this current understanding might not allow us to provide strong evidence of the treatment efficacy of mineral interventions. In the following section, we discuss the involvement of magnesium, zinc, strontium, and sulfur in TMDs and craniofacial pain conditions, as several reports support the hypothesis of their relationship.

1. Magnesium (Mg)

Mg, as a known calcium channel blocker, has garnered significant attention for its potential to alleviate various neurological diseases, including chronic pain [158]. The Western-type diet contains less Mg, whereas green vegetables such as spinach are major sources of this mineral [159]. Studies indicate that Mg plays a protective role against excessive neural excitation, which can lead to excitotoxicity [159]. Accordingly, evidence indicates the alleviating effects of magnesium on conditions such as migraine [160], fibromyalgia [158], low back pain [161], and visceral pain [89]. Local administration of Mg sulfate could prevent spinal sensitization, as indicated by increased glial activation in a rat model of incisional pain [162].

Studies assessing the impact of mineral interventions on TMDs are limited; however, several reports suggested that dietary magnesium might play a role in painful TMDs. A randomized clinical study demonstrated that local injection of Mg is an effective treatment for myofascial trigger points of the masseter muscle, leading to pain reduction and improvements of maximum mouth opening [44]. Likewise, a preclinical study revealed that oral administration of Mg prevented the development of orofacial pain-like behaviors in TMJ-inflamed rats, mimicking the conditions of TMDs, and further, treatment with Mg resulted in a decrease in phosphorylation of NR1 protein, one of the subunits of NMDA receptors [60]. Of note, changes in neural mechanisms related to NMDA receptor-mediated nociceptive processing could be essential to increase deep craniofacial nociception [163,164]. Similarly, two preclinical studies demonstrated that systemic administration of Mg reduced formalin-evoked facial pain-like behaviors [61,62]. Conversely, Mg deficiency caused increases in spontaneous facial pain-like behaviors in TMJ arthritis rats [60].

2. Zinc (Zn)

Zn has been investigated for its potential in treating various chronic pain conditions and could reduce nociceptive responses in preclinical models, possibly due to its anti-

inflammatory properties [114]. In cases of orofacial pain, Zn supplementation therapy could alleviate the burning sensation associated with Burning Mouth Syndrome (BMS) patients [165]. However, it is noteworthy that over 94% of examined BMS patients exhibited normal serum Zn levels [166]. Additionally, studies indicate that administering Zn did not ameliorate symptoms such as oral burning sensation and discomfort in patients with oral lichen planus [167]. Patients with chronic myofascial pain displayed normal and lower levels of Zn in blood serum and intracellular stores (in erythrocytes), respectively [168]. This discrepancy in Zn levels suggests a potential link between Zn metabolism and the development or exacerbation of chronic pain. Our literature search did not yield evidence supporting the supplemental effects of Zn on TMDs. Despite a report with a small sample size (n = 23), Zn deficiencies observed in TMDs suggest that Zn might impact the management of painful TMDs [124].

3. Strontium

Strontium is found naturally in many foods, such as fish, vegetables, grains, and dairy products. Evidence suggested that strontium could exhibit inhibitory impacts on chronic pain conditions, potentially due to the positive effects of bone metabolisms [169,170]. For example, strontium injection can relieve pain in patients with painful bone metastases in a previously irradiated site [171]. While the roles of strontium on painful TMDs are lacking, in preclinical studies using TMD pain, systemic administration of strontium ranelate, a drug usually prescribed to treat osteoporosis, has shown potential in reducing pain-like behaviors. Interestingly, the antinociceptive effects of strontium ranelate observed in the TMJ inflammatory pain model might be attributed to the reduction in inflammatory cytokines, such as TNF-alpha, rather than a decrease in local inflammation [63]. These findings highlight the potential for exploring the effects of strontium in managing craniofacial pain, including TMDs.

4. Sulfur

Sulfur, a mineral found in our bodies and available in our diets, is primarily derived from proteins in various foods [172]. Sulfur sources that could affect chronic diseases include sulfur amino acids, methylsulfonylmethane, sulfur dioxide, and hydrogen sulfide. While the roles of these sulfur compounds in chronic pain are unclear, several reports suggested potential insights. For example, sulfur amino acids like cysteine, as part of protein structures, act as antioxidative metabolic intermediates such as glutathione [172]. Interestingly, therapy using hot springs containing the radioactive gas radon has increased glutathione levels in the brain, providing protective antioxidative functions against brain injury [173]. These findings support the notion that sulfur amino acids might exert antinociceptive effects; however, evidence on pain relief in TMDs is lacking.

Methylsulfonylmethane (MSM) is a notable organic sulfur-containing compound widely used as a dietary supplement for various conditions, such as pain, inflammation, and arthritis [174]. A randomized controlled trial indicated that a 12-week MSM regimen could improve knee quality of life, including pain [175]. However, another randomized controlled trial found no significant difference between MSM supplementation and placebo for knee osteoarthritis pain [176]. These inconsistent findings might be due to differences in pathologies of chronic pain. In the case of TMDs, one report revealed that MSM supplementation could have less beneficial effects on pain in osteoarthritis of the TMJ [39].

4.2.3. Polyunsaturated Fatty Acids

Fatty acids are categorized depending on their length and degree of saturation into saturated fatty acids (FAs), monounsaturated FAs, and polyunsaturated FAs. Polyunsaturated FAs are divided into omega-3 and omega-6 FAs [177]. These FAs are indispensable for human health and show promise as natural remedies for chronic disorders [178–180], which are abundantly found in certain fish, nuts, and seeds.

Research has highlighted the contrasting roles of omega-6 and omega-3 FAs [181]. Notably, omega-3 FAs have been shown to alleviate chronic pain across various conditions,

including musculoskeletal pain [27,98], low back pain [182], osteoarthritis [183], rheumatoid arthritis [184], and headaches [185]. Moreover, several studies have investigated the potential causal relationship between omega-3 FAs and chronic pain, revealing that increased circulating omega-3 FA levels might reduce the risk of low back pain [186] and pelvic pain [187]. Consistent with those clinical reports, omega-3 FAs exerted antinociceptive effects in various preclinical models [188,189]. Additionally, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), major polyunsaturated FAs, can act as substrates for the synthesis of specialized pro-resolving lipid mediators (SPMs) like resolvins, protectins, and maresins [190]. While these SPMs play a pivotal role in resolving inflammation by facilitating the clearance of inflammatory cells and debris, resolvin D1 could decrease hyperexcitability of nociceptive neurons in the trigeminal subnucleus caudalis in the facial inflammation rats [191].

In contrast, omega-6 FAs, enriched in the Western-style diet, are often regarded as detrimental to health promotion due to their potential to elevate levels of various proinflammatory substances such as prostaglandins, inflammatory cytokines, and inflammatory eicosanoids [192]. A randomized clinical trial with a diet low in omega-6 FAs and high in omega-3 FAs displayed decreased pain frequency and intensity in chronic headache patients [193]. A cross-sectional study found that a lower omega-6 to omega-3 FA ratio is linked to clinical and experimental pain responses in knee pain [194]. Recently, Sanders et al. suggested that omega-6 FAs could promote a generalized upregulation of nociceptive processing. They showed that omega-6 FAs were negatively associated with lower mechanical nociceptive thresholds in nociplastic pain conditions [182]. Preclinical studies supported the pronociceptive roles of nociception. Boyd et al. demonstrated that omega-6 FA intakes could induce persistent mechanical and thermal nociceptive hypersensitivity in the hindpaw due to neural changes in primary afferent nerves associated with elevated phospholipase A2-mediated lipid release. Of interest, pronociceptive changes could be rescued by intakes of omega-3 FAs [195].

Consequently, this article will delve into the influence of omega-3 FAs on painful TMDs. The physiological mechanisms of omega-3 FAs have been intensively investigated from a broad perspective, and their safety has been reported [177]. Currently, there is limited research exploring the roles of omega-3 FAs on TMDs; however, two cross-sectional studies have suggested an association between a higher ratio of omega-6 to omega-3 FAs and painful TMDs [196]. Similarly, a higher level of circulating omega-3 FAs has been linked to a reduced likelihood of painful TMDs [197]. Further, considering that psychological distress is a risk factor for TMDs, the preventive effects of omega-3 FAs on depression may support the idea of their inhibitory role in TMDs [178].

Preclinical studies have provided interesting evidence supporting the effectiveness of omega-3 FAs in managing TMJ inflammation. For example, Marana et al. [49] demonstrated that systemic administration of omega-3 FAs led to a decrease in TMJ damage and reduced levels of proinflammatory cytokines, such as IL-1 beta and TNF-alpha, in rats subjected to ovariectomy and rheumatoid arthritis induction. Similarly, systemic administration of omega-3 FAs exhibited anti-inflammatory effects, as evidenced by decreased levels of various proinflammatory cytokines in facial inflammatory models [64,65]. These findings highlight the potential of omega-3 FAs to inhibit local inflammation within the TMJ.

On the other hand, regarding the clinical features of TMDs, it is important to note that TMDs often present with fewer obvious inflammatory signs in craniofacial tissues. In this context, the potential of omega-3 FAs to alleviate craniofacial pain in TMDs might be mediated by mechanisms within the central rather than the peripheral nervous system. This idea finds support in several studies. For example, preclinical studies have shown that omega-3 FA deficiencies can induce emotional and neural disturbances similar to those observed after social defeat stress conditioning [198]. Consistently, supplementation with omega-3 FAs has been documented to have antidepressant effects [199,200]. Furthermore, a neuropathic pain model demonstrated hyperalgesia-like responses alongside decreases in DHA levels, while central administration of DHA decreased central microglia-associated

neuroinflammation and pain-like behaviors [201]. In an intermittent cold stress model, omega-3 FAs exhibited modulatory effects on neuroinflammation related to transient receptor potential V1 signaling in the prefrontal cortex, hippocampus, and periaqueductal gray [202]. Finally, in the case of TMD models, systemic administration of DHA exerted inhibitory effects on neural activities in the trigeminal subnucleus caudalis in the persistent facial inflammatory pain rat [66].

While neural function heavily depends on adequate omega-3 FA levels, the precise mechanisms by which omega-3 fatty acids exert inhibitory effects on nociception remain unclear. Several findings might partially explain them [200,203]. For example, the facilitative effects of EPA and DHA on microglial autophagy might reduce inflammatory processes [204,205], leading to decreased nociceptive responses in the brain. Despite variations in chronic pain conditions, omega-3 FAs could play beneficial roles in alleviating deep craniofacial nociception. Further clinical and preclinical investigations into their effects on TMDs are warranted.

4.2.4. Polyphenols

Polyphenols are natural compounds with phenolic structures found in various foods, such as fruits. Clinical and preclinical studies have evaluated the therapeutic roles of polyphenols on chronic pain. Accordingly, randomized controlled trials have shown that several fruits, such as blueberries [206], passionfruit [207], and strawberries [208], could offer relief for musculoskeletal pain, likely due to the antioxidative effects of various polyphenols that they contain. A meta-analysis demonstrated the beneficial roles of these for fibromyalgia patients [209]. Similarly, consuming fruits and vegetables has been linked to reduced odds of headaches in university students [210]. Polyphenol-rich foods have also been shown to reduce chronic pain in women with fibromyalgia [211]. Further, evidence has revealed that various polyphenols play beneficial roles in improving rheumatoid arthritis [184], irritable bowel syndrome [89], and migraine headaches [117].

While our literature search yields fewer randomized controlled studies assessing the effects of polyphenols on painful TMDs, a report not cited in PubMed has elucidated their beneficial roles in TMDs [212]. Moreover, one randomized control trial revealed the beneficial roles of avocado–soybean unsaponifiable extract that could contain polyphenols [213]. Additionally, several studies have shown that TMD patients experience alterations in oxidative status within the TMJ disc [156,214,215]. These findings suggest that targeting oxidative conditions with polyphenols could help reduce painful TMDs. Despite the limited clinical evidence, accumulating studies emphasize the effects of polyphenols on pain-like behaviors and their neural mechanisms in preclinical models of TMDs. For example, extracts rich in polyphenols from grape seeds [216] and purple corn [217] have shown promise in reducing neuronal and glial responses in critical areas associated with craniofacial nociception. The latter study highlighted the polyphenol content in purple corn extracts [217]. Similarly, citrus fruits [71,72] and cocoa [218,219], which also contain polyphenols, have demonstrated potential antinociceptive properties in facial pain models.

A randomized experimental clinical study revealed that intake of chocolate, a cocoa-derived product rich in polyphenols, could reduce pain sensation caused by the injection of hypertonic saline into the masseter muscle in healthy individuals; however, the authors did not specifically assess the role of polyphenols in the observed reduction in pain responses [220]. An in vitro study demonstrated that cocoa bean extracts containing polyphenols could repress the release of calcitonin gene-related peptides in the trigeminal root ganglion [221], potentially regulating craniofacial nociception. Consistently, repeated administration of the coffee polyphenolic extract (CE) modulated reflexive pain responses, depressive-like behavior, and spinal cord gliosis in a dose-dependent manner in a model of fibromyalgia [222]. These effects are believed to arise from the polyphenols, which impact neural changes associated with orofacial nociceptive processing [29]. In the following paragraph, we demonstrate several preclinical examples of the roles of polyphenols, including

quercetin, (-)-epigallocatechin-3-gallate, resveratrol, and curcumin on facial pain because of the available evidence regarding polyphenols.

Local administration of quercetin, one of the flavonoid phytochemicals, exerted inhibitory effects on nociceptive neural activities evoked by facial stimulation in trigeminal primary sensory neurons in the presence [51] and absence of persistent facial inflammatory conditions [52]. Further, (-)-epigallocatechin-3-gallate, the main catechin contained in green tea, could inhibit nociceptive neural activities in the trigeminal subnucleus caudalis region [55]. Likewise, daily administration of resveratrol contained abundantly in grapes and wine exerted inhibitory impacts on the trigeminal nociception in response to facial stimulation [56,223]. Notably, Ma et al. illustrated that the development of TMJ inflammation, accompanied by increased neural activities in the trigeminal subnucleus caudalis region due to microbiome perturbation, could be mitigated by systemic administration of resveratrol [67]. These findings suggest that the involvement of polyphenols in gut microbiota could have beneficial roles in alleviating deep craniofacial pain. Moreover, curcumin, a naturally occurring polyphenolic compound found in turmeric, exhibits antioxidative and anti-inflammatory properties. Systemic administration of curcumin inhibited pain-like behaviors in the hindpaw [224], trigeminal neuralgia model [225], and orofacial formalin pain model [50]. An in vitro study revealed that curcumin can inhibit key molecular processes in TMJ inflammatory chondrocytes [226], suggesting the antinociceptive roles of curcumin [227].

Collectively, while twelve preclinical studies suggest promising therapeutic roles for polyphenols in managing painful TMDs, further longitudinal and randomized controlled trials are needed to confirm their potential benefits, and the clinical efficacy of polyphenols is unclear.

4.2.5. Isoprenoids

Isoprenoids are found in various common foods such as fruits, vegetables, nuts, and seeds. They are a class of natural compounds, including terpenes, that could exert antioxidative potential and help in pain relief [228]. Although clinical evidence regarding the roles of isoprenoids in TMDs is lacking, preclinical evidence supports their antinociceptive properties. For example, limonene, a monoterpene found in citrus fruits like lemons and oranges, has been shown to inhibit TMJ nociceptive behavior and alter neural responses in the trigeminal subnucleus caudalis [71]. Similarly, limonene's inhibitory effects on nociception have been observed in models of fibromyalgia [229] and neuropathic pain [230]. Furthermore, systemic administration of citral, another monoterpene, has been found to attenuate masseter muscle pain-like behaviors induced by various algesic and inflammatory agents through the regulation of several transient receptor potential channels [73]. Prophylactic and therapeutic administration of citral also inhibited mechanical and heat hypersensitivity of the facial skin in rats with persistent TMJ inflammation [72]. Those findings from craniofacial pain models are consistent with those seen in hindpaw inflammatory, neuropathic, and plantar incisional pain models [231]. Additionally, carvacrol, another type of isoprenoid, has exerted antinociceptive effects in craniofacial pain models [232]. However, that isoprenoid was derived from aromatic and medicinal plants, not from ordinary foods. Based on three preclinical studies and the absence of clinical evidence, it is still too early to make definitive conclusions. However, it is worth considering that isoprenoids might have therapeutic potential in treating painful TMDs. Consequently, the efficacy of isoprenoids for managing painful TMDs remains uncertain.

4.2.6. Carotenoids

Carotenoids are naturally occurring pigments synthesized by plants like carrots, sweet potatoes, avocados, and algae [233]. More than 1000 natural carotenoids have been documented, and only 40–50 of them are consumed in human diets, such as lutein and beta-carotene. They are responsible for the bright red, orange, and yellow colors of many fruits and vegetables and exert critical roles in human health as antioxidant

properties by inhibiting reactive oxygen reactions and sources of vitamin A [234]. Evidence revealed that carotenoids could offer protection against local inflammation associated with rheumatoid arthritis [90]. For example, two preclinical studies have demonstrated the analgesic effects of crocin, a compound found in saffron and gardenia flowers, often used as a flavor. In one study, intra-cerebroventricular administration of crocin reduced pain-like behaviors in sleep-deprived rats [235]. In a relevant context to craniofacial pain, crocin inhibited facial pain-like behaviors triggered by formalin injection in rats [74]. Lutein is the most widespread carotenoid found in fruits and leafy vegetables. Preclinical reports demonstrated that lutein administration could exert inhibitory effects on various pain conditions. For example, lutein could potentially promote antioxidative, antidepressant, and antinociceptive effects in the preclinical models of fibromyalgia [236]. Regarding the conditions of facial pain, two reports revealed that lutein decreased nociceptive neural activities indicated by c-Fos expressions [75] and neural activities [76] in the medullary and upper cervical dorsal horn junction region. Further clinical and preclinical investigations would be needed to confirm and expand upon these findings regarding the potential roles of carotenoids on painful TMDs. Thus, the efficacy of carotenoids on painful TMDs is unclear.

4.2.7. Lectin

Lectins are a heterogeneous group of proteins found in both plants and animals that can bind to carbohydrate molecules. It is well known that seaweed and beans are major sources of lectins, which exhibit antinociceptive and anti-inflammatory properties [237,238]. The biological activities of lectins might be related to their ability to recognize carbohydrates on the surface of cell membranes and trigger intracellular signaling that results in biological responses, including nociceptive processing. Clinical evaluations of the therapeutic roles of lectins in painful TMDs are lacking, yet preclinical studies have shown that lectins derived from seaweed [77,78] and seeds [79–82] could reduce facial nociception and inflammation in craniofacial tissues by regulating molecular mechanisms in TMD models. While the involvement of lectins in nociception is not fully understood, evidence suggests that plant lectins can modulate transient receptor potential vanilloid one function, leading to a reduction in formalin-evoked facial pain-like behaviors [78]. Additionally, the inhibitory effects of lectins on TMJ pain-like behaviors and inflammation could be due to decreased levels of proinflammatory cytokines, such as interleukin-1 beta, in TMJ tissues and the trigeminal ganglion [81]. Because no clinical evidence is available, further clinical and preclinical evaluations are necessary.

4.2.8. Polysaccharide

Polysaccharides are naturally derived from plants and are macromolecular carbohydrates composed of monosaccharides linked by glycosidic bonds. Currently, various types of them have been identified, which can exert a broad range of biological activities [239]. Indeed, sulfated polysaccharides exhibit diverse biological activities, including antioxidant, neuroprotective, and anti-inflammatory effects [228,240]. These findings could align with the notions of their antinociceptive properties. Although evidence for their effectiveness in treating painful TMDs is currently lacking, preclinical studies have indicated the impacts of sulfated polysaccharides on craniofacial pain-like responses. For example, systemic administration of sulfated polysaccharides derived from red seaweed has been shown to reduce TMJ-evoked pain-like behaviors and enhance opioid function, as evidenced by increased levels of endorphins in the trigeminal subnucleus caudalis [83]. The reduction in TMJ formalin-evoked pain-like behaviors by polysaccharides derived from marine red algae is mediated through the modulatory effects of several receptor mechanisms, such as glutamatergic receptors [84]. A report, not cited in PubMed, revealed that sulfated polysaccharides derived from green seaweed could reduce mechanical hyper-nociception in the facial region 4 h after TMJ inflammation evoked by zymosan [85]. Despite a few preclinical reports, further human and preclinical studies are expected to assess the roles of

polysaccharide-based treatments on craniofacial pain conditions. Additionally, polysaccharides derived from various plants have shown promise in preclinical studies for improving mental disorders such as anxiety and depression [239], which are risk factors for painful TMDs, thus supporting further exploration of their therapeutic potential. Collectively, it is too early to describe the definitive efficacy of polysaccharides on TMDs.

4.2.9. Glucosamine

Glucosamine is an amino sugar and a prominent precursor in the biochemical synthesis of glycosylated proteins and lipids [241,242]. While glucosamine is an amino monosaccharide naturally biosynthesized in the human body, it is commonly extracted from the exoskeletons of crustaceans such as shrimp and crabs, as well as from animal cartilage and bone broth. It attaches to protein cores to form proteoglycans, which are essential components of the extracellular matrix in articular cartilage. The therapeutic potential of glucosamine in treating osteoarthritis has been well documented [243].

Indeed, as mentioned earlier, while randomized controlled trials assessing the effects of various nutraceuticals on painful TMDs have been scarce, this might not be the case for glucosamine. Several review articles have demonstrated that certain types of TMDs, particularly those involving degenerative joint diseases and pain, benefit from glucosamine administration [241,242,244]. The clinical efficacy of glucosamine in reducing pain associated with osteoarthritis of the TMJ appears to depend on the duration of administration, with benefits observed after more than 1–3 months of use [40–43,45,46]. However, a randomized clinical trial reported that glucosamine had a less beneficial effect on TMJ osteoarthritis pain compared to placebo controls [47].

Given the high prevalence of females with painful TMDs, a preclinical study using female rats might provide insights into the role of glucosamine in craniofacial pain; however, this study found that glucosamine caused no alterations in proinflammatory cytokine levels in both sham and ovariectomized females (OVX) [245]. These findings indicated that changes in sex hormones played a lesser role in those. Regardless of these findings, it is important to note that these reports did not focus on the therapeutic roles of glucosamine for myogenic TMDs.

Additionally, despite the unclear benefits of glucosamine alone, chicken bone broth, which contains glucosamine, exerted inhibitory effects on pain-like behaviors evoked by facial skin stimulation in a TMD model developed through prolonged jaw opening in rats [246]. These reports supported the notion that glucosamine might alleviate chronic pain in certain types of TMDs. Collectively, the efficacy of glucosamine on TMDs might be present in specific types of this condition. However, further clinical and preclinical evaluations and studies on other types of TMDs, like myogenic pain conditions, are necessary.

4.2.10. Palmitoylethanolamide

Palmitoylethanolamide (PEA) is a naturally occurring fatty acid amide found in various foods, such as tomatoes, soybeans, eggs, and broccoli. It is also synthesized in the body, including the brain, within the lipid bilayer [247]. Due to its antinociceptive, anti-inflammatory, and antioxidative effects [248], PEA has documented benefits in managing various chronic pain conditions. Various molecular mechanisms for its neural involvement have been demonstrated [249]. A randomized controlled trial revealed that the inhibitory effects of daily PEA administration on craniofacial pain, indicated by the maximum mouth opening, appeared to be greater than those of ibuprofen [48]. Additionally, PEA administration could reduce mechanical behavioral sensitivity in the facial region and glial activities in the trigeminal ganglion after the induction of inflammation in the TMJ [86]. Similarly, PEA might improve other types of chronic trigeminal pain conditions, including burning mouth syndrome and periodontal disease [247]. In the case of post-tooth extraction trigeminal neuropathy patients, PEA administration alone significantly alleviated pain as measured by a numeric rating scale [250]. Due to clinical and preclinical assessments of PEA's efficacy

seeming too premature, there is a need for more well-designed randomized controlled trials and animal studies to understand the roles of PEA on painful TMDs.

5. Limitations

While our current investigation shown above could allow us to advocate for clinical investigations into the beneficial role of nutraceuticals in managing TMDs, limitations are identified. First, a systematic review was not conducted. Indeed, due to the limited clinical and preclinical evidence available, this narrative review aims to elucidate current understandings of the relationships between nutrition and chronic craniofacial pain conditions rather than evaluating the efficacy of each intervention. Additionally, the literature search was confined to mainly Medline-PubMed and English-language sources, as outlined in the methods section. However, additional articles were retrieved through reference lists to avoid missing relevant studies. Second, our discussion relies heavily on preclinical evidence (38/50 articles), which could weaken the argument for the effectiveness of dietary and nutraceutical approaches from the clinical aspects. Attention should be paid to the preclinical models, as many experiments conducted pain assessments in the acute stages of pain. Therefore, additional approaches are crucial to assess the effects of dietary and nutraceutical interventions on chronic craniofacial nociception using models like psychological stress models [33,251]. Further, most preclinical studies developed TMD models using male rodents, while only two reports employed females [49,50]. Considering the predominance of females in TMDs, the conclusiveness of these statements based on the preclinical evidence was weakened. Despite those limitations, it is noteworthy that various models for painful TMDs have been well documented. These models share key features of painful TMDs, including behavioral characteristics and neural activities in the brain associated with craniofacial nociception, with or without therapeutic interventions [33]. This indicates that various preclinical models for TMDs have high translational value. Third, almost all preclinical studies reported predominantly positive findings, with little evidence of negative results. This might indicate publication bias, which could limit the comprehensiveness of our perspective. Therefore, future research should aim to standardize experimental protocols and improve reporting clarity to facilitate a more accurate and comprehensive evaluation of nutraceutical efficacy in managing craniofacial nociception. Fourth, while several mechanisms, such as the antioxidative and anti-inflammatory properties of nutraceuticals, were mentioned, detailed mechanisms of action at the molecular level and effective doses of each nutraceutical were not discussed. However, as mentioned earlier, these issues were beyond the scope of this investigation. Finally, randomized controlled trials involving dietary or nutraceutical interventions for managing TMDs were less common before 2015 [32]. These notions indicate that this area of research is still in its early stages and is actively developing. These limitations are important considerations for researchers to keep in mind in order to draw strong conclusions about the therapeutic roles of each nutraceutical on painful TMDs.

6. Future Directions: An Alternative Diet—Rice-Fermented Food

Given the significant role of diet and nutrition in managing chronic pain, it is crucial to explore alternative dietary approaches. Recently, there has been increasing interest in the health-promoting effects of various fermented foods [252]. Among these, we would discuss the potential impacts of several rice-fermented foods, such as Sake lees and Rice-*koji*, on chronic pain. Those foods have been shown to contain various nutraceuticals. For example, Rice-*koji* contains vitamins B and E [253]. Another study has shown that it also contains phenolic acids, flavonoids, vitamin B3, and various amino acids such as tryptophan and GABA [254]. Sake lees consist of macro-nutrients like protein, carbohydrates, and fats [255], along with nutraceuticals including vitamins B3 and B6 [256]. Given the presence of these various nutraceuticals in rice-fermented foods, they hold promise for offering novel therapeutic benefits for painful TMDs. Although exhibiting significant geographical diversity [257], rice-fermented foods such as Sake lees and Rice-*koji* encompass a variety

of traditional Japanese culinary delights renowned for their rich flavor and nutritional benefits [253,258]. Figure 1 illustrates the Japanese Sake brewing process, where the primary goal is to produce sake, the popular and traditional alcoholic beverage in Japan, alongside Rice-*koji* and Sake lees as byproducts, which contain various nutraceuticals. These products are bioactive, acting as probiotics that offer antioxidant and anti-inflammatory effects while also regulating various bodily functions [257,259]. Importantly, rice-fermented diets are known to be safe due to the absence of toxicity and adverse effects observed in in vitro and in vivo studies [260,261].

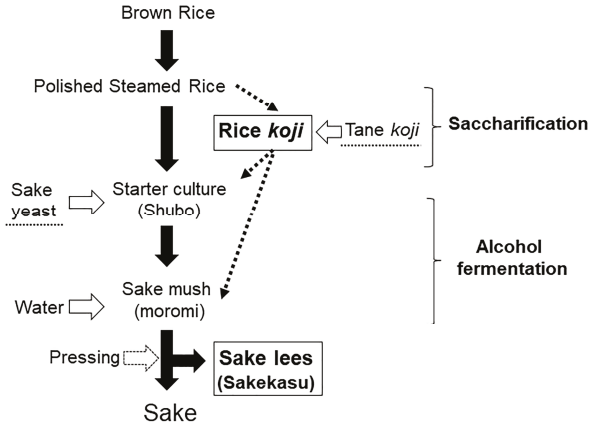


Figure 1. Japanese sake brewing process. Both Rice-koji and Sake Lees are byproducts during the processing.

Our preclinical reports revealed that these rice-fermented foods could have modulatory roles on neural functions associated with nociception. First, daily administration of Sake lees attenuated pain-like hindpaw behaviors with the reduction of neural activities in the lumbar spinal dorsal horn in psychophysical stress rats [262]. Sake lees are a byproduct produced in large quantities during the brewing process of Japanese Sake (Figure 1). However, there are currently few effective methods for utilizing them. If Sake lees, rich in nutrients, could be consumed as functional food, it would reduce waste and align perfectly with the principles of the Sustainable Development Goals. Additionally, we reported an interesting finding [263]. An in vitro experiment demonstrated that Sake lees could induce functional changes in odontoblast-like cells, promoting their mineralization. Furthermore, an in vivo study revealed the facilitatory effects of Sake lees in producing reparative dentin-like hard tissues in the dental pulp of rats [263]. This evidence suggests that Sake lees might have potential applications in treating even toothache. Second, Japanese Sake displayed inhibitory effects on nociceptive neural activities in the trigeminal subnucleus caudalis region [264]. Of note, the results revealed that Sake effects are due to the constituents rather than the 15% ethanol found in Sake [264]. Third, daily consumption of Rice-*koji* prevented anxiety- and pain-like behaviors associated with psychological stress conditionings [260]. This study also revealed that ergothioneine, an antioxidant contained in Rice-*koji*, was well known for its regulatory roles in brain functions associated with pain [260]. While further research is needed to elucidate the precise mechanisms underlying these effects and assess their efficacy in clinical settings, our current studies propose that rice-fermented food could be the additional candidates that benefit from mitigating various chronic pain conditions, including TMDs.

7. Conclusions

Our review suggests that painful TMDs could influence dietary habits, leading to potential deficiencies in nutrients, yet the extent of this impact remains uncertain due to inconsistencies in research findings [108]. The multifactorial nature of TMDs, encompassing various pathologies, complicates efforts to draw definitive conclusions about their nutritional implications. However, given the multifactorial etiologies of TMDs, dietary

improvements and nutraceutical approaches could be reasonable, as the regulatory effects of these interventions might address a wide range of bodily functions through their diverse constituents. This review discussed the roles of several nutraceuticals (Table 1) in chronic pain in the craniofacial tissues associated with TMDs, supported by preclinical evidence. Additionally, various rice-fermented foods might be suggested as supplementary nutritional approaches.

Emerging evidence indicates that supplementation with those nutraceuticals could hold promise in managing painful TMDs, aligning with observations in other chronic pain conditions. However, as mentioned in the section on limitations above, our understanding is limited by the scarcity of evidence from longitudinal and randomized controlled trials. Therefore, further preclinical investigations are required to clarify the therapeutic benefits of specific nutritional interventions to chronic pain in deep craniofacial tissues, which are prominently affected in TMDs.

Finally, a recent review highlighted the need for well-designed clinical trials based on dietary assessments and measurements capable of evaluating food quality and nutrient adequacy to assess the role of nutrition in TMDs [30]. This standpoint aligns with our ongoing inquiries. By advancing our understanding of the interplay between nutrition and craniofacial pain conditions, such as TMDs, through comprehensive research efforts, we can pave the way for personalized therapeutic strategies that enhance the quality of life for individuals affected by these conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu16172868/s1. Table S1: Risk of bias assessment for clinical studies; Table S2: Risk of bias assessment for preclinical studies.

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