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Intervention effects of food-derived polyphenols and bioactive peptides on chronic inflammation

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Intervention effects of food-derived polyphenols and bioactive peptides on chronic inflammation

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Editorial: Intervention effects of food-derived polyphenols and bioactive peptides on chronic inflammation

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Editorial on the Research Topic
Intervention effects of food-derived polyphenols and bioactive peptides on chronic inflammation

Introduction

Chronic inflammation is a pivotal factor in the development of numerous chronic diseases (1), including cardiovascular diseases, neurodegenerative disorders, anemia of inflammation, metabolic syndromes and even cancer (2, 3). Chronic inflammation is even considered the underlying cause of diseases throughout the lifespan (4). The critical link between nutrition and immunity, particularly in the context of chronic illnesses, highlights the importance of diet in controlling chronic inflammation. Micronutrient deficiencies are key drivers of inflammation and are closely associated with increased morbidity and mortality, emphasizing the need for proper nutrient intake to manage inflammatory responses (5). Proper nutritional support, including the intake of anti-inflammatory nutrients, plays a pivotal role in mitigating the effects of chronic inflammation and reducing disease progression. This underscores the importance of exploring dietary interventions that can actively modulate inflammatory pathways and improve health outcomes.

Hence, the growing interest in food-derived polyphenols and bioactive peptides as potential therapeutic agents (Figure 1) for managing chronic inflammation has inspired extensive research into their molecular mechanisms and practical applications (6–8). However, the therapeutic use of polyphenols is often hampered by their poor stability and bioavailability. Nanotechnology-based delivery systems have shown promise in addressing these limitations by improving the stability, bioactivity, bioavailability, and cellular uptake of these compounds (9).

The complex interplay among diet, inflammation, and chronic diseases has emerged as a key focus in nutritional research. This Research Topic, shared across *Frontiers in Nutrition*, *Frontiers in Immunology*, and *Frontiers in Chemistry*—particularly within the *Nutritional Immunology* and *Food Chemistry* sections—addresses the "Intervention

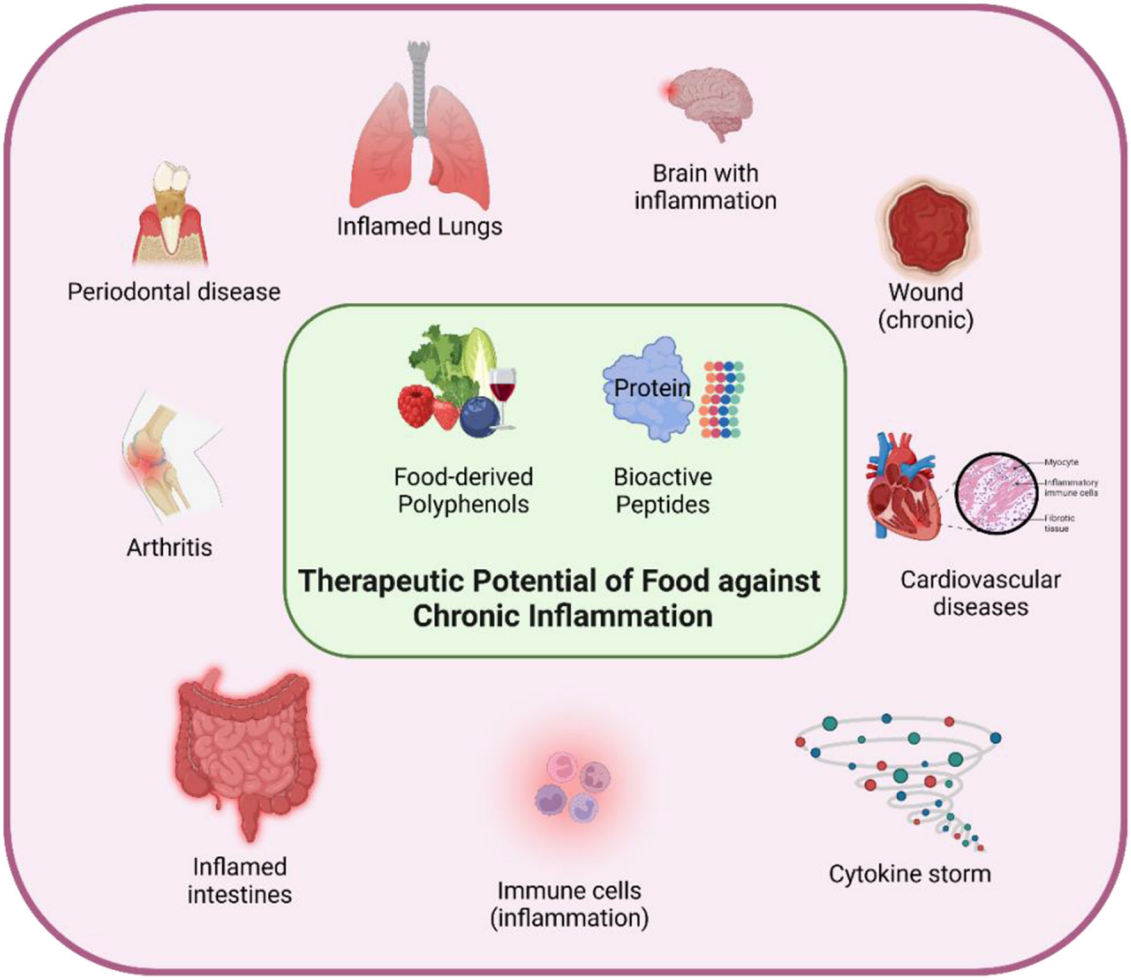

FIGURE 1
The figure illustrates the synergistic potential of food-derived polyphenols and bioactive peptides in mitigating chronic inflammation. The collective manuscripts suggest that incorporating these compounds into the diet could provide a therapeutic strategy for managing chronic inflammatory conditions.

Effects of Food-derived Polyphenols and Bioactive Peptides on Chronic Inflammation." It compiles various studies that investigate how these bioactive compounds can modulate inflammation and enhance health outcomes. The collected manuscripts featured in this Research Topic cover a broad spectrum of topics, ranging from the molecular pathways influenced by specific polyphenols to the therapeutic applications of bioactive peptides in various disease contexts. One of the primary focuses is the modulation of oxidative stress and inflammatory pathways, which are key contributors to chronic inflammation.

Intervention effects of food-derived polyphenols

For instance, one of the notable contributions is the study (Liu M. et al.) on dietary supplementation with mulberry leaf flavonoids and carnosic acid complex. This research delves into the synergistic effects of mulberry leaf flavonoids and carnosic acid in improving growth performance and antioxidant capacity in broilers. The findings reveal that the combination of these bioactive compounds enhances growth performance and antioxidant capacity in broilers by regulating the p38 MAPK/Nrf2 pathway. This research highlights the potential of these compounds as alternatives to antibiotics (particularly at a dosage of 150 mg/kg), promoting intestinal health and systemic antioxidant defenses.

In the context of neurodegenerative diseases, the review (Li S. et al.) titled *Neurodegenerative Diseases and Catechins: (–)-Epigallocatechin-3-gallate as a Modulator of Chronic Neuroinflammation and Oxidative Stress* delves into the neuroprotective effects of catechins, particularly the most abundant polyphenol in green tea, (-)-Epigallocatechin-3-gallate (EGCG). The discussion on how EGCG attenuates neuroinflammatory processes and oxidative stress mechanisms like scavenging free radicals, reducing oxidative stress and attenuating neuroinflammatory processes. Meanwhile, they underscore its potential as a therapeutic agent for conditions like Alzheimer's and Parkinson's diseases.

In a related review (Qi et al.) on *Zanthoxylum bungeanum* Maxim. (Chinese prickly ash) delves into the polyphenolic components of this traditional spice and their promising anti-inflammatory effects. The review synthesizes data from preclinical studies, suggesting that these polyphenols may offer therapeutic benefits for a range of inflammatory diseases, including ulcerative colitis, arthritis, and cardiovascular diseases. This comprehensive analysis not only highlights the potential of *Z. bungeanum* polyphenols as natural anti-inflammatory agents but also calls for further research to elucidate their mechanisms of action and therapeutic efficacy in humans.

While in the context of antimicrobial therapies, another review (Wang et al.) in this Research Topic addresses the use of polyphenolic natural products as photosensitizers in antimicrobial photodynamic therapy (aPDT). Given the rising concern of antibiotic resistance, this review is timely, offering a comprehensive overview of the potential for polyphenols like curcumin, quercetin, and resveratrol to serve as effective photosensitizers in aPDT. The review not only details the antimicrobial properties of these compounds but also explores their mechanisms of action, providing a solid foundation for future research aimed at developing novel, natural antimicrobial therapies.

Expanding on the topic of polyphenols, a significant contribution to this Research Topic is the study (Fu et al.) exploring the J-shaped association of dietary catechin intake with the prevalence of osteoarthritis (OA) in a large American cohort. This study presents intriguing evidence that moderate intake of specific catechins, such as epigallocatechin and EGCG, is associated with a reduced prevalence of OA, particularly when combined with physical activity. However, excessive intake of these catechins was identified as a risk factor, suggesting a nuanced relationship between catechin consumption and OA. This research emphasizes the importance of balanced dietary intake and highlights the potential for dietary interventions to mitigate OA risk.

Continuing the theme of anti-inflammatory properties, the review (Cozmin et al.) titled *Turmeric: From Spice to Cure. A review of the anti-cancer, radioprotective and anti-inflammatory effects of turmeric sourced compounds*. This review delves into the multifaceted pharmacological properties of turmeric, particularly curcumin, which has shown promise in cancer prevention and treatment, as well as in mitigating radiation-induced damage. The authors provide a thorough analysis of the molecular mechanisms by which curcumin exerts its effects, reinforcing the spice's potential as a cornerstone in integrative oncology and radiation therapy.

Moreover, the clinical trial (Karegar et al.) on ellagic acid supplementation in multiple sclerosis (MS) patients adds to the growing body of evidence supporting the anti-inflammatory and neuroprotective effects of polyphenols. The trial demonstrates that ellagic acid can significantly reduce inflammatory cytokines and modulate gene expression related to immune response, leading to improved clinical outcomes in MS patients. These results highlight the therapeutic potential of ellagic acid in managing chronic autoimmune diseases and call for further exploration in larger, more diverse patient populations.

In a similar context, the study (Janilkarn-Urena et al.) on dihydromyricetin (DHM) supplementation and its effects on ethanol-induced lipid accumulation and inflammation in a murine model of alcohol-associated liver disease (ALD) provides promising results. DHM, a bioactive polyphenol, was shown to reduce liver inflammation and improve lipid metabolism, suggesting its potential as a therapeutic agent for ALD. This study contributes valuable preclinical data supporting the use of DHM as a cost-effective and safe dietary supplement for managing ALD and other inflammatory liver conditions.

Intervention effects of bioactive peptides

A review (Liu H. et al.) examines the application and mechanism of bioactive peptides (BAPs), focusing on their immunomodulatory properties. The review provides a comprehensive overview of how BAPs can regulate key signaling pathways such as MAPK and NF-κB, offering a natural alternative to non-steroidal anti-inflammatory drugs (NSAIDs) that avoids their associated adverse effects. This work emphasizes the potential of BAPs in managing chronic inflammation across various medical conditions.

In addition to BAPs, the review (Boboua et al.) titled *Valorization of Animal Waste Proteins for Agricultural Food Production and Medicinal Applications* addresses the sustainable utilization of animal waste proteins, transforming what is often considered a liability into a valuable resource. The review examines the potential of hydrolysates and peptides derived from animal waste proteins in various industries, including agriculture, food production, and medicine. The authors advocate for further research into the bioavailability and structure-activity relationships of these peptides to fully realize their therapeutic potential.

Intervention effects of lifestyle

On the dietary front, a case study (Zhang et al) from Kashi Xinjiang investigated the relationship between the Dietary Inflammatory Index (DII) and metabolic syndrome (MS) among Uygur adults. This case study provides compelling evidence that dietary patterns can significantly influence the risk of developing MS and its components. The findings emphasize that a diet rich in anti-inflammatory foods, such as fruits and milk, correlates with a reduced prevalence of MS and hypertension, while a diet heavy in meat and eggs is associated with increased risks of high fasting glucose and obesity. These insights underscore the importance of dietary interventions in managing metabolic health, particularly in populations with distinct dietary habits.

Besides dietary habits, lifestyle choices can also increase oxidative stress, enhance mitogenic signaling pathways, and lead to genomic and epigenomic disturbances (4). Turning to lifestyle factors, a novel risk factor for knee osteoarthritis (KOA) is presented in a study (Huang et al.) examining the association between alcohol consumption, particularly pea-based alcoholic drinks, and the incidence of knee surgery in KOA patients. This study identifies a clear correlation between high alcohol intake and the increased risk of knee surgery, with pea-based alcoholic beverages emerging as a particularly potent risk factor. These

findings offer new perspectives on dietary and lifestyle factors contributing to KOA progression and suggest potential avenues for patient education and prevention strategies.

Antioxidant effect in modulating ROS homeostasis on plants

Finally, the research (Li N. et al.) on the crosstalk between melatonin and reactive oxygen species (ROS) in fruits and vegetables post-harvest preservation provides an important update on the role of melatonin in modulating ROS homeostasis. This review highlights the dual role of melatonin as both an antioxidant and a signaling molecule, suggesting new strategies for enhancing the post-harvest quality of fruits and vegetables through the application of melatonin.

Conclusions

In conclusion, the studies presented in this Research Topic provide a comprehensive overview of the intervention effects of food-derived polyphenols and bioactive peptides on chronic inflammation. They not only advance our understanding of the molecular mechanisms involved but also pave the way for developing innovative dietary strategies and therapeutic interventions. We anticipate that this Research Topic will serve as a valuable resource for researchers and clinicians alike, driving further exploration and application of these bioactive compounds in promoting human health.

We extend our deepest appreciation to all the authors, reviewers, and editorial team members for their contributions to this Research Topic. Their efforts have been instrumental in advancing the field of nutritional immunology and expanding our knowledge of the health benefits of food-derived bioactive compounds.

Author contributions

JZ: Project administration, Writing – review & editing, Formal analysis, Writing – original draft. DC: Writing – review & editing. MI: Writing – review & editing. LZ: Writing – review & editing, Conceptualization, Project administration, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Crosstalk between melatonin and reactive oxygen species in fruits and vegetables post-harvest preservation: An update

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Fruits and vegetables contain numerous nutrients, such as vitamins, minerals, phenolic compounds, and dietary fibers. They reduce the incidence of cardiovascular diseases and the risk of certain chronic diseases, and improve the antioxidant and anti-inflammatory capacity. Moreover, melatonin was found in various fruits and vegetables species. Melatonin acts as a multifunctional compound to participate in various physiological processes. In recent years, many advances have been found that melatonin is also appraised as a key modulator on the fruits and vegetables post-harvest preservation. Fruits and vegetables post-harvest usually elicit reactive oxygen species (ROS) generation and accumulation. Excess ROS stimulate cell damage, protein structure destruction, and tissue aging, and thereby reducing their quality. Numerous studies find that exogenous application of melatonin modulates ROS homeostasis by regulating the antioxidant enzymes and non-enzymatic antioxidants systems. Further evidences reveal that melatonin often interacts with hormones and other signaling molecules, such as ROS, nitric oxide (NO), hydrogen sulfide (H_2S), and etc. Among these 'new' molecules, crosstalks of melatonin and ROS, especially the H_2O_2 produced by RBOHs, are provided in fruits and vegetables post-harvest preservation in this review. It will provide reference for complicated integration of both melatonin and ROS as signal molecules in future study.

KEYWORDS

fruit, melatonin, post-harvest preservation, reactive oxygen species, signaling networks, vegetable

Introduction

Fruits and vegetables contain numerous nutrients, such as vitamins, minerals, phenolic compounds, and dietary fibers (1–4). They play an essential part of a well-balanced daily food. It is generally recommended to eat more fruits and vegetables to reduce the incidence of cardiovascular diseases and the risk of certain chronic diseases, and improve the antioxidant and anti-inflammatory capacity (3, 5). For example, polyphenols inhibit chronic inflammation through regulating multiple inflammation-associated cell signaling pathways (6). However, fruits and vegetables often generate significant post-harvest losses after harvest (3). They are

vulnerable to mechanical damages, water and phytochemicals loss, microbial infections, thus resulting in a considerable concern during long-term storage (7, 8). To reduce post-harvest losses, several appropriate storage technologies are used, including cold chain management, hypobaric storage, modified atmosphere package (MAP), and ultraviolet treatment (9–13). To some extent, natural/synthetic preservative agent can also preserve fruits and vegetables storage, whereas there are some residues of chemical compounds (14). To date, previous studies also indicate that plant natural hormones (melatonin, ethylene (ET), salicylic acid (SA), and methyl jasmonate (MeJA), etc) and signaling molecules (nitric oxide (NO), hydrogen sulfide (H_2S), and reactive oxygen species (ROS), etc) can play key roles in regulating the maturation and senescence of fruits and vegetables, delaying postharvest senescence and extending shelf life (15–21).

Acting as a pleiotropic compound, melatonin (*N*-acetyl-5-methoxytryptamine) has a wide range of cellular and physiological functions in living organisms (22–24). For example, melatonin modulates sleep and circadian rhythms, enhances immunity and anti-inflammatory activities (23, 24). Melatonin improves the anti-inflammatory activity, particularly against the chronic inflammation which induced by many chronic diseases (25). In plants, melatonin was firstly detected in 1995 (26, 27). Since then, it was found in various plant species and their different tissue parts, such as rice, wheat, tomato, apple, strawberry, grape, pepper, cucumber, and solanaceous, etc (28–36). Melatonin acts a key molecule to mediate multiple physiological processes, such as the alleviation of abiotic and biotic stresses, and plant growth and development (37–42). For example, melatonin obviously promoted the lateral root formation in *Arabidopsis thaliana* (37). Recently, many studies have reported that melatonin plays an vital role in the fruit and vegetable post-harvest preservation (43–46). In general, endogenous melatonin was increased by exogenous application of melatonin in broccoli, pear, and *Zizyphus* jujuba fruit (43, 44, 46). Then, melatonin observably decreased the accumulation of ROS by enhancing antioxidant capacity and total phenolic and ascorbic acid (AsA) content, and improved the quality of fruits and vegetables (43, 44, 46). Besides, melatonin improved the polyphenol accumulation and antioxidant capacity *via* ethylene signaling in grape berries (47).

ROS contain a group of molecules, mainly including hydrogen peroxide (H_2O_2), hydroxyl radical (OH), superoxide anion ($O_2^{\bullet-}$), and singlet oxygen ($^1O_2$) (48). ROS can cause the oxidation of lipids, and damages of proteins and many other small molecules structures (48). Accordingly, plants have evolved sophisticated antioxidant strategies to regulate the ROS homeostasis, such as antioxidant enzymes [catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), and glutathione peroxidase (GPX)] and non-enzymatic antioxidants (glutathione (GSH), AsA, flavonoids, carotenoids, and alkaloids, etc) (40, 41). Moreover, numerous studies revealed that ROS play key dual roles in the signaling networks in plant stress responses and developmental processes (49, 50). Interestingly, several studies have revealed that the signaling crosstalk between melatonin and ROS was also suggested in red pear and strawberry fruits during post-harvest period (51, 52).

In this review, we mainly discuss exogenous application of melatonin in fruits and vegetable preservation, synthesis of endogenous melatonin, effects of melatonin on the quality of postharvest fruits and vegetable, and the mechanism of melatonin-modulated postharvest protection of fruits and vegetables. We further highlight and discuss the vital role of ROS signaling during the processes, so as to provide reference for future complicated integration of both melatonin and ROS as signal molecules.

The changes of phenomenon and quality of fruits and vegetables during the postharvest period

Fruits and vegetables contain diverse nutrients, such as phenolic compounds, AsA, carotenoids, and mineral content, which beneficial for the anti-nflammation, antioxidation, anti-diabetes, cancer prevention, and cardio-protection in human (1, 2). Many popular kinds of fruits and vegetables, such as tomato, apple, banana, papaya, etc., are consumed worldwide with the rapidly increasing demand and production. However, most of these are highly susceptible to soften rapidly and over-ripen, and often accompanying by the chlorophyll degradation and pathogens (53–59). For example, papaya ripened and softened rapidly, and the fruit peel color gradually turned from green to yellow after harvest (53). Meanwhile, the lightness value declined slightly, the chroma value increased, and the hue angle value gradually dropped during late storage. The most serious damage was disease incidence, and thus decreasing the papaya commodity rate. Similar changes of fruit firmness, hue angle, brightness, and color saturation values were also found in guava during the postharvest period (54). After harvest for 11 days, the anthracnose disease index and disease incidence increased rapidly. In cherry tomato and litchi fruits, the weight loss and fruit firmness were declined, accompanied by fruit decay during storage (57, 59). Furthermore, other fruits and vegetables usually encountered the same cases as well (56, 57, 60). Hence, low-temperature preservation for fruits and vegetables has received increasing research attention (61). Nevertheless, storage for long times may cause chilling injury, such as surface pitting and browning, inability to ripen, watersoaking lesions, and rapid decay (62, 63).

The changes of melatonin content in fruits and vegetables during the postharvest period

Our previous reviews systematically summarized the melatonin biosynthesis and catabolism in plant tolerance to abiotic stresses (38, 40–42). In general, various abiotic stresses, such as salinity, heat, cold, drought, and cadmium metal stresses induce melatonin accumulation by the upregulation of genes which encoding tryptamine 5-hydroxylase (T5H), tryptophan decarboxylase (TDC), *N*-acetylserotonin methyltransferase (ASMT), serotonin *N*-acetyltransferase (SNAT), and caffeic acid O-methyltransferase (COMT) (40). Interestingly, the changes of melatonin content have different trends among different kinds of fruits and vegetables, and some findings were listed in Table 1 and (19, 44, 58, 64–71, 73). Wang et al. (19) found that endogenous melatonin was increased at 0 d to 14 d, and decreased at 14 d to 63 d throughout storage period in cherry fruit. Interestingly, it was decreased dramatically from anthesis to maturity period (45).

TABLE 1 Summary table explaining the changes of melatonin content, and genes related to melatonin metabolic pathway in fruits and vegetables during the postharvest period.

Fruit Species	Impact on melatonin content, or/and genes and enzyme activities related to melatonin metabolic pathway	References
Cassava	Melatonin (0–2 h ↑; 2–72 ↓); *TCD1*, *TCD2*, *T5H*, *ASMT1*, *ASMT2*, *ASMT3*, *SNAT*	(64)
Strawberry	Melatonin (0–3 d ↑; 3–12 ↓); *TCD*, *T5H*, *ASMT*, *SNAT*	(65)
Sweet cherry	Melatonin (0–14 d ↑; 14–63 d↓)	(19)
Jujube	Melatonin (0, 14, 28 d no significant changes)	(44)
"Feizixiao" litchi	Melatonin (0–12 d ↑)	(58)
Table grape	Melatonin (0–15 d ↑; 15–25 d↓), 5-methoxytryptamine (5-MT) (0–15 d ↑; 15–25 d↓); *TDC1*, *TDC2*, *TDC3*, *TDC4*, *T5H1*, *T5H2*, *T5H3*, *T5H4*, *T5H5*, *SNAT1*, *SNAT2*, *SNAT3*, *ASMT1*, *ASMT2*, *ASMT3*, *ASMT4*	(66)
"Summer black" grape	Melatonin (0–40 d ↓; 40–50 d ↑)	(67)
Mulberry	*ASMT4*, *ASMT20* genes	(68)
Mango	Melatonin (0–14 d ↑; 14–28 d↓)	(69)
Angeleno plum	Melatonin (0–8 d↓)	(70)
Pakchoi	Melatonin (0–8 d↓)	(71)
Cherry tomato	Melatonin (0–72 h↓); T*CD*, *T5H*, *ASMT*, *SNAT*	(72)

TDC, tryptophan decarboxylase; T5H, tryptamine 5-hydroxylase; SNAT, serotonin N-acetyltransferase; ASMT, N-acetylserotonin methyltransferase.

These results suggested that endogenous melatonin accumulation was regulated by growing and picking storage periods in fruits. Similarly, melatonin content of table grape, mango, cassava, and strawberry was in parallel with the change trend of cherry fruit, and manifested a trend of rising first and then falling (64–66, 69). Nevertheless, in "Summer black" grape, the change of melatonin accumulation showed an contrary tendency (67). Besides, it showed an decreasing trend in angeleno plum, pakchoi, and cherry tomato (70, 71, 73). Moreover, expression of the genes *TDCs*, *T5Hs*, *SNATs*, and *ASMTs* related to melatonin biosynthesis were also differently regulated in table grape, mulberry fruits, cassava, strawberry, and cherry tomato (64–66, 68, 73). Therefore, melatonin accumulation and its biosynthesis genes transcripts are dynamic and highly regulated in various fruits and vegetables during the post-harvest period.

Protective effects of exogenous melatonin on qualities of fruits and vegetables during the postharvest period

Previous studies have shown that hormones, such as ET, SA, gibberellins [GAs, including gibberellin 1 (GA1), gibberellin 3 (GA3), gibberellin 4 (GA4), and gibberellin 7 (GA7)], MeJA, and abscisic acid (ABA), modulate the postharvest preservation of fruits and vegetables (70, 74–77). Over the past several years, numerous reports have proposed that melatonin acts as an important role on qualities of fruits and vegetables during the postharvest period (53, 54, 56–60, 67, 76). For example, exogenous melatonin treatments delayed fruit firmness decrease, maintained higher hue of the peel fruit, and retained greater lightness of papayas than the control group during the later storage period (53, 54). Similarly, it observably alleviated the decrease of fifirmness and the weight loss in cherry tomato (59). Fruit colour index (a*/b*) was also obviously increased by melatonin treatment in both sweet cherry and guava fruits (78). In pepper, broccoli, and Chinese flowering cabbage vegetables, exogenous melatonin application inhibited the degradation of chlorophyll during the postharvest period (43, 56, 79). In addition to the above phenotypic changes, melatonin also reduced the decay and disease index in fruits (41, 53, 54, 80). Moreover, exogenous melatonin also bought about significant increases in total soluble solids, sugar, protein, AsA, carotenoids, and total flavonoid and phenols contents, which were important substances of fruits and vegetables (43, 56, 81–83). Besides, melatonin mediated the aroma volatiles (propyl acetate and hexyl acetate) of postharvest pear fruit (84, 85).

Effects of exogenous melatonin on the redox homeostasis of fruits and vegetables during the postharvest period

In general, ROS (mainly MDA, H_2O_2, and $O_2^{\bullet-}$) are largely caused during fruit ripening period, and induce oxidizing proteins and membrane lipids formation (53). For example, $O_2^{\bullet-}$ produce by the oxygen reduction by the electron transport chain (ETC) (53, 54). They also generate by photorespiration pathway and fatty acid-oxidation reaction (59). Then, H_2O_2 produces from $O_2^{\bullet-}$ by the activity of SOD and/or glycolate oxidases. Moreover, NADPH oxidases, polyamine oxidases (PAO), and cell wall bound peroxidases (POX) induce the ROS generation in cell membrane, cell wall, and apoplast, respectively (7, 57, 58). As toxic byproducts, ROS could cause serious damages to proteins and quality of fruits and vegetables. Combined with the antioxidant capacity of melatonin, these led to study the role of melatonin in the postharvest preservation of fruits and vegetables, especially in recent years (86–110). In this review, the protective impacts of melatonin on the antioxidant capacity of fruits and vegetables during the postharvest period have been summarized in Table 2. In fact, ROS were largely stimulated in fruits and vegetables, including papaya, cherry tomato, pepper, wax apple, Chinese flowering cabbage, pear, peach, litch, pomegranate, sweet cherry, sapota, apple, blueberry, longan, zucchini, guava, rambutan, water bamboo shoot, mango, tomato, eggplant, rosa roxburghii fruit, cucumber, jujube, sweetpotato, avocado, persimmons, and table grape

during the postharvest period (stored at room temperature and/or low temperature; Table 2). Then, the ROS accumulation were significantly decreased by exogenous application of melatonin. Two main pathways might be involved in melatonin-inhibited ROS acumulation. Exogenous application of melatonin improved the antioxidant contents, such as GSH, AsA, proline, flavonoids, carotenoids, anthocyanins, and dehydroascorbate (DHA) through inducing the expression of *GSH*, *GR1*, *GR2*, *GMDH*, *GME*, *GGGT*, *GPP*, *GDH*, and *GLDH* genes (Table 2) and (88, 92, 95, 101). In most of the above fruits and vegetables, the antioxidant enzymes act as key roles in melatonin-downregulated ROS overproduction, such as CAT, SOD, APX, GR, GPX, DHAR, and MDHAR (Table 2). Besides, exogenous application of melatonin enhanced the total antioxidant capacity (T-AOC), cupric-reducing antioxidant power (CUPRAC), ferric-reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), and 1,1-diphenyl-2-trinitrophenylhydrazine (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging capacity. For example, exogenously melatonin obviously induced the expression of *PpAPXs*, *PpSODs*, and *PpCATs*, and thereby activating the antioxidant system in peach fruit during storage for 14 d (88). Furthermore, the expression of AsA biosynthetic genes (including *GMDH*, *GME*, *GGGT*, *GPP*, *GDH*, and *GLDH*) were also stimulated, which increase the content of AsA to inhibit the ROS accumulation (88). In addition, exogenous melatonin interacted with ROS by regulating the expression of genes involved in AsA-GSH cycle, such as *DHA*, *DHAR*, *MDHAR*, *GSH*, *GSSG*, and *GR* in sweet cherry (101). Among the above fruits, blueberry contains high level of bioactive compounds, flavonoids and anthocyanins. These were also increased by exogenous melatonin to improve the nutraceutical traits of blueberry fruit during storage time (98).

The roles of hormones in melatonin-modulated postharvest protection of fruits and vegetables during storage period

In recent years, hormones have been described to regulate fruits and vegetables postharvest performance (112). For example, ET and ABA played central roles in modulating senescence that strongly influence fruits and vegetables shelf-life (21, 113, 114). Ethylene is synthesized from *S*-adenosylmethionine to 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase (ACS), and then ACC is oxidazed by ACC oxidase (ACO) (21). Thus, ACS and ACO are the rate-limiting enzymes involved in this biosynthetic pathway. To reduce the ethylene accumulation through regulating the expression of genes encoding ACS and ACO enzymes might contribute to delay fruits and vegetables senescence (21, 114). Exogenous application of ABA induced flavanols and anthocyanin accumulation to promote the fruit coloration in fruits, including apple, grape, tomato, and litchi (115–118). Meanwhile, JA and SA have been suggested to be involved in the disease resistance during postharvest period (15, 16, 119, 120). MeJA induced the expression of JA synthesis genes, increased the allene oxide cyclase (AOC) activity, and thereby resulting in high endogenous JA generation (119). Nevertheless, DIECA treatment reduced the endogenous levels of JA, and AOC and 12-oxo-phytodienoic acid reductase activities. Then, a significant correlation between JA and chlorophyll content was observed in broccoli flowers, and that was the important reason for broccoli postharvest yellowing (119). Besides, SA-mediated defense response was involved in litchi downy blight possibly *via* modulating fruit senescence (120). Other hormones, such as auxins, cytokinins (CK) or GAs, are usually at very low contents and attributed to the anti-senescence properties as well (74, 121, 122).

Many studies have confirmed the role of melatonin in modulating hormone levels during fruits and vegetables postharvest period (Figure 1) and (123). Melatonin can significantly delay fruit and vegetables senescence through inhibiting ET and ABA accumulation. For example, exogenous application of melatonin inhibited the expression of *ACSs* and *ACOs* genes, and reduced ethylene production to delay the banana and tomato fruits color through (124, 125). It significantly down-regulated the expression of ET synthetase genes (*PcACS* and *PcACO*), reduced ethylene production and rates of respiration, then thereby delaying senescence in pear fruit (126). Correspondingly, melatonin also down-regulated the expression of ET transcription factors (*AdERF4*, *AdERF74*, and *AdERF75*), and inhibited the ET release in kiwifruit during the storage period (127). Interestingly, research studies have showed that exogenous application of melatonin repressed the expression of *BrABF1*, *BrABF4*, *BrABI5* (128). They binded to the promoters of ABA biosynthetic genes (*BrNCED*, *BrABA2*, and *BrAAO*) and chlorophyll catabolic genes, and regulated the expression levels of above genes, thus resulting in a low endogenous ABA level (128). Therefore, melatonin regulated the inhibition of Chinese flowering cabbage senescence by the suppression of *ABFs*-modulated ABA synthesis and chlorophyll degradation (128). Furthermore, exogenous application of melatonin reduced both ET and ABA contents to modulate the softening through inhibiting the activities of ACS, ACO, and 9-cis-epoxycarotenoid dioxygenase (NCED) in "Guifei" mango fruit (73). Additionally, exogenous application of melatonin induced the expression of JA synthesis genes (*VaLOX*, *VaAOS*, and *VaAOC*), and promoted JA accumulation (129). Hence, melatonin modulated the jasmonic acid signaling pathway to enhance the postharvest disease resistance of blueberries fruit (129). Similarly, generation of SA was also promoted by exogenous application of melatonin in tomato. Afterwards, the increase of activities of chitinase (CHI) and β-1,3-glucanase (GLU) inhibited tomato gray mold development, which caused by *B. cinerea* (130). Besides, after melatonin treatment for 4 days, GA1 had a sharp increase, and no differences were observed in the content of GA3, GA4, and GA7 in *Angeleno* plums during postharvest decay (70). Furthermore, it was also suggested that *WRKY*, *MYB*, *ERF*, *ARF* and *bHLH3* transcription factors were mainly involved in auxin and ethylene signalings in postharvest banana fruit peel (131, 132). These transcription factors were also beneficial to maintain redox homeostasis (133). Some others, such as auxin and mitogen-activated protein kinase (MAPK) signaling pathway, might be involved in melatonin-regulated fruits and vegetables postharvest preservation and/or disease resistance during the storage period. In summary, an appropriate amount of melatonin can prolong fruits and vegetables senescence shelf life by regulating the release of ET, ABA, SA, and etc. Additionally, more genetic evidence needs to be explored in future study.

TABLE 2 Summary table explaining the impacts of exogenous melatonin on the antioxidative defense systems of fruits and vegetables during the postharvest period.

Fruit and vegetable names	Treatments	Impact on oxidative markers and antioxidative defense systems	References
Papaya	0, 100, 400, and 800 μM melatonin	H_2O_2, MDA, O_2^-; SOD, CAT, POD, APX, GR, NOX, T-AOC, AsA, flavonoids	(53)
Cherry tomato	0 and 100 μM melatonin	MDA; GSH, AsA, GPX, APX, GR, T-AOC	(59)
Pepper	0 and 100 μM melatonin	H_2O_2, MDA, O_2^-; AsA, DHA, GSH, GSSG, APX, SOD, CAT, POD, GR, MDHAR, DHAR	(56)
Wax apple	0, 800 μM melatonin	MDA, H_2O_2, O_2^-; SOD, CAT, APX, GR; *CAT1*, *SOD2*	(60)
Chinese flowering cabbage	0 and 100 μM melatonin	H_2O_2, MDA, O_2^-; POD, SOD, CAT, APX, GR, DHAR, MDHAR, AsA, DHA, GSH, GSSG; *RBOHB*, *RBOHC*, *RBOHD*, *RBOHD2*, *RBOHE*, *POD*, *SOD*, *CAT*, *APX*, *GR*, *DHAR*, *MDHAR*	(79)
Pear	0, 50, 100, 150, 200, and/or 500 μM melatonin	H_2O_2, MDA; SOD, POD, AsA, DPPH and ABTS scavenging capacity; *POD*	(46, 85)
Peach	0 and 100 μM melatonin	MDA, H_2O_2, O_2^-; AsA, GSH, POD; *SOD1*, *SOD2*, *SOD3*, *SOD4*, *SOD5*, *SOD6*, *SOD7*, *SOD8*, *CAT1*, *CAT2*, *APX1*, *APX3*, *APX6*, *MDHAR1*, *MDHAR2*, *DHAR2*, *DHAR3*, *GR1*, *GR2*, *GMDH*, *GME*, *GGGT*, *GPP*, *GDH*, *GLDH*	(88, 92)
Litch	0, 50, 100, 200, and/or 600 μM melatonin	MDA, H_2O_2, O_2^-; flavonoids, anthocyanin, proline, P5CS, PDH, POD, SOD, CAT, APX, GR; *Fe-SOD*	(58, 95)
Pomegranate	0 and 100 μM melatonin	AsA, AOX, AAO, APX, GR, GSH, anthocyanins	(86)
Sweet cherry	0, 50, 100, 150, 200, 300, and/or 500 μM melatonin	MDA, H_2O_2, O_2^-; SOD, CAT, APX, POD, DHAR, GR, MDHAR, AsA, DHA, GSH, GSSG, flavonoids, anthocyanins; *Cu/Zn-SOD*, *Mn-SOD*, *CAT*, *APX*, *MDHA*, *MDHAR*, *DHA*, *DHAR*, *GSH*, *GSSG*, *GR*	(19, 45, 78, 101)
Sapota	0, 30, 60, and 90 μM melatonin	MDA, O_2^-, H_2O_2; proline, SOD, CAT	(102)
Apple	0 and 1 mM melatonin	MDA; CAT, SOD, POD	(104)
Blueberry	0 and 1 mM melatonin	H_2O_2, MDA; polyphenols, flavonoids, anthocyanins, AsA, SOD, CAT, APX, POD	(98)
Longan	0 and 400 μM melatonin	H_2O_2, MDA, O_2^-; POD, PPO, flavonoids, SOD, CAT, APX, AsA, GSH	(97)
Zucchini	0 and 1 mM melatonin	MDA	(100)
Guava	0, 50, 100, 150, 200, 400, and/or 600 μM melatonin	H_2O_2, MDA, O_2^-; SOD, APX, CAT, T-AOC, AsA, flavonoids, total soluble sugar	(54, 89)
Rambutan	0 and 125 μM melatonin	H_2O_2, MDA, O_2^-; AsA, DHA, GSH, GSSG, POD, PPO, SOD, CAT, flavonoids, anthocyanins, APX, GR, MDHAR, DHAR	(107)
Water bamboo shoot	0 and 500 μM melatonin	AsA, POD; *POD1*, *POD2*, *POD3*, *POD4*, *POD5*	(108)
Mango	0, 100, or 200 μM melatonin	H_2O_2, MDA, O_2^-; carotenoid, SOD, CAT, POD, APX, CUPRAC, TEAC, DPPH, TEAC, FRAP	(69, 87, 91)
Tomato	0 and 10 μM melatonin	SOD, CAT, POD, APX, GSH	(109)
Eggplant	0, 50, 100, 150, and 200 μM melatonin	H_2O_2, MDA; SOD, CAT, anthocyanins; *SOD*, *CAT1*, *CAT2*	(111)
Rosa roxburghii fruit	0, 20, 50, 100, 200, and 400 μM melatonin	H_2O_2; SOD, CAT, POD, APX, GR, MDHAR, DHAR, AsA, GSH; *APX*, *GR*, *MDHAR*, *DHAR*	(90)
Cucumber	0, 50, 100, and 500 μM melatonin	H_2O_2, MDA, O_2^-; AsA, proline	(96)
Jujube	0, 20, 50, 100, 200, and 400 μM melatonin	H_2O_2, MDA, O_2^-; AsA, GSH, APX, SOD, CAT, POD,	(106, 110)
Sweetpotato	0, 200, and 500 μM melatonin	H_2O_2, MDA, O_2^-; SOD, CAT, POD, APX, GR, AsA, vitamin C, *SOD1*, *SOD2*, *CAT1*, *APX1*, *APX3*, *GR1*, *GR2*, *DHAR*	(94)
Avocado	0 and 1 mM melatonin	H_2O_2, MDA, O_2^-; SOD, CAT, APX, POD, flavonoids, AsA	(99)

(Continued)

TABLE 2 (Continued)

Fruit and vegetable names	Treatments	Impact on oxidative markers and antioxidative defense systems	References
Persimmons	0 and 100 μM melatonin	H_2O_2, MDA; flavonoids, AsA, DPPH and ABTS radical scavenging activity, FRAP	(93)
Table grape	0, 50, and 100 μM melatonin	H_2O_2, $O_2^{\cdot-}$; CAT, POD	(103)

H_2O_2, hydrogen peroxide; MDA, malondialdehyde; $O_2^{\cdot-}$ superoxide anion; Cu/Zn-SOD, copper/zinc-superoxide dismutase; Mn-SOD, manganese-superoxide dismutase; POD, guaiacol peroxidase; APX, ascorbate peroxidase; GR, glutathione reductase; T-AOC, total antioxidant capacity; AsA, ascorbic acid; GSH, reduced glutathione; GPX, glutathione peroxidase; DHA, dehydroascorbate; GSSG, oxidized glutathione; CAT, catalase; MDHA; monodehydroascorbate reductase; MDHAR, monodehydroascorbate; DHAR, dehydroascorbate reductase; RBOH, respiratory burst oxidase homologue; DPPH, 1,1-diphenyl-2-trinitrophenylhydrazine; ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); NOX, NADH oxidase; GMPH, mannose-1-phosphate guanylyltransferase; GME, GDP-D-mannose-3′,5′-epimerase; GGGT, GDP-L-galactose guanylyltransferase; GPP, L-galactose-1-phosphate phosphatase; GDH, L-galactose-1-dehydrogenase; GLDH, L-galactono-1,4-lactone dehydrogenase; P5CS, Δ1-pyrroline-5-carboxylate synthetase; PDH, pyruvate dehydrogenase; CUPRAC, Cupric-reducing antioxidant power; FRAP, Ferric-reducing antioxidant power; TEAC, Trolox equivalent antioxidant capacity; FRA, ferric reducing antioxidant power.

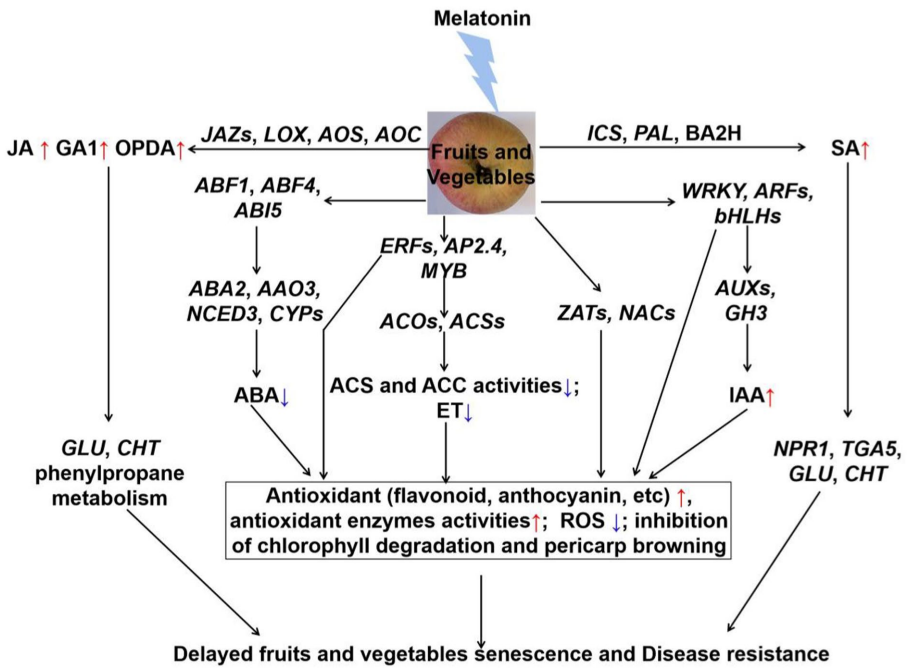

FIGURE 1
The crosstalk between melatonin and hormones of fruits and vegetables during the postharvest period. Exogenous application of melatonin reduced the ABA and ET content, and increased IAA content through regulating the related transcription factors and synthetic genes. Then, the significant antioxidants and antioxidant enzymes activities were induced to reduce the ROS accumulation, and chlorophyll degradation and pericarp browning were also inhibited. Besides, exogenous application of melatonin induced the JA and SA signaling pathways, and activated the proteins in the defense system to enhance the disease resistance in fruits and vegetables during storage. Jasmonic acid (JA), gibberellin 1 (GA1), 12-oxo-phytodienoic acid (OPDA), jasmonate ZIM-domain (JAZ), phenylalanine ammonia lyase (PAL), benzoic acid 2-hydroxylase (BA2H), lipoxygenase (LOX), allene oxide synthase (AOS), allene oxide cyclase (AOC), isochorismate synthase (ICS), salicylate (SA), non-expresser of pathogenesis-related genes 1 (NPR1), basic/leucine zipper-type transcription factor (TAG5), β–1,3-glucanase (GLU), chitinase (CHT), abscisic acid (ABA), ABRE-binding factor (ABF), ABA-insensitive (ABI); 9-cis-epoxycarotenoid dioxygenase (NCED), aldehyde oxidase (AAO), ethylene-response factor (ERF), adipocyte protein (AP), v-myb avian myeloblastosis viral oncogene homolog (MYB), zinc finger protein (ZAT), NAM/ATAF/CUC (NAC), basic/helix–loop–helix (bHLH), 1-aminocyclopropane-1-carboxylic acid (ACC), ACC oxidase (ACO), ACC synthase (ACS), ethylene (ET), auxin response factor (ARF), indole-3-acetic acid-amido synthetase (GH3), indole-3-acetic acid (IAA).

The crosstalk between melatonin and signal molecules (NO, H_2S, and ROS) in the postharvest protection of fruits and vegetables during storage period

Numerous studies showed that signal molecules, such as ROS, NO, and H_2S, play key roles in resistances to biotic and abiotic damages in plants (134–139). Recent studies have shown that there are interactions between melatonin (applied exogenously) and the signalig molecules (37, 40–42, 126, 130, 140). For example, our previous studies revealed that H_2O_2 signaling was required for melatonin-promoted root growth and melatonin-improved salinity tolerance in alfalfa and Arabidopsis, respectively (37, 38). NO signaling was also involved in melatonin-regulated salinity tolerance in *Brassica napus*

L. and sunflower seedlings (140, 141). Furtherly, melatonin induced H_2S generation through increasing L-/D-cysteine desulfhydrase (LCD/DCD) activity. Similarly, it also stimulated NO generation. However, the H_2S and NO induced by melatonin were inhibited by H_2S scavenger (hypotaurine, HT) and NO scavenger (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, cPTIO), respectively. Therefore, the H_2S and NO jointly were participated in the melatonin-enhanced salinity tolerance in cucumber (34). In fact, the complex regulatory function of melatonin and its crosstalk with H_2O_2, NO and H_2S is existed in many cases.

Interestingly, these signal molecules were also involved in exogenous melatonin-modulated fruits and vegetables postharvest protection, and thus improving their quality and yield (Figure 2) and (72, 142, 143). For instance, exogenous melatonin treatment rapidly elicited ROS burst. These ROS acted as signaling molecules to enhance SA accumulation and improve the expression of related defense genes in cherry tomato fruit during the storage (94). In litchi fruit, exogenous application of melatonin activated the NR and NOS activities and triggered NO accumulation (142). Endogenous NO mediated the melatonin-enhanced cold tolerance *via* regulation of redox status (142). Similarly, exogenous melatonin increased NOS activity, and induced endogenous NO production to maintain normal mitochondrial function in lotus seeds (144). Besides, it also induced *NOS* gene expression and enzyme activity to keep safe membrane integrity in tomato fruit (145). Furthermore, H_2S has been reported to regulate the process by delaying senescence (146). However, more studies should be investigated on the crosstalks among melatonin, NO, and H_2S in the postharvest preservation of fruits and vegetables using pharmacological, genetic, and proteomic approaches.

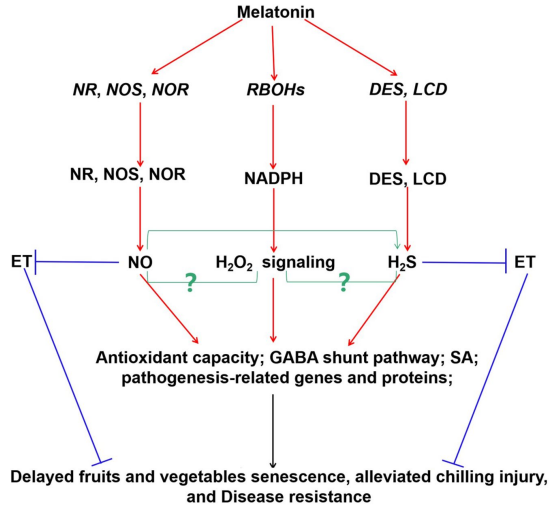

FIGURE 2
Probable integrative model of melatonin and signaling molecules (H_2O_2, NO, and H_2S) in postharvest protection of fruits and vegetables during storage period. Increasing evidences showed that melatonin enhanced the expression of *RBOHs*, *NR*, *NOS*, *NOR*, *DES*, and *LCD* genes, and induced H_2O_2, NO, and H_2S generation, thereby activating the signaling pathways in fruits and vegetables. Besides, hormones were also involved in these pathways to regulate fruits and vegetables quality. Interaction between NO and H_2S was also suggested. The relationships between the H_2O_2 signaling and NO/H_2S in postharvest protection of fruits and vegetables are still largely unknown (green arrow, yet largely unknown). Red arrow, induced; blue arrow, inhibited. Respiratory burst oxidase homologs (RBOHs), nitrate reductase (NR), nitric oxide synthase (NOS), desulfhydrase, (DES), *L*-cysteine desulfhydrase (LCD), nitric oxide (NO;), hydrogen peroxide (H_2O_2), hydrogen sulfide (H_2S), ethylene (ET), salicylic acid (SA), γ-aminobutyric acid (GABA).

Crosstalk between the RBOH-regulated ROS signaling and melatonin in the postharvest protection of fruits and vegetables during storage period

Previous studies suggested that melatonin is a potent free radical scavenger, and reacts with ROS *via* the addition of a hydroxyl group (-OH) in position 2, 4, or 6 to form a family of molecules (147). Among the hydroxymelatonin metabolites, 2-hydroxymelatonin (2-OHMel) and 4-hydroxymelatonin (4-OHMel) were found in 24 plant species and predicted to have the antioxidant protection (147–149). For example, 4-OHMel reacted with ROO• about 200 times faster than trolox. Furthermore, ROS act as key signaling molecules at low concentrations in regulating plant biotic and abiotic stress (150, 151). Recent studies have shed new light on the interactions of melatonin and ROS in higher plants development and growth (37, 38, 41). For example, Bian et al. (111) identified that melatonin acted as upstream signaling of ROS to facilitate lateral root development. Besides, the phytomelatonin receptor (PMTR) sensed and binded with melatonin to release G-protein α (Gα), and activated Ca^{2+} signaling. Afterwards, the Ca^{2+} signaling activated H_2O_2 production, while H_2O_2 worked with Ca^{2+} signaling to induce the expression of cell cycle regulatory genes, and thereby promoting the lateral root development.

Previous reviews summarized the pathways of ROS generation in plant organs, including cell membrane, peroxisome, mitochondria, chloroplast, apoplast, and etc (150, 151). Among these, respiratory burst oxidase homolog (RBOH) proteins localize on plasma membrane, and encode the NADPH oxidases, which associate with the signal transduction (152). There are several *RBOHs* genes encoding NADPH oxidase in various plants (150, 151). Recently, many studies have revealed the vital roles of *RBOH*-regulated ROS signaling in melatonin-enhanced plant abiotic stress tolerance (41). Furthermore, it is necessary to balance intracellular ROS homeostasis to maintain to the quality of postharvest fruits and vegetables. Recently, the functions of H_2O_2 signaling in melatonin-mediated fruits and vegetables postharvest protection were also preliminarily studied (Figure 2) and (72, 130, 132, 153, 154). For example, $O_2^{\cdot-}$ and H_2O_2 generation of cherry tomato fruit increased to a maximum by exogenous melatonin treatment at 12 h and 36 h, respectively, and then decreased during the storage period (130). Exogenous melatonin treatment significantly up-regulated the expression of respiratory burst oxidase homolog protein B (*RbohB*) gene, which accelerated the response signaling in banana peel in banana during postharvest storage period (132). Similarly, melatonin treatment also up-regulated the *RBOH1* expression in tomato, however, it was significantly attenuated by treatments of diphenyleneiodonium (DPI, an NADPH oxidase inhibitor) and dimethylthiourea (DMTU, a ROS scavenger) (153). Exogenous melatonin elevated $O_2^{\cdot-}$ and H_2O_2 accumulation by upregulating the *SlNOX* expression and NOX activity for the first 36 h in cherry tomato fruit during storage (94). These results were further confirmed by the transcriptome analysis in cherry tomato

fruit (155). Besides, the positive crosstalks between melatonin and H_2O_2 have also been observed in apple and strawberry fruits against *Diplocarpon mali* infection and decay, respectively (51, 156). Moreover, SA signaling acted as the downstream pathway of the crosstalk between melatonin and H_2O_2 signaling to modulate the postharvest protection of fruits and vegetables during storage period (94, 156). Therefore, ROS generation-induced transiently by melatonin serve as the key signal in fruits and vegetables, especially in resistance to various diseases. However, it is important to further clarify the roles of this crosstalk on the quality and extending storage times in diverse fruits and vegetables species.

Conclusion and perspectives

Melatonin is ubiquitous in fruits and vegetables. This reviews describes the changes of melatonin content and synthesis sites in fruits and vegetables during the postharvest period. Exogenous melatonin can increase endogenous melatonin accumulation, alleviate the weight loss, fruit firmness decrease and discoloration, reduce the decay incidence, decay and disease index, and improve the quality of fruits and vegetables. In addition, it increases GSH, AsA, DHA, anthocyanins, carotenoids, and total flavonoid and phenols contents, and decreases MDA, H_2O_2, and $O_2^{\bullet-}$ contents. It has also been noted that melatonin enhances the CAT, SOD, APX, GR, GPX, DHAR, and MDHAR activities to improve the antioxidant capacity. Application of exogenous melatonin increases proline content and decreases the membrane lipid peroxidation to protect cell membrane integrity in fruits and vegetables during the cold storage. Furtherly, exogenous melatonin regulates hormones, such as ethylene, salicylic acid, and abscisic acid, to delay postharvest senescence and protect fruits and vegetables aganist bacterial invasion. However, the effective concentrations of melatonin are different for postharvest protection of different fruits and vegetables species. Therefore, it is important to use the appropriate melatonin concentrations to prolong fruits and vegetables postharvest shelf life.

ROS signaling during fruit and vegetable ripening has been extensively studied (147). Recently, several studies revealled that ROS signaling is involved in melatonin-modulated fruits and vegetables post-harvest preservation. In particular, the vital role of RBOHs-regulated H_2O_2 generation during these processes are shown. However, there are still many questions that should be characterized to understand the crosstalk of melatonin and ROS. For example, it is necessary to focus more attention on the signaling role of ROS produced by PAO in melatonin-modulated fruits and vegetables post-harvest preservation in future studies. Since the transmembrane receptor of melatonin (PMTR1/CAND2) were found in plants, researches focus on the mechanisms that the interaction between *PMTR1/CAND2* and Gα subunits acts on the expression of the *RBOHs* in plant responses to abiotic stress (56, 71, 157). In this review, it is urgent to deeply study whether or how Gα directly regulates the crosstalk between melatonin and reactive oxygen species in fruits and vegetables post-harvest preservation.

Author contributions

NL, KZ, QY, and QG: writing—original draft preparation. XZ: writing—provided deeply discussion and sorted out the references. MM and ZC: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Dihydromyricetin supplementation improves ethanol-induced lipid accumulation and inflammation

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Introduction: Excessive alcohol consumption leads to a myriad of detrimental health effects, including alcohol-associated liver disease (ALD). Unfortunately, no available treatments exist to combat the progression of ALD beyond corticosteroid administration and/or liver transplants. Dihydromyricetin (DHM) is a bioactive polyphenol and flavonoid that has traditionally been used in Chinese herbal medicine for its robust antioxidant and anti-inflammatory properties. It is derived from many plants, including *Hovenia dulcis* and is found as the active ingredient in a variety of popular hangover remedies. Investigations utilizing DHM have demonstrated its ability to alleviate ethanol-induced disruptions in mitochondrial and lipid metabolism, while demonstrating hepatoprotective activity.

Methods: Female c57BL/6J mice (n = 12/group) were treated using the Lieber DeCarli forced-drinking and ethanol (EtOH) containing liquid diet, for 5 weeks. Mice were randomly divided into three groups: (1) No-EtOH, (2) EtOH [5% (v/v)], and (3) EtOH [5% (v/v)] + DHM (6 mg/mL). Mice were exposed to ethanol for 2 weeks to ensure the development of ALD pathology prior to receiving dihydromyricetin supplementation. Statistical analysis included one-way ANOVA along with Bonferroni multiple comparison tests, where $p \leq 0.05$ was considered statistically significant.

Results: Dihydromyricetin administration significantly improved aminotransferase levels (AST/ALT) and reduced levels of circulating lipids including LDL/VLDL, total cholesterol (free cholesterol), and triglycerides. DHM demonstrated enhanced lipid clearance by way of increased lipophagy activity, shown as the increased interaction and colocalization of p62/SQSTM-1, LC3B, and PLIN-1 proteins. DHM-fed mice had increased hepatocyte-to-hepatocyte lipid droplet (LD) heterogeneity, suggesting increased neutralization and sequestration of free lipids into LDs. DHM administration significantly reduced prominent pro-inflammatory cytokines commonly associated with ALD pathology such as TNF-α, IL-6, and IL-17.

Discussion: Dihydromyricetin is commercially available as a dietary supplement. The results of this proof-of-concept study demonstrate its potential utility and functionality as a cost-effective and safe candidate to combat inflammation and the progression of ALD pathology.

KEYWORDS

Dihydromyricetin, polyphenols, flavonoids, ethanol, lipids, inflammation, lipophagy

1. Introduction

Alcohol use disorder (AUD) affects over 280 million people worldwide, and in the United States alone, it affects over 18 million people, leading to approximately 140,000 deaths annually (1, 2). This ranks AUD third on the list of preventable causes of death and morbidity. Unfortunately, the rates of alcohol misuse are on the rise, with unhealthy drinking patterns contributing to a higher incidence of mortality, particularly due to alcohol-associated liver disease (ALD) (3–5). The liver is the primary site of alcohol metabolism, and when ALD manifests, it is in a progressive order. This progression includes alcohol-associated fatty-liver disease (AFLD), alcohol-associated steatohepatitis (ASH) and fibrosis, to ultimately cirrhosis. The stages of ALD are characterized by disruptions in lipid metabolism and transport, altering the levels of free fatty acids, triglycerides, total cholesterol, and lipoproteins that result in injury due to lipotoxicity, oxidative stress, and inflammation (6). Available FDA-approved medications have limited success in treating patients for AUD, and there are no approved pharmaceutical or nutritional therapies for ameliorating ALD beyond the administration of corticosteroids as anti-inflammatory agents or in the worst-case scenarios, a liver transplant (7). The lack of effective therapies for ALD is due, in part, to the multifactorial systemic responses that are associated with heavy ethanol (EtOH) intake and the multi-organ damage that can result from excessive EtOH consumption.

Plant-derived products, including members of the polyphenol families, are traditionally used worldwide for the treatment of liver disorders that include hepatic-driven metabolic imbalances (8–10). Polyphenols can regulate homeostasis by acting on nuclear receptors in response to the cellular environment and metabolic sensors. Emerging studies have demonstrated the effects of dietary polyphenols on dyslipidemia by reducing circulating levels of low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), and promoting high density lipoprotein (HDL) levels, while improving liver function as noted by improved aspartate and alanine aminotransferase (AST and ALT) levels (10–13). Dihydromyricetin (DHM), a polyphenol and bioactive flavonoid found in many plants such as *Hovenia dulcis*, has been used for centuries in Traditional Chinese Medicine and is still used today (14), namely, as the active ingredient of popular hangover remedies.

In 2021, the global natural product (i.e., herbal medicine) market was valued at nearly $152 billion, highlighting the growing consumer preference for natural remedies over synthetic products (15, 16). In fact, the market for hangover cures was valued at $1.8 billion in 2021, and is expected to grow over 14.6% by 2028 (17), led by plant-based herbal products. Building evidence suggests that DHM improves steatosis (18–20) while providing hepatoprotective effects and restoring metabolic processes (19, 21). Regarding alcohol, commercially available DHM is used for its anti-veisalgia effect and is instructed to be administered before, during, and after consuming large amounts of ethanol.

To expand on this further, our group has begun to investigate the effects of DHM on ethanol-induced disturbances in lipid metabolism, steatosis, and inflammation. We recently reported that administration of 5 and 10 mg/kg of DHM delivered via intraperitoneal (i.p.) injection significantly protected the livers of mice from ethanol-induced steatosis and improved mitochondrial health via the AMP-activated protein kinase (AMPK), sirtuin-1 (SIRT1), PPARG coactivator-1α (PGC-1α) signaling pathway (18, 21). The AMPK-SIRT1-PGC-1α pathway is a key regulator of energy homeostasis through its effects on metabolic and mitochondrial activity, namely, lipid oxidation, mitochondrial biogenesis, and autophagy (22–26). Autophagy is an evolutionarily conserved process that plays an important role in liver physiology, and is induced through AMPK pathway activation (27). Typically, autophagy promotes the proteolytic degradation and recycling of damaged proteins and organelles, including lipid droplets (LDs), in response to environmental cues, such as starvation and energy requirements. LD catabolism is mediated by lipolysis and lipophagy, a form of selective macro-autophagy that targets lipid droplets (28).

Along with steatosis, inflammation plays a critical role in the development and progression of ALD. Chronic alcohol consumption leads to the activation of several inflammatory pathways including NF-κB and toll-like receptor 4 (TLR4) signaling pathways, and inflammasome activation (29). These pathways are responsible for elevation of pro-inflammatory cytokines which promote liver inflammation and injury via increased oxidative stress and mitochondrial dysfunction (30). Furthermore, chronic alcohol consumption leads to disruption of the gut barrier, leading to bacterial translocation and release of endotoxins into the

Abbreviations: AFLD, alcohol-associated fatty-liver disease; ALD, alcohol-associated liver disease; ALT, Alanine aminotransferase; AMPK, AMP-activated protein kinase; ASH, alcohol-associated steatohepatitis; AST, Aspartate aminotransferase; Atg, autophagy-related genes; AUD, alcohol use disorder; CE, cholesteryl ester; DHM, Dihydromyricetin; EtOH, ethanol; FFA, free fatty acid; HDL, high density lipoprotein; LC3B, microtubule-associated protein 1 light chain 3 beta; LD, lipid droplet; LDC, Lieber DeCarli; LDL, low-density lipoprotein; mTOR, mammalian target of rapamycin; OXPHOS, oxidative phosphorylation; PCC, Pearson's Correlation Coefficient; PGC-1α, PPARG coactivator-1α; PKA, protein kinase A; PLIN-1, perilipin-1; p62/SQSTM-1, sequestosome-1; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SIRT1, sirtuin-1; TG, triglyceride; VLDL, very low-density lipoprotein.

liver. These endotoxins activate Kupffer cells, which leads to further increases in production of pro-inflammatory cytokines and oxidative stress resulting in exacerbated liver inflammation and injury (31). Overall, the inflammatory response in ALD is a complex process which involves multiple cell types, mediators, and pathways. Targeting inflammatory responses early could prove to be an important therapeutic strategy for ALD.

Alcohol-associated fatty-liver disease and ASH are characterized by the accumulation of fat primarily found in the form of lipid droplets and increased inflammatory signaling through TNF-α, IL-1β, IFN-γ, IL-17, and IL-6 (32, 33). In the current investigation, we tested the hypothesis that oral DHM improves ethanol-induced disruptions in lipid homeostasis by reducing levels of harmful lipids, leading to decreased levels of circulating pro-inflammatory cytokines.

2. Materials and methods

2.1. Lieber DeCarli Diet (LDC)

Female wild-type c57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) weighing ≥ 19 g and ≥ 10 weeks of age at the beginning of the study were individually housed in cages with shredded filter paper and wooden blocks for enrichment and to prevent malocclusion from receiving a liquid-only diet. Mice were acclimated for 2 weeks in temperature (22°C), light, and humidity-controlled (40–60%) conditions with a 12 h light/dark cycle. During acclimation weeks, mice were given free access to the liquid Lieber DeCarli diet with no ethanol (Bio-Serv, Flemington, NJ, USA) following the model described by Bertola et al. (34), with modifications. After the acclimation period, mice were randomly assigned to groups (where feed was given *ad libitum*): (1) No-EtOH (n = 12); (2) EtOH [(n = 12) 5.5% (v/v)]-containing LDC diet; and (3) DHM (n = 12; 6 mg/mL) + EtOH-containing LDC diet, for a total of 5 weeks. Every morning, fluid intake was recorded by measuring the meniscus on the graduated feed tube. Mice in the DHM group were exposed to ethanol-only for 2 weeks prior to DHM supplementation, which lasted for the remainder of the study; the feeding paradigm was isocaloric between groups. DHM, [(2R, 3R)-3, 5, 7-trihydroxy-2-(3, 4, 5-trihydroxyphenyl)-2,3-dihydrochromen-4-one], HPLC grade, >98%, MW 320.25 was purchased from Master Herbs Inc., Pomona, CA. The LDC diet is a robust forced-drinking model that induces severe liver disease with a potential for a high mortality rate. Therefore, for this proof-of-concept study, a single dose of 6 mg/mL was used as a comparison to the 10 mg/kg dose delivered via i.p. in our previous publications. After the study period ended, mice were euthanized via CO_2 exposure followed by cardiac puncture. Blood was collected and kept at room temperature for 45 min and serum was separated by centrifugation for 10 min at 10,000 × g in 4°C and stored at −80°C until use; livers were harvested and frozen in nitrogen-isopentane and stored at −80°C until use or fixed in 10% formalin and embedded in paraffin. Animals used in the study were considered and handled in adherence to the University of Southern California's Department of Animal Resources Institutional Animal Care and Use Committee (IACUC) policies and guidelines.

2.2. Immunohistochemistry

Lipid droplets were stained using Oil Red O Staining Kit (Lifeline Cell Technology, San Diego, CA, USA) on frozen liver sections (10 μm thick). Liver sections were also stained with Hematoxylin and Eosin (H&E) staining kit (Abcam, Boston, MA, USA). Antibodies against p62/SQSTM-1 (1:400, Cell Signaling Technology, Danvers, MA, USA); LC3B (1:1,000, Cell Signaling Technology, Danvers, MA, USA); PLIN-1 (1:200, Cell Signaling Technology, Danvers, MA, USA); anti-CD68 (1:250, Cell Signaling Technology, Danvers, MA, USA); and Alexa Fluor 405, 488, and 647 secondary antibodies (1:250, Cell Signaling Technology, Danvers, MA, USA) were used for visualization. Images were acquired using Cytation 5 Cell Imaging Multi-Mode Reader (BioTek, Winooski, VT, USA) and Zeiss LSM880 w/Airyscan Confocal Laser Scanning Microscope (Carl Zeiss Microscopy, White Plains, NY, USA), and were analyzed using ImageJ software (ImageJ; Coloc2 Fiji software) and Zen (Black and Blue versions) imaging analysis software (Carl Zeiss Microscopy, White Plains, NY, USA). Lipid droplet density and size were analyzed using whole image analysis on the ImageJ Color Threshold software.

2.3. Immunoblotting

Protein expression (Atg7, PLIN-1, p62, CETP, and LCAT) was analyzed using protein extracts (60–125 mg for immunoprecipitation) from liver homogenates that were isolated using Dynabeads Magnetic Beads (Thermo-Fisher Scientific, MA, USA), and visualized via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), where bands were detected by chemiluminescent reaction. Signal density was quantified by densitometry using ImageJ software: Atg7, PLIN-1, p62/SQSTM-1, CETP, and LCAT were analyzed using immunoprecipitation, while LC3B and Beta-actin antibodies purchased from Cell Signaling Technology, CA were analyzed from Western blots (diluted 1:1,000, while 10 μg of antibody was used for IP).

2.4. Biochemical assays

The following assays were measured from serum: aspartate and alanine aminotransferase (AST and ALT) levels were measured using AST and ALT activity assays (Sigma Aldrich, St. Louis, MO, USA). Free cholesterol and cholesteryl esters were measured using the Cholesterol Fluorometric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). LDL/VLDL levels were measured using the Cholesterol Assay Kit (Abcam, Boston, MA). Circulating triglyceride levels were measured from liver homogenates (∼100 mg) and serum using Triglyceride Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA).

Mitochondrial oxidative phosphorylation system (OXPHOS) in complexes I, II, and IV were analyzed using Complex I Enzyme Activity Colorimetric Assay Kit, Complex II Enzyme Activity Microplate Assay Kit, and the Complex IV Rodent Enzyme Activity Microplate Assay Kit (Abcam, Boston, MA, USA) using isolated mitochondria that were purified from the liver tissues using

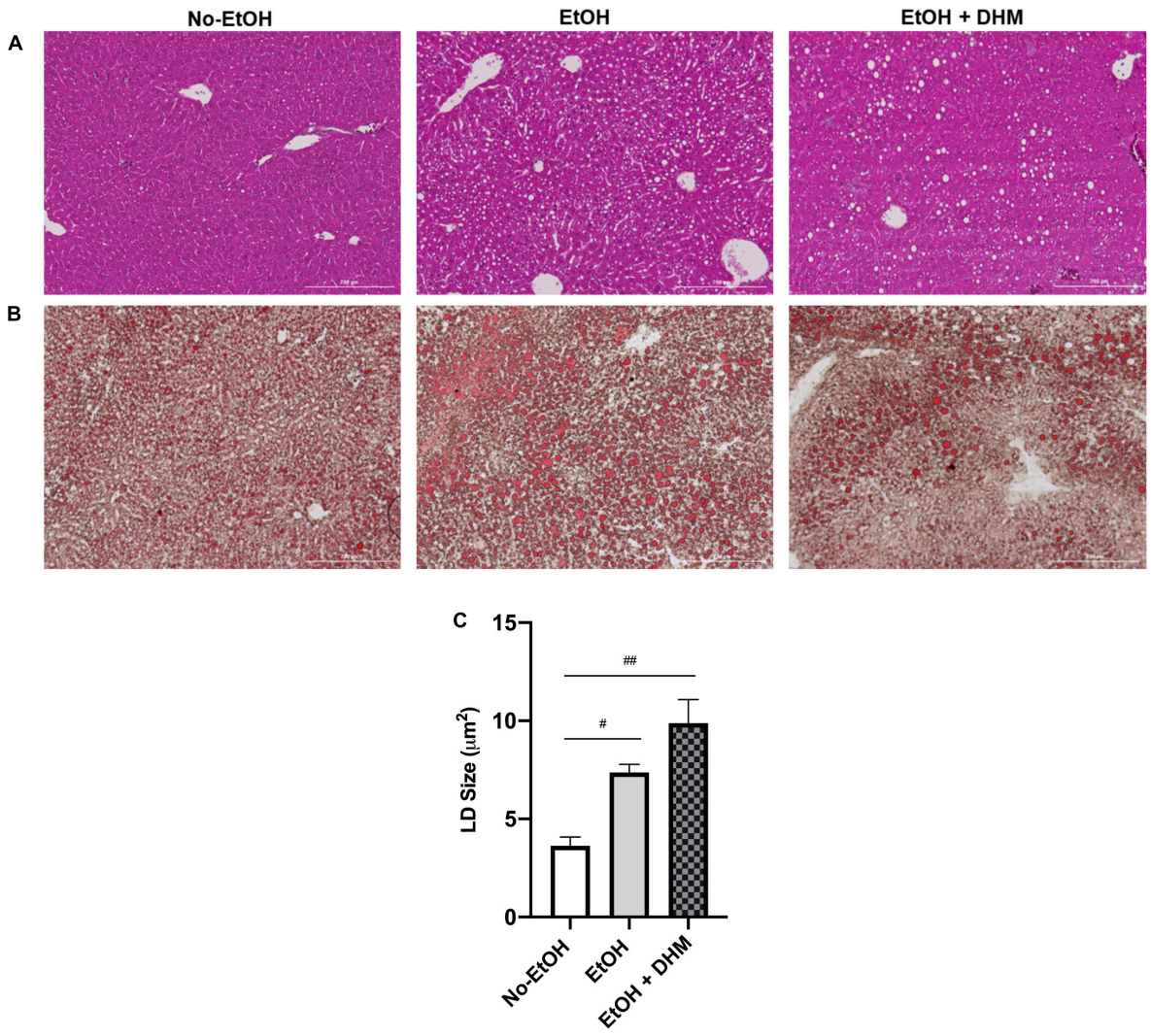

FIGURE 1
DHM administration results in heterogeneous lipid droplet size and distribution. Histology images (scale bars: 200 μm) shown are **(A)** Hematoxylin and Eosin and **(B)** Oil Red-O-stained liver sections demonstrating heterogeneity in LD size and distribution between groups (white circles); **(C)** Lipid droplet size in each group ($^{\#}0.0063$, $^{\#\#} < 0.0001$).

the Mitochondria Isolation Kit for Tissue (Abcam, Boston, MA, USA).

Cytokine levels were measured using Proteome Profiler Array Mouse Cytokine Array Kit Panel A (R&D Systems, Minneapolis, MN, USA) and signal density was quantified by densitometry using ImageJ software.

2.5. Statistical analysis

Immunohistochemistry images were analyzed using n = 3–4 from each group and 4 different sections were analyzed per sample. Biochemical assays were conducted using 3–4 samples from each group. Data are presented as means ± standard deviation. Statistical analysis included one-way ANOVA along with Bonferroni multiple comparison tests using Prism 9.3 (GraphPad Software, Inc., San Diego, CA, USA), where $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. DHM administration ameliorates ethanol-induced changes in hepatic and circulating lipid content while improving aminotransferase levels

To investigate the utility of oral DHM, mice in the DHM group received ethanol-only treatment for 2 weeks prior to DHM supplementation to assure the initiation and development of ALD pathology. A hallmark of early ALD is hepatic steatosis, characterized by the accumulation of LDs throughout the liver and disruptions in lipid homeostatic conditions (35–37). H&E and Oil Red O-staining of liver tissue sections demonstrated increased steatosis in the EtOH group which was alleviated by DHM treatment (**Figures 1A, B**; scale bars 200 μm). LDs are synthesized by nearly all cells, and size varies considerably among

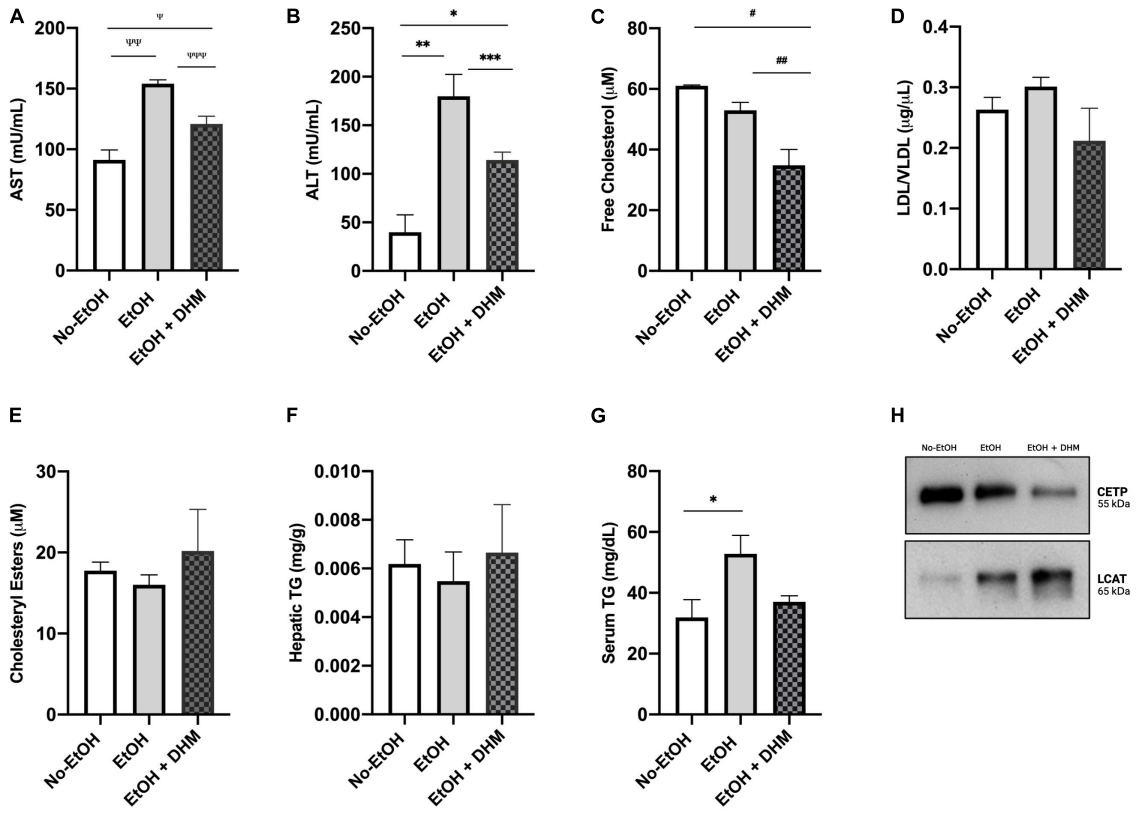

FIGURE 2
DHM administration ameliorates ethanol-induced changes in circulating and hepatic lipid content and improves aminotransferase levels. DHM effect on levels of circulating: **(A)** Aspartate aminotransferase (AST) levels ($^{\Psi}$0.029, $^{\Psi\Psi}$0.0006, and $^{\Psi\Psi\Psi}$0.024), **(B)** Alanine aminotransferase (ALT) levels (*0.027, **0.0014, and ***0.0046), **(C)** Circulating levels of free cholesterol ($^{\#}$0.0097 and $^{\#\#}$0.0227), **(D)** Levels of LDL/VLDL are reduced in the DHM group, **(E)** Levels of cholesteryl esters are increased in the DHM group, **(F)** Levels of hepatic triglycerides are increased in the DHM group, **(G)** Levels of circulating triglycerides are reduced in the DHM group (*0.022), and **(H)** immunoprecipitation of CETP expression is reduced with DHM and levels of LCAT are increased in the group fed DHM.

different cell types in response to environmental cues, particularly in the liver. Chronic ethanol consumption alters hepatocyte LD properties, including increased size and cellular distribution (38). Interestingly, mice in the DHM group exhibited a wide range of lipid accumulation and distribution in addition to significantly larger LDs, compared to all groups. In a previous study, it was shown that heterogeneous lipid distribution within the hepatocyte population, similar to what is observed in the DHM group, is a potential hepatoprotective social organization that reduces lipotoxicity within the overall region, compartmentalizing lipids within a cell population (39). The authors also reported that LD heterogeneity is not only reversible and variable (depending on intracellular-environmental factors) but also allows for the reduction of lipotoxicity between cells by exchanging LD content over time. We found a wide range of noticeable hepatocyte-to-hepatocyte LD heterogeneity, which was more prominent in the group receiving DHM. LD size was also found to be varied across groups, with the mean LD sizes in the No-EtOH group measuring at 3.64 μm^2, EtOH-only at 7.37 μm^2, and DHM measuring at 9.88 μm^2. Mice receiving EtOH had larger LDs than the No-EtOH group ($^{\#}$0.0063; Figure 1C), and the difference was even greater in the mice fed DHM ($^{\#\#} < 0.0001$; Figure 1C).

As a measure of overall hepatic health and function following DHM administration, we next analyzed the levels of circulating aspartate and alanine aminotransferases (AST and ALT). As shown in Figure 2A, there was a significant decrease in AST levels in mice receiving oral DHM compared to mice in the EtOH-only group ($^{\Psi}$0.029, $^{\Psi\Psi}$0.0006, $^{\Psi\Psi\Psi}$0.024); Figure 2B shows significantly lowered levels of ALT in mice receiving oral DHM when compared to the EtOH-only fed mice (*0.027, **0.0014, and ***0.0046). The levels of circulating lipids were measured and show that DHM administration significantly lowers total free cholesterol levels, ($^{\#}$0.0097, $^{\#\#}$0.0227; Figure 2C). Although not significant, the levels of LDL/VLDL were reduced with DHM administration, like those measured in the No-EtOH group (Figure 2D). When interpreting the results obtained from this study, it is important to note that the LDC diet is considered as a high fat diet, where 35% of calories are derived from FFAs: 23.5 g/L of monounsaturated and 5.2 g/L unsaturated fats. The No-EtOH group is isocaloric to the other groups and therefore is also receiving the high fat diet, which may influence the amount and types of lipids in circulation compared to the EtOH-receiving groups.

Lipid droplets are primarily composed of triglycerides (TGs) and cholesteryl esters (CE) (37). Although TGs are not considered determinants of lipotoxicity (6), conversion of free fatty acids (FFAs) into TGs, as well as FFA utilization in CEs via esterification (40), essentially acts to neutralize the reactivity of and damage caused by excessive FFAs (41, 42). Accordingly, cholesteryl esters

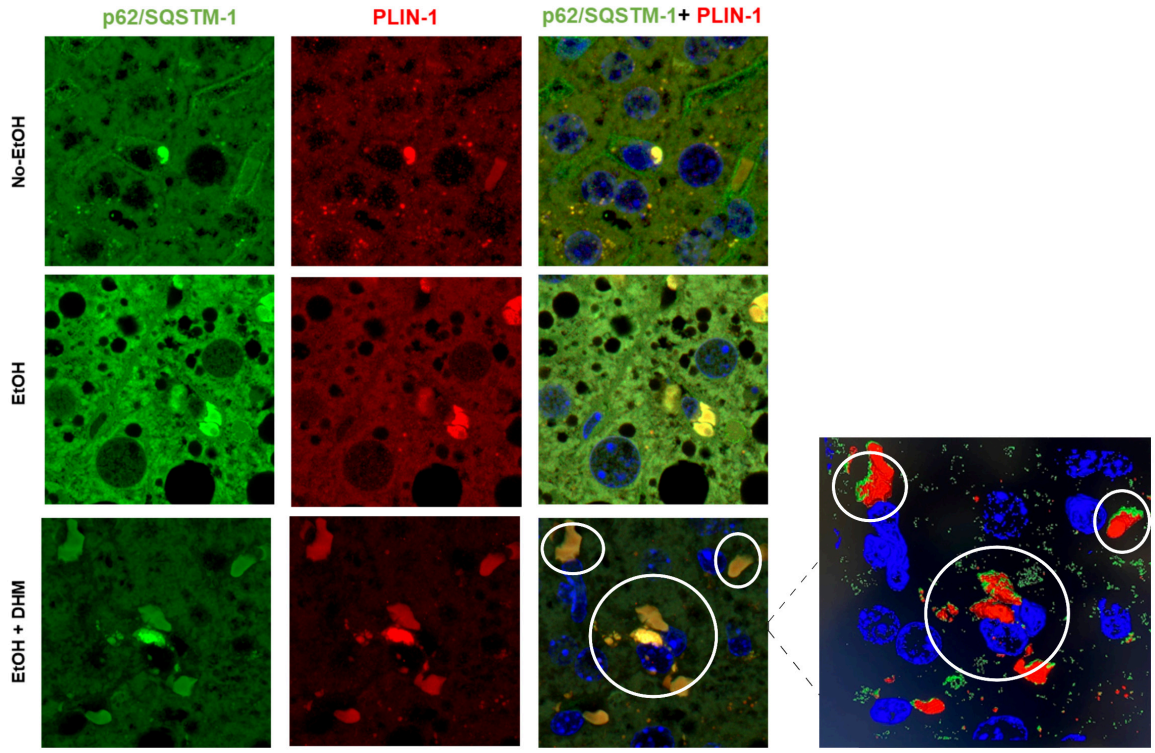

FIGURE 3
DHM increases ethanol-induced colocalization events between p62/SQSTM-1 and perilipin 1 (PLIN-1). Confocal images (scale bars 5 μm) from the livers of mice receiving the LDC diet confirm the presence and interaction between p62/SQSTM-1 and perilipin 1 (PLIN-1). As shown: p62/SQSTM-1 (green), perilipin 1 (red), and nuclei (blue). Colocalization events are circled between p62/SQSTM-1 + PLIN-1 (yellow/brown). The magnified image from the DHM group highlights the nuclei (blue), p62/SQSTM-1 (green), and PLIN-1 (red) and the magnitude and distribution of colocalization events on lipid droplets.

and hepatic TG levels were found to be increased in the group receiving DHM (Figures 2E, F), while circulating TG levels were normalized by DHM to levels similar to those measured in the No-EtOH group (*0.022; Figure 2G). Two facilitators of cholesterol exchange and transport are cholesteryl ester transfer protein (CETP) and lecithin-cholesterol acyltransferase (LCAT). CETP is a known mediator in the transfer of cholesteryl esters from HDL to LDL/VLDL (43). LCAT is a key enzyme involved in the esterification of free cholesterol into cholesteryl esters and facilitates the metabolism of cholesterol (44). As demonstrated in Figure 2H, we found that CETP expression was increased in the EtOH-only group compared to the DHM group, and found that LCAT expression was highest in the DHM fed group.

3.2. DHM fed mice demonstrate increased colocalization of lipophagy proteins, p62/SQSTM-1, perilipin 1 (PLIN-1) and LC3B

Lipid droplet membranes are coated with various components, including lipid droplet-associated proteins belonging to the perilipin family (PLIN-1-5) that assist in the regulation of LD synthesis and cytosolic lipase activity. Lipophagy involves the recruitment of selective autophagy proteins such as sequestosome-1 (p62/SQSTM-1), microtubule-associated protein 1 light chain 3 beta (LC3B), and PLIN-1, which when combined are recognized as defense mechanisms against oxidative stress (45–48). Ethanol is known to trigger the selective interactions of p62/SQSTM-1, LC3B, and PLIN-1 using *in vitro* models during LD clearance (45). To assess the effect of DHM on ethanol-induced interactions between selective autophagy-associated proteins, we analyzed the presence and interactions between p62/SQSTM-1; LC3B; and PLIN-1. We began by confirming the interaction between p62/SQSTM-1 and PLIN-1 using liver sections that were stained for p62/SQSTM-1 (green), PLIN-1 (red), and nuclei (blue). As illustrated in Figure 3 (scale bars 5 μm), we confirmed the presence of and interactions between p62/SQSTM-1 and PLIN-1 in all three groups as shown from their colocalization. Mice receiving ethanol had noticeably higher levels of p62/SQSTM-1 + PLIN-1 interactions than the No-EtOH group (Figure 4B). DHM-fed mice had even greater colocalization events between PLIN-1 and p62/SQSTM-1, where the interactions were widely distributed across LD surfaces, compared to the EtOH-only group (Figure 3 inset).

Autophagy is a diverse mechanism that follows several pathways based on cellular demands. There are over 32 different autophagy-related genes (Atg) that activate the formation of double-membrane structures that deliver cytoplasmic components to lysosomes for degradation. Atg7 is a ligase that has ubiquitin E1-like activity which facilitates interactions and complexations between other autophagy-related genes. These subsequently interact with other Atg proteins, forming a much larger complex

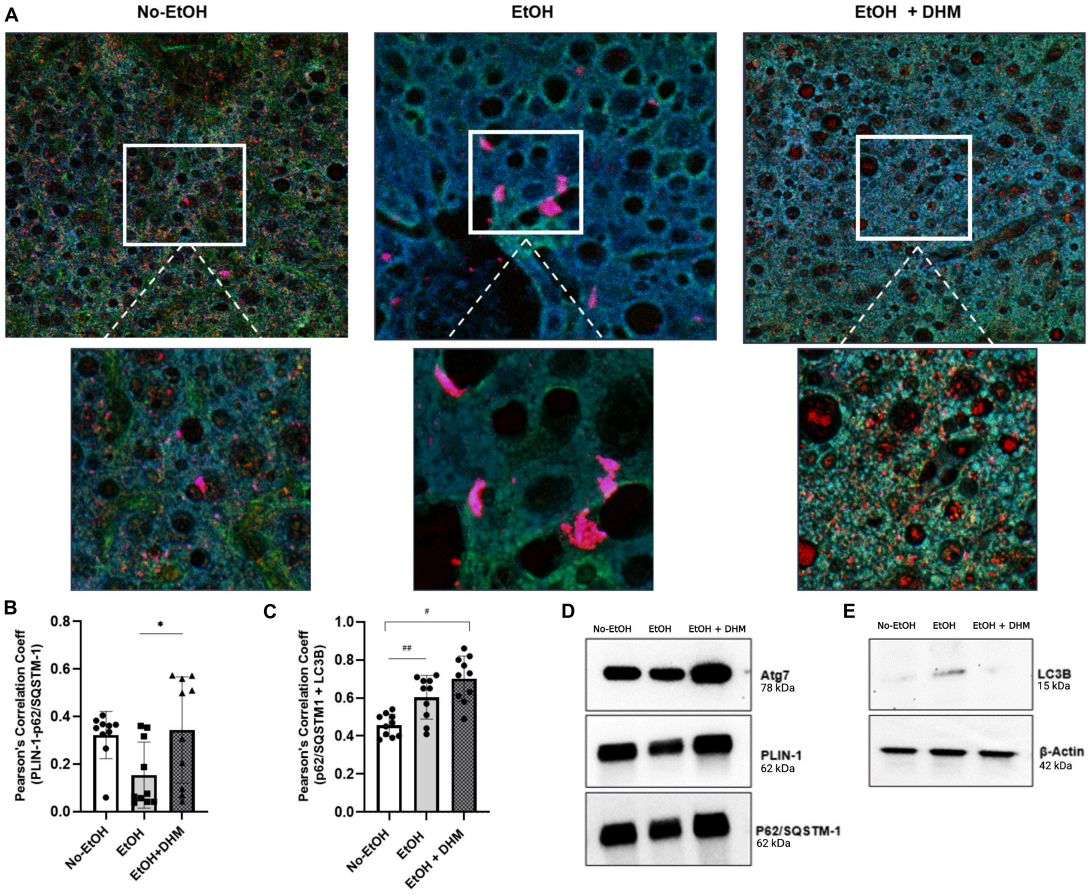

FIGURE 4
DHM enhances the colocalization and expression of lipophagy-associated proteins in mice exposed to chronic ethanol. DHM administration increases the colocalization and interaction between p62/SQSTM-1 + PLIN-1 + LC3B. **(A)** Confocal images (upper and lower (magnified) scale bars on images are 10 and 5 μm, respectively) show the expression and colocalization of p62/SQSTM-1 (green), PLIN-1 (red), and LC3B (blue) between groups. **(B)** Colocalization of p62/SQSTM-1 + LC3B + PLIN-1 as quantified by Pearson's Correlation Coefficient (PCC) shows a significant increase in colocalization between PLIN-1 + p62/SQSTM-1 (*0.04; brown puncta). **(C)** Colocalization of p62/SQSTM-1 + LC3B is increased in mice fed EtOH (##0.008; cyan puncta) and highest in EtOH + DHM fed mice (# < 0.0001). **(D)** Differences in (immunoprecipitated) protein expression levels of Atg7, PLIN-1, and p62/SQSTM-1; **(E)** LC3B protein expression.

that binds to LC3B, a molecule that is essential for autophagosome structure, formation, and cargo recognition (47, 49). LC3B interacts with cargo adaptor protein (p62/SQSTM-1) that binds to poly-ubiquitinated cargo, and is a classical selective autophagy receptor (48).

Selective autophagy occurs when p62/SQSTM-1 and LC3B interact (50), resulting in the formation of an autolysosome that is directed to ubiquitinated PLIN-1 proteins found on LDs, resulting in lipophagy activity. The colocalization of p62/SQSTM-1 + PLIN-1 + LC3B is illustrated in Figure 4A (upper and lower magnified images scale bars are 10 and 5 μm, respectively). Additionally, we quantified levels of interactions by measuring the correlation between two proteins using Pearson's Correlation Coefficient (PCC) analysis, where values closer to 1.0 confirm the strength of correlation. As shown, interactions between PLIN-1 + p62/SQSTM-1 were significantly higher in mice receiving DHM with a mean PCC of 0.344 (*0.04; brown puncta), compared to the EtOH-only group with a mean PCC of 0.154 (Figure 4B). Our results also show that mice in the group receiving EtOH-only demonstrated an increased colocalization of p62/SQSTM-1 + LC3B (## 0.008; cyan puncta) when compared to the No-EtOH group; the increase was more apparent when comparing the No-EtOH group to mice fed DHM (# < 0.0001) (Figure 4C). Our data show that DHM-fed mice had a greater and wider range of colocalization events and activity. Quantification (via immunoprecipitation) of the expression levels of Atg7, PLIN-1, p62/SQSTM-1 were analyzed (Figure 4D), and LC3B: β-Actin (via Western blots) are shown in Figure 4E, supporting the findings from histological colocalization analyses.

3.3. DHM reverses ethanol-induced reductions in mitochondrial oxidative phosphorylation activity

Chronic ethanol consumption leads to loss of mitochondrial function and increased production of reactive oxygen species (ROS), promoting oxidative stress, particularly in the mitochondria. Damage, brought on by increases in ROS to mitochondrial proteins and DNA, decreases mitochondrial

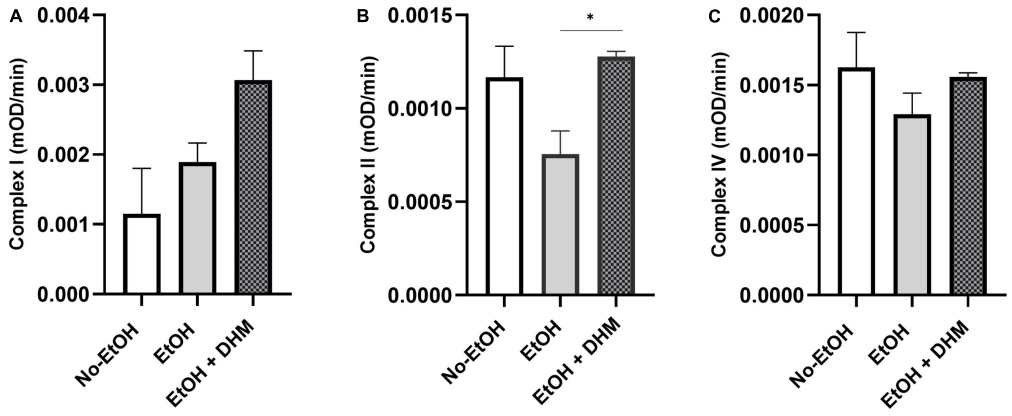

FIGURE 5
DHM reverses ethanol-induced reductions in mitochondrial function. DHM-fed mice demonstrate improvement in mitochondrial oxidative phosphorylation systems as shown by restored activity in **(A)** complex I, **(B)** complex II (*0.03), and **(C)** complex IV.

function due to the breakdown of these complexes (51). Lipid metabolism takes place in the mitochondria, where fatty acids undergo β-oxidation. We measured mitochondrial health by analyzing the activity of complexes I, II, and IV from isolated mitochondria. Our results show that DHM had a significant effect on restoring complex II activity (*0.03; Figure 5B). Although the changes were not significant, DHM led to increases in complex I activity (Figure 5A), while normalizing the activity of complex IV (Figure 5C).

3.4. DHM supplementation reduces pro-inflammatory and hematopoietic cytokines

Next, we investigated the effects of DHM on ethanol-induced inflammation. The livers of mice were stained with CD68, a biomarker for immune cells of the monocyte lineage, such as monocytes and macrophages. As illustrated in Figure 6, mice in the EtOH-only group had larger bursts of monocyte/macrophage infiltration clouds (green puncta) when compared to the No-EtOH and DHM-fed mice. These observations were further confirmed by conducting a cytokine panel assay that measured several different circulating pro-inflammatory cytokines from serum (Supplementary Figure 1). We found that oral DHM administration reduced circulating levels of pro-inflammatory cytokines and immune cell chemokines that are traditionally associated with ethanol-induced inflammation. TNF-α promotes acute inflammation and is one of the critical inflammatory cytokines in ALD progression and liver injury, as it contributes to the production of other pro-inflammatory cytokines (33). DHM supplementation reversed the significant elevations in TNF- α levels in mice receiving ethanol-only as demonstrated in Figure 7A (*, **, and ***< 0.0001), while also normalizing levels of IFN-γ ($^{\#}$< 0.0001, $^{\#\#}$0.0018, and $^{\#\#}$0.001) closer to those of the No-EtOH group (Figure 7B). DHM receiving mice had reduced levels of IL-1β ($^{\Psi}$< 0.0001, $^{\Psi\Psi}$0.027, and $^{\Psi\Psi\Psi}$0.0007) compared to the EtOH-only mice, with levels close to the No-EtOH group (Figure 7C). Although IL-1β is not produced in a healthy liver, it is secreted by activated inflammasomes during excessive alcohol consumption and is an essential cytokine in giving rise to Th17 cells that subsequently secrete IL-17 (52, 53).

Mice receiving EtOH-only had significantly higher levels of circulating IL-17 overall, which was ameliorated with DHM supplementation (Figure 7D; ^{ŧ}< 0.0001, ^{ŧŧ}0.003, and ^{ŧŧŧ}< 0.0001). IL-17 is a potent pro-inflammatory cytokine that has received much attention for its synergistic effects with other inflammation promoting cytokines during ALD pathogenesis that were also reduced in the DHM-fed group, such as IL-6 (Figure 7E; *0.007), IL-1β, and IL-1α (Figure 7F; ^{ŧ}< 0.0001, ^{ŧŧ}0.047, and ^{ŧŧŧ}< 0.0001) (52–54). IL-17 induces the expression of hematopoietic cytokines and chemokines such as granulocyte-macrophage-colony stimulating factor (Figure 8A: GM-CSF; $^{\#}$< 0.0001, $^{\#\#}$ 0.003, and $^{\#\#\#}$0.0001), macrophage-colony stimulating factor (Figure 8B: M-CSF; $^{ŧ,ŧŧ,\ ŧŧŧ}$< 0.0001), granulocyte-colony stimulating factor (Figure 8C: G-CSF; *< 0.0001, **0.005, and ***0.0004), neutrophil activating and chemotactic chemokine, CXCL1 (Figure 8D; $^{\#}$< 0.0001, $^{\#\#}$0.0001, and $^{\#\#\#}$< 0.0001), and B-cell recruiting CXCL13 (Figure 8E; *0.023, and **0.002) (53, 55–58). DHM-fed mice had lower levels of IL-3 (Figure 8F; $^{\#}$0.035), a cytokine that amplifies acute inflammation (59) and works in coordination with GM-CSF to promote pathogenic clearance during chronic inflammation (60). Additionally, CXCL2/MIP-2 (macrophage inflammatory protein-2), is synthesized by a variety of immune cells to recruit neutrophils in response to damage and acute liver injury (61). CXCL2 was significantly increased in mice receiving EtOH-only, and that increase was reversed with DHM supplementation (Figure 8G; $^{\#,\#\#}$< 0.0001).

3.5. DHM supplementation increased production of protective anti-inflammatory cytokines in mice treated with ethanol

Mice receiving DHM supplementation had increased anti-inflammatory cytokine levels compared to mice fed EtOH-only. IL-1ra is a receptor antagonist to members of the IL-1 family of

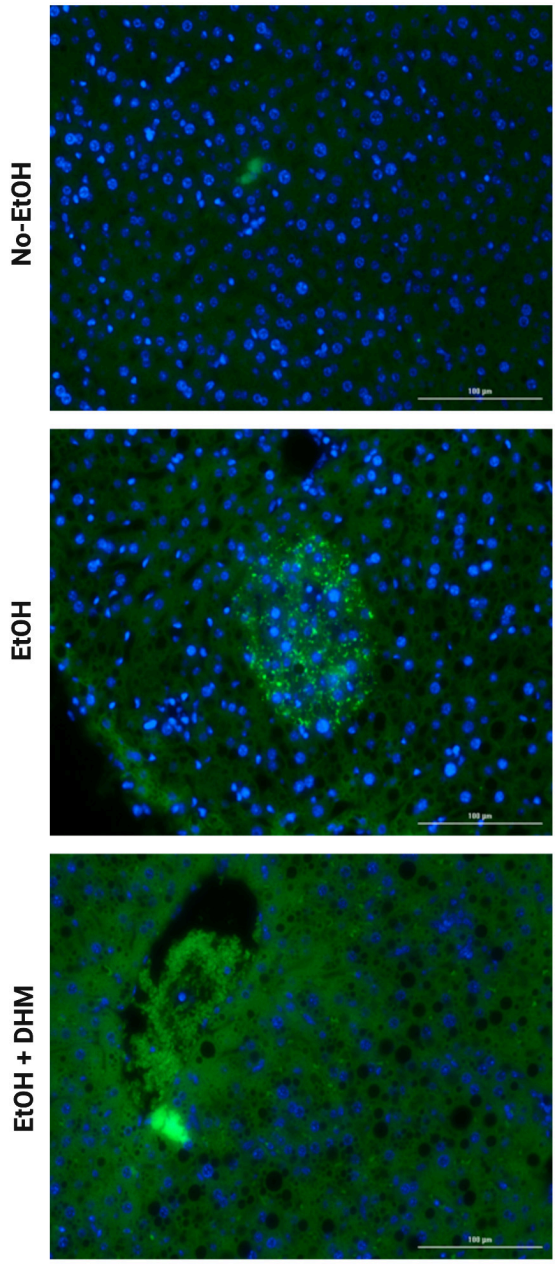

FIGURE 6
DHM reduces the increase in monocyte infiltration seen in mice exposed to chronic EtOH. Images (scale bars: 100 μm) demonstrating smaller bursts of monocyte infiltration clouds (green puncta) when compared to mice in the EtOH-only group.

pro-inflammatory cytokines, and has neutralizing and protective effects against IL-1 activity (62). Mice in the DHM group had levels of IL-1ra that were nearly identical to the No-EtOH group and significantly lower than the EtOH-only group (**Figure 9A**; *, ** < 0.0001). Following excessive ethanol intake and burn injury, IL-27 has been shown to promote liver regeneration by enhancing liver progenitor cell expansion and differentiation as well as intestinal barrier repair following ethanol intoxication (63, 64). In our study, as shown, DHM-fed mice had significantly higher levels of IL-27 in circulation than that of the No-EtOH group (**Figure 9B**; *0.034) and nearly twice as much as those in the EtOH-only group.

4. Discussion

Despite the detrimental health effects associated with high ethanol intake, individuals continue to partake in excessive drinking behavior, as evidenced by the increasing rates of alcohol sales and alcohol-related mortality and morbidity. ALD is the leading cause of liver disease in the United States, where alcohol accounts for up to 50% of cirrhosis-related mortality (65) and 20% of mortality worldwide (66). Therefore, targeting ethanol-induced steatosis and the mechanisms that lead to the dysregulation of lipid homeostasis are key for preventing lipotoxicity and the systemic metabolic dysfunction that eventually affects multiple organ systems (6). Considering the growing interest and consumer preference for herbal therapies (i.e., polyphenols and flavonoids), the present study tested the hypothesis that the bioactive polyphenolic-flavonoid DHM, improves ethanol-induced lipid imbalance and steatosis in part by restoring lipophagy activity and reducing pro-inflammatory cytokines. The data presented here supports the hypothesis that DHM can counteract the progression of ALD pathology caused by damage due to inflammation and the dysregulation in lipid homeostasis due to chronic ethanol consumption.

The liver is the primary site for the breakdown of ethanol and is one of the major organs for lipid metabolism and is, therefore, highly susceptible to damage and lipotoxicity. Elevated levels of lipids is a major factor that leads to hepatic injury caused by lipotoxicity and oxidative stress. Cellular defense mechanisms neutralize FFAs via their conversion into TGs through esterification. Lipid droplets are primarily composed of TGs and CEs, acting as energy stores, subsequently minimizing the lipotoxicity of FFAs that would otherwise occur in the cell (37, 42). Cholesteryl esters are reverse transported to the liver from circulation and peripheral tissues via high-density lipoproteins, where they are stored in LDs or metabolized for bile acid synthesis (67). The increased levels of CEs, hepatic TGs, and LD size in our study, combined with the reduction in circulating TGs, suggest increased synthesis and hepatic sequestration of TGs and CEs in the DHM-fed mice. Taken together, the results from our study indicate the possibility of increased FFA neutralization and containment in LDs as a protective measure against lipotoxicity by FFAs. Chronic ethanol consumption disturbs metabolic flux through various pathways. As mentioned earlier, the LDC diet is regarded as a high fat diet, providing excess dietary free fatty acids to all groups. Future studies will also consider the effect of a high fat diet when measuring circulating lipid content in the No-EtOH group(s) and comparing them to EtOH-fed group(s).

Chronic ethanol consumption alters metabolic processes, including hepatocyte LD properties that include LD membrane protein composition, resulting in increased size and differential tissue distribution (38). Lipophagy, a subtype of macroautophagy, is associated with the degradation of LDs via engulfment by autophagosomes and subsequent fusion with lysosomes. Ethanol can stimulate autophagy through multiple mechanisms, including the modulation of mammalian target of rapamycin (mTOR) through AMPK signaling pathways. Excessive ethanol consumption is associated with decreased AMPK activation, which in turn activates mTOR in the liver and inhibits autophagy (21, 68). Previous work found that DHM can improve autophagy

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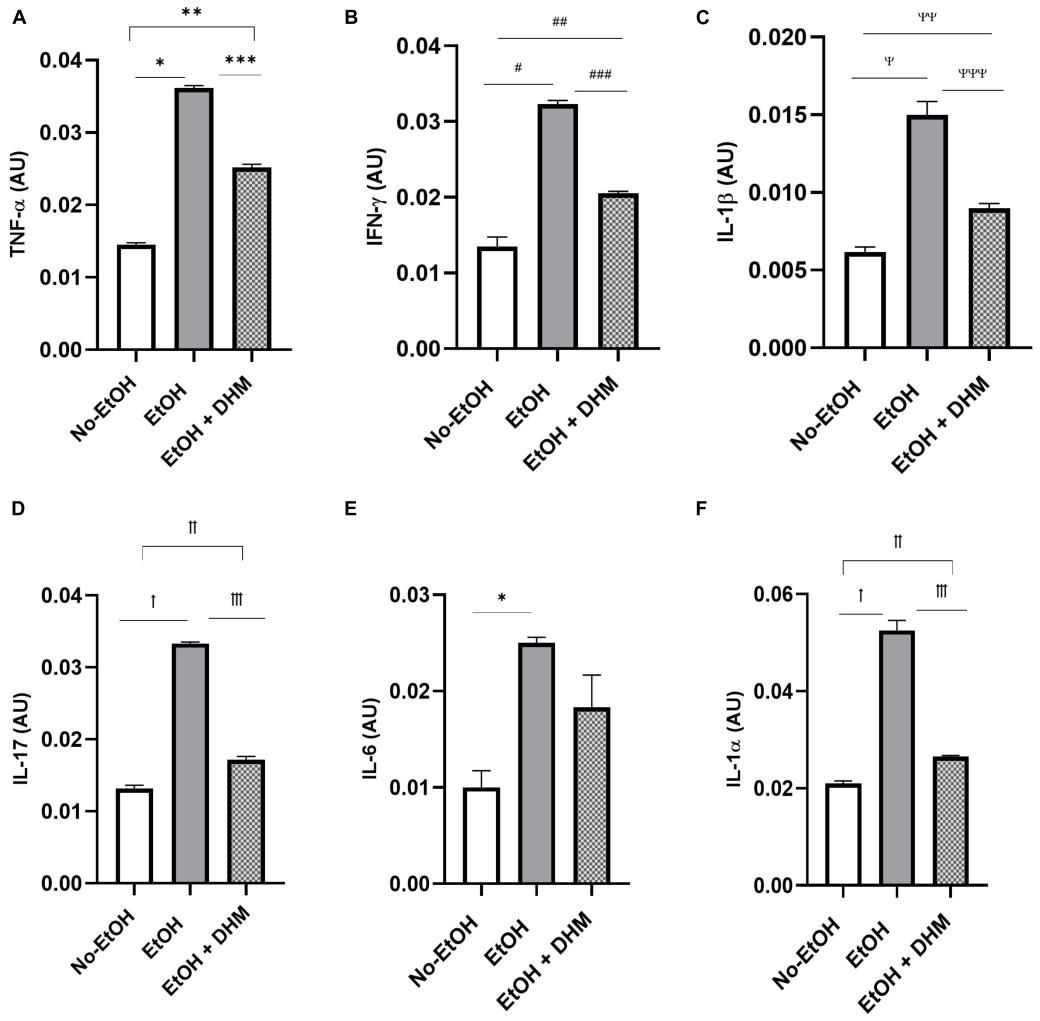

FIGURE 7
DHM supplementation ameliorates elevations in pro-inflammatory cytokines seen in mice given chronic EtOH. **(A)** DHM-fed mice show significant decreases in levels of TNF-α compared to EtOH-only mice (*, **, and *** < 0.0001). Normalization of levels of **(B)** IFN- γ ($^{\#}$ < 0.0001, $^{\#\#}$0.0018, and $^{\#\#}$0.001) and **(C)** IL-1β ($^{\Psi}$ < 0.0001, $^{\Psi\Psi}$0.027, and $^{\Psi\Psi\Psi}$0.0007) to those similar to those in the No-EtOH group is shown. DHM-fed mice show significant decreases in **(D)** IL-17 (‡ < 0.0001, ‡‡0.003, and ‡‡‡ < 0.0001) compared to EtOH-only mice. EtOH-only mice show a significant increase in **(E)** IL-6 (*0.007) expression compared to No-EtOH mice. **(F)** IL-1α (‡ < 0.0001, ‡‡0.047, and ‡‡‡ < 0.0001) levels are significantly reduced in DHM-fed mice compared to EtOH-only mice.

activity by activating AMPK, inhibiting mTOR, and reversing ethanol-induced AMPK-deficiency (21, 69, 70). Results from the current study demonstrate the downstream activity of AMPK-autophagy related activity and offer a glimpse into the possible downstream effects on lipophagy, as demonstrated by the enhanced expression and interactions between lipophagy protein complexes p62, LC3B, and PLIN-1.

Members of the PLIN family of LD-associated proteins are essential for regulating triglyceride synthesis, packaging TGs into LDs, and lipolysis. PLIN-1 positively contributes to the formation of larger LDs and is expressed on the membranes of larger, more mature LDs (36, 38, 71, 72). Studies have also shown that lipolysis is enhanced and regulated via proteasomal degradation of PLIN1 (73–75). Activation of protein kinase A (PKA) via AMPK signaling leads to the phosphorylation of PLINs (76), which are then subjected to ubiquitination and are tagged for proteasomal degradation. This results in effective priming of LD surfaces for recognition and recruitment of autolysosomal bodies through the activities of selective mechanisms such as those directed by p62/SQSTM-1 (46, 77). Lipolysis and lipophagy are tandem pathways in hepatocytes. Lipolysis is the process in which FFAs are released from TGs, which takes place during lipophagy (in lysosomes), and preferentially targets the degradation of large LDs. The increased presence of PLIN-1 and interactions with p62 and LC3B in the DHM-fed mice demonstrates the possibility of enhanced lipophagic activity, which potentially results in greater lipid clearance over time.

Released FFAs are then further broken down in the mitochondria, where they undergo β-oxidation. This system works in tandem with the oxidative phosphorylation system (OXPHOS), which is located in the mitochondrial inner membrane, composed of four respiratory chain complexes (I-IV), and is key for driving ATP production (51). As such, mitochondrial health and function is determined by the analysis of OXPHOS-complex

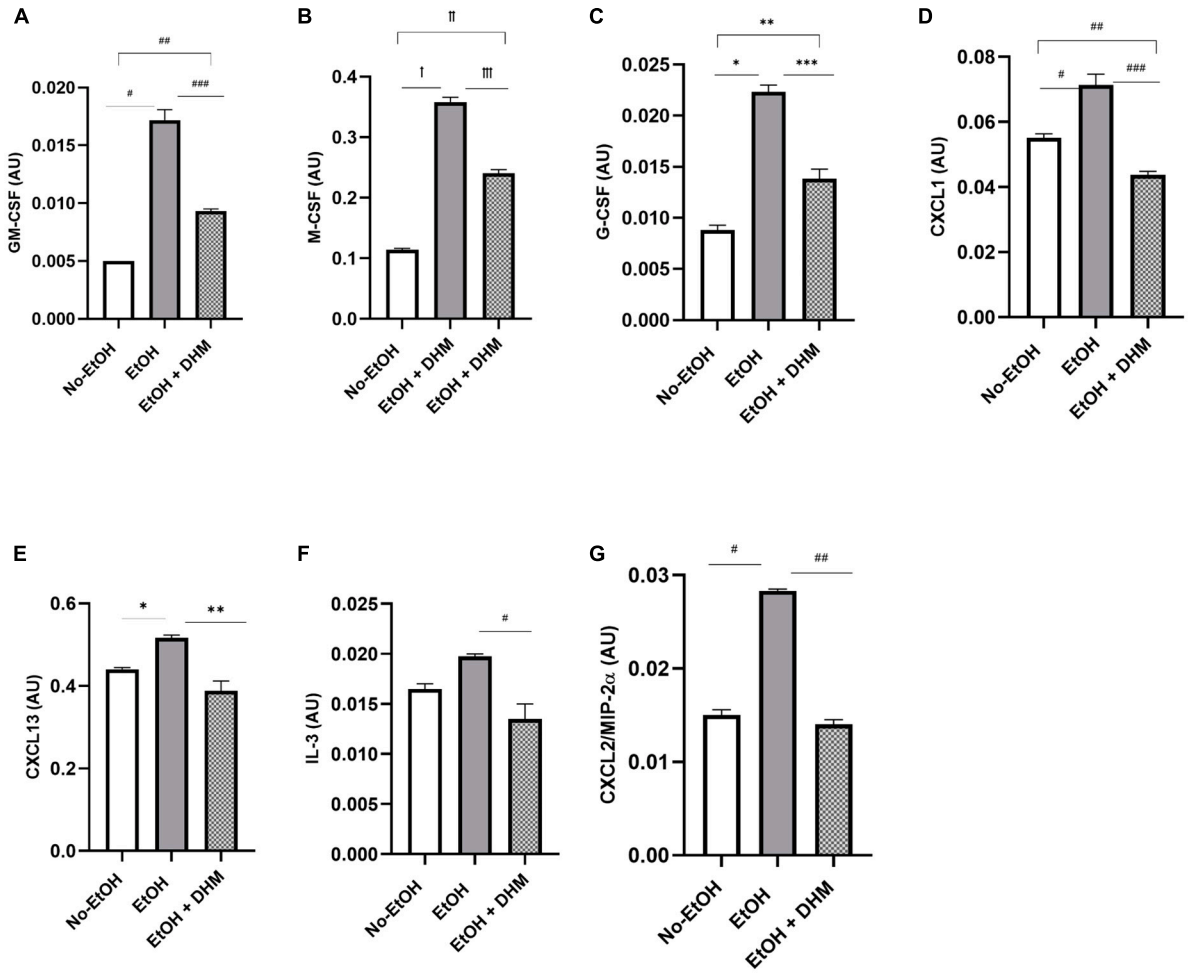

FIGURE 8
DHM administration reduces levels of hematopoietic cytokines and chemokines which are increased during chronic alcohol consumption. Expression of **(A)** granulocyte-macrophage-colony stimulating factor (GM-CSF; $^{\#} < 0.0001$, $^{\#\#}$ 0.003, and $^{\#\#\#}0.0001$), **(B)** macrophage-colony stimulating factor (M-CSF; $^{\dagger,\dagger\dagger,\ \dagger\dagger\dagger} < 0.0001$), and **(C)** granulocyte-colony stimulating factor (G-CSF; $^{*} < 0.0001$, $^{**}0.005$, and $^{***}0.0004$) significantly decreases with DHM administration. Expression of chemokines **(D)** CXCL1 ($^{\#} < 0.0001$, $^{\#\#}0.0001$, and $^{\#\#\#} < 0.0001$) and **(E)** CXCL13 ($^{*}0.023$ and $^{**}0.002$) is also reduced in DHM-fed mice. Expression of pro-inflammatory cytokine **(F)** IL-3 ($^{\#}0.035$) and pro-inflammatory chemokine **(G)** CXCL2 ($^{\#,\#\#} < 0.0001$) is reduced following DHM administration.

activity (78). Previous studies have demonstrated the effect of DHM on mitochondrial health, effectively reversing stress-induced deficiencies in mitochondrial function (19, 21, 79, 80). Our data demonstrates a positive effect of DHM on mitochondrial function restoration, particularly in Complex II. The increase in lipophagy activity is possible when mitochondrial function is efficient, as measured by complexes I, II, and IV. This data further supports the potential benefit of DHM on reversing ethanol-induced OXPHOS deficiencies and in turn, improving overall function.

In addition to direct induction of oxidative stress-induced inflammation, alcohol disrupts gut permeability, causing endotoxin/lipopolysaccharide (LPS) translocation to interact with TLR4 which results in the generation of inflammatory cytokines via NF-κB signaling pathway activation (31, 81). The increased oxidative stress and increased TLR4/NF-κB transcription upregulates and activates inflammasome, an intracellular protein complex that leads to the cleavage of pro-inflammatory cytokines like IL-1β (82). In ALD, pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-17 are produced by alcohol-induced activation of liver innate immunity (30). In addition, leukocyte chemoattractants and hematopoietic cytokines can recruit and proliferate immune cells in the liver, exacerbating the inflammatory response. IL-3 is a hematopoietic cytokine that regulates the differentiation, proliferation, and survival of various immune cells. DHM has been shown to suppress the production of hematopoietic cytokines that regulate the differentiation, proliferation, and survival of various immune cells such as IL-3, M-CSF, and G-CSF in LPS-stimulated macrophages (83). Furthermore, our study shows that DHM decreases the expression of CXCL1 and CXCL13, chemokines that are involved in the recruitment and activation of neutrophils and B cells, respectively, in hepatic inflammatory processes (84, 85). Evidence shows that inflammation plays an essential role in the initiation and progression of ALD (86). Results from our study support the published reports on the immuno-modulatory activity of DHM, and offer a glimpse on the protective effects of DHM

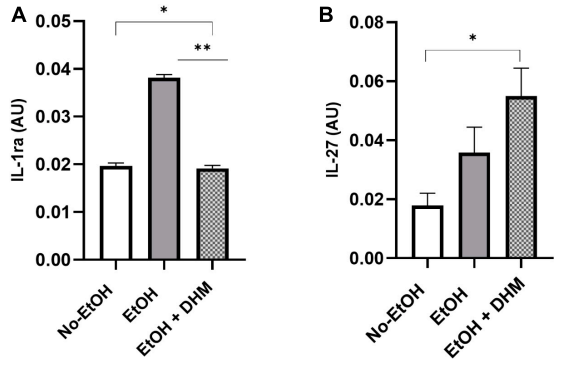

FIGURE 9
DHM increases production of anti-inflammatory cytokines compared to mice fed EtOH-only. Expression of **(A)** IL-1ra (*, ** < 0.0001) is significantly reduced in mice receiving DHM compared to those fed EtOH-only. In addition, levels of circulating **(B)** IL-27 (*0.034) in the DHM-fed group are significantly higher than that in the No-EtOH group.

against the damaging effects of ethanol-induced inflammation and injury (87, 88). The mechanism of action of DHM in reducing inflammation in ALD is believed to be multifactorial. As demonstrated in this study, DHM supplementation led to significant decreases in inflammatory signaling through reductions in the prominent pro-inflammatory cytokines associated with ALD pathology. Interestingly, DHM supplementation led to a significant increase in IL-27, a cytokine that has demonstrated protective action on the gut barrier by promoting anti-inflammatory functions, regenerative activity in the liver and intestines, and promoting intestinal barrier repair following ethanol intoxication and burn injury (63, 64).

The results from our study align with various animal and human studies investigating DHM for its robust antioxidant activity (79, 89), ability to reverse dyslipidemia (18, 21, 90, 91), having anti-alcohol intoxication effects (92), and amelioration of non-alcohol-associated fatty liver disease (19).

With the rates of ethanol-related health effects continuing to rise, particularly ALD, the need for therapeutic intervention is imperative. DHM is a natural compound that is widely available as a dietary supplement and has demonstrated the potential to mitigate the progression of ALD development caused by disruptions in lipid metabolism and transport in mice. As a natural product that is readily and commercially available, our findings help set the stage for the rapid advancement of DHM to improve liver health against the damaging effects of excessive ethanol consumption.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

Author contributions

IJ-U served as the project lead and conceptualized the contents and the project related to this manuscript. EC and DD contributed to the conception and design of the study. AI, MZ, MV, NS, SS, SC, ZZ, JW, and LA contributed either by gathering, analyzing, and/or interpreting data, as well as to the writing and intellectual content of the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1201007/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Cytokine Profile Arrays. Cytokines were measured from serum using a cytokine profile array such as the one shown here, where each dot blot represents treatment groups (U0781: No-EtOH; U0782: EtOH; U0784: EtOH + DHM).

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Ellagic acid effects on disease severity, levels of cytokines and T-bet, RORγt, and GATA3 genes expression in multiple sclerosis patients: a multicentral-triple blind randomized clinical trial

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Background: Multiple sclerosis (MS) is a chronic autoimmune disease. Ellagic acid is a natural polyphenol and affects the fate of neurons through its anti-inflammatory and antioxidant properties. The present study aimed to investigate ellagic acid effects on disease severity, the expression of involved genes in the pathogenesis of MS, and the levels of related cytokines.

Methods: The present study was a triple-blind clinical trial. Eligible patients were randomly assigned to two groups: Ellagic acid (25 subjects) for 12 weeks, receiving 180 mg of Ellagic acid (Axenic, Australia) and the control group (25 subjects) receiving a placebo, before the main meals. Before and after the study, the data including general information, foods intake, physical activity, anthropometric data, expanded disability status scale (EDSS), general health questionnaire (GHQ) and pain rating index (PRI), fatigue severity scale (FSS) were assessed, as well as serum levels of interferon-gamma (IFNγ), interleukin-17 (IL-17), interleukin-4 (IL-4) and transforming growth factor-beta (TGF-β), nitric-oxide (NO) using enzyme-linked immunoassay (ELISA) method and expression of T-box transcription factor (Tbet), GATA Binding Protein 3 (GATA3), retinoic acid-related orphan receptor-γt (RORγt) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were determined using Real-Time Quantitative Reverse Transcription PCR (RT-qPCR) method.

Findings: Ellagic acid supplementation led to a reduction in IFNγ, IL-17, NO and increased IL-4 in the ellagic acid group, however in the placebo group no such changes were observed (-24.52 ± 3.79 vs. -0.05 ± 0.02, $p < 0.01$; -5.37 ± 0.92 vs. 2.03 ± 1.03, $p < 0.01$; -18.03 ± 1.02 vs. -0.06 ± 0.05, $p < 0.01$, 14.69 ± 0.47 vs. -0.09 ± 0.14, $p < 0.01$, respectively). Ellagic acid supplementation had no effect on TGF-β in any of the study groups ($p > 0.05$). Also, the Tbet and RORγt genes expression decreased, and the GATA3 gene expression in the group receiving ellagic acid compared to control group significantly increased (0.52 ± 0.29 vs.

1.51 ± 0.18, $p < 0.01$, 0.49 ± 0.18 vs. 1.38 ± 0.14, $p < 0.01$, 1.71 ± 0.39 vs. 0.27 ± 0.10, $p < 0.01$). Also, ellagic acid supplementation led to significant decrease in EDSS, FSS and GHQ scores ($p < 0.05$), and no significant changes observed in PRI score ($p > 0.05$).

Conclusion: Ellagic acid supplementation can improve the health status of MS patients by reduction of the inflammatory cytokines and Tbet and RORγt gene expression, and increment of anti-inflammatory cytokines and GATA3 gene expression.

Clinical trial registration: (https://en.irct.ir/trial/53020), IRCT20120415009472N22.

KEYWORDS

multiple sclerosis, ellagic acid, pathogenesis, inflammation, disease severity

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease that leads to a gradual damage and loss of the myelin sheath of neurons in the spinal cord, brain and optic nerve (1). These injuries then lead to atrophy of the affected nerves over time. The atrophy that occurs at the onset of the disease is mild but progresses over time, eventually leading to numerous disabilities in these patients (1). Among the Middle Eastern countries, Iran has the highest prevalence rate of MS (2). The onset of the disease usually occurs in early to middle adulthood, between 20 and 40 years old, and the prevalence is higher in women (3).

MS disease has different forms with varying severity. The five main types of MS are relapsing–remitting (RR), progressive-remitting (PR), progressive-remitting (RP), primary progressive (PP), and secondary progressive (SP) MS. In approximately 85% of MS patients, the RR phase occurs first and then the SP phase (4). The RR type is the most common form of MS, in which inflammatory attacks on myelin and nerve fibers lead to deterioration of nerve function. In the RR type, symptoms vary from one patient to another, sometimes intensifying unexpectedly (this is called relapse and exacerbation) and then subsiding. The RR phase involves T-helper 1 (Th1) and Th17 cells that invade the central nervous system (CNS), and the SP phase results from inflammation caused by activation of innate immunity (5).

Th1 and Th17 cells play the main role in the pathophysiology of MS, and the inflammatory cytokines they produce lead to an increase in the permeability of the blood–brain barrier (BBB) to monocytes and macrophages (6). Th1 cells are actively present in the bloodstream of MS patients. These cells are also present in the damaged parts of the CNS and cause the production of inflammatory cytokines, including interferon-gamma (IFNγ) (7). The differentiation of immature T cells is under the influence of transcription factors that affect the expression of cytokine genes in these cells. T-bet is a transcription factor of the T-box transcription factor family that causes differentiation of immature T cells into Th1 cells and prevents differentiation of immature T cells into Th2 cells. The activation of T-bet is also the result of the action of IFNγ and IL-12, and after this activation, the number of Th1 cells and cytokines increases (8, 9). The conversion of immature T cells into Th17 cells is influenced by signal transducer and activator of transcription-3 (STAT3), and retinoic acid-related orphan receptor-γt (RORγt). Th17 cells adhere to the BBB (10), and their major cytokine, IL-17, increases BBB permeability and induces neutrophil transfer to the CNS. As a result, antigen (Ag)-specific CD4+ and CD8+ T cells are secreted into the CNS. The produced T cells pass through the BBB *via* the immune pathway and form the basis for the passage of monocytes into the CNS. Following this process, remnant microglia and astrocytes are activated, antigen-specific Th cells are differentiated, and the release of inflammatory cytokines leads to axon damage and loss (11, 12).

Th2 and Treg cells, which produce anti-inflammatory cytokines, play a modulatory and protective role against MS progression. To differentiate immature T cells into Th2 cells, IL-4 affects and activates the Th2 transcription factor (GATA3). As a result, the concentration of Th2 cytokines, including IL-4, increases (12, 13). Many drugs, including glatiramer acetate, reduce relapse in MS patients by altering the differentiation of immature T cells toward Th2 production and increasing IL-4 levels and inhibiting IFNγ secretion. This shows that increasing anti-inflammatory cytokines has a positive effect on the healing process of MS patients (13–15). Treg cells include a subset of CD4+ T lymphocytes that have immunoregulatory effects due to their ability to inhibit Th1 and Th17 cells. Treg cells can protect a person from autoimmune diseases. This is because CD4+ Treg cells inhibit inflammatory processes, and their cytokines, such as TGF-β, are considered a therapeutic target in MS patients (16). IL-10 and TGF-β are regulatory cytokines whose function affects Treg cell differentiation. IL-10 inhibits the secretion of Th1 cytokines and the progression of MS (17). Thus, the mice whose IL-10 gene was knocked out had a higher susceptibility to the development of MS, and in contrast, the mice whose expression of the IL-10 gene was overexpressed showed resistance to the development of MS (18).

Therapeutic strategies that shift immune system responses from Th1 to Th2 and Th17 to regulatory T cells may be effective in treating MS. Drugs approved by the Food and Drug Administration (FDA) for MS patients include beta-interferon, glatiramer acetate (GA), mitoxantrone, and natalizumab. All of these drugs can only affect MS disease to some degree, suggesting that more effective ways need to be found to affect disease progression as soon as possible (19, 20). Inflammation and apoptosis have been shown to have detrimental effects on brain cell function, and natural antioxidants play an important protective role in controlling this process (21).

Ellagic acid is a polyphenolic lactone found in a variety of vegetables and fruits, including pomegranates, strawberries, eucalyptus leaves, green tea, raspberries, and blackberries (22). The most

important polyphenol in pomegranate is punicalagin, which is not absorbed in its healthy and intact form in the intestine but can be hydrolyzed and converted to ellagic acid. When ellagic acid is orally ingested, it is converted by the intestinal microbiota under the influence of a specific metabolism into urolithins, which are much better absorbed in the digestive tract (22–24). Ellagic acid is a natural tannic acid derivative and influences the fate of neurons through its anti-inflammatory (25), antioxidant (26), and antidepressant effects (27). The limited pharmacological data on ellagic acid indicate that its serum elimination half-life in humans is 8.4 ± 1.4 h (200 ng/mL, oral). Serum elimination was also rapid when taken orally in animal studies (28). Previous studies have shown that ellagic acid reduces the inflammatory response in animal models of colitis (29), acute lung injury (30), and acute inflammation (31). Thus, treatment with ellagic acid leads to a reduction in the level of IL-17, IFNγ, and suppression of inflammatory cytokines (32, 33). in animal studies, ellagic acid has been shown to lead to an increase in some anti-inflammatory cytokines, including IL-4 (33, 34). Some experimental studies have also shown that ellagic acid decreases BBB permeability and TNF-α levels in the CNS (35). In clinical trials, the highest dose studied was 180 mg per day, and no side effects were reported (36). In addition, some studies have shown the neuroprotective effects of ellagic acid (37). Therefore, the aim of the present study was to investigate the effect of ellagic acid on disease severity, MS patienst disability status, the expression of genes involved in the pathogenesis of MS, the levels of associated inflammatory cytokines and oxidative stress in these patients.

2. Methods

2.1. Type of the study and participants

The present study was a triple-blind, multicentral, placebo-controlled clinical trial conducted in patients with MS. The population study is patients with MS, who were referred to Firouzgar and Hazrate Rasoule Akram hospitals (Tehran, Iran). Patients with MS of either sex who met the criteria for participation in the study and agreed to cooperate were enrolled in the study under the supervision of a neurologist after confirming the disease.

The inclusion criteria for participation in the present study were as follows: MS confirmation getting based on McDonald criteria (38) and magnetic resonance imaging (MRI) by neurologist, clinical status of relapse-remittance based on the criteria proposed by Lublin and Reingold (39), age between 18 and 55 years and EDSS score of less than 5.5.

The exclusion criteria were: refusal to continue participation in present study, change in the severity of the disease during the study, relapse occurrence during the intervention period, changes in dosage and type of medication consumed during the study, changes in the physical activity of the patients, no use of less than 90% ellagic acid supplements, ellagic acid or other supplements usage within1 month before the start of the study and within the study, estrogen, progesterone, diuretics, and corticosteroids within 1 month prior to the study, suffering from autoimmune disease, and pregnancy or breastfeeding. Also, patients with history of allergy and smokers excluded. The study flow diagram including different stages of study are presented in Figure 1.

The study protocol was approved by Ethics Committee of Iran University of Medical Sciences (Ethics code: IR.IUMS. REC.1399.1000). The study protocol was registered on website of the Iranian Registry of Clinical Trials (identifier: IRCT20120415009472N22, at the date of 19/12/2020). Written informed consent was provided from the participants.

2.2. Sample size calculation and

In this study, in order to determine the number of required patients, according to type I error equal to 5%, a power of 90% and EDSS as one the primary outcomes, the standard deviation for the EDSS was considered for calculation (40). Considering the 20% possibility for sample dropout, the volume of studied patients is estimated to be 29 people in each group and 58 people in total.

2.3. Sampling, blinding and randomization

Sampling was done by convenience sampling method. The selected patients based on the inclusion criteria were randomly assigned to two groups receiving ellagic acid and placebo. The method of random allocation was done by the balanced block method. None of the patients and the interviewer and analysis consultant knew the sample in which the group were placed (triple-blind randomized trial). The manufacturer was responsible for blinding the supplements by coding.

Then, a total of 58 MS patients were randomly assigned to ellagic acid ($n = 29$) and placebo ($n = 29$) groups. Each ellagic acid capsules (90 mg) and placebo capsules (maltodextrin) were taken twice a day by MS patients in intervention and control groups, respectively. Ellagic acid and placebo capsules were similar in shape, weight, taste, size, odor and color and produced by Axenic Company, Australia. Ellagic acid purity was 99.9% and the dosage of ellagic acid was chosen based on previous studies (37).

Patients were given packets of ellagic acid or placebo sufficient for 4 weeks of consumption in the order they entered the study, and at the fourth and eighth weeks they were again given supplements or placebo. They were told to take two capsules of ellagic acid or placebo daily after lunch, and this process continued for 12 weeks. At each visit, patients were asked to bring the packet of supplements or placebo. If patients did not consume less than 90% of the supplements or placebo at each of the four-, eight-, or 12-week visits, they were excluded from the study.

During the study, patients were reminded to take the supplements by phone call and text message. During the intervention period, patients were asked not to change their diet or physical activity and not to take any dietary supplements without the advice of their treating physician. They were also asked to inform us of any change in the dosage of their medications during the study period.

2.4. Questionnaires

In the phase before the start of the intervention, general characteristics including age, sex, duration of disease, and dosage of medication taken by participants were recorded. To assessment of the patients' food intake, three 24-h food intake questionnaire was completed on the first, sixth and twelfth weeks of the study (two normal days and one day off) and analyzed by Nutritionist 4

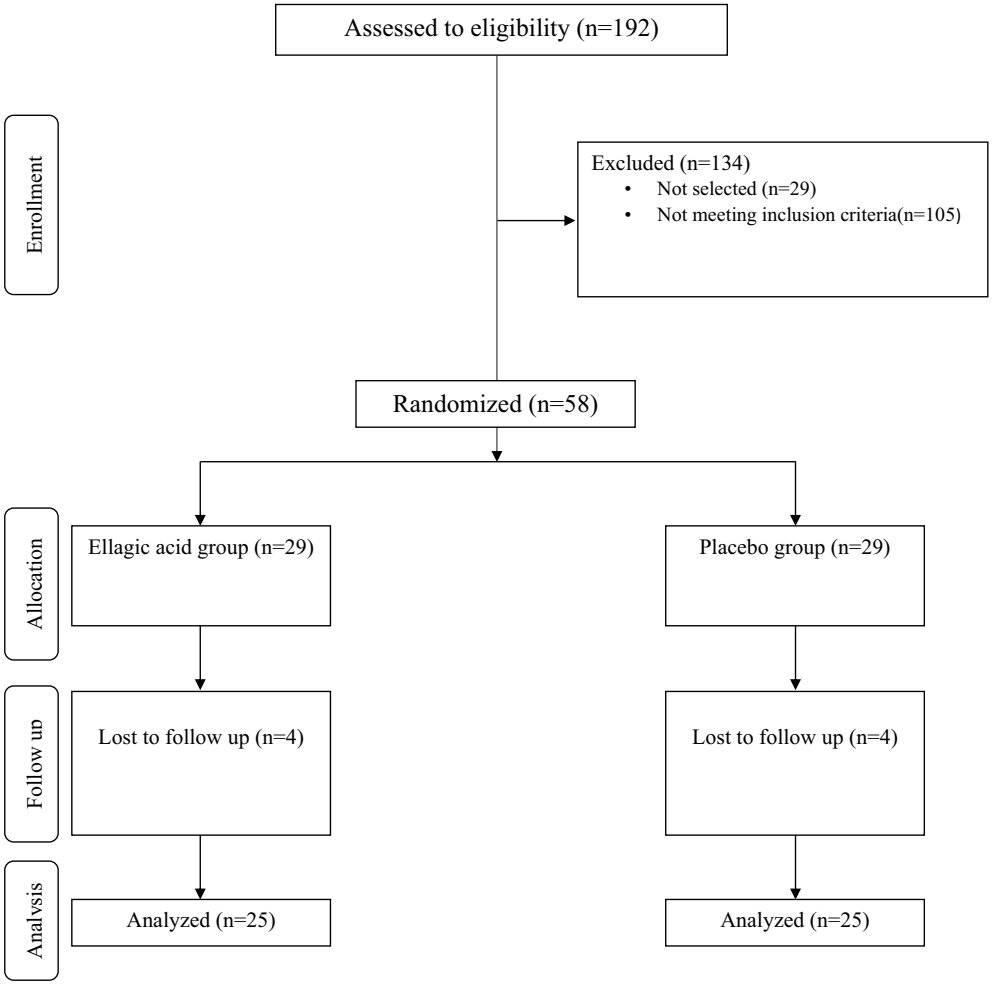

FIGURE 1
Summary of participants recruitment based on the consolidated standards of reporting trials (CONSORT) flow diagram.

software (USA). In addition, the International Physical Activity Questionnaire (IPAQ) was completed to check the level of physical activity as a confounding factor before and after the intervention. The anthropometrical questionnaire is included height and weight. Height measurement was performed using a strip meter with a precision of 0.1 cm and weight measurement, using a digital scale of 100 grams (Seca, Germany) under standard conditions. Also, the body mass index was calculated through the formula. GHQ questionnaire consisting of 28 questions were used to assess the general health status (41). The McGill Pain Questionnaire was also used to calculate the pain rating index (PRI) score, which has 78 items and 20 groups that examine the different dimensions of pain (42). Fatigue severity scale (FSS) questionnaire was used to assess the fatigue severity in MS patients (43). All questionnaires validity and reliability were approved in Iranian population in recent studies (41–43).

2.5. Immunological assessments

Blood samples were drawn from the patients at the pre- and post-intervention and centrifuged at a speed of 2000 rpm for 10 min until the serum was separated. Then, the serum of the samples for measuring the indices of interleukin 4, interleukin 17, TGF-β, and IFNγ were kept in a freezer at-80°C until assay. The levels of IL-4 (Zellbio, Germany), IL-17 (Zellbio, Germany), TGF-β (Zellbio, Germany), IFNγ (R&D Systems, USA), and nitric oxide (NO) (Zellbio, Germany) were measured using enzyme-linked immunoassay (ELISA) kits based on the instructions in the kit guidelines. The protocol of the measurement method was as follows: First, the reagents, samples, and standards were prepared according to the instructions. Then, 100 microliters of the standard solution and sample were added to each well of the 96-well plate and incubated at 37°C for 2 h, and the liquid in each well was drained. Then, 100 microliters of biotin antibody (x1) were added to each well and incubated at 37°C for 1 h. Then, the wells were emptied and washed three times with PBS solution (phosphate-buffered saline). Then, 100 microliters of HRP-avidin (x1) were added to each well and incubated at 37°C for 1 h. Again, the wells were emptied and washed 5 times with PBS solution. Then, 90 microliters of TMB (tetramethylbenzidine) substrate were added to each well and incubated at 37°C (in the dark) for 30 min. Finally, 50 microliters of the stop solution were added to each well and the light absorbance (OD) of the samples was evaluated within 5 min using an ELISA reader (Hyperion, MPR4++, USA) at a wavelength of 450 nm.

2.6. Gene expression assessment

The expression of Tbet, RORγ, GATA3, and GAPDH genes was also measured using the real-time Quantitative Reverse Transcription (RT-qPCR) method. Peripheral blood mononuclear cells (PBMCs) were first isolated to assess gene expression. Ficoll and concentration gradients were used to isolate PBMCs. To isolate PBMCs, 10 mL of peripheral blood was first poured into a heparin-containing tube and the same volume of PBS was added at a temperature of 4°C. Mixing was performed with slow and circular movements. In a separate tube, 3 mL of Ficoll was added and half of the diluted blood was added to the Ficoll in the tube. After the addition of blood, the tube was placed in a refrigerated centrifuge (temperature 4°C) and centrifuged at 800 g for 40 min. After centrifugation, four different layers, including plasma, PBMC, Ficoll, and red blood cells, were observed from top to bottom. The PBMC layer was separated from each tube and poured into a 50 mL tube. Then, up to 40 mL of PBS solution was added and placed in a refrigerated centrifuge (temperature 4°C) and centrifuged at a speed of 600× *g* for 10 min. After centrifugation, the supernatant was discarded. Then, 2 mL of PBS solution was added to the cells located at the bottom of the tube. After adding the PBS solution, the cell layer was dissolved and homogenized by pipetting in the solution. Finally, the homogenized solution was transferred to a 1.5 mL microtube and centrifuged again (4°C at 600× *g* speed, 10 min). After centrifugation, the supernatant was discarded, and the PBMCs were simultaneously used for RNA extraction. RNA extraction was performed using the Rneasy plus mini kit (Qiagen, Germany) according to the kit instructions. This step was performed with nuclease-free equipment under a hood disinfected with alcohol and sterilized with UV light. A Nano Drop device (Thermo Scientific, USA) was used to determine the purity of RNA, and the absorbance ratio of 260/280, 1.8–2.2 was considered high purity based on the kit protocol. RNA concentration was also determined using nanodrops. The extracted RNA was stored in a freezer at-80°C until cDNA synthesis. For cDNA synthesis, 500 ng of RNA was used with the Quantitect reverse transcriptase commercial kit from Qiagen (Qiagen, Germany). Then, the prepared cDNA was stored in the freezer at-20°C until gene expression was measured. Subsequently, the synthesised cDNAs were stored in a freezer at-20°C until the time of gene expression measurement. To select the appropriate primer, the sequence of the primers was taken from reliable articles that similarly investigated the expression of the genes in this study in PBMC, and the properties of the primers were checked using Gene Runner software. The website www.ensembl.org was used to check the sequence of the desired genes and also whether the forward and reverse primers were located in two exons. The site http://www.ncbi.nlm.nih.gov/tools/primer-blast was also used to control the specificity of the primers. For the GAPDH gene (housekeeping gene), primers were selected from the studies in the same way (Table 1). After primer preparation, gene expression was measured using the Rotor-Gene Q (Qiagen, Hilden, Germany) instrument and the SYBR Green method. All experiments were performed with nuclease-free devices under a hood disinfected with alcohol and sterilized with ultraviolet (UV) light. Real-time PCR analysis was done by fold change calculation based on $2^{-\Delta\Delta Ct}$.

TABLE 1 Primers used for quantitative real-time PCR analysis.

Gene	Type	Sequence
GATA3	Forward	5′- ACCACAACCACACTCTGGAGG A-3′
	Reverse	5′- TCGGTTTCTGGTCTGGATGCC T-3′
RORγt	Forward	5′- GCCAAGGCCGGCAGAGCCAA-3′
	Reverse	5′- AAGAAGCCCTTGCACCCCTCACA-3′
Tbet	Forward	5′- CCACCTGTTGTGGTCCAAGT –3′
	Reverse	5′- AACATCCTGTAGTGGCTGGTG-3′
GAPDH	Forward	5′-GCACCGTCAAGGCTGAGAAC-3′
	Reverse	5′-TGGTGAAGACGCCAGTGGA-3′

2.7. Data analysis

Data analysis was performed using SPSS version 24 software. Descriptive statistics methods including frequency distribution tables and central and dispersion indices were used to describe the samples. The Kolomogorov-Smirnov test was performed to determine distribution of variables. Levene's test was used to determine equality of variances.

In this study, to compare the quantitative variables at baseline and to compare the average changes in these variables during the study between groups, the independent t test was used. In addition, to compare the quantitative variables within each group before and after the intervention, the paired t test was used. If there was a confounder variable, covariance analysis was used. Quantitative variables were reported as mean (standard deviation) and 5% was considered as a significant level.

3. Results

3.1. Baseline characteristics of study participants

Among the 192 people with MS referred to the neurology clinics of Rasool Akram Hospital and the MS Clinic of Firozgar Hospital (Tehran, Iran) from January 2019 to the end of September 1,400, 58 patients with MS were eligible to enter the study, in order to perform Intervention were invited. Based on the Stratified Permuted Block Randomization method, patients were assigned into two groups: (1) group receiving ellagic acid supplement (180 mg per day), (2) control group receiving placebo containing maltodextrin. In the second visit of the patients in the middle of the intervention, 3 patients in the group receiving ellagic acid (3 women) and 4 patients in the group receiving placebo (4 women) were excluded from the study due to changes in the course of the disease and the drugs received. In the last visit, one patient in the group receiving ellagic acid was excluded from the study due to a change in the medications received. Thus, in both groups, 25 patients entered the final stage and data analysis (Figure 1).

The rate of patient compliance with the intervention was 92.23 and 92.42% in the ellagic acid and placebo groups, respectively.

The baseline characteristics of the subjects in the present study are provided in Table 2. In both groups, 12% of the participants were male and 88% were female. The average age of people in the ellagic acid

TABLE 2 Baseline characteristics of participants in ellagic acid and control groups.

Variable		Total (n = 50)	Ellagic acid (n = 25)	Control (n = 25)	p-value[a]
Age (years)		39.51 ± 9.15	42.89 ± 9.48	37.98 ± 9.02	0.047
Height (cm)		164.88 ± 8.22	164.29 ± 7.93	165.02 ± 8.44	0.539
Gender Number (%)	Male	6(12)	3(12)	3(12)	–
	Female	44(88)	22(88)	22(88)	–
Weight (kg)		68.51 ± 12.37	69.10 ± 12.52	67.93 ± 12.58	0.482
BMI (kg/m2)		25.51 ± 3.37	25.42 ± 3.52	25.38 ± 3.29	0.891
MS duration (years)		5.27 ± 0.54	4.42 ± 0.61	6.18 ± 0.49	0.013
Physical activity (MET-h/week)		33.48 ± 5.12	32.99 ± 5.26	33.89 ± 5.02	0.274
EDSS score		2.59 ± 0.35	2.60 ± 0.38	2.58 ± 0.31	0.750
MS drugs Number (%)	Resigen	6(12)	3(12)	3(12)	–
	CinnoVex	38(76)	19(76)	19(76)	–
	Betaferon	6(12)	3(12)	3(12)	–

Data are presented as mean ± SD for quantitative and frequency (%) for qualitative variables.
BMI, body mass index; EDSS, Expanded Disability Status Scale.
[a]Independent Sample t-test.
p value < 0.05 considered significant.

group was 42.89 ± 9.48 and in the control group it was 37.98 ± 9.02, which were statistically significantly different from each other. Also, there were significant difference between two study groups regarding the disease duration ($p < 0.05$). The patients in the present study were not significantly different in terms of the EDSS score, and the drugs used (Table 2).

3.2. Effect of ellagic acid supplementation on dietary intake

In Table 3, the findings related to energy, carbohydrate, fat, protein, fiber, and micronutrients has been shown. There were no significant differences between the two groups regarding any of the findings of energy, macronutrients, fiber and micronutrients at baseline and end of the intervention ($p > 0.05$). Also, there were no significant differences regarding dietary intake within ellagic acid and control groups at the end of the study compared to their respective baselines ($p > 0.05$).

3.3. Effect of ellagic acid supplementation on anthropometric measurements and physical activity

The findings of anthropometric measurements showed no significant differences between two groups regarding weight, BMI, WC and physical activity at baseline and end of the intervention ($p > 0.05$). Also, there were no significant differences regarding weight, BMI, WC and physical activity within ellagic acid and control groups at the end of the study compared to their respective baselines ($p > 0.05$; Table 4).

3.4. Effect of ellagic acid supplementation on EDSS and general health

Based on the findings of the present study, the average changes of the EDSS index in the ellagic acid group had a significant decrease, the changes of the ellagic acid and control groups were significantly different from each other (−1.06 ± 0.09 vs. 0.04 ± 0.02, $p < 0.01$). The average changes of GHQ and FSS indices in the ellagic acid group had a significant decrease compared to the control group (−5.35 ± 1.94 vs. 0.08 ± 0.04, $p = 0.032$, −1.51 ± 0.42 vs. −0.20 ± 0.08, $p = 0.028$, respectively). The mean changes of PRI index in both ellagic acid and control groups were not significantly different, and the changes of both groups were also insignificant ($p > 0.05$). Also, there were significant differences regarding EDSS, GHQ, and FSS within ellagic acid group ($p < 0.05$) and there were no significant changes in control group at the end of the study compared to their respective baselines ($p > 0.05$; Table 5).

3.5. Effect of ellagic acid supplementation on IFNγ, IL-17, IL-4 and TGF-β cytokines and NO

The results indicated that supplementation with ellagic acid caused a significant decrease in the level of IFNγ (−24.52 ± 3.79 vs. −0.05 ± 0.02, $p < 0.01$) and interleukin-17 (−5.37 ± 0.92 vs. 2.04 ± 1.03, $p < 0.01$) in the ellagic acid group and it has caused significant changes between the ellagic acid and control groups. Also, Ellagic acid supplementation led to significant increase in IL-4 levels and there was significant difference between two groups (14.69 ± 0.47 vs. −0.09 ± 0.14, $p < 0.01$). Ellagic acid supplementation led to no significant changes in TGF-β levels in both groups ($p > 0.05$). Besides, our results indicated significant decreasing changes in serum NO (−18.03 ± 1.02 vs. −0.06 ± 0.05, $p < 0.01$) levels after intervention in ellagic acid and control groups. Moreover, there were significant differences regarding IFNγ, IL-17, IL-4 and NO within ellagic acid group ($p < 0.05$) and there were no significant changes in control group at the end of the study compared to their respective baselines ($p > 0.05$). However, there were no significant changes in TGF-β within both ellagic acid and control groups ($p > 0.05$) (Table 6).

3.6. Effect of ellagic acid supplementation on Tbet, RORγt, and GATA3 gene expression

The findings of the present study showed that supplementing with ellagic acid caused a significant decrease in the expression level of tbet and RORγt genes in the group receiving ellagic acid compared to the control group, so that the fold change in the expression of tbet genes in the ellagic acid and control groups was 0.52 ± 0.29 and 1.51 ± 0.18 ($p < 0.01$), respectively. The fold change in the expression of RORγt genes in the ellagic acid and control groups was 0.49 ± 0.18 and 1.38 ± 0.14 ($p < 0.01$), respectively. The Fold change in the expression of GATA3 gene increased significant in ellagic acid group compared to control group (1.71 ± 0.39 vs. 0.27 ± 0.10, $p < 0.01$; Figures 2–4).

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TABLE 3 Dietary intake of participants in ellagic acid and control groups at weeks 0, 6 and 12 of the intervention.

Variable	Time	Ellagic acid group (n = 25)	Control (n = 25)	Mean differences (95% CI)	p-value[a]
Energy (Kcal)	Week 0	2214.00 ± 426.61	2134.50 ± 293.73	80.92 (−10, 177)	0.18
	Week 6	2202.00 ± 389.31	2109.50 ± 269.41	93.55 (−9, 158)	0.28
	Week 12	2182.00 ± 408.01	2124.31 ± 124.58	58.04 (−21,92)	0.13
	p-value[b]	0.06	0.09		
Carbohydrate (g/d)	Week 0	317.15 ± 46.33	320.00 ± 41.31	−3.12 (−4.18, 5.27)	0.82
	Week 6	303.40 ± 37.80	309.60 ± 40.95	−6.2 (−10.63, 9.82)	0.74
	Week 12	323.00 ± 29.70	325.18 ± 57.69	−1.81 (−3.46, 5.39)	0.19
	p-value[b]	0.86	0.74		
Protein (g/d)	Week 0	70.30 ± 34.76	70.85 ± 18.64	−0.55 (−1.77, 1.42)	0.91
	Week 6	71.65 ± 28.98	69.40 ± 18.27	2.25 (−0.67, 3.09)	0.82
	Week 12	69.95 ± 27.12	71.25 ± 17.20	−1.31 (−2.55, 1.40)	0.06
	p-value[b]	0.38	0.06		
Total fat (g/d)	Week 0	84.60 ± 17.99	83.85 ± 9.10	0.75 (−0.08, 1.17)	0.76
	Week 6	80.25 ± 14.25	81.30 ± 14.25	−1.15 (−2.45, 1.32)	0.49
	Week 12	84.81 ± 18.17	84.00 ± 10.81	0.81 (−0.94, 1.42)	0.09
	p-value[b]	0.45	0.14		
SAFA (g/d)	Week 0	22.98 ± 6.34	20.00 ± 4.12	2.98 (−1.57, 3.93)	0.18
	Week 6	19.34 ± 3.91	22.29 ± 5.44	−2.95 (−5.48, 3.28)	0.28
	Week 12	21.47 ± 2.11	21.94 ± 4.85	−0.47 (−1.55, 0.39)	0.13
	p-value[b]	0.34	0.17		
MUFA (g/d)	Week 0	19.75 ± 4.33	16.08 ± 2.38	3.67 (−2.75, 5.21)	0.82
	Week 6	18.33 ± 4.57	18.72 ± 4.29	−0.39 (−1.15, 1.44)	0.74
	Week 12	19.81 ± 5.15	18.66 ± 4.62	1.15 (−0.28, 2.19)	0.19
	p-value[b]	0.98	0.25		
PUFA (g/d)	Week 0	11.10 ± 2.42	11.39 ± 1.67	−0.19 (−0.78, 0.83)	0.91
	Week 6	11.11 ± 1.34	11.57 ± 2.23	−0.46 (−0.99, 1.12)	0.82
	Week 12	11.08 ± 2.07	11.44 ± 2.17	−0.36 (−1.02, 0.82)	0.06
	p-value[b]	0.27	0.09		
Vitamin A (mg)	Week 0	415.25 ± 66.74	390.85 ± 48.69	24.47 (−11, 39)	0.76
	Week 6	386.15 ± 51.46	397.85 ± 62.35	−11.7 (−17, 23)	0.49
	Week 12	367.27 ± 45.34	400.21 ± 51.47	−32.94 (−52, 101)	0.09
	p-value[b]	0.07	0.09		
Vitamin E (mg)	Week 0	4.41 ± 0.89	4.89 ± 0.80	−0.48 (−1.26, 1.73)	0.17
	Week 6	4.89 ± 0.80	4.67 ± 0.93	0.22 (−0.18, 0.67)	0.29
	Week 12	4.47 ± 0.99	4.80 ± 0.12	−0.33 (−0.92, 1.15)	0.10
	p-value[b]	0.63	0.08		
Vitamin D (μg)	Week 0	5.15 ± 0.51	4.81 ± 0.70	0.34 (−0.16, 0.91)	0.81
	Week 6	5.00 ± 0.62	4.82 ± 0.67	0.18 (−0.27, 0.59)	0.58
	Week 12	5.08 ± 0.81	4.20 ± 0.90	0.88 (−0.08, 1.12)	0.92
	p-value[b]	0.58	0.25		
Vitamin C (mg)	Week 0	17.75 ± 3.70	16.84 ± 3.03	0.91 (−0.22, 1.70)	0.77
	Week 6	16.72 ± 3.17	16.19 ± 2.76	0.53 (−0.11, 0.92)	0.93
	Week 12	17.28 ± 1.18	16.96 ± 2.55	0.32 (−0.62, 1.39)	0.07
	p-value[b]	0.45	0.07		

(Continued)

TABLE 3 (Continued)

Variable	Time	Ellagic acid group (n = 25)	Control (n = 25)	Mean differences (95% CI)	p-value[a]
Calcium (mg)	Week 0	1056.30 ± 167.68	892.20 ± 168.80	164 (−83, 226)	0.08
	Week 6	1019.00 ± 127.36	917.50 ± 174.85	102 (−107, 261)	0.59
	Week 12	1087.01 ± 133.36	908.63 ± 185.25	179 (−114, 293)	0.32
	p-value[b]	0.08	0.59		
Iron (mg)	Week 0	13.56 ± 2.69	10.85 ± 2.38	2.71 (−1.43, 3.82)	0.12
	Week 6	10.90 ± 2.10	12.60 ± 2.49	−1.70 (−3.71, 2.99)	0.92
	Week 12	10.74 ± 10.54	11.91 ± 1.24	−1.17 (−4.12, 2.31)	0.06
	p-value[b]	0.06	0.25		
Selenium (μg)	Week 0	0.05 ± 0.01	0.04 ± 0.00	0.01 (−0.02, 0.04)	0.77
	Week 6	0.04 ± 0.01	0.04 ± 0.00	0 (−0.01, 0.03)	0.73
	Week 12	0.04 ± 0.18	0.04 ± 0.01	0 (−0.01, 0.03)	0.09
	p-value[b]	0.09	0.39		
Zinc (μg)	Week 0	7.78 ± 1.15	6.01 ± 1.00	1.77 (−0.23, 2.18)	0.37
	Week 6	7.31 ± 1.09	6.06 ± 1.09	1.25 (−1.15, 2.19)	0.45
	Week 12	7.97 ± 1.85	6.04 ± 1.02	1.93 (−1.81, 3.32)	0.06
	p-value[b]	0.12	0.81		
Fiber (g/d)	Week 0	15.70 ± 2.60	15.19 ± 2.80	0.51 (−0.77, 1.42)	0.52
	Week 6	14.37 ± 2.50	15.17 ± 3.18	−0.80 (−1.83, 2.49)	0.88
	Week 12	15.98 ± 1.84	15.22 ± 3.84	0.76 (−0.75. 1.88)	0.15
	p-value[b]	0.74	0.89		

Data are presented as mean ± SD.
[a]Independent sample t-test.
[b]Value of p for repeated measures ANOVA performed to assess variations in dietary intakes across periods.
p value < 0.05 considered significant.

4. Discussion

The results showed that daily supplementation with 180 milligrams of pure ellagic acid in MS patients decreased the level of inflammatory cytokines, including IL-17 and IFNγ, and increased the serum level of anti-inflammatory cytokines, including IL-4. In addition, ellagic acid supplementation in the present study decreased the tbet and RORγt genes expression and increased the GATA3 gene expression.

MS is an autoimmune disease of the CNS, and the protein components of myelin are the target of the immune system attacks. The role of various immune system factors in the onset of MS has been investigated in several studies (44).

The present study showed that daily intake of 180 mg ellagic acid led to a significant reduction in the serum levels of IFNγ and the tbet gene expression. Noh et al. (45) also reported a decrease in the level of proinflammatory cytokines, including IFNγ, in their study on the ellagic acid effects on dendritic cell maturation. Allam et al. (34), in a study on the potential effect of ellagic acid in the schistosomiasis mansoni treatment in mice, reported a significant decrease in IFNγ with the addition of 600 mg of ellagic acid. In one study, ellagic acid effects at doses of 5, 10, and 20 μg/mL on the immunologic balance of mononuclear cells and colon carcinoma cells were investigated, which at high doses the production of IL-6, TNF-α, IL-1, and IL-10 cytokines suppressed, while showing no effect on IFNγ (46). Some studies found significant increase in IFNγ levels following ellagic acid supplementation. In 2016, Allam et al. conducted a study to investigate the ellagic acid potential effect on the adjuvant induced arthritis (AIA) model in mice and found that supplemenation of 700 mg/kg body weight of ellagic acid let to an increase in IFNγ levels, while TGF-β levels did not change (47). Similarly, Kang et al. (48) reported an increase in IFNγ levels when investigating the effects of ellagic acid on immunologic resistance in transgenic rats carrying hepatitis B virus antigen (48). The reason for this discrepancy with our results is likely due to differences between studies, including differences in the samples studied, the disease studied, the dose of ellagic acid, and the study design. Differentiation of Th1 cells from immature T cells depends on IFNγ and IL-12, which cause expression of T-bet factor *via* activation of the signal transducers STAT1 and STAT4, respectively (49). T-bet causes the production of Th1 cytokines, particularly IFNγ, and in this way enhances the differentiation of Th1 cells. At the same time, T-bet suppresses the differentiation of other subsets of Th cells (50).

The results of studies in the animal model of experimental autoimmune encephalomyelitis (EAE) show that transfer of myelin-specific activated Th1 cells to healthy mice induces EAE in them, and the infiltrated T cells in the CNS mainly produce Th1 cytokines (51). An increase in Th1 cytokines has also been observed in MS patients (52). In studies, an increase in serum levels of the main cytokine of Th1 cells, i.e., IFNγ, was observed in mice with EAE, confirming the pathogenicity of these cells (52). The results of some studies have also shown that disruption of the T-bet gene renders the animals resistant

TABLE 4 Anthropometric measurements and physical activity status in ellagic acid and control groups in the beginning and at the end of the study.

Variable	Time	Ellagic acid group (n = 25)	Control (n = 25)	Mean differences (95% CI)	p-value[a]
Weight (Kg)	Before	69.10 ± 12.52	67.93 ± 12.58	1.17 (−0.52, 1.93)	0.482
	After	69.54 ± 11.32	68.41 ± 12.62	1.12 (−0.88, 1.79)	0.065
	Mean change	0.43 ± 0.09	0.46 ± 0.02	−0.03 (−0.08, 0.05)	0.931
	p-value[b]	0.778	0.591		
BMI (Kg/m^2)	Before	25.42 ± 3.52	25.38 ± 3.29	0.13 (−0.01, 0.28)	0.891
	After	25.41 ± 3.53	25.40 ± 3.27	0.11 (−0.01, 0.32)	0.938
	Mean change	0.01 ± 0.02	0.02 ± 0.01	−0.01 (−0.09, 0.07)	0.289
	p-value[b]	0.089	0.142		
Waist circumference (cm)	Before	89.44 ± 6.86	90.16 ± 6.85	−0.75 (−1.21, 1.77)	0.184
	After	89.09 ± 6.83	90.24 ± 6.92	−1.15 (−2.33, 2.91)	0.059
	Mean change	−0.35 ± 0.04	0.08 ± 0.04	−0.44 (−1.04, 1.25)	0.184
	p-value[b]	0.312	0.571		
Physical activity (MET-min/week)	Before	32.99 ± 5.26	33.89 ± 5.02	0.10 (−0.01, 0.23)	0.274
	After	32.82 ± 5.35	33.93 ± 5.12	−0.11 (−0.23, 0.01)	0.063
	Mean change	−0.17 ± 0.08	0.04 ± 0.09	−0.21 (−0.42, 0.29)	0.081
	p-value[b]	0.584	0.617		

Data are presented as mean ± SD.
BMI, body mass index.
[a]Independent Sample t-test.
[b]Paired t-test.
p value < 0.05 considered significant.

to the induction of EAE (53). Clinical studies have shown that the exacerbation of MS is often associated with the proliferation of myelin-specific Th1 cells in the CSF, and based on pathological observations, the accumulation of Th1 cells and the production of IFNγ in sclerotic plaques are directly related to the demyelination process (54). Moreover, treatment of MS patients with IFNγ increases disease severity, whereas treatment with IFN- neutralising antibodies improves disease progression (17). However, in the present study, ellagic acid was found to decrease Th1 cell activity, as evidenced by a decrease in Tbet gene expression and IFNγ cytokine. Ellagic acid through decreasing the IFNγ, reduces the expression of the death receptor (FAS) on the surface of oligodenocytes and prevents their apoptosis (37, 55). Accordingly, ellagic acid seems to affect Th1 cells, which are responsible for a large proportion of the immunopathologic responses in MS, and to reduce the immunopathologic lesions in MS by preventing the differentiation of naive T cells into Th1 cells, preventing the activation of Th1 cells, and targeting cytokines secreted by Th1 cells or their receptors (56).

Our study showed that daily consumption of 180 mg ellagic acid has immunomodulatory effects on Th17 cells, as evidenced by a significant decrease in Th17 cytokine production. Many studies have investigated the immune system modulating effects of polyphenols, but there are few studies that have investigated the effect of ellagic acid on the immune system. Sanadgol et al. investigated the neuroprotective effect of ellagic acid on acute demyelination by cuprizone with daily supplementation of 40 or 80 mg/kg body weight ellagic acid and observed a significant reduction in IL-17 gene expression (32). Lu et al. showed that pomegranate peel extract exhibited preventive and therapeutic effects in EAE animal models, and this effect is achieved by modulating the gut microbiota, and furthermore, these effects were achieved by inhibiting the filtration of peripheral inflammatory cells into the CNS by reducing the amount of CD4+ IL-17+ and CD4 + IFNγ+ cells (57). In another study, Petrou et al. (58) showed that 6 months of pomegranate seed oil intake in 30 MS patients improved the cognitive characteristics of them. Kiasalari et al. (59) obtained a significant decrease in IL-17 levels following supplementation with 10 or 50 mg/kg body weight ellagic acid in an EAE animal model. Parisi et al. (60) showed in a study that propolis, pomegranate, and grape marc improved RA symptoms and disease severity by lowering the levels of IL-17, IL-1b, and IL-17 stimulating cytokines. Also, in the study on the effect of peel extract of pomegranate on the animal model of EAE and type 1 diabetes, improvement of the symptoms of the disease obtained, and these changes were caused by the inhibition of the filtration of immune cells into the pancreatic islet cells and the reduction of the production of IL-17 and IFNγ (61).

Studies have shown that polyphenols from the tannin family, such as elagitanin, can prevent the production of cytokines by T cells. In addition, these polyphenols can bind to inflammatory cytokines, including IL-17 and IFNγ, or their receptors and prevent their signal transduction, which should be further investigated in future studies. IL-17 is the specific cytokine of Th17 cells. IL-17A induces the production of pro-inflammatory mediators in various cells, all of which demonstrate the pro-inflammatory nature of Th17 cells (62). Th17 differentiation depends on the expression of the RORγt gene. It has been shown that genetic defect of RORγt in mice leads to disruption of Th17 differentiation and protects the mice from induction of EAE disease (63). The results showed that daily supplementation with 180 mg ellagic acid resulted in a significant decrease in levels of IL-17 and RORγt gene expression. In humans, the effects of IL-17 on the

TABLE 5 EDSS, PRI and GHQ status in ellagic acid and control groups in the beginning and at the end of the study.

Variable	Time	Ellagic acid group (n = 25)	Control (n = 25)	Mean differences (95% CI)	p-value[a]	P2[b]
EDSS	Before	2.60 ± 0.38	2.58 ± 0.31	0.02 (−0.17, 0.21)	0.750	
	After	1.54 ± 0.32	2.62 ± 0.29	−1.08 (−1.25, −0.90)	0.001	0.001
	Mean change	−1.06 ± 0.09	0.04 ± 0.02	−1.10 (−1.28, −0.08)	0.001	
	p-value[c]	0.001	0.157			
PRI	Before	36.92 ± 5.29	35.83 ± 5.48	1.09 (−1.18, 1.65)	0.882	
	After	36.01 ± 5.32	36.14 ± 5.20	−0.13 (−0.55, 0.29)	0.938	0.927
	Mean change	−0.91 ± 0.94	0.31 ± 1.02	−1.22 (−1.83, 0.54)	0.289	
	p-value[c]	0.061	0.096			
GHQ	Before	35.44 ± 6.72	36.16 ± 6.95	−0.72 (−0.94, 0.35)	0.184	
	After	30.09 ± 5.73	36.24 ± 6.83	−6.15 (−8.27, −4.12)	0.027	0.032
	Mean change	−5.35 ± 1.94	0.08 ± 0.04	−5.43 (−7.43, −3.52)	0.032	
	p-value[c]	0.018	0.571			
FSS	Before	5.52 ± 0.73	5.82 ± 0.48	−0.30 (−0.67, 0.02)	0.572	
	After	4.01 ± 0.29	5.62 ± 0.55	−1.61 (−2.22, −0.79)	0.041	0.045
	Mean change	−1.51 ± 0.42	−0.20 ± 0.08	−1.71 (−2.39, −0.83)	0.028	
	p-value[c]	0.001	0.353			

Data are presented as mean ± SD.
EDSS, Expanded Disability Status Scale; PRI, Pain Rating Index; GHQ, general health questionnaire; FSS, Fatigue Severity Scale.
[a]Independent Sample t-test.
[b]General linear model adjusted for baseline differences between groups, age, MS duration.
[c]Paired t-test.
p value < 0.05 considered significant.

process of demyelination of neurons in MS patients are well known, and furthermore, the exacerbation of the disease is related to the increase in the number of Th17 cells in the serum of patients (12). Th17 cells are the first cells to encounter myelin antigens presented by antigen-presenting cells (APC) in the subcranial space. After recognizing the antigen, Th17 cells release several proinflammatory mediators such as IL-17A and create an inflammatory environment that can cause tissue damage in the CNS (64). By stimulating the production of matrix metalloproteinase (MMP) enzymes, IL-17A also causes the destruction of cell junction proteins. IL-17 and ROS also lead to increased expression of endothelial adhesion molecules, resulting in large numbers of inflammatory cells migrating into the CNS (65). Myelin-specific Th17 cells can interact directly with neurons. It seems that ellagic acid by reducing Th17 cells prevents the change of calcium level in the neuron and thus the destruction of neurons. It is also possible that ellagic acid reduces oxidative stress and apoptosis by reducing Th17 cells and IL-17 cytokine levels in oligodendrocytes. By decreasing IL-17 levels, ellagic acid also facilitates the regeneration process of myelin and removes obstacles to the maturation of oligodendrocytes and increases their survival (66, 67).

In the present study, ellagic acid supplementation increased IL-4 levels and GATA3 gene expression. Kang et al. (48) also showed an increase in IL-4 level (48). Rogerio et al. (68), found that a dose of 10 mg/kg ellagic acid inhibited the production of IL-4 in a model of athma. Tanner et al. (69), examined the response of systemic inflammation to ellagic acid in marathon runners and found a significant increase in IL-4 and GATA3 gene expression within 24 h of supplementation. Anderson et al. (70) showed that pure ellagic acid and walnut kernel polyphenols decreased the synthesis of the cytokines IL-13, TNF-a, and increased the production of IL-2, with no effect on IL-4. However, in our study, Th2 cells differentiation increased, as determined by increase in GATA3 expression and IL-4 levels. The increase in the response of Th2 cells to ellagic acid supplementation, which was characterized by the increase in the level of IL-4, is a new phenomenon, and it is likely that it is due to the effect of ellagic acid on the inhibition of Th1 and Th17 responses, because Th1 and Th17 responses are antagonistic with Th2 cells (71, 72). Th2 lymphocytes produce IL-4, IL-5, IL-13, IL-9, and IL-10. GATA-3 inhibits the expression of IFNγ, thereby reducing the Th1 cells response (73). The protective role of Th2 cells dependent on Th1 and Th17 cells has been established; therefore, when the brain is damaged, the immune response tends to favor Th2 cells, which suppress Th1 and Th17 dependent responses and prevent further autoimmune damage in the CNS. Th2 cytokines play a role in reducing the destructive effect of Th1 cells in the EAE (74). A decrease in CNS inflammation and minimal clinical symptoms were also observed in transgenic mice with high expression of GATA3 (leading to a deviation of their immune response toward Th2) after EAE induction. By releasing IL-4, Th2 cells can directly inhibit autoimmune diseases. In the EAE model, the protective effect of Th2 cells has been demonstrated, making it possible to cure EAE by increasing the expression of Th2 cytokines in the brain (75). It appears that ellagic acid, through its effect on naive T cells, induces them to differentiate into Th2 cells, which was observed in the present study by increasing IL-4 levels and GATA3 gene expression. Through the secretion of IL-4, Th2 cells are involved in preventing the free radicals and their propagation through microglial cells (76). Increasing the expression of the GATA3 gene is very important for improving inflammation and disease severity in MS patients. In MS patients, the

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TABLE 6 Cytokines and NO status in ellagic acid and control groups in the beginning and at the end of the study.

Variable		Ellagic acid group (n = 25)	Control (n = 25)	Mean difference (95% CI)	p-value[a]	P2[b]
IFNγ (pg/ml)	Before	54.24 ± 3.83	53.14 ± 5.06	1.09 (−1.46, 3.64)	0.394	
	After	29.72 ± 5.89	53.09 ± 5.14	−23.37 (−26.51, −20.22)	0.001	0.001
	Mean change	−24.52 ± 3.79	−0.05 ± 0.02	−24.47 (−28.29, −19.71)	0.001	
	p-value[c]	0.001	0.830			
IL-17 (pg/ml)	Before	25.59 ± 5.92	24.48 ± 5.22	1.10 (−2.07, 4.28)	0.489	
	After	19.95 ± 5.99	26.52 ± 8.59	−6.56 (−10.78, −2.34)	0.003	0.003
	Mean change	−5.37 ± 0.92	2.04 ± 1.03	−7.41 (−11.33, −3.47)	0.001	
	p-value[c]	0.001	0.065			
IL-4 (pg/ml)	Before	29.32 ± 5.96	29.93 ± 6.80	−0.61 (−4.25, 3.02)	0.735	
	After	44.01 ± 7.87	29.84 ± 6.58	14.16 (9.98, 18.35)	0.001	0.001
	Mean change	14.69 ± 0.47	−0.09 ± 0.14	14.78 (10.04, 18.62)	0.001	
	p-value[c]	0.001	0.095			
TGF-β (pg/ml)	Before	1.42 ± 0.05	1.43 ± 0.05	−0.01 (−0.04, 0.01)	0.508	
	After	1.42 ± 0.05	1.43 ± 0.06	−0.01 (−0.04, 0.02)	0.394	0.399
	Mean change	0.00 ± 0.004	−0.02 ± 0.003	0.02 (−0.01, 0.04)	0.639	
	p-value[c]	0.285	0.387			
NO (μm)	Before	53.91 ± 1.22	54.05 ± 1.12	−0.14 (−0.09, 0.24)	0.411	
	After	35.88 ± 2.29	53.99 ± 1.18	−18.11 (−22.37, −16.82)	0.001	0.001
	Mean change	−18.03 ± 1.02	−0.06 ± 0.05	−17.97 (−19.21, −15.09)	0.001	
	p-value[c]	0.001	0.308			

Data are presented as mean ± SD.

IFNγ, Interferon-gamma; IL-17, Interleukin-17; IL-4, Interleukin-4; TGF-β, Transforming Growth Factor-beta; NO, Nitric Oxide.

[a]Independent Sample t-test.

[b]General linear model adjusted for baseline differences between groups, age, MS duration.

[c]Paired t-test.

p value < 0.05 considered significant.

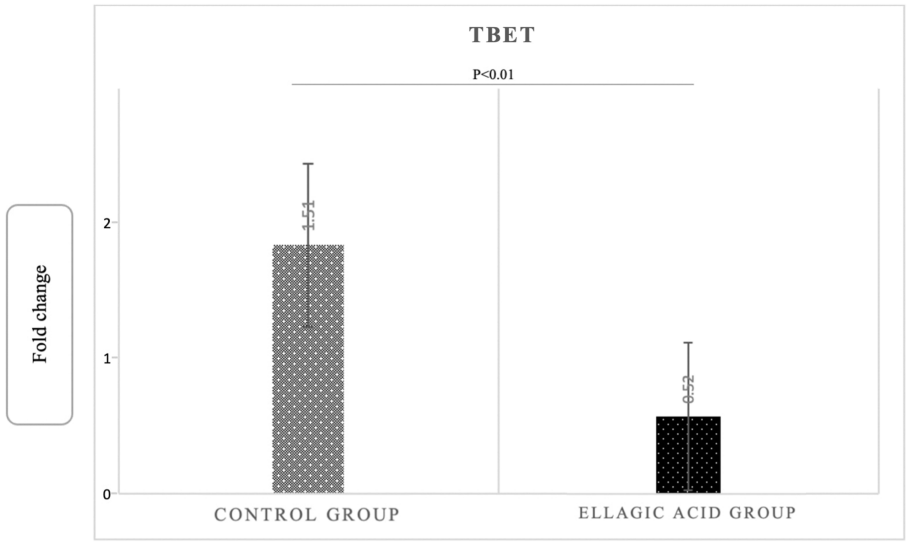

FIGURE 2
Gene expression of Tbet in ellagic acid and control groups.

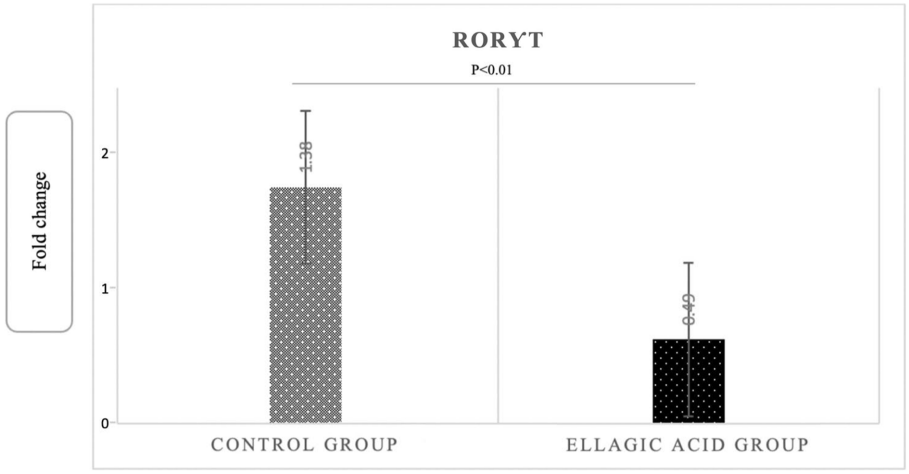

FIGURE 3
Gene expression of RORγt in ellagic acid and control groups.

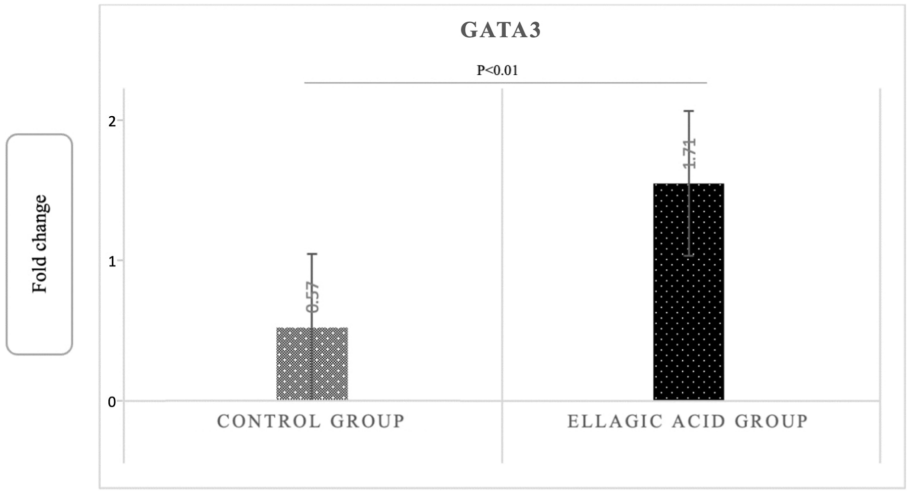

FIGURE 4
Gene expression of GATA3 in ellagic acid and control groups.

decrease of Th2 cells and the decrease of the corresponding transcription factor (GATA3) leads to an increase of Th1 cells and their inflammatory cytokines, IFNγ. On this basis, effective treatments also trigger anti-inflammatory responses induced by Th2 cytokines that increase the differentiation of naïve T cells into Th2, for which the transcription factor GATA3 is required. Increasing the expression of the GATA3 gene increases the differentiation of naïve T cells to Th2 and the amount of IL-4, whereupon the number of Th1 cells and inflammatory cytokines and the severity of MS disease decrease. Considering that the absence of phagocytosis of dead and damaged cells leads to disruption of inflammatory processes and remyelination, ellagic acid promotes phagocytosis by M2-type microglia by influencing this process, thus exerting its protective effect (77). Therefore, more attention can be paid to strengthening the activity of Th2 cells in the treatment of MS.

Jha et al. achieved neuroprotective and cognitive improvements in rats suffering from Alzheimer's disease when they investigated the neuroprotective and cognitive effects of ellagic acid (50 mg/kg), which were confirmed in their study (78).

Another finding in the present study concerned Treg cells activity, which did not change under the influence of ellagic acid and showed no effect on TGF-β levels. Align with our results, Čolić et al. (79), who investigated the immunomodulatory effects of pomegranate peel extract, showed that the Treg cells activity and the levels of TGF-β and IL-10 cytokines decreased in the supernatant of the culture medium. The reason for this finding is probably the increased use of Treg cytokines to downregulate Th1 and Th17 cytokines (79).

4.1. Strengths and weaknesses

To our knowledge, our study was the first study to assessed the effect of pure ellagic acid on immunologic parameters, pathogenic genes expression and disease severity in MS. The strength of our study

is the ellagic acid purity level (99.9%). In addition, this study discussed the immunological aspects of MS influenced by ellagic acid. However, this study has some limitations. Among them, it was not possible to check the all indicators of oxidative stress in patients in this study. Also, it was not possible to study different doses of ellagic acid. It is suggested that these limitations be addressed in further studies. It would be better if gene expression results were confirmed by measuring protein content, however, in our study, this was not possible due to equipment and budget constraints. It is suggested that in the next studies, a protein measurement method, including Western blotting, should be performed to confirm the gene expression results.

5. Conclusion

The present study has shown that supplementation with pure ellagic acid at a dose of 180 mg per day for 12 weeks lowers the levels of the cytokines IFNγ, IL-17, and increases IL-4 so decrease the TH1/Th2 ratio in the group receiving ellagic acid, whereas no significant changes were seen in the placebo group. Supplementation with ellagic acid did not affect TGF-β in any of the study groups. In addition, supplementation with ellagic acid led to the Tbet and RORγt genes expression reduction and GATA3 gene expression increment.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Ethics Committee of Iran University of Medical Sciences (Ethics code: IR.IUMS.REC.1399.1000). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

SJK: hypothesis, writing the draft, laboratory experiments, sampling, and data analysis. NA and A-AD: supervision and edit. FS: edit and review. BA and GH: sampling. MS: data analysis. A-AD and PF: laboratory experiments, KS: data analysis, native edit of manuscript and conceptualization. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Alcoholic drink produced by pea is a risk factor for incident knee surgery in patients with knee osteoarthritis

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Objective: The objective of this study is to investigate whether alcohol exposure and specific alcoholic drinks are independent risk factors for incident knee surgery in knee osteoarthritis (KOA) patients.

Methods: We identified all patients who were clinically diagnosed as KOA between January 2010 and January 2018 in our outpatient department. Demographic, clinical, and radiographic data were collected from the database of our hospital. Next, we analyzed the association between alcohol consumption and incident knee surgery.

Results: A total of 4,341 KOA patients completed the current study and were included in the final analysis. Incident knee surgery for the purpose of treating osteoarthritis was observed in 242 patients. Incident knee surgery was significantly associated with age (OR [95%CI], 1.023 [1.009–1.039], $P = 0.002$), BMI (OR [95%CI], 1.086 [1.049–1.123], $P < 0.001$), baseline K-L grade 3 (OR [95%CI], 1.960 [1.331–2.886], $P = 0.001$), baseline K-L grade 4 (OR [95%CI], 1.966 [1.230–3.143], $P = 0.005$), 7.1–14 drinks per week (OR [95%CI], 2.013 [1.282–3.159], $P = 0.002$), >14 standard drinks per week (OR [95%CI], 2.556 [1.504–4.344], $P = 0.001$), and the most common alcoholic drink produced by pea (OR [95%CI], 3.133 [1.715–5.723], $P < 0.001$).

Conclusion: KOA patients who consumed more than seven standard drinks per week were at substantial risk of incident knee surgery. In addition, alcoholic drink produced by pea is also an independent risk factor.

KEYWORDS

knee osteoarthritis, alcoholic drink, Kellgren-Lawrence (K-L) grades, total knee arthroplasty (TKA), unicompartmental knee arthroplasty (UKA), high tibial osteotomy

1. Introduction

Knee osteoarthritis (KOA) is characterized by three core symptoms (pain, stiffness, and limited function) and accompanied by many structural alterations including degradation of cartilage subchondral bone remodeling, meniscal degeneration, and Hoffa's and effusion synovitis, affecting more than 10% of the overall population globally as estimated (1–3). The disease burden of KOA had been projected to double in the following decades because of the increasing aging of the population (4). Many risk factors for KOA development and progression including female sex, aging, and overweight/obesity have been well-established by previous studies (5, 6). Lifestyle intervention is the cornerstone of KOA management.

According to a previous study, weight loss could be beneficial for KOA patients in the long term (7). In addition, increasing physical activities appropriately is also important for KOA patients by increasing lower-limb muscle strength (8–10).

KOA could also be treated with surgical procedures. The efficacy and safety of total knee arthroplasty (TKA), unicompartmental knee arthroplasty (UKA), and high tibial osteotomy (HTO) have been well-established and generally recommended for KOA management (11, 12). In contrast, many high-quality, multicenter, randomized clinical trials have consistently and repeatedly demonstrated that arthroscopic procedures, including lavage, debridement, and arthroscopic partial meniscectomy, are ineffective and even harmful in the long term for KOA patients (13–16). However, this high-quality evidence failed to curb the increase in arthroscopic procedures in KOA patients (13–16). Nevertheless, incident arthroscopic procedures at least reflected poor symptom control and were reasonably considered clinically important events for disease progression.

Excessive alcohol consumption and alcoholism are major global risk factors for increased all-cause mortality and incident morbidities but not limited to cardiovascular diseases, malignancies, neurological diseases, and accidental injuries (17–20). For KOA, a previous study revealed that excessive alcohol drinking was associated with an increased risk of KOA (21). Furthermore, the mechanistic link between alcohol intake and KOA development has been elucidated by a preclinical study (22). A population-based study concluded that alcohol consumption contributed to radiographic change in KOA in Korea (23). Notably, previous studies in KOA patients only focused on the amount of alcohol consumption rather than specific types of alcoholic drinks. This study aimed to investigate whether alcohol exposure and specific alcoholic drinks are independent risk factors for incident knee surgery in KOA patients.

2. Patients and methods

2.1. Study population

This study followed the Declaration of Helsinki and all local laws and regulations during design and conducted data analysis. We obtained ethics approval for collecting all related data from patients and medical records. We identified all patients who were clinically diagnosed as KOA at a visit to our outpatient department between January 2010 and January 2018 via the hospital information system (HIS). The

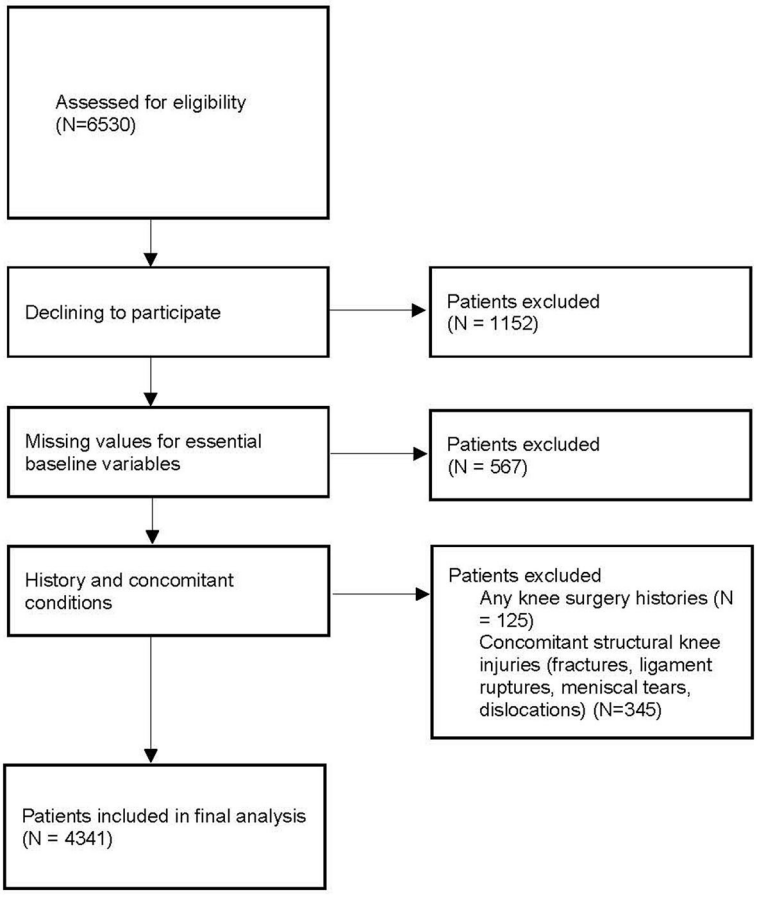

FIGURE 1
Study screening and enrollment.

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TABLE 1 Univariable analysis on characteristics grouped by incident knee surgery.

	No incident knee surgery ($n = 4,099$)	Incident knee surgery ($n = 242$)	*P*-value
Age, years	61.02 ± 8.83	62.93 ± 8.46	<0.001
Sex, No. (%)			
Male	1,068 (26.1%)	70 (28.9%)	0.324
Female	3,031 (73.9%)	170 (71.1%)	
Baseline BMI, kg/m^2	25.07 ± 3.59	26.26 ± 4.39	<0.001
Alcohol consumption, No. (%)			
None	775 (18.9%)	29 (12.0%)	<0.001
≤1 standard drink/week	784 (19.1%)	29 (12.0%)	
1.1–7 standard drinks/week	1,146 (28.0%)	68 (28.1%)	
7.1–14 standard drinks/week	1,008 (24.6%)	79 (32.6%)	
>14 standard drinks/week	386 (9.4%)	37 (15.3%)	
Most common type of alcoholic beverage*, No. (%)			
Beer	1,003 (30.2%)	59 (27.7%)	0.204
Chinese rice wine	318 (9.6%)	16 (7.5%)	
Wine	675 (20.3%)	37 (17.4%)	
Chinese distilled spirit	981 (29.5%)	81 (38.0%)	
Others	180 (5.4%)	10 (4.7%)	
Multiple	167 (5.0%)	10 (4.7%)	
Most common type of alcoholic beverage produced by kaoliang, No. (%)			
Yes	1,012 (24.7%)	86 (35.5%)	0.233
No	3,087 (75.3%)	156 (64.5%)	
Most common type of alcoholic beverage produced by rice, No. (%)			
Yes	1,068 (26.1%)	70 (28.9%)	0.324
No	3,031 (73.9%)	172 (71.1%)	
Most common type of alcoholic beverage produced by barley, No. (%)			
Yes	1,300 (31.7%)	81 (33.8%)	0.569
No	2,799 (68.3%)	161 (66.2%)	
Most common type of alcoholic beverage produced by wheat, No. (%)			
Yes	551 (13.4%)	39 (16.1%)	0.238
No	3,548 (86.6%)	203 (83.9%)	
Most common type of alcoholic beverage produced by pea, No. (%)			
Yes	79 (1.9%)	14 (5.8%)	<0.001
No	4,020 (98.1%)	228 (94.2%)	
Most common type of alcoholic beverage produced by grape, No. (%)			
Yes	406 (9.9%)	22 (9.1%)	0.680
No	3,693 (90.1%)	220 (90.9%)	
Most common type of alcoholic beverage produced by corn, No. (%)			
Yes	205 (5.0%)	16 (6.6%)	0.268
No	3,894 (95.0%)	226 (93.4%)	
Hypertension, No. (%)			
Yes	1,335 (32.6%)	86 (35.5%)	0.339
No	2,764 (67.4%)	156 (64.5%)	

(Continued)

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TABLE 1 (Continued)

	No incident knee surgery (n = 4,099)	Incident knee surgery (n = 242)	P-value
K-L grades, No. (%)			
0 or 1	1,134 (27.7%)	48 (19.8%)	0.001
2	1,766 (43.1%)	96 (39.7%)	
3	833 (20.3%)	66 (27.3%)	
4	366 (8.9%)	32 (13.2%)	
Diabetes, No. (%)			
Yes	505 (12.3%)	32 (13.2%)	0.678
No	3,594 (87.7%)	210 (86.8%)	
Smoking, No. (%)			
Yes	357 (8.7%)	24 (9.9%)	0.519
No	3,742 (91.3%)	218 (90.1%)	
Education, No. (%)			
More than 9 years	1,036 (25.3%)	66 (27.3%)	0.488
Not more than 9 years	3,063 (74.7%)	176 (72.7%)	

Data are shown as means (±SD) unless otherwise indicated.
BMI, body mass index; K-L, Kellgren–Lawrence.
*For drinkers only.

clinical diagnosis of KOA in the current study was defined as those made by clinical specialists in orthopedic and/or sports medicine. It was generally determined based on patient history, physical examination, and laboratory and radiographic findings (24). As shown in Figure 1, patients were excluded from this study if they had any knee surgery histories (n = 125), concomitant structural knee injuries (fractures, ligament ruptures, meniscal tears, and dislocations; n = 345), missing values for essential baseline variables (n = 567), and declining to participate (n = 1,152).

2.2. Baseline patient data

We defined the baseline as the time of performing the first knee plain radiograph during the study period. All baseline demographic and clinical data were retrieved from HIS, and the information was further confirmed by contacting patients through on-site interview, telephone, email, and instant message software. The baseline demographic data consisted of demographic information (sex, age, and body mass index [BMI]) calculated by weight, height, and education. The Kellgren–Lawrence (K-L) grades (25) were rated by a radiographic evaluation committee consisting of three radiologists specialized in musculoskeletal radiology. The rating process was conducted without grouping information. The consensus on grading was achieved by the majority of people. When the two knees had different K-L grades, the final K-L grade was recorded according to the more severe side.

2.3. Alcohol consumption and details of alcoholic drinks

The alcohol consumption was self-reported by patients based on their recalling for the last 12 months. Show cards were used to prompt recalling of the number of standard drinks usually consumed per week. Each show card, respectively, illustrated the typical volume of Chinese distilled spirit (25 ml of 50% alcohol/volume), Chinese rice wine (90 ml of 15% alcohol/volume), wine (120 ml of 11% alcohol/volume), and beer (285 ml of 4.5% alcohol/volume) equivalent to 10 g of ethanol, defined as a standard drink (26). For those who reported frequent consumption of other types of alcoholic beverages during the previous year, researchers calculated the weekly consumption after collecting the label information of these alcoholic beverages. The weekly number of standard drinks was recorded in a categorical manner as none, ≤1, 1.1–7, 7.1–14, and >14 standard drinks per week. The researchers also asked the patients for his or her most common alcoholic beverage type (beer, Chinese distilled spirit, Chinese rice wine, wine, and others). We collected label information of the most commonly consumed alcoholic beverages and recorded the raw materials (barley, wheat, grape, rice, kaoliang, pea, and corn) of these alcoholic beverages.

2.4. Definition of incident knee surgery

In this study, incident knee surgery was defined as any surgical procedure performed for the purpose of treating KOA no matter whether this type of surgical procedure was recommended or not. The incident knee surgery and types of surgery were reported by

patients. In the current study, incident knee surgery included TKA, arthroscopic procedures (ineffective and not recommended), UKA, and HTO.

2.5. Statistical analysis

All statistical analyses were performed using SPSS software (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, version 26.0. Armonk, NY: IBM Corp). The statistical significance was set at a two-sided 0.05. We first tabulated descriptive statistics to summarize the characteristics of the subjects. Continuous and categorical variables were, respectively, presented as means ± standard deviations and counts (percentage), unless otherwise indicated. When the *P*-value was <0.2 in univariable analysis, the variables along with demographic variables (age, sex, and BMI) were further included in logistic regression for multivariable analysis.

3. Results

A total of 4,341 KOA patients were included in the final analysis. Incident knee surgery for the purpose of treating osteoarthritis was observed in 242 patients out of 4,341 patients during the study period. Specifically, 65 patients had TKA, 5 had UKA, 162 had arthroscopic procedures, and 10 had high tibial osteotomy. For univariable analyses, incident knee surgery was significantly associated with age, BMI, baseline K-L grades, alcohol consumption, and the most common type of alcoholic beverage produced by pea (Table 1). The logistic regression model included sex, baseline BMI, baseline age, alcohol consumption, most common type of alcoholic beverage produced by pea, and baseline K-L grades.

After adjustment with the multivariable logistic regression, incident knee surgery was significantly associated age (OR [95%CI], 1.023 [1.009–1.039], $P = 0.002$), BMI (OR [95%CI], 1.086 [1.049–1.123], $P < 0.001$), baseline K-L grade 3 (OR [95%CI], 1.960 [1.331–2.886], $P = 0.001$), baseline K-L grade 4 (OR [95%CI], 1.966 [1.230–3.143], $P = 0.005$), 7.1–14 drinks per week (OR [95%CI], 2.013 [1.282–3.159], $P = 0.002$), >14 standard drinks per week (OR [95%CI], 2.556 [1.504–4.344], $P = 0.001$), and the most common alcoholic drink produced by pea (OR [95%CI], 3.133 [1.715–5.723], $P < 0.001$; Table 2).

TABLE 2 Multivariable analysis on characteristics grouped by incident knee surgery.

	Odds ratio (95% confidence interval)	*P*-value
Baseline BMI	1.086 (1.049–1.123)	<0.001
Male Sex (Reference: female)	0.918 (0.676–1.247)	0.585
Age	1.023 (1.009–1.039)	0.002
Most common type of alcoholic beverage produced by pea	3.133 (1.715–5.723)	<0.001
Alcohol consumption ≤1 standard drink/week (Reference: none)	0.939 (0.551–1.603)	0.819
Alcohol consumption 1.1–7 standard drinks/week (Reference: none)	1.536 (0.976–2.417)	0.064
Alcohol consumption 7.1–14 standard drinks/week (Reference: none)	2.013 (1.282–3.159)	0.002
Alcohol consumption >14 standard drinks/week (Reference: none)	2.556 (1.504–4.344)	0.001
K-L grade 2 (Reference: K-L grade 0 or 1)	1.339 (0.935–1.919)	0.111
K-L grade 3 (Reference: K-L grade 0 or 1)	1.960 (1.331–2.886)	0.001
K-L grade 4 (Reference: K-L grade 0 or 1)	1.966 (1.230–3.143)	0.005

The logistic regression model included baseline sex, baseline BMI, age, alcohol consumption, most common type of alcoholic beverage produced by pea, and K-L grades.

4. Discussion

A previous study using data from the Osteoarthritis Initiative study revealed that excessive alcohol drinking was associated with an increased risk of both radiographic and symptomatic KOA (21). Similarly, a population-based and longitudinal study conducted in Korea found that alcohol consumption contributed to the radiographic progression of KOA (23). For osteoarthritis in other anatomic sites, researchers reported that alcohol exposure is associated with structural destruction and inflammatory features of hand osteoarthritis (27). Notably, previous studies in KOA patients only focused on the amount of alcohol consumption rather than specific types of alcoholic drinks.

The underlying mechanism between alcohol drinking and osteoarthritis remains greatly unclear, while many plausible hypotheses and theories have been proposed. As revealed by many preclinical investigations, alcohol intake is capable of inducing pro-inflammatory states in joints and is thus believed to be a contributing factor to the development and progression of KOA (22). In a mouse model, chronic alcohol consumption also increases cartilage loss in large joints by impairing extracellular matrix production and accelerating the degradation (28). In addition, alcohol could increase the level of inflammatory mediator interleukin-6 (IL-6), an important cytokine in KOA development and progression (29).

The most important and novel finding of the current study is the unexpected association between incident knee surgery and exposure to pea-derived alcoholic beverages in KOA patients. To the best of our knowledge, only some types of Chinese distilled spirit (most of them are made by a fermentation technique called "Daqu") use pea as a major raw material worldwide. Daqu is one of the oldest and most widely used fermentation technique for spirit-making (30). In addition to alcohol, fermentation with the Daqu technique often produces substantial amount and various

types of chemicals with unknown effects on humans (30–32). Clearly, microbiota (molds, yeasts, and bacteria) are responsible for the final chemicals. However, in the current study, we are unable to further determine whether certain microorganisms are involved in this phenomenon. Nevertheless, our finding provides a unique and exciting insight into the pathogenesis of osteoarthritis.

The current study had several limitations. First, future confirmation of our observation by prospective and larger cohort studies should be performed. If so, mechanistic studies are urgently needed to explore why pea-derived alcoholic beverages are associated with osteoarthritis progression. Notably, this is the first study reporting this phenomenon, and thus, we are currently unable to propose a reasonable hypothesis without future mechanistic studies. Second, because of the observational nature, the decision on whether to receive surgical treatment in this study lacked transparency for us and readers. Finally, because the drinking pattern and specific types of alcoholic beverages may largely vary by age, sex, and socioeconomic status in a general population (33, 34), future studies on alcohol consumption and KOA should further explore these factors. Extrapolation of our conclusion to a different setting should be cautious.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by the Ethics Committee at Jinjiang Municipal Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

XH: Data curation, Investigation, Writing—original draft. JZ: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing—review and editing. YZ: Data curation, Investigation, Writing—original draft. XL: Data curation, Investigation, Writing—original draft. YX: Data curation, Investigation, Writing—original draft. YF: Data curation, Investigation, Writing—original draft. ZL: Data curation, Investigation, Writing—original draft. LL: Data curation, Investigation, Writing—original draft. HZ: Data curation, Investigation, Writing—original draft. ZW: Data curation, Investigation, Writing—original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Polyphenolic natural products as photosensitizers for antimicrobial photodynamic therapy: recent advances and future prospects

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Antimicrobial photodynamic therapy (aPDT) has become a potent contender in the fight against microbial infections, especially in the context of the rising antibiotic resistance crisis. Recently, there has been significant interest in polyphenolic natural products as potential photosensitizers (PSs) in aPDT, given their unique chemical structures and inherent antimicrobial properties. Polyphenolic natural products, abundant and readily obtainable from natural sources, are generally regarded as safe and highly compatible with the human body. This comprehensive review focuses on the latest developments and future implications of using natural polyphenols as PSs in aPDT. Paramount polyphenolic compounds, including curcumin, hypericin, quercetin, hypocrellin, celastrol, riboflavin, resveratrol, gallic acid, and aloe emodin, are elaborated upon with respect to their structural characteristics, absorption properties, and antimicrobial effects. Furthermore, the aPDT mechanism, specifically its targeted action on microbial cells and biofilms, is also discussed. Polyphenolic natural products demonstrate immense potential as PSs in aPDT, representing a promising alternate approach to counteract antibiotic-resistant bacteria and biofilm-related infections.

KEYWORDS

polyphenols, natural products, photodynamic therapy, photosensitizers, antibacterial

1 Introduction

The pervasive phenomenon of antimicrobial resistance (AMR) in a broad array of pathogenic microorganisms presents a grave and pressing concern for global health and developmental progress (1). Multidrug resistance (MDR) in bacteria culminates in hundreds of thousands of deaths annually (2), underscoring AMR's role as a profound international health concern (3). Concurrently, the limited availability of effective drugs for fungal infections and the rising resistance to these drugs have led to a distressingly high mortality rate (4–6). Additionally, the unprecedented emergence of SARS-CoV-2 has introduced a global threat to human life and health. The severe implications of antimicrobial resistance on human health and economic systems necessitate the accelerated development of innovative strategies to counteract this formidable issue effectively (7, 8). As shown in Figure 1, in response to this, there has been a surge of research interest dedicated to developing alternative solutions to combat antimicrobial resistance, such as cationic polymers, peptidoglycans, metal nanoparticles, nanocarriers, photodynamic therapy (PDT), and photothermal therapy (PTT) (9, 10).

PDT is a therapeutic modality that employs low-energy light to activate photosensitizers (PSs) for both diagnostic and therapeutic purposes. Antimicrobial photodynamic therapy (aPDT), a specific application of PDT, serves as a chemical treatment method to control infections caused by bacteria, fungi, and viruses. As a potent and promising alternative, aPDT strives to mitigate the proliferation of pathogenic microorganisms, encompassing both gram-positive and gram-negative bacteria, fungi, viruses, and parasites. This is achieved by curtailing microbial growth, preventing biofilm formation, and potentially resolving antibiotic resistance issues (11, 12). One notable advantage of aPDT is its noninvasive or minimally invasive nature, which enables a targeted approach primarily against the microorganisms, sparing animal tissue cells from unnecessary damage. This relatively simple and selective approach ensures effective pathogen elimination while minimizing harm to the host (13). The fundamental components of aPDT include the light, PSs, and ambient oxygen. Independently, these elements are benign, but their amalgamation can render a potent antimicrobial effect. This process entails the use of a PS, which, when activated by a particular wavelength of light in the presence of oxygen, generates a copious amount of reactive oxygen species (ROS). These ROS, in turn, interact with multiple targets within microbial cells, inducing the oxidation of biomolecules and ultimately causing cell death.

PSs play an instrumental role in aPDT because they are responsible for absorbing light energy. Various synthetic compounds such as tetrapyrrole macrocycles (porphyrins, phthalocyanines), heterocyclic compounds (methylene blue, toluidine blue O), indocyanine green, and psoralens have been extensively studied for their antibacterial potency in aPDT (14–16). In contrast to synthetic compounds, natural products are generally imbued with more complex chemical structures, granting them unique capabilities in moderating physiological processes and contending with external threats. Derived from natural sources such as plants, animals, and microorganisms, these products acquire unique chemical structures through prolonged evolutionary processes. These structures can engage with molecular entities within organisms, thus intervening in and regulating numerous physiological processes. Among these natural products, polyphenols represent a noteworthy class of compounds found abundantly in various plant-based products, such as vegetables, fruits, seeds, and legumes. Characterized by a series of molecules bearing one or more phenolic rings (17, 18),

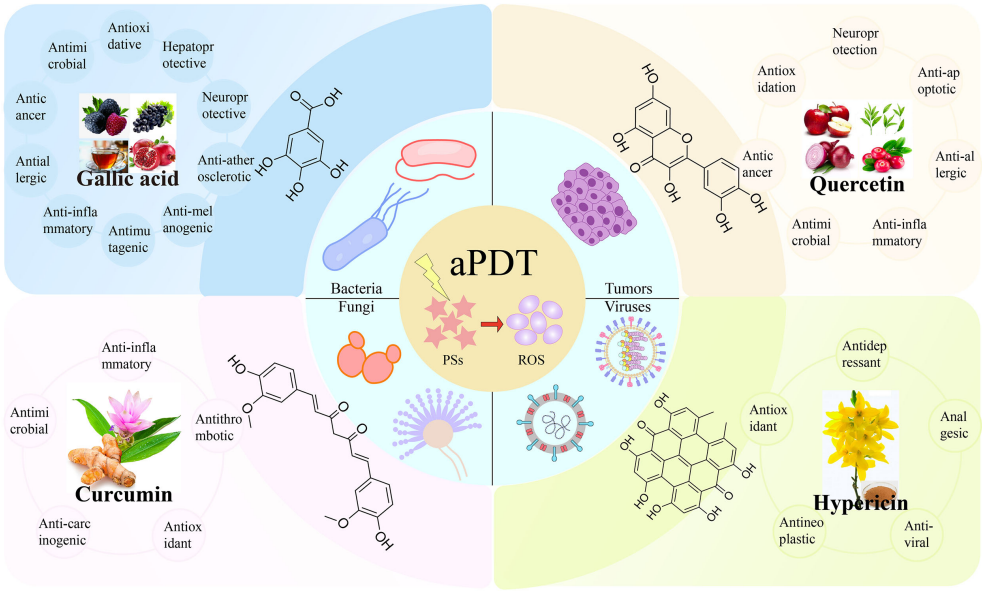


FIGURE 1
The representative polyphenolic natural products as photosensitizers for antimicrobial photodynamic therapy.

polyphenols frequently exhibit a diverse array of biological activities, including antioxidant, anticancer, antibacterial, antiviral, and anti-inflammatory properties, which render them potent candidates for the treatment of infections and other diseases (19–21). The significance of polyphenolic natural products in aPDT is underscored by their traditional role as a source for modern drug discovery, offering potential drug leads due to their unique structures, diverse chemical and biological properties, and antimicrobial and anti-inflammatory characteristics (22, 23). Consequently, polyphenolic natural products as PSs have gained considerable attention in the field.

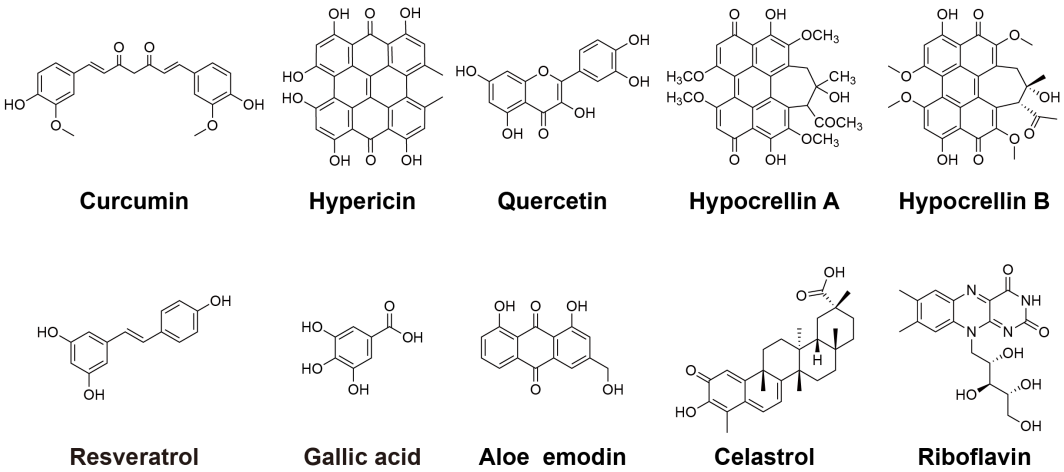
This review focuses on recent advances and future prospects of aPDT for treating microbial infections, with a specific emphasis on the application of polyphenolic natural product PSs (Scheme 1). The unique properties and promising potential of these compounds in combating infections warrant further exploration and development to identify effective therapeutic interventions.

2 Polyphenolic natural PSs

2.1 Curcumin

Curcumin (CUR), a natural polyphenol extracted from the dried rhizomes of the ginger plant turmeric (*Curcuma longa L.*), has a long history of culinary, traditional medicinal, cosmetic, and herbal supplement use (24). Chemically, curcumin is a diarylheptanoid, a polyphenol, with beta-diketone and enone functionalities, and its structure is related to a dimer of ferulic acid (25). Natural curcumin consists of three distinct curcuminoids: curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC) (21). The compound demonstrates a broad spectrum of pharmacological effects, including anti-inflammatory, antimicrobial, anticarcinogenic, antioxidant, and antithrombotic activities (26, 27). Curcumin is known for its safety, efficacy, and environmentally friendly characteristics (28). It has also been extensively investigated as a highly effective PS in the field of photodynamic therapy due to its broad absorption range between 300 and 500 nm and its nontoxicity in cell culture models and animal studies (29). Due to its favorable properties, curcumin has been extensively researched for its therapeutic potential and supportive care in clinical conditions such as breast cancer, multiple myeloma, non-small cell lung cancer, and depression (30–33).

As a natural compound, curcumin has been widely investigated as a PS in aPDT. For instance, Li et al. demonstrated the effective eradication of *Bacillus subtilis* (*B. subtilis*) through curcumin-mediated PDT by inducing an imbalance in the cellular redox state, causing DNA damage and disrupting membrane structures (13). Wang et al. demonstrated that curcumin (25 μM)-mediated aPDT could inhibit 5 log CFU/ml of *Staphylococcus saprophyticus* (*S. saprophyticus*) with the irradiation parameters (430-470 nm, 4.32 J/cm^2 10 min) in food production (34). Abdulrahman et al. concluded that curcumin-mediated aPDT inhibits the biofilm formation by 70% of *Pseudomonas aeruginosa* (*P. aeruginosa*) with 10 J/cm^2 laser light and 6.75 mM of curcumin (35). However, the use of curcumin in aPDT is currently limited to local applications on superficial wounds, such as the skin and oral cavity, primarily due to its absorption of blue light within the light spectrum (300-500 nm), which has restricted tissue penetration capabilities. Muniz et al. demonstrated that curcumin (100 μg), as a PS being activated *ex vivo* by LED (450 nm, 13.5 J/cm^2), effectively controlled *Staphylococcus aureus* (*S. aureus*) infections in mice with type 1 diabetes mellitus (36). Méndez et al. found that curcumin-mediated aPDT effectively reduced the viability of microbial cells and compromised the vitality of intact biofilms of infected dentin caries microcosms (37). Moreover, curcumin-mediated aPDT has shown efficacy against various pathogens, including *Escherichia coli* (*E. coli*, inactivated up to 3 log CFU/mL), *Listeria innocua* (*L. innocua*, inactivated more than 5 log CFU/mL) in food systems (38), *Propionibacterium acnes* (*P. acnes*, inhibition ratio was 100%) associated with acne vulgaris (39), significantly decreased planktonic *Streptococcus mutans* (*S. mutans*) and *S. mutans*



SCHEME 1
The chemical structures of polyphenols.

biofilm (2 log10 CFU/mL reductions) in dental caries (40, 41), complete kill of *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) (42), methicillin-resistant *S. aureus* biofilm (2.03 log10 CFU/mL reductions) (24), and fungi such as *Candida albicans* (*C. albicans*, 1 log reductions) and other stains of the *Candida* spp. (43) Table 1 for a detailed description of the application of polyphenols as PSs in aPDT.

However, the excellent biological and pharmacological activities of curcumin are hindered by its inherent physicochemical properties, including low solubility, rapid metabolization, instability, and the presence of a negative charge state, which hampers effective contact and adhesion to the surfaces of bacteria with negative charge (72). Extensive research has been conducted to address these challenges, particularly through the exploration of an ideal nanocarrier for curcumin (73–75). Additionally, optimizing the formulation and delivery methods of curcumin-based aPDT is crucial to overcome limitations related to tissue penetration. Further research is necessary to improve the bioavailability and absorption of curcumin, maximizing its efficacy in medical and health applications.

2.2 Hypericin

Hypericin (HYP), a naturally occurring pigment isolated from hypericum plants of the genus *Hypericum perforatum* (commonly referred to as Saint John's Wort), is well-known for its antidepressant, antioxidant, antineoplastic, potential antiviral and analgesic activities. It has recently been recognized as an effective and promising PS agent found in nature (44, 45). HYP, an anthraquinone derivative exhibits a high quantum yield for the generation of ROS and a slow rate of photobleaching (49, 76). It can also be synthesized from emodin, another anthraquinone derivative (77). The optical properties of HYP enable its absorbance of electromagnetic radiation within the visible spectrum range of 500-620 nm, with a peak absorbance at 595 nm. Upon light exposure, it displays strong red fluorescence, typically emitted at approximately 603 nm, contributing to its intense red fluorescence characteristics (78). HYP exhibits high lipophilicity and poor water solubility, displaying multiple absorption peaks in organic solvents within its visible spectrum, notably at 550 nm and 588 nm in ethanol. Additionally, emodin in ethanol exhibits fluorescence emission at approximately 600 nm. However, when dissolved in aqueous solutions, HYP tends to form nonfluorescent high-molecular-weight aggregates (79, 80).

Recently, there has been increasing interest in investigating the pharmaceutical potential of HYP as a PS in aPDT. Barroso et al. demonstrated effective antimicrobial activity of aPDT using HYP as a PS against *P. acnes* biofilms and highlighted its potential for clinical treatment of acne vulgaris (44). Kashef et al. investigated the high phototoxicity of HYP against *S. aureus*, *Enterococcus faecalis* (*E. faecalis*), and *E. coli* at extremely low drug concentrations. While observing minimal cytotoxic effects on cultured human fibroblast cells (46). Aponiene et al. showed efficient elimination of food-borne pathogen *Bacillus cereus* (*B. cereus*) through hypericin-based photosensitization in both *in vitro* experiments and on the surfaces of fruits and vegetables (45). Paz-Cristobal et al. confirmed the greater efficacy of HYP at lower concentrations against azole-resistant *C. albicans* (47). In a study by Alam et al., the effectiveness of PDT against *P. aeruginosa*, a gram-negative bacterium with limited PS penetration, was enhanced by combining HYP with ampicillin. This combination acted as a permeabilizing agent, disrupting the bacterial cell wall and increasing cell permeability, thereby maximizing the efficacy of PDT (48). Additionally, Kashef et al. demonstrated the efficacy of combining HYP with acetylcysteine in reducing biofilm formation and disrupting mature biofilms across various bacterial strains, notably, against *S. aureus*, a prominent pathogen (49).

Despite its desirable properties such as a high quantum yield of singlet oxygen generation, low dark toxicity, a high extinction coefficient near 600 nm, and significant inhibition of gram-positive bacterial growth, the utilization of HYP in biological applications is limited by its high lipophilicity and water insolubility in its natural form. Consequently, its potential in biopharmaceuticals is constrained, and its clinical implementation faces substantial hurdles. Therefore, the development of a delivery system is crucial to overcome these limitations. Various delivery systems, including polymeric nanoparticles and liposomes, have been extensively explored for HYP, showing promising results (76, 81–83).

2.3 Quercetin

Quercetin (QCT), a natural polyphenol, belongs to the subclass of flavonols, one of the six subclasses of flavonoid compounds (84). It is abundantly found in various fruits and vegetables such as apples, grapes, onions, and tomatoes, as well as beverages such as tea and red wine, nuts and honey, from different plant sources (50, 84). As a secondary metabolite, QCT exhibits a diverse array of pharmacological activities, including neuroprotection, antioxidation, antimicrobial, anticancer, anti-inflammatory, and anti-allergic and anti-apoptotic effects (50, 51). QCT demonstrates distinct absorption peaks at 380 and 258 nm (85), and its biological efficacy is significantly enhanced at micromolar concentrations when activated by light within the range of 405 ± 10 nm (51).

Despite limited research on the application of QCT as a PS in aPDT, some studies have explored its correlation and potential. One study demonstrated that QCT-mediated aPDT significantly reduced the growth of *E. coli* and *Listeria monocytogenes* (*L. monocytogenes*) in a buffer solution, indicating its potential as an antimicrobial agent against these bacteria (52). Pourhajibagher et al. utilized QCT with a light-emitting diode to effectively reduce the growth of *A. baumannii* biofilms and downregulate genes involved in the biofilm formation (53). Condat et al. developed synthetic photoactivable glycerol-based coatings incorporating QCT, which demonstrated a remarkable 99% inhibition of *S. aureus* proliferation after 2 and 6 hours of incubation under light activation (50). Another study conducted by Pourhajibagher et al. demonstrated that the synergistic combination of blue laser and low-concentration nanoquercetin can disrupt the microbial biofilm

TABLE 1 Polyphenols as PSs for aPDT.

Polyphenols	The absorption range/peak	Light type and parameters (wavelength, power/power density, irradiation time) *	Microorganisms	Concentration and incubation time of PSs	Efficacy	Reference
Curcumin	420-470 nm	Blue LED, 470 nm, 120 W, 6 min	*B. subtilis*	50 μM, 15 min	Effectively kill	(13)
		Blue LED, 430-470 nm, 4.32 J/cm^2 10 min	*S. saprophyticus*	25 μM, 15 min	5 log CFU/ml reductions	(34)
		405 nm light, 10 J/cm^2 26 s	*P. aeruginosa*	6.75 mM, 10 min,	4.62 $\log_{10}$ planktonic cell reductions	(35)
		Blue LED, 450 nm, 13.5 J/cm^3 180 s	*S. aureus* (MRSA)	100 μg/mice, NR	Effectively control the burden of MRSA in type 1 diabetes mellitus mice	(36)
		Blue LED, 455 ± 30 nm, 75 J/cm^3 1870 s	Intact microcosm biofilms of dentin caries	NR	Reduced substantially the vitality of intact microcosm biofilms	(37)
		UVA, 320-400 nm, 32 W/m^2 5 min	*E. coli O157:H7*	1~10 mg/L, 5 min,	Inactivate up to 3 log CFU/mL	(38)
		UVA, 320-400 nm, 32 W/m^2 5 min	*L. innocua*	1~10 mg/L, 5 min,	Inactivate more than 5 log CFU/mL	(38)
		LED, 410-510 nm, 0.09 (0.18) J/cm^2, 0.5 (1) min	*P. acnes*	1.56~100 μM, NR,	Inhibition ratio was 100%	(39)
		LED, 405 nm, 25.3 J/cm^2, 300 s	*S. mutans*	10^4 ng/mL, NR,	Significantly decreased	(40)
		Blue Light, 385-515 nm, 14.6 J/cm^2, 60 s	*S. mutans* biofilm	0.10wt% CUR loading on resin physicochemical, 6 h or 24 h	2 $\log_{10}$ CFU/mL reductions	(41)
		LED, 420-480 nm, 16.8 J/cm^2, 1 min	*A. actinomycetemcomitans*	0.78 μg/mL Curcuma longa extract, 48 h	Complete kill	(42)
		LED, 450 nm, 50 J/cm^2, 455 s	*MRSA* biofilm	80 μg/mL, 20 min	2.03 $\log_{10}$ CFU/mL reductions	(24)
		Blue LED, 450 ± 5 nm, first:10 J/cm^2, 91 s; second: 25 J/cm^2, 228 s	*C. albicans*	200 μg/mL, 20 min	1 log reductions	(43)
		Blue LED, 450 ± 5 nm, first:10 J/cm^2, 91 s; second: 25 J/cm^2, 228 s	*C. tropicalis*	200 μg/mL, 20 min	5 log reductions	(43)
Hypericin	590-595 nm	LED, 660 nm, 100 J/cm^2, 30 s	*P. acnes* biofilms	15 μg/mL, 3 min	14.1% reductions	(44)
		BL-300 LED, 585 nm, 9.2 J/cm^2, 40 min	*B. cereus*	10^{-7} M, 60 min	4.4 log CFU/mL reductions	(45)
		LED, 590 nm, 48 J/cm^2, 10 min	*S. aureus*	1 μg/mL, 5 min	6.3 log killing	(46)
		LED, 590 nm, 48 J/cm^2, 10 min	*E. faecalis*	1 μg/mL, 5 min	6.5 log killing	(46)
		LED, 590 nm, 48 J/cm^2, 10 min	*E. coli*	1 μg/mL, 5 min	6.2 log killing	(46)
		LED, 590 nm, 48 J/cm^2, 10 min	*P. aeruginosa*	1 μg/mL, 5 min	0.7 log killing	(46)
		LED, 602 ± 10 nm, 18 or 37 J/cm^2, 10 min	Azole-resistant and sensitive *C. albicans*	5 or 10 μM, 5 h	5 $\log_{10}$ CFU/mL reductions	(47)
		LED, 590 nm, 150 ± 20 W/m^2,3 h	Ampicillin-resistant *P. aeruginosa*	10 μM + ampicillin (100μg/mL), 30 min	3.4 log reductions	(48)

(Continued)

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TABLE 1 Continued

Polyphenols	The absorption range/peak	Light type and parameters (wavelength, power/power density, irradiation time) *	Microorganisms	Concentration and incubation time of PSs	Efficacy	Reference
		LED, 590 nm, 150 ± 20 W/m^2,1 h	*C. albicans*	10 μM, 30 min	4.8 log reductions	(48)
		LED, 590 nm, 16 J/cm^2, 10 min	*S. aureus* biofilms	0.5 μg/mL + 10 mg/mL acetylcysteine, 5 min,	5.7 log killing	(49)
Quercetin	405nm	Xenon lamp, 365 nm, 70 mW/cm^2, 240 s	*E. coli*	500 mM, 2 h or 6 h,	No effect	(50)
		Xenon lamp, 365 nm, 70 mW/cm^2, 240 s	*S. aureus*	500 mM, 2 h or 6 h	Total death	(50)
		Blue laser, 405 ± 10 nm, 150 mW/cm^2, 60 s	*S. mutans* biofilms	64 μg/mL, 5 min,	4 $\log_{10}$ CFU/mL reductions	(51)
		LED, 405 nm, 80 J/cm^2, 68 min 21 s	*E. coli* O157:H7	75 μM, 68 min 21 s	6.20 log reductions	(52)
		LED, 405 nm, 80 J/cm^2, 68 min 21 s	*L. monocytogenes*	75 μM, 68 min 21 s	>7.55 log reductions	(52)
		LED, 435 ± 10 nm, 300-420 J/cm^2, 5 min	*A. baumannii* biofilms	500 μg/mL, 2 h	40.8% reductions	(53)
Hypocrellin A	400-700nm	Incandescent lamp,400-780 nm, 1128 lux, 30 min	*C. albicans*	1.0 μg/mL, 30 min	Approximately 50% reductions	(54, 55)
		Laser, 470 nm, 100 mW/cm^2, 30 min	*C auris*	With polylactic acid, 30 min	>99.9% mortality	(56)
		Laser, 470 nm, 100 mW/cm^2, 30 min	Multidrug-resistant *Candida* spp.	12.5 μg/mL with polyethylene glycol, 30 min	Completely kill	(57)
		NR, 470 nm, 90 mW/cm^2, 60 min	Methicillin-resistant *S. aureus*	1.38 mg/L with mPEG-PCL, 24 h	Minimum bactericidal concentration	(58)
Hypocrellin B	450-550nm	Xenon lamp, 400-780 nm, 72 J/cm^2, 15 min	*C. albicans*	100 μM, 30 min	No viable cells	(59)
		Xenon lamp, 400-780 nm, 72 J/cm^2, 15 min	Azole-sensitive clinical isolate of *C. albicans*	100 μM, 30 min	6.01 $\log_{10}$ reductions	(59)
		Xenon lamp, 400-780 nm, 72 J/cm^2, 15 min	Azole-resistant clinical isolate of *C. albicans*	100 μM, 30 min	7 $\log_{10}$ reductions	(59)
		LED, 460 ± 20 nm/645 ± 20 nm, 24 J/cm^2, 3 min	Drug-resistant *P. aeruginosa*	10 μM (HB: La^{+3})$^{\&}$, 5 min	5 log reductions	(60)
Resveratrol	200-330nm	Blue LED,450 ± 20 nm/, 54 J/cm^2, 5 min	*S. aureus*	2 mg/mL, 5 min,	Approximately 75% reductions	(61)
Gallic acid	273nm	UVA-light, 2646 ± 212 μW/cm^2, 15 min	*E. coli O157:H7*	10 mM, 15 min	4.95 ± 0.19 log CFU/mL reductions	(62)
		UVA-light, 3.2 ± 0.2 mW/cm^2, 30 min	*E. coli O157:H7*	1 mM with 5 mM lactic acid, 30 min	4.7 ± 0.5 log CFU/ml reductions	(63)
		LED, 400 nm, 80 mW/cm^2, 15 min	*S. aureus*	4 mmol/L, 15 min	>5 log reductions	(64)
Aloe emodin	250nm, 284nm, 430nm	Xenon lamp, 435 ± 10nm, 96 J/cm^2, 20 min	Multidrug-resistant *A. baumannii*	100 μM, 20 min	4.50~6.89 $\log_{10}$ reductions	(65)

(Continued)

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TABLE 1 Continued

Polyphenols	The absorption range/peak	Light type and parameters (wavelength, power/power density, irradiation time) *	Microorganisms	Concentration and incubation time of PSs	Efficacy	Reference
		Xenon lamp, 400-780 nm, 24 J/cm^2, 5 min	*C. albicans* (a standard strain)	5 μM, 30 min	5.84 $\log_{10}$ reductions	(66)
		Xenon lamp, 400-780 nm, 24 J/cm^2, 5 min	Azole-sensitive *C. albicans*	5 μM, 30 min	5.56 $\log_{10}$ reductions	(66)
		Xenon lamp, 400-780 nm, 24 J/cm^2, 5 min	Azole-resistant *C. albicans*	5 μM, 30 min	4.69 $\log_{10}$ reductions	(66)
		Xenon lamp, 435 ± 10nm, 72 J/cm^2, 30 min	*T. rubrum* (control strain)	1 μM, 2 h	Decreased survival rate to 17.10%	(67)
		Xenon lamp, 435 ± 10 nm, 72 J/cm^2, 30 min	*T. rubrum* (clinical strain)	1 μM, 2 h	Decreased survival rate to 18.63%	(67)
		Xenon lamp, 400-780 nm, 96 J/cm^2, 20 min	*Malassezia furfur*	10 μM, 30 min	No viable cells	(68)
Celastrol and *T. wilfordii extract*	425nm	LED, 660 nm, 120 ± 20 W/m^2, 15 min	*S. aureus*	20 μg/mL (TWE), 30 min	3.3 log reductions	(69)
		LED, 660 nm, 120 ± 20 W/m^2, 10 min	MRSA	20 μg/mL (TWE), 30 min	3.4 log reductions	(69)
		LED, 660 nm, 120 ± 20 W/m^2, 30 min	*C. albicans*	20 μg/mL (TWE), 30 or 60 min	2.0 log reductions	(69)
Riboflavin	270 nm, 366 nm, and 445 nm	LED, 365 nm, 30 J/cm^2, 1 h	*S. aureus*, *P. aeruginosa* *E. coli*	0.1 mg/mL with PEG, 15 min	Approximately 4 log reductions	(70)
		LED, 365 nm, 30 J/cm^2, 1 h	*S. typhimurium*, Coliphage	0.1 mg/mL with PEG, 15 min	Approximately 3 log reductions	(70)
		Blue light, 460 nm, 80 mW/cm^2, 10 min	*S. aureus, E. coli*, MRSA	100 μL (Riboflavin-loaded supramolecular hydrogels), NR,	Inhibition ratio over 99.999%	(71)

NR, not reported; *The irradiation frequency is 1 without special explanation; [&]Hypocrellin B with lanthanide ions; MRSA,: methicillin-resistant *Staphylococcus aureus*; LED, light-emitting diode; UVA, ultraviolet A; CUR, curcumin; mPEG-PCL, methoxy poly (ethylene glycol)-block-poly(ϵ-capro-lactone); TWE, ethanolic extract of *T. wilfordii*; PEG, polyethylene glycol; CFU, colony forming units.

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of *S. mutans* and reduce its metabolic activity (51). However, further research is necessary to evaluate the antibacterial pharmacological activity of QCT and determine its potential value in clinical applications.

2.4 Hypocrellins

Hypocrellins, primarily composed of hypocrellin A and B, which are perylenoquinone derivatives, are obtained from the fruiting bodies of the traditional Chinese medicine fungi *Hypocrella bambusae* and *Shiraia bambusae* (86, 87). Hypocrellins, structurally related to HYP, are predominantly lipophilic, although a few hydrophobic hypocrellin derivatives have been synthesized, with limited studies on their properties (86, 88). Structurally, hypocrellin A (HA) and hypocrellin B (HB) exhibit a high degree of similarity, differing only by the presence of a single hydroxyl group (59, 89). Hypocrellins exhibit several advantageous characteristics, including a notable quantum yield for singlet oxygen ($^1O_2$) generation, strong generation of anionic free radicals in deoxygenated environments, rapid clearance from normal tissues, minimal dark toxicity, and existence in a pure monomeric form. These exceptional attributes have led to the extensive utilization of hypocrellin as a PS in photodynamic therapy (89). In ethanol, HA exhibits three distinct absorption peaks at 581 nm, 542 nm, and 463 nm, within the visible light spectrum range of 400 - 700 nm (54). The absorption wavelength of HB ranges from 450 nm to 550 nm (90).

Hypocrellins have been extensively studied for their potential applications in treating various dermatological conditions, and viral infections, including human immunodeficiency virus (HIV), and even cancer (91). Due to their unique characteristics, such as ease of preparation and purification, high photoreactivity with low dark toxicity (92), and rapid tissue clearance, hypocrellins have garnered significant attention as novel therapeutic agents and/or diagnostic tools (87, 91). In PDT, HA plays a crucial role in anticancer treatment (93). However, research on the antimicrobial photodynamic activity of HA is limited and primarily focused on *C. albicans* (55), *Candida auris* (*C. auris*) (56, 57), and methicillin-resistant *S. aureus* (58). Nonetheless, the efficacy of HA is limited by certain characteristics, including poor water solubility, tendency to aggregate under physiological conditions, and limited absorption within the phototherapeutic window, which restricts its clinical application in PDT. To overcome these limitations, Guo and colleagues developed a self-assembled amphiphilic micelle that is sensitive to lipase, enabling efficient delivery of HA. The micelles composed of mPEG-PCL/HA demonstrated promising antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) (58). In another study, Liu et al. prepared a recyclable and light-triggered nanofibrous membrane of polylactic acid conjugated with HA and modified porous organic cages with HA for targeting *C. auris* and multidrug-resistant *Candida* species, respectively (56, 57). Similarly, research on HB primarily revolves around its antitumor and antiviral properties. Studies have revealed that HB demonstrates potent photodynamic effects against malignant tumors, human immunodeficiency virus type I (HIV-I), and herpetic stomatitis (90). In their *in vitro* experiments, Hu et al. demonstrated that HB-LED PDT triggers apoptosis in human keloid fibroblasts through the mitochondrial apoptotic pathway (89). Moreover, Hashimoto et al. found that HB-mediated aPDT exhibits promise as a viable alternative treatment for *P. aeruginosa*-infected burns, as it effectively reduces *P. aeruginosa* at the infection site, delays bacteremia, maintains lower bacterial levels in the bloodstream compared to untreated groups, and significantly increases the lifespan of mice (60). The Jan group investigated the photodynamic inactivation effects of HB on both azole-sensitive and azole-resistant strains of *C. albicans in vitro*. HB exhibited negligible dark toxicity and efficiently deactivated *C. albicans* cells in a light-dose and PS concentration-dependent manner (59). Recently, Law et al. proposed HB as a potential PS for PDT in the treatment of SARS-CoV-2 (94). These innovative approaches hold great potential for enhancing therapeutic outcomes in the treatment of microbial infections.

2.5 Resveratrol

Resveratrol, also known as trans-3,4,5-trihydroxystilbene, is a naturally derived polyphenolic compound and phytoalexin. It is synthesized in response to various stressors, including plant damage or microbial infections caused by bacteria or fungi (95). Resveratrol is commonly found in a variety of dietary substances, such as grapes, berries (cranberries), red wine, nuts (peanuts) and other foods (96–98). Chemically, it belongs to the stilbene family and acts as a fundamental precursor for the synthesis of other stilbenes, such as piceatannol and pterostilbene (specifically trans-3,5-dimethoxy-4'-hydroxystilbene) (96). Resveratrol presents a diverse array of biological activities, encompassing antimicrobial, antiviral, antioxidant, anti-aging, anti-inflammatory, and anticancer properties. Moreover, it has been recognized for its cardioprotective and neuroprotective attributes (99). These notable biological functions can be attributed to its unique molecular structure, which enables effective interactions with various biomolecules. Resveratrol displays a wide absorption spectrum ranging from 290 nm to 360 nm, with a peak wavelength observed at approximately 320 nm (100).

The antimicrobial activity of resveratrol has been studied extensively. Klančnik et al. reported a minimum inhibitory concentration (MIC) of 0.313 mg/ml for resveratrol against *Campylobacter jejuni* (101). In contrast, Duracka et al. found no significant bactericidal activity of resveratrol against *Enterococcus faecalis* in rabbit ejaculates (102). Li et al. discovered that resveratrol, at a concentration of 800 μg/mL, significantly inhibits the growth of *S. mutans* (96). Furthermore, Kugaji et al. demonstrated remarkable antibacterial and anti-biofilm activity of resveratrol against *Porphyromonas gingivalis* (*P. gingivalis*), a bacterium associated with gum disease (99). Dos Santos et al. were the first to establish a connection between aPDT and resveratrol, highlighting its effective inhibition of *S. aureus* when used as a PS (61). Resveratrol as a natural polyphenol compound, exhibits therapeutic potential. However, it is pertinent to acknowledge that the stability of the resveratrol can be influenced by factors such as UV radiation, pH, and temperature (103).

2.6 Gallic acid

Gallic acid (GA) (3,4,5-trihydroxybenzoic acid), a natural polyphenolic compound, is abundant in various plants, including trees, herbs, fruits, and nuts, as well as processed beverages such as red wine and green tea (104). Recognized for its inherent and potent biological activities, GA exhibits a diverse range of effects, encompassing antioxidative, antimicrobial, antiallergic, anticancer, anti-inflammatory, antimutagenic, anti-melanogenic, anti-atherosclerotic, neuroprotective, and hepatoprotective properties (105, 106). Its versatile applications span multiple fields, such as medicine, chemical research, pharmaceuticals, cosmetics, and the food industry (107). The polyphenolic functional groups present in GA contribute to its remarkable ability to scavenge oxygen-derived free radicals (108). Moreover, GA is commonly employed as a standard compound for quantifying phenol content using the Folin-Ciocalteu method (109). Derived from protocatechuic acid, GA serves as an intermediate in the secondary metabolism of plants (108). Structurally, GA is a phenolic acid consisting of benzene ring with a carboxyl group and three hydroxyl groups attached to it. Its formation can be obtained through the acid hydrolysis of hydrolysable tannins (110). It has the capability to absorb ultraviolet (UV) irradiation and light in the visible spectrum (111).

GA has demonstrated remarkable inhibitory effects on the motility, adhesion, and biofilm formation of *S. aureus*, *S. pyogenes*, *P. aeruginosa*, and *L. monocytogenes* (112–114). In an insightful study by Cossu et al., GA treatment combined with UV-A irradiation significantly inactivated metabolically active *E. coli* O157:H7 (62). Furthermore, De Oliveira et al. demonstrated that the synergistic combination of GA with lactic acid (LA) and UV-A was specifically effective against *E. coli* O157:H7 (63). A study conducted by Nakamura et al. investigated the antibacterial effect of GA (4 mmol/L) on *S. aureus* under LED light irradiation, resulting in a 99.9% reduction in bacteria. Notably, the authors suggest that the antibacterial action is induced by photooxidation and automatic oxidation of GA, as its individual bactericidal effect is less pronounced (64).

2.7 Aloe emodin

Aloe emodin (AE) is a naturally occurring anthraquinone derivative with structural similarity to HYP. It is extracted from traditional Chinese medicine (TCM) plants such as *Aloe vera*, *Rheum officinale Baill.*, *Rumex patientia Linn.*, *Cassia mimosoides L.* and *Polygonum multiflorum Thunb* (115, 116). AE shares a remarkable chemical structure resemblance to HYP, an extensively studied classical PS, and exhibits light absorption capability in the ultraviolet-visible regions. AE displays three primary absorption bands centered at 250 nm, 284 nm and 430 nm. Light sources within the blue region, including lasers emitting wavelengths of 405 nm, 430 nm, and 473 nm, as well as broadband light using suitable filters, effectively activate AE (65). The maximum absorption band of AE in the blue region makes AE-mediated PDT particularly advantageous for the treating of superficial diseases, including skin cancer, oral disorders, and ocular conditions. The singlet oxygen quantum yield ($^1O_2$) of AE was determined to be 0.57 (2) in methanol, which is marginally higher than that of methylene blue (117).

Recently, AE has gained increasing attention due to its potential applications in the treatment of various diseases. Several studies have indicated that aloin, a compound found in aloe vera, possesses various biological properties, including antiviral, antibacterial, anti-inflammatory, and hepatoprotective activities (118–120). Moreover, AE has demonstrated anticancer activity against lung squamous cell carcinoma, neuroectodermal tumors, hepatocellular carcinoma cells, gastric cancer cells, and colon cancer cells (121, 122). However, AE exhibits low solubility in aqueous medium (~19 μM), leading to poor oral absorption and bioavailability (123). Furthermore, long-term administration of AE may result in genotoxicity, including gene lesions and mutations, and pose potential risks such as the occurrence of acute renal failure. These factors constrain the widespread application of AE in the medical field. Consequently, research efforts aimed at enhancing the aqueous solubility of AE assume significant importance as they can substantially improve its bioavailability (124–126).

Nanomaterials are widely recognized as exceptional drug carriers due to their good biodistribution, enhanced bioavailability, and low drug toxicity. Li et al. developed AE-encapsulated nanoliposomes using reverse evaporation to improve the bioavailability of AE against human gastric cancer cells (126). Unfortunately, there have been few studies on nanomaterials for AE-mediated aPDT. AE has emerged as a promising agent for aPDT, garnering considerable attention for the treatment of surface or localized bacterial infections in recent years. Studies conducted by Li and Wang et al. provide evidence that AE-mediated aPDT is highly effective in inactivating *in vitro* isolates of MDR *Acinetobacter baumannii (A. baumannii)* and successfully treating infections caused by MDR *A. baumannii* following thermal burn injuries in mice. In summary, AE, as an exceptionally promising PS, exhibits tremendous potential in the context of managing of superficial infections caused by MDR *A. baumannii* through aPDT (65, 127). Ma et al. confirmed that AE-aPDT exhibited significant efficacy in the inactivation of *C. albicans* cells in a concentration-dependent manner by causing damage to the cell wall, cytoplasm, and nuclei (66). Additionally, the research conducted by Ma et al. demonstrated that AE is highly effective in inactivating *Trichophyton rubrum* (*T. rubrum*) microconidia in a light dose-dependent manner, exhibiting substantial inhibitory effects on the growth of *T. rubrum* (67). Cui et al. reported the *in vitro* photodynamic antimicrobial efficacy of AE on *Malassezia furfur* (*M. furfur*), a lipo-dependent yeast fungus frequently found on the skin. The findings revealed that AE-mediated aPDT demonstrated remarkable effectiveness in inactivating the fungal cells in a concentration- and light energy dose-dependent manner (68). These results suggest the potential application of AE-aPDT as a promising therapeutic option for addressing *M. furfur*-related skin conditions.

2.8 Celastrol

Tripterygium wilfordii Hook F. (*Tripterygium wilfordii*), is an ivylike vine belonging to the *Celastraceae* family, widely employed as a traditional natural medicine in Chinese traditional medicine

(128). The main chemical constituents of *Tripterygium wilfordii* include diterpenoids, triterpenoids and alkaloids, with triptolide and celastrol being the most studied and clinically applied components (129). *Tripterygium wilfordii* exhibits a range of pharmacological activities, including anti-inflammatory, immunomodulatory, anticancer, and anti-rheumatic effects. As a result, it finds extensive application in the treatment of autoimmune diseases, encompassing rheumatoid arthritis and systemic lupus erythematosus (130, 131). Furthermore, *Tripterygium wilfordii* has demonstrated anticancer activity and is currently under investigation as a potential anticancer drug (128). Alam et al. conducted a study exploring the application of a natural PS derived from the medicinal plant *Tripterygium wilfordii* for aPDT. The ethanolic extract and PS-enriched fraction contained six demethylated chlorophyll derivatives as active compounds. The combined treatment of red light (660 nm) and the natural PS effectively eradicated pathogenic bacteria and fungi, particularly various skin pathogens *in vitro*. The *in vivo* efficacy and adverse reactions of aPDT were evaluated using a nematode model infected with *S. aureus* and *Streptococcus pyogenes* (69).

Celastrol is a quinone methide triterpenoid natural compound that possesses a broad range of antiviral, anti-inflammatory, and anticancer properties (132). In a previous investigation, titanium dioxide (TiO_2) nanofibers conjugated with celastrol were employed for the treatment of HepG2 cancer cells with ultraviolet A (254 nm) (128). Caruso et al. conducted a study investigating the mechanism of action of celastrol at the active site of the main SARS-CoV-2 protease, 3CLpro, employing various techniques. Their findings suggest that celastrol could potentially serve as a PS in photodynamic therapy against SARS-CoV-2 (132, 133).

2.9 Riboflavin

Riboflavin, scientifically termed vitamin B2, is a water-soluble vitamin with inherent photodynamic properties. It can be found in various food sources such as dairy products (milk and cheese), meat, fish, fruits, dark green leafy vegetables, bread, grains, and grain products (134). Chemically, riboflavin comprises an isoalloxazine ring attached to a ribitol side chain and exists in two coenzyme forms: flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). These key cofactors play a pivotal role in energy metabolism as indispensable components of oxidation-reduction enzymes, reductases, and dehydrogenases (134, 135). Riboflavin, a potent light-activated free-radical producer, exhibits absorption maxima at 270 nm, 366 nm, and 445 nm, facilitating efficient generation of ROS (135, 136).

Riboflavin plays an indispensable role in maintaining human health and has exhibited the ability to hinder the growth of a diverse spectrum of microorganisms, encompassing bacteria, viruses, fungi, and parasites, suggesting its potential as an effective antimicrobial agent (134). Its biocompatibility, nontoxic characteristics, and ROS generation capacity have attracted significant attention among researchers, particularly in the field of dentistry (135). In aPDT, riboflavin serves as both a photosensitizer and a crosslinking agent. Its multifunctional properties extend beyond reducing inflammation and eradicating microbial biofilms to preserving adhesive strength in orthodontic brackets (135, 136). Studies by Maisch et al. and Mahsa et al. have showcased the safety and effectiveness of riboflavin-based aPDT in eradicating multidrug-resistant bacteria such as *S. aureus*, *E. coli*, *P. aeruginosa*, *A. baumannii*, and *E. faecalis* biofilm. Despite the widespread use of riboflavin as a PS in aPDT, its water-soluble nature limits its incorporation rate in diverse biological tissues. Consequently, numerous studies have focused on enhancing its bioavailability by employing riboflavin derivatives or nanodelivery systems. Zhang et al. demonstrated that riboflavin formulated into a nanoemulsion exhibited potent bactericidal effects against *S. aureus* cell membranes (70, 71, 137). Additionally, Du et al. found that supramolecular materials loaded with riboflavin were capable of killing gram-positive bacteria (*e.g.*, *S. aureus*), gram-negative bacteria (*e.g.*, *E. coli*), and multidrug-resistant *S. aureus* (71). These approaches aim to overcome the challenges associated with riboflavin solubility and improve its effectiveness in aPDT.

3 The photochemical mechanism and targets of aPDT

aPDT relies on the generation of ROS by PSs upon exposure to specific wavelengths of light. This process involves the transfer of electrons or energy from the excited PSs to molecular oxygen (138), leading to photochemical reactions of Type I or Type II (139). In type I reactions, the excited PS transfers high-energy electrons to nearby molecules, often molecular oxygen, resulting in the production of ROS, including hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), and hydroxyl radical (·OH), among others (134, 140). Type II reactions involve the transfer of energy from the PS to oxygen, generating highly reactive singlet oxygen ($^1O_2$) (52). These two reaction types induce oxidative stress and cellular damage, ultimately leading to cell death. The equilibrium between Type I and Type II reactions can be influenced by specific substrates, PSs, and oxygen levels (141). Recently, a novel mechanism termed the "Type III photochemical pathway" has been proposed, which is an oxygen-independent mechanism for antimicrobial photoinactivation. Currently, this mechanism has been primarily observed under anaerobic/hypoxic conditions, involving PSs such as psoralens and tetracyclines, as well as the addition of organic salts such as potassium iodide and sodium azide (142, 143).

aPDT is a multitarget process that inflicts damage on multiple levels. Natural product PSs can be categorized into three distinct types according to their proximity and interaction with bacterial cells: (i) PSs positioned in close proximity to the bacterial cell wall, (ii) PSs exhibiting affinity for bacterial cells, potentially causing oxidative damage to extracellular structures, and (iii) PSs capable of penetrating bacterial cells and reaching the cytoplasm, thereby exerting detrimental effects on intracellular components such as cytoplasmic proteins or DNA (144). Overall, aPDT operates through ROS generation and subsequent oxidative damage, with PSs targeting various cellular components depending on their location and interaction with bacterial cells. Understanding the mechanism and targets of aPDT is crucial for optimizing

treatment strategies and developing effective antimicrobial interventions (Figure 2).

3.1 biofilm

The formation of biofilms involves the adhesion and aggregation of bacteria on living or nonliving surfaces. Biofilms exhibit a complex and organized structure, providing protection and facilitating the survival and growth of the microorganisms within the community (145, 146). They represent a distinct lifestyle from planktonic states and serve as a survival strategy for microorganisms in challenging environments (15, 147). Extracellular polymeric substances (EPS), comprising proteins, extracellular DNA (eDNA), polysaccharides, humic substances, and water-insoluble compounds, such as cellulose, amyloid proteins, nonamyloid protein fibers, and lipids, surround and immobilize biofilm cells (148). Biofilms shield microorganisms from host defense systems, increasing their tolerance to various antibiotics and disinfectants. which can result in persistent and difficult-to-treat infections (149, 150). However, polyphenolic natural product-mediated aPDT has shown significant potential in targeting biofilms and inactivating clinically relevant microorganisms. Minhaco et al. reported that curcumin-loaded PLGA nanoparticles presented effective antimicrobial activity against endodontic biofilms. Notably, encapsulated curcumin demonstrated potent antibacterial effects on both mono- and multispecies biofilms (*e.g.*, *E. faecalis, S. mutans, and Streptococcus oralis*) at a lower concentration (29). A study by Ribeiro et al. demonstrated that curcumin-mediated aPDT, when irradiated with LED light, effectively generated photoproducts, and ROS, such as singlet oxygen and free radicals, inducing phototoxicity. Thus, PDT with curcumin significantly reduced the viability of MRSA strains in biofilms (24).

Hypericin-mediated aPDT has shown effective activity against both methicillin-susceptible and methicillin-resistant *S. aureus* biofilms, as evidenced in the study conducted by García et al. (151); nevertheless, inactivation of *S. aureus* biofilms was not achieved with HYP alone, as shown in the study by Kashef et al. Interestingly, the combination of HYP with acetylcysteine exhibited remarkable efficacy in eradicating the preformed mature biofilms of *S. aureus* strains. The authors hypothesized that acetylcysteine's ability to degrade the extracellular polysaccharide matrix of the biofilm enhances the susceptibility of biofilm-associated bacteria to the phototoxic properties of HYP (49). Xiang et al. observed that AE does not disrupt the anchoring of surface proteins to the cell wall. Instead, its inhibitory effect on biofilm development was attributed to the downregulation of specific surface protein expression or the direct obstruction of adhesion of these proteins to other matrix components (119).

3.2 Cell wall and cell membrane

Bacteria consist of three primary components: the cell wall, cell membrane, and cytoplasm (152). The cytoplasmic membrane

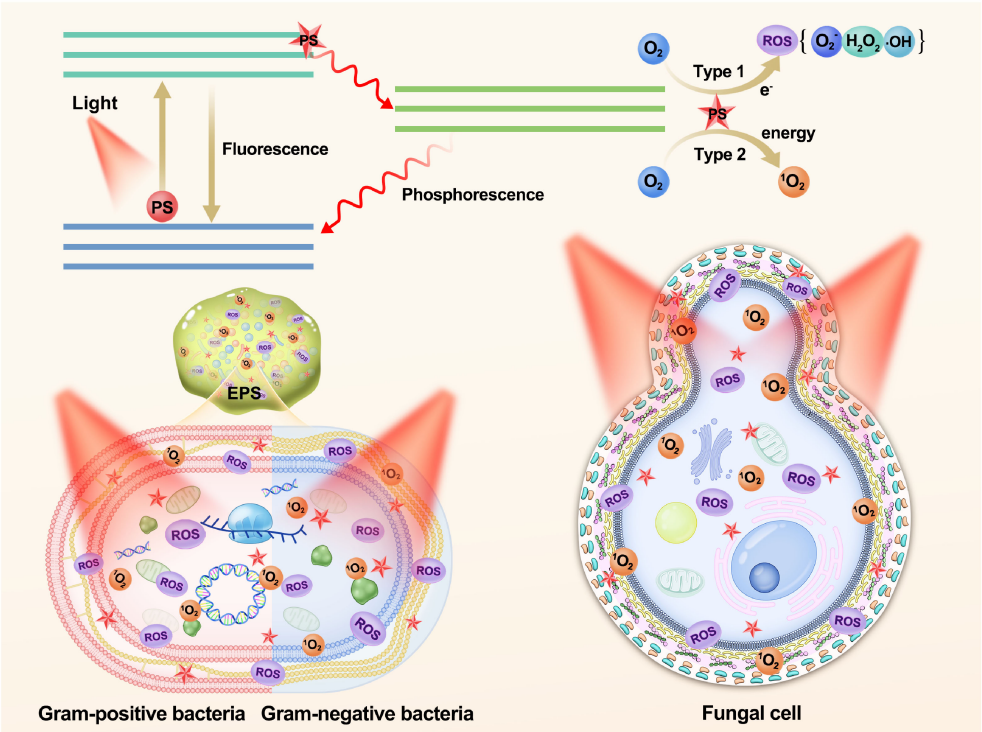

FIGURE 2
Schematic illustration of the photochemical mechanisms and the role of polyphenols as photosensitizers in aPDT targeting biofilms, Gram-positive bacteria, Gram-negative bacteria, and fungal cell. PS, photosensitizer; ROS, reactive oxygen species; EPS, extracellular polymeric substances.

shares a similar structure in both gram-negative bacteria and gram-positive bacteria, consisting of a phospholipid bilayer along with minor lipids and proteins (153). However, extensive research has demonstrated that gram-positive bacteria exhibit higher sensitivity to aPDT than gram-negative bacteria due to differences in their cell wall structures. Gram-positive cells have a single thick peptidoglycan layer surrounding their cytoplasmic membrane, resulting in higher porosity of their cell walls. Consequently, this increased porosity facilitates easier diffusion of the PSs into the intracellular space. In contrast, gram-negative bacteria possess a highly selective and complex outer membrane composed of lipopolysaccharides, lipoproteins and lipoteichoic acids, along with a thin peptidoglycan layer. These factors collectively make the penetration of PSs significantly more challenging (154, 155). In the study, Wang et al. demonstrated that quercetin had the ability to disrupt the cell wall and cell membrane structures in both gram-positive and gram-negative bacteria. This disruption increased the permeability of these structures, leading to the release of cellular cytoplasmic contents and impairment of adenosine triphosphate (ATP) activity (152). Furthermore, Lee et al. illustrated that the inactivation process of aPDT mediated by quercetin involved damage to *E. coli* O157:H7 and *L. monocytogenes* membranes through the generation of ROS. The predominant mechanism observed was type I, with $O_2^{\bullet-}$ and H_2O_2 identified as the main ROS involved (52). The fungal cell wall consists of a cell membrane containing various membrane proteins. At the outermost layer, mannoproteins form a protective fibrous layer that conceals the underlying β-glucan layer, while chitin is situated in close proximity to the cell membrane (156, 157). In their investigation, Jan et al. discovered that HB-mediated aPDT resulted in significant impairment to the cell wall, cell membrane, cytoplasm, and nucleus of *C. albicans*, suggesting that ROS might be accountable for the damage observed in the cytoplasm and cell wall components, signifying a distinct mechanisms from that of antifungal drugs (59) (Figure 2).

3.3 Nucleic acids, proteins and lipids

To date, there have been relatively few studies investigating the direct influence of polyphenolic natural product PSs on bacterial nucleic acids, proteins, and especially lipids in aPDT. Previous research suggested that the DNA of microorganisms was primarily affected when they were either inactivated or nonviable, rendering the probability of developing resistance mechanisms against aPDT extremely low (155, 158). In a study by Lee et al., quercetin was identified as an exogenous PS located outside bacterial cells that generates ROS. This process initiated the attack on bacterial cells from the outermost structures. Subsequently, quercetin diffused into the damaged bacteria, and the ROS generated upon its entry resulted in the degradation of bacterial DNA (52). Furthermore, quercetin exhibited the ability to reduce bacterial protein synthesis, thereby affecting protein expression within the cell. Ultimately, this disruption led to cell lysis and death (152). Despite the lipid-rich composition of the bacterial cytoplasm and outer membranes, our understanding of the lipid-related mechanisms underlying natural product-mediated aPDT remains limited. The complexity associated with identifying and characterizing lipid damage has contributed to this gap (155).

4 *In vivo* aPDT with polyphenols

Currently, research on polyphenol-based natural product-mediated aPDT is primarily focused on oral and skin diseases in both *in vivo* (Table 2). In a study conducted by Dascalu Rusu LM and colleagues, utilizing curcuma extract, arnica oil, and oregano essential oil, novel natural PSs mediated aPDT effectively improved induced periodontal disease in rats and reduced inflammation (12). Paolillo FR et al. discovered that a combination of curcumin (0.06 mL of 1.5% curcumin gel) and blue light (450 nm, 80 mW/cm^2, at the dose of 60 J/cm^2)-mediated aPDT, with artificial skin, accelerated bacterial inactivation (*S. aureus* 4.14 log10) and enhanced wound healing in Wistar rats without inducing adverse effects on the tissue (159). Muniz IPR et al. demonstrated that *ex vivo* activation of curcumin (100 μg) by blue LED light (450 nm) at a fluence of 13.5 J/cm^2 effectively controlled *S. aureus* cutaneous infection in type I diabetic mice (36, 160). Alam et al. achieved significant eradication of Ampicillin-Resistant *P. aeruginosa* in the *Caenorhabditis elegans* (*C. elegans*) model by using HYP in conjunction with ampicillin and subsequent orange light treatment (48). Liu et al. assessed the antibacterial capabilities of Poly (lactic acid)-Hypocrellin A (PLA-HA) nanofiber membranes through *in vivo* photodynamic therapy in rats infected with *C. albicans*. The study revealed that PLA-HA-mediated aPDT significantly promoted wound healing, reduced the infected wound area, and increased the wound healing rate by approximately 10% compared to other groups (56). Guo et al. discovered that lipase-sensitive methoxy poly (ethylene glycol)-block-poly(ϵ-caprolactone) (mPEG-PCL)/HA micelles mediated aPDT (470 nm, 90 mW/cm^2, 60 min) effectively eradicated MRSA in the abdominal cavity of mice, increasing the survival rate to 86% at a low concentration of 10 mg/kg (HA concentration) (58). Hashimoto et al. treated burn mice infected with *P. aeruginosa* with HB: La^{+3} and aPDT (LED, 24 J/cm^2). They found that aPDT reduced bacterial burden at the burn wound, delayed bacteremia, and lowered bacterial levels in the blood by 2-3 logarithmic units. Survival rates of mice increased 24 hours after treatment (60). Dos Santos et al. observed that blue LED light (54 J/cm^2) enhanced the antimicrobial effect of resveratrol (2 mg/mL, 100 μL) against MRSA. In a mouse abscess model, it induced the production of TNF-α and IL-17A cytokines, reduced bacterial burden, and consequently decreased inflammation 24 hours after infection (61). Ma et al. demonstrated that AE-mediated aPDT effectively treated tinea corporis caused by *T. rubrum* in a guinea pig model and tinea unguium in an *ex vivo* model (67). *In vivo* studies reported by Wang et al. showed that AE-mediated aPDT effectively treated skin infections caused by multidrug-resistant *A. baumannii* in mice following burn injuries (127). Alam et al. evaluated the efficacy of ethanol extract of *Tripterygium wilfordii* (TWE)-mediated aPDT against various pathogens (*E. coli*, *S. aureus*, MRSA, *S. pyogenes*, and *C. albicans*) in a nematode model. Their findings indicated that it

effectively controlled the pathogens without inducing strong adverse effects. TWE-mediated aPDT reversed the growth inhibition caused by pathogen infection in the nematodes, reduced the viable pathogen count associated with *C. elegans*, and improved the survival rate of the nematodes infected with *Pyogenic Streptococcus*, in conjunction with aPDT (69). Du et al. uniformly applied riboflavin G4 hydrogel (2 mL) onto sterile dressings and treated wounds infected with MRSA in rats by irradiating them with blue light at a wavelength of 460 nm and a light power density of 80 mW/cm^2 for 10 min. Their results revealed that the hydrogel exhibited robust antimicrobial activity in the rat infection wounds after irradiation (71).

5 Conclusions and perspectives

In recent years, aPDT has emerged as a pioneering modality specifically formulated for the inactivation of an extensive array of microorganisms, including bacteria, fungi, and viruses. Its application has grown progressively in diverse fields, notably in dermatology for conditions such as acne, in oral health for issues such as tooth decay and halitosis, and in managing fungal infections and viral diseases, notably COVID-19. Additionally, aPDT's effectiveness in eliminating pathogens has paved its way into the food industry, bolstering food safety measures. PSs, a crucial component of aPDT, are responsible for generating ROS. Natural polyphenolic compounds derived from plants, fruits, vegetables, and other natural sources are increasingly used as PSs in aPDT due to their lower toxicity, structural diversity, and excellent biocompatibility. However, their clinical application is limited by factors such as water solubility. To overcome these limitations, innovative techniques such as nanotechnology have been employed. Nanoparticles, in particular, have proven to be efficacious drug delivery systems for hydrophobic PSs, facilitating their effective transport both *in vitro* and *in vivo*. They enable circumvention of physiological and biological barriers, thereby enhancing bacterial cell uptake. Despite these advancements, further research and technological innovation are imperative to fully exploit the potential of natural polyphenolic PSs and enhance their efficacy in treating a plethora of infectious diseases. Overcoming their

TABLE 2 *In vivo* aPDT with polyphenols.

Authors	Polyphenols	Disease Models	Effects	References
Dascalu Rusu LM et al.	CUR extract	Rats' periodontal disease	Effectively improved periodontal disease and reduced inflammation	(12)
Paolillo FR et al.	CUR	Wistar rats wound healing	Accelerated bacterial inactivation and enhanced wound healing	(159)
Muniz IPR et al.	CUR	*S. aureus* cutaneous infection of type I diabetic mice	Effectively controlled S. aureus cutaneous infection	(36)
Galinari CB et. al.	HYP	Mouse dermatophytosis caused by *M. canis*	After three treatment, a rapid improvement in clinical symptoms at the infection site;After six treatments,a statistically significant reduction in fungal burden compared to untreated infected animals	(160)
Alam et al.	HYP	*C. elegans* of Ampicillin-Resistant *P. aeruginosa* infection	Achieved significant eradication of Ampicillin-Resistant P. aeruginosa	(48)
Liu et al.	HA	Rats infected with *C. albicans*	Significantly promoted wound healing, reduced the infected wound area	(56)
Guo et al.	HA	Mouse abdominal MRSA infection model	Effectively eradicated MRSA in the abdominal cavity of mice	(58)
Hashimoto et al.	HB	Burn mice infected with *P. aeruginosa*	Reduced bacterial burden at the burn wound, delayed bacteremia, and lowered bacterial levels	(60)
Dos Santos et al.	Resveratrol	A mouse abscess model of MRSA infection	induced the production of TNF-α and IL-17A, reduced bacterial burden, and decreased inflammation	(61)
Ma et al.	AE	Tinea corporis caused by *T. rubrum* in a guinea pig model	Effectively treated tinea corporis	(67)
Wang et al.	AE	A mouse skin infection model caused by *A. baumannii* multidrug after burn	Effectively treated skin infections	(127)
Alam et al.	*Tripterygium wilfordii*	Pathogen-infected nematode model	Effectively controlled the pathogens and improved the survival rate of the nematodes infected with *Pyogenic Streptococcus*	(69)
Du et al.	Riboflavin	A rat model of wound infection with MRSA	Exhibited robust antimicrobial activity in the rat infection wounds	(71)

CUR, curcumin; HYP, hypericin; HA, hypocrellin A; HB, hypocrellin B; AE, aloe emodin; *S. aureus*, *Staphylococcus aureus*; *M. canis*, *Microsporum canis*; *C. elegans*, *Caenorhabditis elegans*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *C. albicans*, *Candida albicans*; MRSA: methicillin-resistant Staphylococcus aureus; *T. rubrum*, *Trichophyton rubrum*; *A. baumannii*, *Acinetobacter baumannii*.

limitations and achieving enhanced efficacy in the treatment of various infectious diseases will require continuous exploration and innovation.

Overall, natural polyphenolic PSs-mediated aPDT, in combination with nanoparticle-based drug delivery systems, holds great potential in combating microbial infections and advancing the field of infectious disease treatment. With concerted efforts and ongoing research, it is expected that aPDT will continue to evolve and find wider applications in the future.

Author contributions

GH: Funding acquisition, Investigation, Writing – review & editing. XYW: Conceptualization, Visualization, Writing – original draft. RF: Writing – review & editing, Investigation. LW: Writing – original draft, Funding acquisition, Investigation. LZ: Writing – original draft, Investigation, Visualization. XJ: Funding acquisition, Writing – review & editing. XW: Conceptualization, Funding acquisition, Investigation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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J-shaped association of dietary catechins intake with the prevalence of osteoarthritis and moderating effect of physical activity: an American population-based cohort study

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Background: Catechins are a class of natural compounds with a variety of health benefits, The relationship between catechins and the prevalence of osteoarthritis (OA) is unknown. This study investigated the associations between daily intake of catechins and the prevalence of OA among American adults and assessed the moderating effect of physical activity (PA).

Methods: This study included 10,039 participants from the National Health and Nutrition Examination Survey (2007–2010,2017-2018). The logistic regression, weighted quantile sum (WQS) regression, and restricted cubic spline (RCS) regression models were conducted to explore the associations between daily intake of catechins and the prevalence of OA. Moreover, interaction tests were performed to assess the moderating effect of PA.

Results: After multivariable adjustment, the weighted multivariable logistic regression and RCS regression analyses revealed significant J-shaped non-linear correlations between intakes of epigallocatechin and epigallocatechin 3-gallate had significant associations with the prevalence of OA among in U.S. adults. WQS regression analysis showed that excessive epigallocatechin intake was the most significant risk factor for OA among all subtypes of catechins. In the interaction assay, PA showed a significant moderating effect in the relationship between epigallocatechin intake and OA prevalence.

Conclusions: The intake of gallocatechin and gallocatechin 3-gallate had a significant negative correlation with the prevalence of OA and the dose-

response relationship was J-shaped.PA below 150 MET-min/week and the threshold intakes of 32.70mg/d for epigallocatechin and 76.24mg/d for epigallocatechin 3-gallate might be the targets for interventions to reduce the risk of developing OA.

KEYWORDS

osteoarthritis, catechins, physical activity, epigallocatechin, epigallocatechin 3-gallate

Introduction

Osteoarthritis (OA) is a degenerative and inflammatory joint disease caused by multiple factors, which is characterized by joint swelling, pain and cartilage destruction (1, 2). Although the underlying mechanism is not fully understood, its impact on the quality of life of patients is beyond doubt. According to epidemiological statistics, the age-standardized prevalence rate of OA in the United States increased by 23.2% from 1999-2017, which is one of the highest age-standardized prevalence rate increases of OA in the world (3). It is estimated that the number of OA patients in the USA will increase to 67 million by 2030 (4). The financial expenditure on OA care is estimated at US $15.5-28.6 billion per year (2). OA is becoming a disease that attracts more and more attention.

Flavonoids are a group of polyphenolic compounds derived from plant secondary metabolism. They are widely present in a variety of foods, such as vegetables, fruits, tea and wine (5, 6). According to their chemical structure, they can be divided into six subgroups, namely flavonoids, flavanones, flavonols, isoflavones, anthocyanins and flavanols (7–11). Catechins are a subgroup of flavonols. There were eight monomers of catechins, catechin (C), epicatechin (EC),gallocatechin (GC), epigallocatechin (EGC), catechin gallate (CG), epicatechin gallate (ECG), gallocatechin gallate (GCG) and epigallocatechin gallate (EGCG) (12–14). In recent years, with the deepening of research, catechins have been proven to have a kind of biological activities such as anti-inflammatory, anti-oxidation, anti-cancer and bone protection (6, 12, 15, 16). Therefore, supplemental catechins are increasingly recognized as nutritional supplements for the treatment of many diseases, including OA.

The relationship between physical activity and OA has been well-paid attention to in recent years. Homeostasis of joints and joint damage are regarded as the main causes of OA caused by PA (17–20). In addition, the loss of muscle strength caused by low-intensity PA is also the key to the elderly getting OA (21). However, for the general population, daily PA does not increase the risk of joint OA, and moderate levels of PA can also improve soft tissue ductility, blood flow, and synovial fluid mobility, maintain normal joint range of motion, and provide essential nutrients to the cartilage matrix (18, 22, 23).

Since catechins are easily accessible in daily life and have shown excellent medical effects, the present study, which used the NHANES database, attempted to investigate the relationship between daily intake of catechins and the prevalence of OA and to explore whether physical activity plays a moderating role.

Methods

Study population

The National Health and Nutrition Examination Survey (NHANES) is a periodic cross-sectional random sample survey of the non-institutional population in the United States. Survey data are compiled by professionals and released on the NHANES official website for public access (https://www.cdc.gov/nchs/nhanes/). NHANES was approved by the Division of Health and Nutrition Examination Surveys (DHANES) and the National Center for Health Statistics (NCHS), and all participants provided written informed consent.

This study included all 29,940 participants in the “2007-2008”, “2009-2010” and “2017-2018” survey cycles, which have published data on dietary flavonoid intake. After excluding individuals with missing osteoarthritis, catechins, and covariates data, a total of 10039 participants were included in this study.

OA status

Participants with OA were defined by previous physician diagnosis, and collected by trained interviewers through questionnaires. Shortly, participants aged ≥20 years were asked two questions related to arthritis: “Has a doctor or other health professional ever told you that you have arthritis?” and “Which type of arthritis was it?” Participants who responded “yes” and “osteoarthritis” were divided into the osteoarthritis group and the other participants were divided into the Non-osteoarthritis group.

Dietary catechins intake assessment

Catechins are widely found in plants and can be consumed in the daily diet through fruits, tea, coffee, and so on (24). Dietary

catechins intake data were extracted from the Food and Nutrient Database for Dietary Studies (FNDDS), which used 24-hour dietary recall interview data from NHANES to calculate participants' nutrient intakes over 24 hours. In FNDDS, flavonoids were subdivided into 29 species, of which (-)-Epicatechin, (-)-Epicatechin 3-gallate, (-)-Epigallocatechin, (-)-Epigallocatechin 3-gallate, (+)-Catechin, (+)-Gallocatechin were included in the "total catechins" category.

Physical activity assessment

Physical activity (PA) data were collected by trained interviewers through a global physical activity questionnaire survey. The metabolic equivalent (MET) of weekly PA was calculated by multiplying the weekly minutes of moderate or vigorous PA, frequency, and MET value recommended by NHANES. According to the WHO guidelines, participants who achieved moderate intensity (150 min per week) were categorized into the sufficiently PA group (>600 MET-min/wk) and the rest into the insufficiently PA group (600-150 MET-min/wk), low PA group (<150 MET-min/wk), and inactive PA group (PA=0) (25).

Covariates

Demographic information, including age, gender, ethnicity, education level, marital status, and poverty income ratio (PIR), was collected by trained interviewers using a questionnaire standardized. History of hypertension and diabetes were determined by the use of prescribed medications and previous physician diagnosis. Body mass index (BMI) was measured by professionals in the Mobile Examination Center (MEC). Defined as a drinker based on ≥ 12 drinks per year and < 12 drinks per year as a non-drinker. Smokers were defined as having smoked more than 100 cigarettes in the past.

Statistical analyses

Considering the complex sampling design of NHANES, the dietary day one sample weights were included in all analyses of this study. In the characterization of osteoarthritis and non-osteoarthritis participants, normally distributed continuous variables were presented as mean (standard deviation), catechins intake was presented as median (25th, 75th) due to non-normal distribution, and categorical variables were expressed as absolute values (weighted percentages). Statistical differences between the two groups were tested by t-tests, Wilcoxon rank sum tests, and Rao-Scott Chi-square tests., respectively. Univariate and multivariate logistic regression analyses adjusting for the confounding factors and trend tests for the Q group of catechins were used to investigate the 6 catechins and total catechins intake in relation to the prevalence of OA. Model 1 was a univariate logistic regression model without adjustment. Model 2 was the multivariate logistic regression model adjusted for age (20–59 years, ≥60 years), gender (male, female), and ethnicity (non-Hispanic White, non-Hispanic Black, Mexican American, Other race). Model 3 was additionally adjusted for education level (less than high school, high school or equivalent, and college or above), marital status (married/cohabiting, widowed/divorced/separated, never married), PIR, BMI, smoke, alcohol drinking, and history of diabetes or hypertension. The restricted cubic spline (RCS) regression model was performed to investigate the non-linear relationship between the intake of Epigallocatechin and Epigallocatechin 3 gallate with the risk of OA. The number of knots for the RCS regression was three, with the smallest Akaike information criterion to ensure the best fit. Weighted quantile sum (WQS) regression models were performed to evaluate the relationship between indexes representing six catechins or Epigallocatechin and Epigallocatechin 3 gallate co-exposure and the risk of OA. The likelihood ratio test was used to test the significance of the effect on the risk of OA caused by the multiplicative interaction term of PA with Epigallocatechin or Epigallocatechin 3 gallate. Multiple sensitivity analyses are conducted to check the robustness of our results, including logistic regression models adjusting for different covariates, weighted regression models, stratified analyses, and interaction tests. All analyses were conducted using R software (version 4.2.1), and a bilateral *P* value less than 0.05 was considered statistically significant.

Result

Basic characteristics and catechins intake of study participants

As shown in Table 1, a total of 10,039 volunteers from the NHANES were enrolled in our study, including 8839 non-osteoarthritis and 1,200 osteoarthritis patients. Interestingly, in addition to catechin and gallocatechin, we found that intakes of epigallocatechin, epigallocatechin 3-gallate, epicatechin, epicatechin 3-gallate, and total Catechins were statistically different between the OA and non-OA populations (P < 0.05). In addition, there were significant differences in age, gender, ethnicity, marital status, BMI, poverty income ratio, smoke, diabetes, hypertension, and physical activity between the OA and non-OA populations (P < 0.05). Among them, those aged ≥60 years old, female, divorced, widowed, obesity, smoke, diabetes, and hypertension were more likely to have osteoarthritis.

The associations between six catechins subclass intakes and the prevalence of OA

Firstly, we used logistic regression models to analyze the single effect of each catechin subclass on the prevalence of OA (Table 2). Interestingly, we found, in model 3 adjusted for all covariates, intakes of epigallocatechin (P for trend = 0.02) and epigallocatechin 3-gallate (P for trend = 0.01) showed significant associations with OA in the third Qs. Compared to the Q 1 group, epigallocatechin (OR: 1.76, 95% CI: 1.28, 2.42) and epigallocatechin 3-gallate (OR: 1.338, 95% CI: 1.08, 1.77) in the Q 3 group presented a higher risk of

TABLE 1 Characteristics and catechins intake in participants stratified by OA in the US (NHANES 2007-2010 and 2017-2018).

Characteristic	All participants (10039)	Osteoarthritis (1200)	Non- Osteoarthritis[a] (8839)	*p* value[b]
Age (years), n (%)				< 0.0001
20-59	7170 (71.42)	393 (42.38)	6777 (84.38)	
> = 60	2869 (28.58)	807 (57.62)	2062 (15.62)	
Gender, n (%)				< 0.0001
Female	4969 (49.5)	773 (65.41)	4196 (48.80)	
Male	5070 (50.5)	427 (34.59)	4643 (51.20)	
Ethnicity, n (%)				< 0.0001
Non-Hispanic Black	1891 (18.84)	170 (5.88)	1721 (10.85)	
Non-Hispanic White	4614 (45.96)	784 (82.96)	3830 (66.64)	
Mexican American	1684 (16.77)	96 (3.02)	1588 (9.36)	
Other Hispanic	973 (9.69)	79 (2.32)	894 (5.74)	
Other Race	877 (8.74)	71 (5.82)	806 (7.40)	
Education level, n (%)				0.76
Less than high school	2333 (23.24)	245 (13.29)	2088 (14.04)	
High school or equivalent	2333 (23.24)	288 (25.58)	2045 (24.62)	
College or above	5373 (53.52)	667 (61.13)	4706 (61.34)	
Marital status, n (%)				< 0.0001
Married/cohabiting	6102 (60.78)	712 (64.90)	5390 (61.90)	
Widowed/divorced/separated	2031 (20.23)	418 (28.05)	1613 (15.23)	
Never married	1906 (18.99)	70 (7.05)	1836 (22.87)	
Poverty income ratio	3.13 (0.04)	3.10 (0.04)	3.34 (0.09)	0.01
Body mass index (kg/m2)	28.73 (0.12)	28.49 (0.13)	30.42 (0.32)	< 0.0001
Smoke, n (%)				< 0.001
no	5665 (56.43)	573 (49.99)	5092 (58.48)	
yes	4374 (43.57)	627 (50.01)	3747 (41.52)	
Alcohol drinking, n (%)				0.28
Non-drinker	1249 (12.44)	162 (10.57)	1087 (9.29)	
Drinker	8790 (87.56)	1038 (89.43)	7752 (90.71)	
Diabetes, n (%)				< 0.0001
no	8957 (89.22)	965 (84.36)	7992 (93.43)	
yes	1082 (10.78)	235 (15.64)	847 (6.57)	
Hypertension, n (%)				< 0.0001
no	6188 (61.64)	394 (39.38)	5794 (70.60)	
yes	3851 (38.36)	806 (60.62)	3045 (29.40)	
Physical activity, n (%)				< 0.0001
Inactive	2435 (24.26)	2024 (18.42)	411 (26.27)	
Low	1061 (10.57)	913 (10.08)	148 (12.68)	
Insufficiently	176 (1.75)	138 (1.53)	38 (3.82)	

(Continued)

TABLE 1 Continued

Characteristic	All participants (10039)	Osteoarthritis (1200)	Non- Osteoarthritis[a] (8839)	*p* value[b]
Sufficiently	6367 (63.42)	5764 (69.97)	603 (57.22)	
Catechin (mg/day)	4.99 (1.13, 11.03)	6.05 (1.49, 12.21)	4.84 (1.08, 10.87)	0.05
Gallocatechin (mg/day)	0.00 (0.00, 1.08)	0.01 (0.00, 1.08)	0.00 (0.00, 1.08)	0.21
Epicatechin (mg/day)	4.74 (0.79, 15.33)	7.05 (1.42, 16.28)	4.46 (0.72, 15.20)	0.003
Epicatechin 3-gallate (mg/day)	0.01 (0.00, 1.38)	0.02 (0.00, 10.38)	0.00 (0.00, 1.03)	0.001
Epigallocatechin (mg/day)	0.40 (0.06, 3.73)	0.73 (0.20, 16.30)	0.35 (0.04, 3.06)	< 0.0001
Epigallocatechin 3-gallate (mg/day)	0.00 (0.00, 3.37)	0.11 (0.00, 24.34)	0.00 (0.00, 2.76)	< 0.001
Total Catechins (mg)	12.41 (3.19, 54.31)	16.38 (4.95, 79.50)	12.01 (3.04, 52.35)	0.004

[a]The non-osteoarthritis group was defined as participants who had not self-reported any kind of arthritis.
[b]Statistical differences between the two groups were tested by t-tests for normally distributed continuous variables, Wilcoxon rank sum tests for non-normal distributed continuous variables, and Rao-Scott Chi-square tests for categorical variables.

OA. In addition, we further used the WQS regression model to analyze the mixed effects of total catechins subclass intakes on the risk of OA (Table 3), and the results showed that the WQS index had no statistical significance with OA risk reduction. Subsequently, we selected epigallocatechin and epigallocatechin 3-gallate for re-analysis, and the results showed that the WQS index was significantly correlated with increased OA risk (OR: 1.04, 95% CI: 1.00,1.08). The estimated weights of epigallocatechin and epigallocatechin 3-gallate in the WQS regression model are shown in Figure 1B.

As shown in Figure 1, in the WQS regression model containing all catechins, the highest contribution to the WQS index was made by epigallocatechin (40%), and in the WQS regression model consisting of intakes of epigallocatechin and epigallocatechin 3-gallate, the highest contribution to the WQS index was still made by epigallocatechin (98.5%). Moreover, we further explored the associations with the regression coefficients assumed to be negative in the two WQS models. Unsurprisingly, the weight of Epigallocatechin in the models was zero and neither model is statistically significant (Figure S1 and Table S3). Furthermore, the restricted cubic spline (RCS) modes adjusted for all confounders demonstrated a nonlinear and J-shaped association between the intakes of epigallocatechin (P for non-linearity = 0.0016) and Epigallocatechin 3-gallate (P for non-linearity = 0.0004) and risk of OA (Figure 2). The risk of OA reached a nadir when epigallocatechin at approximately 32.70 mg/day and epigallocatechin 3-gallate at approximately 76.24 mg/day, followed by a gradual increase in OR with increasing daily intake.

Subgroup analysis

In the subgroup analyses, we stratified all covariates and used multifactorial logistic regression models adjusted for all confounders except the variables themselves to analyze the association between daily dietary epigallocatechin and epigallocatechin 3-gallate intake and the prevalence of OA. In addition, a multiplicative interaction term was added to each model for testing potential interactions, and the results indicated no significant interactions between daily dietary intake of epigallocatechin and epigallocatechin 3-gallate and the stratification variables (Tables S1, 2).

Interaction effect between physical activity and epigallocatechin intake on the prevalence of OA

In the interaction assay, as shown in Table 4, we found a significant moderating effect of PA in the relationship between epigallocatechin intake and the prevalence of OA (P for interaction = 0.03), whereas this interaction was not observed in the relationship between epigallocatechin 3-gallate and OA prevalence (P for interaction = 0.58). Specifically, in the Low PA group, the results of the multivariate-adjusted logistic regression model showed ORs (95%CI) were 0.02 (0.00, 0.21) and 0.05 (0.01, 0.27) for Q 2 and 3, respectively, compared to the Q 1 group (Figure 3). While in the sufficiently PA group, with Q 1 group being the reference, the ORs (95%CI) were Q 2 1.55 (1.09, 2.22) and Q 3 2.03 (1.25, 3.30). In addition, the results of the trend test were statistically significant in the Low PA group (P for trend = 0.004) and sufficiently PA group (P for trend = 0.03).

Discussion

In this study, the relationship between dietary catechins intake and the prevalence of OA was investigated for the first time, using the study cohort of 10039 participants from the NHANES database, including both OA and non-OA individuals. Our results suggest that a J-shaped nonlinearly correlation between the intakes of epigallocatechin and epigallocatechin 3-gallate with the risk of OA, in which PA played a significant moderating effect.

Most studies have found that catechins have a positive effect on OA treatment. The mechanisms are not fully understood, but several possible mechanisms have been suggested. Catechins could

TABLE 2 The association between the catechins intake and osteoarthritis in the US (NHANES 2007-2010 and 2017-2018).

Variable	Q 1	Q 2		Q 3		*p* for trend
Catechin (mg/day)						
Model 1 [OR (95% CI), *p*]	Reference	1.15 (0.92, 1.43)	0.22	1.25 (0.95, 1.66)	0.11	0.15
Model 2 [OR (95% CI), *p*]	Reference	0.81 (0.66, 0.99)	0.95	1.09 (0.80, 1.50)	0.56	0.52
Model 3 [OR (95% CI), *p*]	Reference	1.10 (0.85, 1.42)	0.46	1.26 (0.90, 1.77)	0.18	0.18
Gallocatechin (mg/day)						
Model 1 [OR (95% CI), *p*]	Reference	1.78 (1.36, 2.31)	< 0.0001	1.07 (0.85, 1.34)	0.57	0.50
Model 2 [OR (95% CI), *p*]	Reference	1.40 (1.03, 1.90)	0.03	1.06 (0.81, 1.40)	0.65	0.86
Model 3 [OR (95% CI), *p*]	Reference	1.56 (1.11, 2.18)	0.01	1.12 (0.85, 1.47)	0.40	0.99
Epicatechin (mg/day)						
Model 1 [OR (95% CI), *p*]	Reference	1.47 (1.15, 1.88)	0.003	1.45 (1.06, 1.97)	0.02	0.13
Model 2 [OR (95% CI), *p*]	Reference	1.25 (0.96, 1.62)	0.09	1.21 (0.86, 1.72)	0.27	0.54
Model 3 [OR (95% CI), *p*]	Reference	1.36 (1.03, 1.80)	0.03	1.35 (0.93, 1.94)	0.11	0.31
Epicatechin 3 gallate (mg/day)						
Model 1 [OR (95% CI), *p*]	Reference	1.50 (1.15, 1.94)	0.003	1.53 (1.22, 1.92)	< 0.001	0.001
Model 2 [OR (95% CI), *p*]	Reference	1.19 (0.88, 1.60)	0.25	1.18 (0.91, 1.53)	0.20	0.29
Model 3 [OR (95% CI), *p*]	Reference	1.30 (0.93, 1.80)	0.12	1.28 (0.96, 1.72)	0.09	0.16
Epigallocatechin (mg/day)						
Model 1 [OR (95% CI), *p*]	Reference	2.07 (1.69, 2.54)	< 0.0001	2.55 (1.95, 3.33)	< 0.0001	< 0.0001
Model 2 [OR (95% CI), *p*]	Reference	1.40 (1.11, 1.78)	0.01	1.68 (1.25, 2.25)	< 0.001	0.01
Model 3 [OR (95% CI), *p*]	Reference	1.37 (1.07, 1.77)	0.02	1.76 (1.28, 2.42)	< 0.001	0.02
Epigallocatechin 3 gallate (mg/day)						
Model 1 [OR (95% CI), *p*]	Reference	1.52 (1.16, 1.99)	0.003	1.60 (1.32, 1.94)	< 0.0001	< 0.0001
Model 2 [OR (95% CI), *p*]	Reference	1.17 (0.82, 1.67)	0.37	1.28 (1.02, 1.62)	0.03	0.03
Model 3 [OR (95% CI), *p*]	Reference	1.31 (0.91, 1.88)	0.14	1.38 (1.08, 1.77)	0.01	0.01
Total catechins (mg/day)						
Model 1 [OR (95% CI), *p*]	Reference	1.31 (1.05, 1.63)	0.02	1.41 (1.06, 1.87)	0.02	0.08
Model 2 [OR (95% CI), *p*]	Reference	1.19 (0.93, 1.52)	0.17	1.17 (0.85, 1.61)	0.33	0.59
Model 3 [OR (95% CI), *p*]	Reference	1.30 (1.00, 1.70)	0.05	1.31 (0.93, 1.85)	0.12	0.31

Model 1: No adjustment.
Model 1: Adjusted for age, gender, ethnicity.
Model 2: Adjusted for age, gender, ethnicity, education level, marital status, poverty income ratio, body mass index, smoke, alcohol drinking status, and history of diabetes or hypertension.
CI, confidence interval; OR, odds ratio.

up-regulate the expression of nuclear factor erythrocyte 2-related factor 2 (Nrf2), oxygenase 1 (HO-1), NADPH quinone oxidoreductase 1 (NQO1), and other antioxidant enzymes, and improve the oxidative stress-induced chondrocyte dysfunction (26). Moreover, catechins can effectively clear excessive ROS in cells, significantly reduce the expression of pro-inflammatory cytokines, reduce the expression of M1-type macrophages, and show an excellent promotion effect on the transformation of macrophages to M2 phenotype (27, 28). However, the relationship between catechins intake and the risk of OA has not been evaluated in any study. In this research, we explored the associations between six catechins subclasses and the prevalence of OA, with multifactorial logistic regression and WQS regression model, and found that excessive intake of epigallocatechin and epigallocatechin 3-gallate increases the risk of OA in the general US population.

Several experimental animal studies and epidemiological studies have shown that tea polyphenols have dose-dependent toxicology and low and medium doses (0.01-0.25%) of tea polyphenols show beneficial effects in the large intestine, liver,

TABLE 3 The association between the catechins intake and osteoarthritis in the US by WQS analysis in the US (NHANES 2007-2010 and 2017-2018).

	Adjusted OR (95% CI)	*p*-value
WQS (all catechins)[a]	1.03 (0.99, 1.07)	0.11
WQS (Epigallocatechin and Epigallocatechin 3-gallate)[b]	1.04 (1.00, 1.08)	0.04

[a]The WQS regression model containing all catechins as exposure factors.
[b]The WQS regression model containing epigallocatechin and epigallocatechin 3-gallate intake as exposure factors.
All the models wereadjusted for age, gender, ethnicity, education level, marital status, poverty income ratio, body mass index, smoke, alcohol drinking status, and history of diabetes or hypertension.
WQS, weighted quantile sum; CI, confidence interval; OR, odds ratio.

and kidney (29). Conversely, a high dietary dose (0.5-1%) of GTP reduced the expression of antioxidant enzymes and heat shock protein (HSP), leading to the worsening of colitis and colorectal cancer in mice, and also causing liver and kidney dysfunction (30). In addition, there have been case reports that excessive consumption of tea extract can cause liver damage (31, 32). It has been suggested that this may be related to the properties of tea polyphenols. Mechanistically, studies have found that tea polyphenols can produce reactive oxygen species (ROS) through auto-oxidation. Low and medium doses of tea polyphenols produce low levels of ROS, which can activate Nrf2 to reduce oxidative stress, while high doses of tea polyphenols produce high levels of ROS, leading to apoptosis and tissue damage (33–35). All the above research evidence suggests that the daily intake of dietary catechins should take into account the complementary and toxicological effects of dose relationships. Noteworthy, our findings suggest that although catechins have some adjunctive therapeutic effects in the OA population, gallocatechin intake greater than 32.70 mg/d or gallocatechin 3-gallate intake greater than 76.24 mg/d significantly increased the risk of OA in the general population.

Interestingly, in the present study, using the WQS regression model, we first explored the mixed effect of intake of all catechins on the prevalence of OA and the results showed that this model was

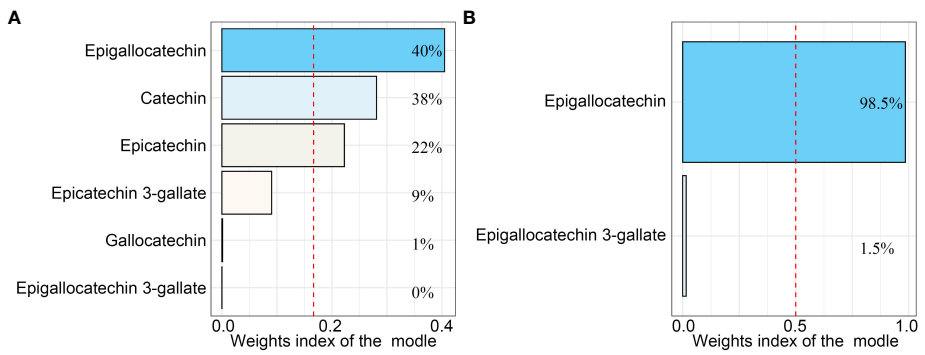

FIGURE 1
The weighted quantile sum (WQS) regression model index weights for the WQS regression model that included **(A)** all catechins and **(B)** only Epigallocatechin and Epigallocatechin 3-gallate. The red dashed line indicates the inverse of the number of exposed variables in the model. All the models were adjusted for age, gender, ethnicity, education level, marital status, poverty income ratio, body mass index, serum cotinine, alcohol drinking status, and history of diabetes or hypertension.

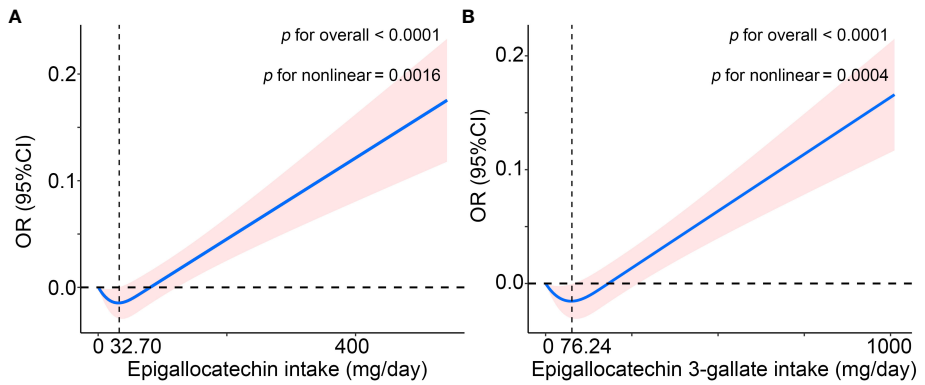

FIGURE 2
The non-linear association of osteoarthritis with **(A)** Epigallocatechin intake and **(B)** Epigallocatechin 3-gallate intake in the US (NHANES 2007-2010 and 2017-2018), using the restricted cubic spline (RCS) regression analysis. The solid red line is the OR value and the shadowed area is the corresponding 95% CI. The model was adjusted for age, gender, ethnicity, education level, marital status, poverty income ratio, body mass index, serum cotinine, alcohol drinking status, and history of diabetes or hypertension. CI, confidence interval; OR, odds ratio.

TABLE 4 Interaction effect between physical activity and Epigallocatechin or Epigallocatechin 3-gallate intake on the risk of osteoarthritis in the US (NHANES 2007-2010 and 2017-2018).

Variable	Adjusted OR (95% CI)			*p* for trend	*p* for interaction
	Q 1	Q 2	Q 3		
Epigallocatechin (mg/day)					0.03
Inactive	Reference	1.45 (0.94, 2.23)	1.90 (1.17, 3.09)	0.22	
Low	Reference	0.02 (0.00, 0.21)	0.05 (0.01, 0.27)	0.004	
Insufficiently	Reference	1.44 (0.79, 2.63)	1.68 (0.80, 3.53)	0.36	
Sufficiently	Reference	1.55 (1.09, 2.22)	2.03 (1.25, 3.30)	0.03	
Epigallocatechin 3-gallate (mg/day)	Reference				0.58
Inactive	Reference	1.46 (0.81, 2.66)	1.57 (1.02, 2.42)	0.07	
Low	Reference	2.31 (0.18, 29.41)	1.69 (0.58, 4.92)	0.66	
Insufficiently	Reference	0.66 (0.25, 1.75)	1.07 (0.71, 1.62)	0.38	
Sufficiently	Reference	1.49 (1.01, 2.21)	1.43 (1.02, 1.99)	0.08	

The model was adjusted for age, gender, ethnicity, education level, marital status, poverty income ratio, body mass index, smoke, alcohol drinking status, and history of diabetes or hypertension. CI, confidence interval; OR, odds ratio.

not associated with the prevalence of OA. Subsequently, we focused on the mixed effect of intake of epigallocatechin and epigallocatechin 3-gallate, and unsurprisingly, there was significance between the model and the prevalence of OA. Therefore, the results of the WQS regression model showed that the overall effect of all six catechins subclasses or epigallocatechin and epigallocatechin 3-gallate mainly resulted from Epigallocatechin suggesting that Epigallocatechin intakes are more important for studying the prevalence of OA in the general U.S. population. Not alone, we found that there was no significant interaction effect in the intake of epigallocatechin and OA prevalence in age, gender, and ethnicity subgroups, while a significant interaction effect was found in the PA subgroup. According to the 2018 Physical Activity (PA) Guidelines from the U.S. Department of Health and Human Services (DHHS), maintaining a moderate intensity of physical activity each week can reduce OA risk and also have a positive effect on OA recovery (18, 36). Many reports have shown that high-intensity exercise itself is easy to cause joint strain, which has shown that high-intensity exercise is an increased risk of OA (19, 37, 38). Similarly, our study found that intake of epigallocatechin hardly affected the protective effect of low PA on the risk of OA in the Low PA group. However, in the Sufficiently PA group, the prevalence of OA was significantly higher in the epigallocatechin Q 3 group compared to the Q 1 group, and OA prevalence increased with the higher daily intake of epigallocatechin.

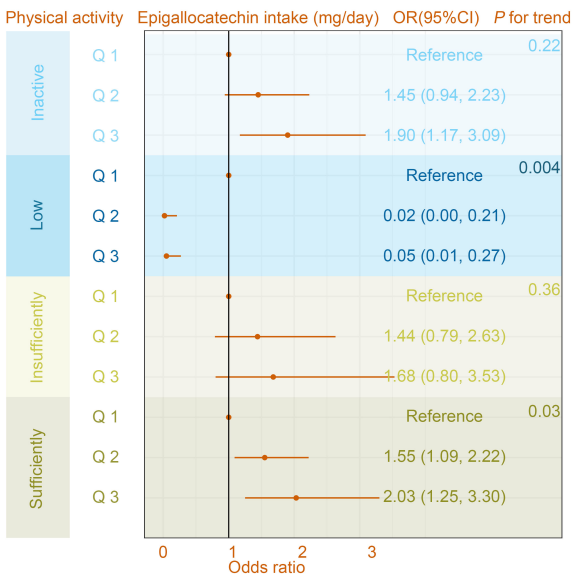

FIGURE 3
The association between osteoarthritis and Epigallocatechin intake in different physical activity subgroups. The model was adjusted for age, gender, ethnicity, education level, marital status, poverty income ratio, body mass index, serum cotinine, alcohol drinking status, and history of diabetes or hypertension. CI, confidence interval; OR, odds ratio.

This study is a relatively large population study using three complementary methods to reveal the relationship between dietary catechin intake and the prevalence of OA in American adults. Under the premise that dietary catechins are now recommended as natural health products in daily life, we first found that excessive daily catechins intake will lead to an increase in the risk of OA. However, further large-scale prospective studies and clinical trials are needed to confirm our findings and their underlying mechanisms. In addition, there are some limitations to our study. First, we used data from a cross-sectional survey. The assessment of dietary catechin intake in this study can only reflect current intake status, but OA is a long-term developing disease, which may have biased our results. Second, dietary catechins intake data were collected through a 24-hour dietary recall survey, which could lead to recall bias. Third, our analysis was unable to conclude a

causal relationship between dietary catechin intake and OA. Fourth, our population inclusion is limited by the NHANES database. It is unclear whether the relationship between dietary catechin intake and OA applies to other populations.

Conclusion

In summary, the results of this study suggested that epigallocatechin intake greater than 32.70 mg/d or epigallocatechin 3-gallate intake greater than 76.24 mg/d significantly increases the risk of OA in the general US population. In addition, PA showed a significant moderating effect on the relationship between epigallocatechin intake and the prevalence of OA.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

YF: Conceptualization. LL: Conceptualization. JG: Writing – original draft. FW: Writing – review & editing. ZZ: Writing – review & editing. YZ: Project administration.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1287856/full#supplementary-material

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Research advances of *Zanthoxylum bungeanum Maxim.* polyphenols in inflammatory diseases

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Zanthoxylum bungeanum Maxim., commonly known as Chinese prickly ash, is a well-known spice and traditional Chinese medicine ingredient with a rich history of use in treating inflammatory conditions. This review provides a comprehensive overview of the botanical classification, traditional applications, and anti-inflammatory effects of *Z. bungeanum*, with a specific focus on its polyphenolic components. These polyphenols have exhibited considerable promise, as evidenced by preclinical studies in animal models, suggesting their therapeutic potential in human inflammatory diseases such as ulcerative colitis, arthritis, asthma, chronic obstructive pulmonary disease, cardiovascular disease, and neurodegenerative conditions. This positions them as a promising class of natural compounds with the potential to enhance human well-being. However, further research is necessary to fully elucidate their mechanisms of action and develop safe and effective therapeutic applications.

KEYWORDS

***Zanthoxylum bungeanum Maxim.*, inflammation, polyphenols, inflammatory disease, NF-κB**

1 Introduction

Chinese prickly ash, also known as Hua Jiao in Mandarin, belongs to the genus *Zanthoxylum* in the Rutaceae family (1). Widely cultivated in Asia, including China, Japan, India, and Korea (2), the genus comprises approximately 250 species, with 41 found in China (Table 1) (3). Chinese prickly ash, or Hua Jiao, is a popular spice and traditional Chinese medicine ingredient specifically derived from *Zanthoxylum bungeanum Maxim.* and *Zanthoxylum schinifolium*, according to the Pharmacopoeia of the People's Republic of China (4). This review, we will focus on *Zanthoxylum bungeanum Maxim. (Z. bungeanum)*.

TABLE 1 Species of the genus *Zanthoxylum* in China.

Z. acanthopodium	*Z. collinsiae*	*Z. khasianum*	*Z. molle*	*Z. pilosulum*	*Z. stipitatum*
Z. ailanthoides	*Z. dimorphophyllum*	*Z. kwangsiense*	*Z. motuoense*	*Z. pteracanthum*	*Z. tomentellum*
Z. armatum	*Z. dissitum*	*Z. laetum*	*Z. multijugum*	*Z. rhombifoliolatum*	*Z. undulatifolium*
Z. austrosinense	*Z. echinocarpum*	*Z. leiboicum*	*Z. myriacanthum*	*Z. scandens*	*Z. wutaiense*
Z. avicennae	*Z. esquirolii*	*Z. liboense*	*Z. nitidum*	*Z. schinifolium*	*Z. xichouense*
Z. bungeanum	*Z. glomeratum*	*Z. macranthum*	*Z. oxyphyllum*	*Z. simulans*	*Z. yuanjiangense*
Z. calcicola	*Z. integrifolium*	*Z. micranthum*	*Z. piasezkii*	*Z. stenophyllum*	

Zanthoxylum bungeanum Maxim., commonly known as Honghuajiao, is a deciduous shrub with a height range of 3-7 meters, bearing small, crimson fruits measuring 4-5 mm in diameter. The flowering period spans from April to May, while fruit ripening occurring between August and October. *Z. bungeanum* holds significant importance in both traditional Chinese medicine and cuisine. The earliest record of its use in China can be traced back to the "Book of Songs," a compilation of folk poetry from the Western Zhou period, underscoring a history of over two thousand years of utilization (5). The dried fruit follicles of *Z. bungeanum* are integral to Chinese cuisine, often incorporated for their distinctive flavor and numbing taste (1). Additionally, leaves at various stages of maturity serve as ingredients and seasonings in Chinese culinary practices (6).

In traditional Chinese medicine, *Z. bungeanum* is esteemed for its properties in warming the spleen and stomach, alleviating pain, and demonstrating anthelmintic and antipruritic effects (4). It is also recognized for promoting the flow of Qi and dispelling coldness (5). Decoctions of *Z. bungeanum* find primary application in treating conditions such as stomachaches accompanied by sensations of coldness and dampness, vomiting, intestinal disorders, diarrhea, ascarid infections, schistosomiasis, and rheumatic joint inflammations (5, 7). Externally, the plant is used to address issues like bruises, eczema, and snakebites (2).

Z. bungeanum also features prominently in Indian and Nepalese folk medicine. Its decoction serves as an aromatic tonic for fevers, and as a carminative and stomachic remedy for dyspepsia, cholera, and toothaches (7).

Current research endeavors have demonstrated the pharmacological effects of *Z. bungeanum* on the gastrointestinal, neurological, and cardiovascular systems. Additionally, it exhibits anti-inflammatory and analgesic properties, along with displaying antioxidant, anti-tumor, antibacterial, antifungal, and insecticidal effects (2) (Figure 1).

Inflammation constitutes an adaptive response of the immune system to deleterious stimuli, encompassing pathogens, cellular injury, and toxic agents. Its principal role is protective, expelling these detrimental agents from the body and instigating the recovery process. However, unbridled inflammation can also be deleterious, culminating in conditions such as atherosclerosis, type 2 diabetes, and rheumatoid arthritis (8). Empirical evidence corroborates the noteworthy anti-inflammatory attributes of polyphenols. They possess the capacity to ameliorate inflammation in various diseases induced by inflammation, such as inflammatory bowel disease and acute pancreatitis (9). The molecular mechanisms underlying the anti-inflammatory activities of polyphenols involve scavenging free radicals, modulating the activity of inflammatory cells, inhibiting enzymes linked to pro-inflammatory attributes like COX2, iNOS, and LOX, suppressing NF-κB and AP-1, and impeding the activation of MAPK, protein kinase C, and Nrf2 (10).

Currently, more than 140 constituents have been identified in *Zanthoxylum bungeanum*, encompassing polyphenols, alkaloids, lignans, coumarin, fatty acids, essential oils, and others (2, 11, 12). Among these, more than 40 polyphenols have been ascertained in *Z. bungeanum*, categorized into various types based on their chemical structures, including flavonoid glycosides, flavonoids, glycosides, phenylpropanoid, anthocyanin and non-glycosides. These polyphenolic compounds have exhibited promising anti-inflammatory effects on disorders affecting diverse organs and systems, comprising ulcerative colitis, arthritis, pain, asthma, UVB-induced skin damage, and cognitive function of the brain ulcerative colitis (13), arthritis (14), pain (15), asthma (16), UVB skin damage (17), and cognitive function of the brain (18). Polyphenols derived from *Z. bungeanum* proficiently inhibit inflammatory cytokines and modulate NF-κB, p38-MAPK, TLR4, Erk1/2, JNK, and Nrf2/HO-1 pathways to exert their anti-inflammatory effects.

In this review, we summarize the polyphenolic compounds present in *Zanthoxylum bungeanum* (*Z. bungeanum*) and the therapeutic effects of *Z. bungeanum* on inflammation, with a particular emphasis on the polyphenols. Recent research suggests that *Z. bungeanum* polyphenols have the potential to significantly contribute to the management and prevention of inflammatory conditions. Further in-depth research is needed to promote their health benefits.

2 Composition and structure of polyphenols in *Z. bungeanum*

Both the leaves and seeds of *Z. bungeanum* contain polyphenolic compounds, predominantly comprising flavonoid glycosides. Research conducted by three independent groups (19–21) provides substantial evidence of the polyphenol richness in the leaves, characterized by potent antioxidant properties. Noteworthy

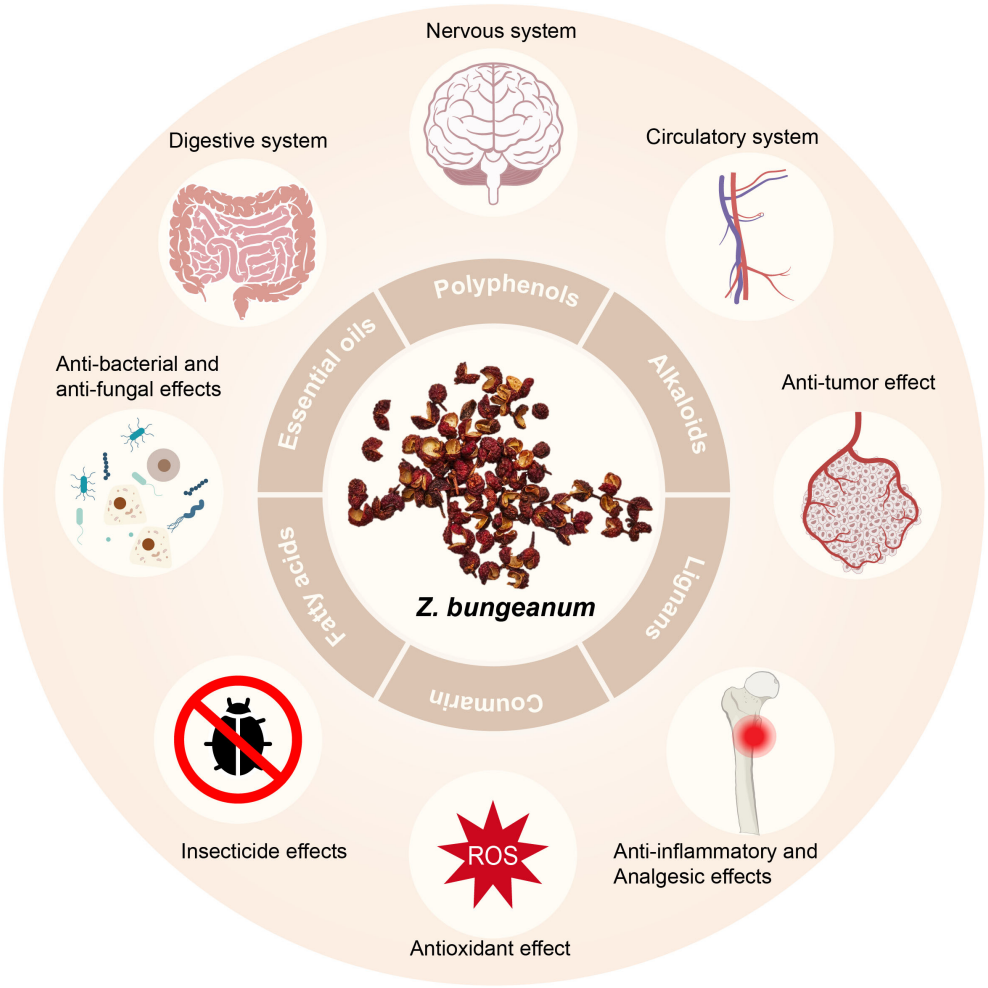

FIGURE 1
Constituents of *Z. bungeanum* and their pharmacological effects.

constituents include 5-feruloyquinic acid, vanillic acid-4-glucoside, quercetin-3-arabinoside, chlorogenic acid, epicatechin, quinic acid, syringetin-3-glucoside, quercetin, isorhamnetin-3-glucoside, trifolin, afzelin, hyperoside, isovitexin, quercitrin, trifolin, rutin, isorhamnetin 3-O-α-L-rhamnoside, astragalin, and isoquercitrin (19–21). In the outer coverings of *Z. bungeanum* fruits, Xiong et al. have identified tamarixetin 3,7-bis-glucoside, quarcetin 3',4'-dimethyl ether 7-glucoside, 3,5,6-trihydroxy-7,4'-dimethoxyflavone, hyperoside, sitosterol β-glucoside, quercetin, quercitrin, isorhamnetin 7-glucoside, rutin, arbutin, and L-sesamin (22). Additionally, the research conducted by Jia's group has revealed the presence of epigallocatechin, dihydrorobinetin, naringenin, catechin, kaempferol, catechin gallate, and isorhamnetin are identified by Jia's group (23). Recently, with the advancement of technology such as the application of high-throughput sequencing techniques, a series of polyphenolic compounds with lower concentrations in *Z. bungeanum* have been identified. The identification of polyphenols in *Z. bungeanum* has expanded from approximately 40 types to over 150 types (24), thanks to these technological developments. Due to words limit, our review specifically revisits polyphenols with higher concentrations in *Z. bungeanum*, focusing on those extensively studied for their anti-inflammatory activities (Figure 2).

Investigation of the structure-activity relationships of *Z. bungeanum* polyphenols reveals a correlation between elevated antioxidant efficacy and the presence of a hydroxyl (-OH) group at both the 4' position on the B ring and the 7 position on the A ring. Moreover, adjacent -OH groups on the B and/or A rings significantly enhanced antioxidant capabilities. Additionally, the diverse structures of these polyphenols suggest that they may display different antioxidant capacities in solution or oil-in-water emulsion reactions (20). *Z. bungeanum* polyphenols have demonstrated effective radical scavenging activities in DPPH, ABTS (21), FRAP, lipid peroxidation inhibition assays (20), and superoxide anion (19). Furthermore, polyphenols have been reported to protect Escherichia coli under peroxide stress (20) and concurrently reduce reactive oxygen species (ROS) levels in HT-29 cells without inducing any cell toxicity (19). Moreover, polyphenols have a cell-protective impact, mitigating oxidative damage in PC12 cells caused by H2O2 (21).

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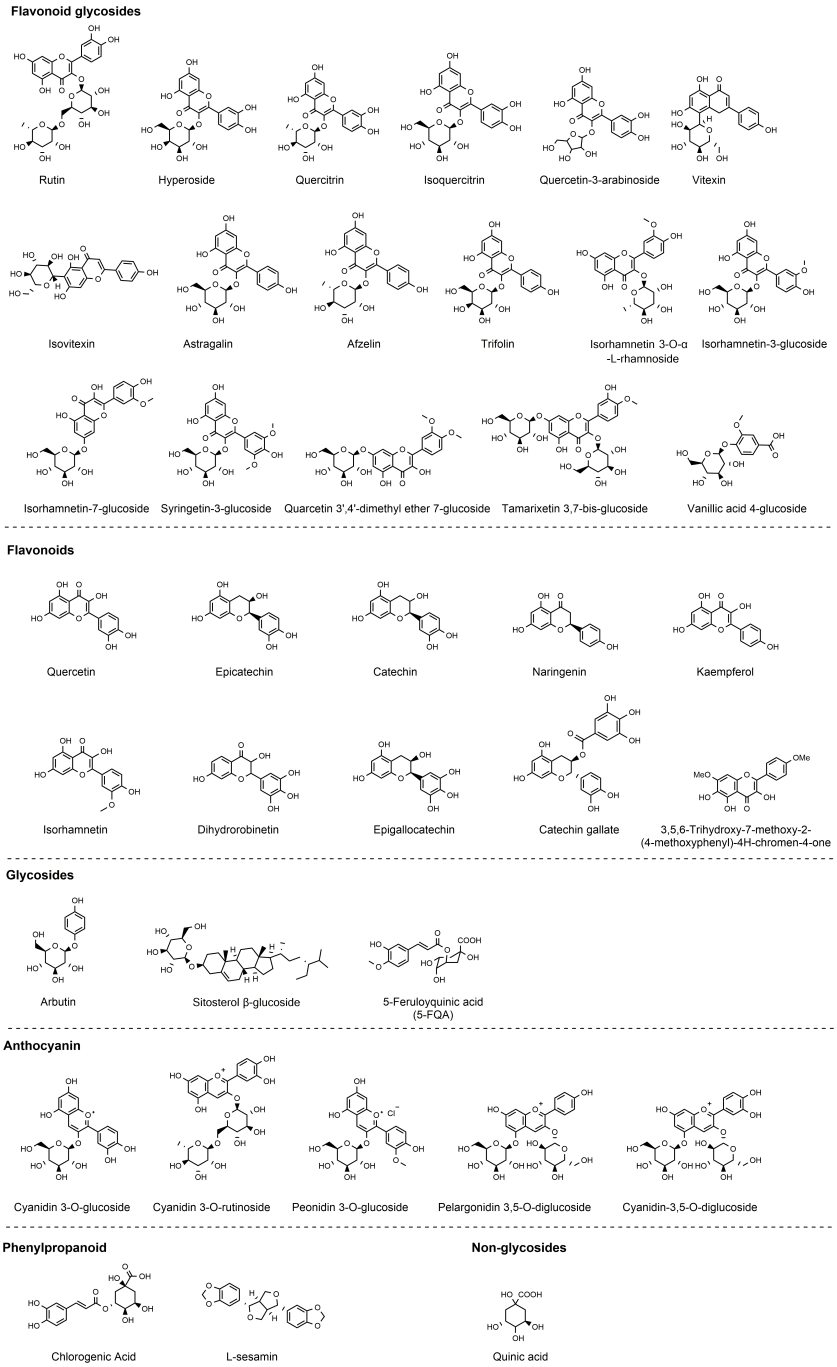

FIGURE 2
Polyphenols identified in *Z. bungeanum*.

3 Inflammatory diseases and polyphenols in *Z. bungeanum*

A combination of polyphenols found in *Zanthoxylum bungeanum* has demonstrated anti-inflammatory effectiveness in both *in vivo* and *in vitro* experiments. The ethyl acetate fraction of *Z. bungeanum* has been identified as the primary active component in enhancing cognitive function in aging mice with D-galactose-induced cognitive decline. This fraction contains several polyphenols, such as hyperoside, chlorogenic acid, quercetin-3β-d-glucoside, rutin, and epicatechin. It aids in reducing neuroinflammation, inhibiting the NLRP3/caspase-1 pathway, GSDMD, and downstream pyroptosis, both in the mouse model and in BV-2 cells subjected to LPS and ATP treatment, leading to overall cognitive improvements (25).

The treatment with *Z. bungeanum* pericarp extract (ZBE), predominantly composed of rutin, isoquercitrin, and quercitrin, has demonstrated effectiveness in protecting mice with dextran sulfate sodium (DSS)-induced ulcerative colitis (UC). It has been observed to mitigate body weight loss, prevent colonic shortening,

reduce disease activity index scores, and inhibit myeloperoxidase activity. ZBE is found to inhibit caspase-1, ASC, NLRP3, TLR4, subsequent MAPK and NF-κB pathways, and the production of TNFα, IL-12, and IL-1β, both *in vitro* in the LPS-triggered J774.1 cell model and *in vivo*. Concurrently, activation of PPARγ is detected (13).

In the subsequent section, we will individually discuss the research pertaining to the anti-inflammatory effects of each polyphenolic component found in *Z. bungeanum*. We will categorize the 40 polyphenolic constituents of *Z. bungeanum* into various groups based on their chemical compositions: flavonoids, flavonoid glycosides, glycosides, phenylpropanoid, anthocyanin, and nonglycosides (Figure 2). Please note that we do not aim to provide an exhaustive or comprehensive list of all anti-inflammatory studies for each component here. Instead, we have selected those with high citation counts or the most recent research to provide an overview of the association between inflammation and polyphenols in *Z. bungeanum*.

3.1 Flavonoid glycosides

3.1.1 Rutin

Rutin is a flavonoid with well-established anti-inflammatory properties (26) Administered at doses of 50-100 mg/kg, rutin exhibits protective effects against hepatotoxicity induced by cyclophosphamide (CP), a potent anticancer agent, in rats. This protection is associated with decreased levels of pro-inflammatory cytokines and signaling molecules, including IL-6, TNFα, iNOS, COX2, p38-MAPK, and NF-κB. Histopathological analysis reveals substantial structural damage to the liver caused by CP, effectively reversed through prior administration of rutin (27). Rutin has also demonstrated the preservation of the vascular barrier integrity in human umbilical vein endothelial cells stimulated by LPS and in an acetic acid-induced mouse mode (28). It effectively reduced hyperpermeability induced by LPS, TNFα, and HMGB1, and suppressed both TNFα production and NF-κB activation triggered by LPS (28). Beyond its anti-inflammatory and vascular protective effects, rutin has demonstrated neuroprotective and anti-colitic properties. In a rat model of spinal cord injury, rutin administration significantly attenuated histological alterations and reduced tissue damage. This was associated with decreased levels of oxidative stress markers, pro-inflammatory cytokines, and caspase-1 (29). In a mouse model of DSS-induced colitis, rutin significantly improved several key indicators of disease severity, including the disease activity score, colon length, and the integrity of goblet cells and colon epithelium. Rutin also reduced the expression of a range of oxidative-inflammatory markers, including IgE, IgM, iNOS, HO-1, and ICAM-1, and restored the balance among effector cells, regulatory cells, and B cells. The study revealed a substantial increase in the activation of the PI3K/Akt/GSK3β/MAPKs/NF-κB and p38/MK2 pathways during DSS-induced colitis in the animal subjects, a condition that rutin treatment effectively mitigated. In silico studies supported the specificity of rutin's interaction with these pathways (30).

In terms of pharmacokinetics, orally administered rutin is absorbed in the small intestine, transferred to the liver via the bloodstream, and eliminated through bile and the kidneys (31). Major metabolites include sulfates and glucuronides of quercetin (32). In rats, Zhang et al. reported elimination rate half-life, area under the curve, and plasma clearance values of 3.345 minutes, 5750 μg min/ml, and 5.891 mL/min/kg, respectively (33). Intravenous rutin accumulates in the liver, with a significant portion then transferred to the small intestine, and is also detected in the lung post-injection (31). Interactions between rutin and drugs were studied as well. Rutin reduces the anticoagulant effect of racemic warfarin by 31% when co-administered orally. This outcome was ascribed to a noteworthy 77% rise in the unbound formation clearance of both oxidative and reductive metabolites, coupled with an elevation in the unbound renal clearance of the more potent S-enantiomer of warfarin (34). Rutin also significantly decreases the oral Cmax and AUC of cyclosporine by 63.2% and 57.2%, respectively, through the activation of Pgp transporter and CYP3A enzyme (35).

3.1.2 Hyperoside

Hyperoside is another flavonoid known for its anti-inflammatory properties. In mouse peritoneal macrophages subjected to LPS stimulation, hyperoside inhibited TNFα, IL-6, and NO production by 32.3%, 41.3%, and 30%, respectively. Moreover, hyperoside reduced NF-κB activation and IκB-α degradation (36). This compound also exhibits anti-neuroinflammation effect *in vitro* and *in vivo* (37, 38). In the LPS-induced HT22 murine neuronal cell line, hyperoside enhances cell survival and mitigates inflammation, oxidative stress, and apoptosis. This effect is achieved by amplifying SIRT1, triggering the activation of both Wnt/β-catenin and sonic hedgehog pathways (38). In rats, 50 mg/kg hyperoside protected against cerebral ischemia-reperfusion injury by mitigating oxidative stress, inflammation, and cell death. Rats treated with hyperoside exhibited significantly enhanced neurological function and a substantial reduction in the ratio of cerebral infarction volume (37).

Hyperoside also attenuate several vascular inflammatory responses initiated by elevated glucose levels in human umbilical vein endothelial cells and mice. These responses include vascular permeability, monocyte attachment, CAMs expression, ROS formation, and NF-κB activation (39). Furthermore, hyperoside's anti-arthritic properties have also been verified both *in vitro* and *in vivo*. It can suppress inflammation and prevent cartilage breakdown by influencing the PI3K/AKT/NF-κB and MAPK signaling pathways, as well as the interplay between the Nrf2/HO-1 and NF-κB signaling pathways (40). Hyperoside also inhibited OVA-induced airway hyperresponsiveness in mice through activation of Nrf2/HO-1 (41). In a rat model of antiphospholipid syndrome (APS), hyperoside at a dose of 40 mg/kg led to increased fetal weight, reduction of fetal resorption rates, and reduced pregnancy loss by modulating the mTOR/S6K and TLR4/MyD88/NF-kB signaling pathways (42).

3.1.3 Quercitrin

Quercitrin demonstrates the ability to attenuate carbon tetrachloride (CCl4) induced brain injury by suppressing ROS,

MDA, TNFα, and IL-6 (43). Furthermore, it exhibits protective effects against skin damage induced by UVB damage. This protection is achieved through the reduction of ROS, NF-κB activation, and DNA damage triggered by UVB exposure. Quercitrin also restores the diminished expression of catalase and the GSH/GSSG ratio due to UVB exposure (17). In a study involving mice with Alzheimer's disease, quercitrin inhibits the activation and proliferation of microglia, decreases the accumulation of amyloid-β plaques, and improves cognitive impairment by inhibiting inflammation. Specifically, this compound inhibits the level of IL-1α, IL-17A, IL-6, and G-CSF in peripheral blood, as well as IL-1α, IL-4, IL-6, Eotaxin, CXCL-1, MIP-1α, MIP-1β and G-CSF in the brain, thereby alleviating systemic inflammation in the 5XFAD mice (44).

Quercetin and quercitrin, common flavonoids in vegetables, are frequently compared (45). Theoretical calculations clarify that the oxygen atom located on the B rings could serve as the primary site for alterations in electron cloud density, providing insights into how quercetin and quercitrin exert their anti-inflammatory and ROS scavenging effects (46). In LPS-stimulated RAW264.7 cells, both compounds markedly decrease NO and ROS production, as well as the expression of TNFα, IL-1β, and IL-6 (46). However, Comalada et al. reported that unlike quercitrin, quercetin can reduce the expression of cytokines and iNOS by inhibiting the NF-κB pathway *in vitro* in bone marrow-derived macrophages, without affecting c-Jun N-terminal kinase activity. The group revealed that quercitrin's *in vivo* impact in a rat colitis model induced by DSS may be attributed to the liberation of quercetin, which occurs following the breakdown of glycosides by intestinal microbiota. In other words, quercitrin releases quercetin to exert its anti-inflammatory influence, achieved by inhibiting the NF-κB pathway (45).

3.1.4 Isoquercitrin

Isoquercitrin has undergone tested in an LPS-stimulated RAW264.7 cell model, revealing its ability to decrease NO production, downregulate the expression of PGE2, COX2, iNOS, and NF-κB p65 protein, and reduce the mRNA levels of IL-1, IL-6, PTGES2, and MCP-1 (47). Moreover, at a dosage of 20 mg/kg, isoquercitrin has demonstrated the capacity to protect denervated muscle from atrophy. This protective effect is achieved by reducing the levels of IL-1β, TNFα, and IL-6 and inactivating the JAK/STAT3 signaling pathway in the target muscle (48).

3.1.5 Vitexin

Vitexin exhibits anti-inflammatory properties in the OVA-induced mouse allergic asthma model at doses of ranging from 0.2 to 5 mg/kg. Specifically, vitexin mitigates the migration of eosinophils, neutrophils, and mononuclear cells prompted by OVA within bronchoalveolar lavage fluid (BALF). Examination of lung tissue reveals that vitexin effectively suppresses the invasion of leukocytes, mucus production, and development of pulmonary edema. It also moderates the escalation of Th2 cytokines in BALF and reduces the concentration of IgE in the plasma (49). Vitexin has also demonstrated anti-inflammatory effects in chronic cerebral hypoperfusion injury in a rat model of persistent bilateral common carotid artery occlusion and in HT22 mouse hippocampal neuronal cells exposed to oxygen and glucose deprivation followed by reoxygenation injury. The findings confirm vitexin's ability to modulate Epac and NLRP3. Additionally, in the rat model, vitexin has shown the potential in diminishing the severity of ongoing pathological harm in the cortex and hippocampus and preventing further decline in cognitive function (18). Moreover, vitexin inhibits inflammatory pain in various mouse models of inflammation-related pain, including acetic acid-induced writhing, pain-like behavior prompted by phenyl-p-benzoquinone, capsaicin, complete Freund's adjuvant (CFA), and both phases of the formalin test. It also alleviates mechanical and thermal hyperalgesia triggered by capsaicin, carrageenan, and chronic CF. TRPV1 is considered the key target (50). Additionally, vitexin alleviates liver inflammation in a DSS-induced colitis model by inhibiting the TLR4/NF-κB signaling pathway activation. Administration of vitexin results in lower ALT and TC levels in the livers of mice suffering from liver injury. It also reduces the release of IL-6, TNFα, and IL-1β induced by DSS (51). Furthermore, vitexin inhibits the movement of neutrophils toward areas of inflammation by suppressing the p38, ERK1/2, and JNK pathways (52).

3.1.6 Isovitexin

Isovitexin effectively alleviates contact dermatitis in mice triggered by ginkgolic acids, leading to a significant reduction in ear swelling, splenomegaly, and inflammatory cell infiltration. Subsequent investigations have revealed that isovitexin can impede the MAPK and STAT signaling pathways, along with the phosphorylation of SHP2 (53). In the mouse models of kidney injury induced by cyclophosphamide (CP) (54), liver injury triggered by LPS/d-galactosamine (55), and acute lung injury induced by LPS (56), isovitexin demonstrates its therapeutic effects via inhibiting NF-κB activation and inducing Nrf2 and HO-1 expression. In the kidney injury model, isovitexin mitigates CP-induced increases in serum BUN and creatinine, and curbs TNFα, IL-1β, and IL-6 (54). Isovitexin substantially diminishes liver injury, evidenced by reduced histopathological changes and lower AST and ALT levels. It also reduces TNFα levels, MPO activity, and MDA content (55). Pretreatment with isovitexin significantly alleviates acute lung injury, as demonstrated by reduced histopathological changes, diminished granulocyte infiltration, and subdued endothelial activation. Additionally, it lowers VCAM-1 and ICAM-1 expression, reduces MPO and MDA levels, and enhances GSH and SOD (56).

3.1.7 Astragalin

Astragalin notably alleviates inflammatory reactions and bone damage in both DBA/1J mice with collagen-induced arthritis and human fibroblast-like synoviocytes. It reduces joint swelling, arthritis index, and bone erosion, while also inhibiting the production of IL-1β, TNFα, IL-6, and IL-8. Moreover, a decrease in MMP-1, MMP-3, and MMP-13 levels has also been observed in chondrocytes, synovial cells, and TNFα-induced MH7A cells. Additionally, astragalin inhibits p38, JNK phosphorylation, and c-Jun/AP-1 activation (57). Furthermore, through the ROS and

MAPK signaling pathway, the process of osteoclastogenesis in inflammatory osteolysis is alleviated by astragalin (58). In an OVA-challenged mouse model, astragalin at doses of 10-20 mg/kg impedes mast cell recruitment, preventing airway thickening and alveolar emphysema (59).

3.1.8 Afzelin

Afzelin performs anti-inflammatory effect in two *in vitro* experiments (60, 61). In human keratinocytes exposed to particulate matter (PM), a widespread airborne contaminant, afzelin mitigates inflammation and ROS production. It also inhibits p38 kinase, as well as the transcription factors c-Fos and c-Jun (61). The inhibitory effect of afzelin on the p38 kinase pathway contributes to its protective effect of human keratinocytes and epidermal equivalent models exposed to UVB, resulting in a reduction of IL-6, TNFα, and PGE2 release induced by UVB (60).

3.2 Flavones

3.2.1 Quercetin

Quercetin stands out as one of the extensively researched polyphenols in *Z. bungeanum*. showcasing therapeutic potential in addressing inflammatory conditions, particularly arthritis (62, 63). In a study involving women with rheumatoid arthritis, a daily supplement of 500mg quercetin over 8 weeks resulted in significant improvements in the clinical symptoms, disease activity, hs-TNFα levels, and health assessment questionnaire outcomes (62). For rabbits with surgically-induced osteoarthritis (OA), a 4-week gavage treatment of 25 mg/kg quercetin demonstrated increased SOD and TIMP-1 expressions, reduced MMP-13 expression, and mitigation of OA degeneration, comparable to the effects observed in the celecoxib-treated group (63). Quercetin's impact extends to inflammation-based pain models, as intraperitoneal and oral administrations significantly suppressed pain induced by phenyl-p-benzoquinone and acetic acid. It also mitigated the second phase of pain intensity escalation caused by formalin and carrageenin. This compound further demonstrated its efficacy in curtailing hypernociception stimulated by TNFα and CXCL1, along with reducing carrageenin-induced IL-1β production (15). Moreover, in RAW264.7 cells stimulated with LPS, quercetin significantly reduced the production of NO, inducible NO synthase, and IL-6. It also hindered the relocation of NF-κB to the cell nucleus and suppressed the activation of Erk1/2 and JNK. In DNCB-induced atopic dermatitis mouse model, quercetin exhibited anti-inflammatory effects, as evidenced by improvements in ear thickness, serum IgE levels, and histological analysis (64).

Regarding the pharmacokinetic aspects of quercetin, initial metabolism occurs in the small intestine through processes like glucuronidation and O-methylation. The subsequent breakdown and processing take place in the liver after reaching it through the hepatic portal vein. Notably, gut bacteria, especially clostridium orbiscindens, play a role in the breakdown process in the large intestine. Key metabolites found in human plasma include quercetin-3-glucuronide, quercetin-3-sulfate, and isorhamnetin-3-glucosidic acid. Quercetin distribution involves various organs (lungs, kidneys, heart, and liver), with the lungs exhibiting the highest concentrations. Conjugates are predominantly present in the blood and are excreted in urine (65).

Pharmacokinetic and pharmacodynamic interactions between quercetin and drugs have been unveiled in studies. Competitive binding to serum albumin influence on cytochrome P450, glycoproteins, and other factors modify drug profiles, affecting treatment outcomes for infectious diseases, cardiovascular diseases, diabetes, and cancer (65). For example, quercetin competes with erlotinib for binding to bovine serum albumin, potentially contributing to increased adverse events associated with erlotinib use (66). Additionally, combined treatment with quercetin and methotrexate significantly reduces inflammatory mediators in collagen-induced arthritis mice, suggesting quercetin's potential as an adjuvant to enhance anti-rheumatic monotherapy (67).

3.2.2 Epicatechin

Epicatechin exhibits dose-dependent reduction in TNFα-induced increase of JNK, p38, and ERK1/2 phosphorylation, nuclear AP-1-DNA interaction, activation of the NF-κB signaling pathway, nuclear NF-κB-DNA binding, p65 nuclear translocation, and PPARγ expression in 3T3-L1 adipocytes (68). A dosage of 20 mg/kg epicatechin proves effective in mitigating inflammation in the renal cortex of fructose-fed rats (69), while a higher dose of 80 mg/kg demonstrates efficacy in alleviating LPS-induced renal inflammation in rats (70). In both studies, downregulation of TNFα, iNOS and IL-6 are observed (69, 70). Furthermore, a dosage of 15 mg/kg epicatechin exhibits anti-inflammatory properties in mice experiencing LPS-induced acute lung injury, achieved by directly impeding the function of the p38-MAPK signaling pathway (71). Epicatechin also shows significant effects in mitigating atherosclerosis, specifically reducing severe lesions by 27% in ApoE*3-Leiden mice, without affecting plasma lipids. Additionally, it successfully countered diet-induced increases in inflammatory markers such as serum amyloid A and human C-reactive protein (72).

Concerning the pharmacokinetic parameters, orally administered epicatechin is initially absorbed in the duodenum, with the majority (70%) being absorbed in the lower intestine after catabolism by the gut microbiome. Over 80% of ingested epicatechin is absorbed, and the gut microbiome plays a crucial role in its metabolism, yielding more than 20 identifiable metabolites. These metabolites are then mainly excreted through urine (73).

3.2.3 Catechin

Catechin mitigates coronary heart disease in rats induced by pituitrin injection and a high-fat diet by inhibiting, lipoprotein-associated phospholipase A2, C-reactive protein, TNFα, and IL-6. Simultaneously, catechin treatment also demonstrates the inhibition of NF-κB and upregulation of FXR, p-STAT3, and p-Akt expression levels (74). High fructose consumption over a six-

week period in rats induces a series of metabolic problems, including insulin resistance, dyslipidemia, obesity, reduced plasma adiponectin, and inflammation of adipose tissue. Supplementing their diet with 20 mg/kg/day of catechin effectively enhances all these parameters. In the TNFα induced 3T3-L1 adipocyte model, catechin inhibits inflammation by suppressing MAPKs, JNK and p38 activation, and preventing PPAR-γ reduction (75). At a dose of 75-300 mg/kg, catechin alleviates allergic symptoms such as sneezing and nose rubbing in mice suffering from OVA-induced allergic rhinitis. It reduces the levels of ovalbumin-specific IgE, IL-5, IL-13, restoring the balance between Th2 and Th1 cells. The potential mechanism of action involves the inhibition of TSLP expression in epithelial cells through the modulation of the NF-κB/TSLP pathway by catechin (76).

3.2.4 Naringenin

Naringenin significantly inhibits paw swelling and pathological changes in the joint tissue in the SD rat model of complete Freund's adjuvant-induced arthritis. Additionally, IL-1β, TNFα, and IL-6 in serum are notably suppressed (14). Naringenin demonstrates neuroprotective effects by ameliorating neuroinflammation through the inhibition of p38-MAPK and STAT-1. In neuroglial cells induced by LPS/IFN-γ, this compound reduces the production of TNFα and NO, along with the expression of iNOS, thereby preventing neuron death induced by inflammation (77). Furthermore, naringenin inhibits pain behavior in mice triggered by various inflammatory stimuli, including acute pain caused by the use of acetic acid, PBQ, formalin, capsaicin, and CFA, as well as the provocation of mechanical hyperalgesia through subplantar injection of capsaicin, CFA, carrageenan, or PGE2. The mechanism of naringenin involves the activation of NF-κB and the inhibition of IL-1β, IL-33, TNFα, and oxidative stress. Additionally, naringenin activates the analgesic NO-cyclic GMP-PKG-ATP sensitive K+ channel pathway (78). Naringenin also exhibits anti-inflammatory effects in respiratory inflammation. In a murine COPD model, characterized by 90 days of cigarette smoke exposure-induced initiation, 20-80mg/kg of naringenin significantly improves pulmonary function, reduced inflammatory cells, and inhibits IL-8, TNFα, and MMP-9 in mouse BALF and serum. Suppression of the NF-κB pathway is also observed in mice treated with naringenin (79).

Delving into the pharmacokinetic characteristics, orally administrated naringenin exhibits limited absorption in the human gastrointestinal tract, yielding a modest 15% oral bioavailability. The absorption process encompasses both passive diffusion and active transport mechanisms. Once absorbed, naringenin swiftly distribute to vital organs such as the liver, cerebrum, kidney, spleen, and heart, suggesting potential neuroprotection within the central nervous system. Remarkably, naringenin demonstrates high permeability across blood-brain barrier models. The enterohepatic recycling of naringenin plays a crucial role, contributing to hepatic conjugate excretion in bile and participating in the enteric excretion of phase II conjugation. Post-absorption, Naringenin undergoes a significant metabolic process involving glucuronidation, resulting in the detection of 98% of naringenin–o–β–d–glucuronide in plasma. Before absorption in the caecum, naringenin undergoes hydrolysis by beta–glucosidase in the small intestine. Further metabolism by intestinal bacterial microflora produces p–hydroxybenzoic acid, p–hydroxyphenylpropionic acid, and p–coumaric acid, which manifest in plasma and urine. Ultimately, flavonoid excretion primarily occurs through two pathways: the biliary and urinary pathways (80).

3.2.5 Kaempferol

In OVA challenged asthmatic mouse models, oral intake of kaempferol mitigated the increase in eosinophil major basic protein and eotaxin-1 expression, achieve through the transactivation inhibition of NF-κB. Consequently, this reduction leads to decreased accumulation of eosinophils in the airways and lung tissue (16). Furthermore, kaempferol demonstrates the ability to control vascular inflammation in an atherosclerosis rabbit model with a high-cholesterol diet for ten weeks. Following treatment with kaempferol, decreased levels of IL-1β, TNFα, and MDA, an increase in serum SOD activity, and a reduction in the gene and protein expression of aortic E-selectin, ICAM-1, VCAM-1, and MCP-1 are observed (81). In a rat model simulating cerebral ischemia/reperfusion by occluding the middle cerebral artery for 60 minutes and then reperfusion, kaempferol is administered at doses of 25-100 mg/kg. The treatment significantly reduces the volume of cerebral infarction following cerebral ischemia-reperfusion, alleviated inflammation, and prevented the breakdown of the blood-brain barrier, thereby improving the neurological outcome on the 7th day after cerebral ischemia reperfusion. Additionally, reduced nuclear translocation and phosphorylation of the transcription factor NF-κB p65 are observed (82). What's more, kaempferol exerts a protective effect on osteoarthritis chondrocytes by regulating the XIST/miR-130a/STAT3 axis, thereby inhibiting inflammation and extracellular matrix degradation (83).

Limited absorption and minimal oral bioavailability are observed with kaempferol. Its lipophilic nature allows for passive absorption, diffusion facilitation, and active transport. Metabolism in the liver results in the formation of glucuronic acid and sulfate conjugates, while intestinal enzymes in the small intestine contribute to its processing. Aglycogens, produced through the metabolism of kaempferol by colonic microbiota, are further transformed into 4-hydroxyphenylacetic acid, 4-methylphenol, and phloroglucinol. These metabolites undergo absorption into the systemic circulation, distribution to tissues, and eventual excretion in feces or urine (84).

Notably, the administration of a 12 mg/kg kaempferol dose demonstrated a substantial improvement in oral etoposide bioavailability in rats, showing a 64% enhancement compared to lower doses of 47% and 15%. At the highest dose, 12 mg/kg kaempferol exhibited a 26% increase in intravenous etoposide bioavailability. This intriguing finding suggests potential hepatic CYP3A4 inhibition and implicates kaempferol in reducing the unpredictable oral bioavailability of etoposide (85).

3.2.6 Isorhamnetin

Isorhamnetin possesses the ability to inhibit inflammation and provide renal protection. In a rat model of type 2 diabetes induced by a high-fat diet and streptozotocin, isorhamnetin significantly improved the renal function. The study reported that Isorhamnetin inhibited NF-κB signaling activity, resulting in reductions in IL-1β, IL-6, TNFα, TGF-β1, and ICAM-1 levels, as well as the mitigation of oxidative stress in diabetic rats and glomerular mesangial cells (86). Research conducted by Dou's team demonstrated that isorhamnetin exerts beneficial effects on TNBS- and DSS-induced mouse inflammatory bowel disease (IBD) by upregulating xenobiotic metabolism mediated by PXR and concomitantly downregulating NF-κB signaling. Isorhamnetin inhibited the expression of IL-6 and TNFα, as well as the mRNA levels of ICAM-1, iNOS, TNFα, COX2, IL-6, IL-2, through the aforementioned pathways (87). Isorhamnetin has been found to inhibit neuroinflammation. In BV2 microglial cells stimulated with LPS, isorhamnetin significantly inhibits NO and PEG2, as well as IL-1β, TNFα, iNOS and COX2. Research on its anti-inflammatory mechanism indicates that isorhamnetin controls neuroinflammation by inhibiting the TLR4/MyD88/NF-kB pathway (88). Moreover, isorhamnetin exhibits efficacy in asthma. In TNFα-induced human bronchial epithelial cell line BEAS-2B, isorhamnetin at concentrations of 20-40 μM can reduce cellular proliferation and notably suppress the expression of CXCL10, IL-1β, IL-6, and IL-8. Furthermore, treatment with isorhamnetin downregulates the phosphorylation of the NF-κB and MAPK pathways in this model (89).

In the context of collagen-induced arthritis, isorhamnetin at doses ranging from 10 to 20 mg/kg significantly alleviate arthritis, improving arthritis score, joint damage score, and inflammation score. Isorhamnetin can also regulate the production of cytokines such as IL-1β, TNFα, IL-6, IL-10, IL-17A, IL-17F, and IL-35, while mitigating oxidative stress (90).

3.3 Glycosides

Arbutin significantly enhances kidney function in rats experiencing LPS-induced acute kidney damage. It reduces inflammation and cell death by modulating the PI3K/Akt/Nrf2 pathway after LPS exposure both *in vivo* and *in vitro*. Moreover, the Akt inhibitor GDC effectively inhibits this arbutin-induced improvement *in vitro* (91). Additionally, arbutin protects mice from isoproterenol (ISO)-induced cardiac hypertrophy. Pre-treatment with arbutin notably inhibits the TLR4/NF-κB pathway, resulting in decreased IL-6 and TNFα (92). In a DSS-induced mouse colitis model, arbutin significantly mitigates symptoms such as elevated disease activity index, loss of body weight, and increased colon weight-to-length ratio. This anti-inflammatory impact is contingent upon the control of JAK2 and the suppression of IL-1β, TNFα, and IL-6. Arbutin also suppresses inflammatory responses in epithelial (IEC6) and immune (RAW264.7) cells triggered by LPS. However, these benefits, both *in vitro* and *in vivo*, can be negated by the JAK2 inhibitor AG490 (93). In addressing metabolic issues, arbutin is found to suppress high-glucose-induced inflammation in adult human retinal pigment epithelial cells via upregulation of SIRT1, which provides a novel therapeutic target for diabetic retinopathy management (94). In arbutin-treated LPS-triggered BV2 murine microglial cells, inhibition of NO production, and reduced expression of COX2 and iNOS are observed. Arbutin significantly diminishes the expression of IL-1β, IL-6, MCP-1, and TNFα. Additionally, it impedes the nuclear transcriptional and translocation activity of NF-κB (95).

Jin's group developed arbutin-loaded gelatine methacryloyl-Liposome microspheres (GM-Lipo@ARB), offering extended arbutin release and notable cartilage targeting. The microspheres decrease inflammation in IL-1β-stimulated arthritic chondrocytes and maintain cartilage matrix equilibrium through NF-κB inhibition and Nrf2 pathway activation. Application of the GM-Lipo@ARB lessens inflammation and oxidative stress in articular cartilage, effectively decelerating osteoarthritis progression in a mouse model (96).

3.4 Phenylpropanoid

The anti-inflammatory effects of chlorogenic acid (CGA) have been investigated in LPS-stimulated RAW 264.7 macrophages and BV2 microglial cells. CGA inhibits the production of NO, IL-1β, IL-6, TNFα, CXCL1, COX2, and iNOS. A possible mechanism of action involves the reduction of ninjurin1 level and nuclear translocation of NF-κB (97). CGA also downregulates the TLR4/MyD88/NF-κB signaling pathway (98, 99). Through this pathway, CGA can potently inhibit CCl4-induced liver fibrosis in rats (98), and alleviate renal inflammation in a mouse model of hyperuricemia induced by hypoxanthine and potassium oxonate (99). Animal experiments have confirmed that the systemic administration of CGA can help alleviate both inflammatory and neuropathic pain (100).

The hydrophilic nature of CGA essentially impedes its passage through the lipophilic membrane barrier, resulting in low absorption. Absorption likely occurs in the stomach rather than the small intestine. Caffeic acid is detected in plasma and urine 1.5 hours after a CGA-supplemented meal, along with derivatives like ferulic acid and isoferulic acid. These derivatives result from CGA hydrolysis in the small intestinal mucosa. CGAs' absorption and metabolism are relatively low, constituting about one-third of total intake in the upper gastrointestinal tract. The remaining two-thirds reach the colon, where intense microbial metabolism occurs. Microflora-derived esterase hydrolyzes CGA, producing microbial metabolites, comprising 57.4% of the total CGA consumed, emphasizing the crucial role of gut microbiota in CGA metabolism and biological properties (101).

3.5 Anthocyanin

Anthocyanins, a member of the polyphenolic family in *Z. bungeanum*, contribute to the crimson coloration of its fruit peel. (102). In total, five types of anthocyanins with clear chemical structure have been identified in *Z. bungeanum* (24, 102–104).

The anti-inflammatory efficacy of cyanidin 3-O-glucoside (C3G) has been demonstrated across various *in vivo* and *in vitro* models. C3G exhibits the ability to safeguard mice from chronic skin damage induced by UVB exposure, leading to notable improvements in UVB-induced epidermal hyperplasia, collagen fiber preservation, ROS levels, and the expression of COX-2 and IL-6 (105). Furthermore, C3G demonstrates protective effects in rats against cecal ligation and puncture (CLP)-induced acute lung injury (ALI), enhancing their survival rate. C3G treatment results in reduced serum levels of TNF-α, IL-1β, and IL-6, along with the inhibition of COX-2 protein expression and PGE2 production in the lung, potentially through the suppression of the NF-κB signaling pathway (106). C3G also exerts anti-neuroinflammatory effects. In LPS-stimulated BV2 microglia, C3G effectively suppresses microglial activation and the levels of neurotoxic mediators and pro-inflammatory cytokines. Moreover, there is observed suppression of the NF-κB and p38 MAPK signaling pathways (107). Additionally, in TNBS-challenged mice, C3G significantly ameliorates clinical symptoms and mitigates histological damage, possibly by protecting the intestinal barrier and suppressing inflammatory cytokine secretion (108).

Cyanidin 3-O-rutinoside, peonidin 3-O-glucoside, pelargonidin 3,5-O-diglucoside, cyanidin-3,5-O-diglucoside have limited study in inflammatory disorders. Only a few *in vitro* studies were found (109, 110).

3.6 Non-glycosides

Research on the anti-inflammatory effects of quinic acid is limited. However, one study shown that quinic acid mitigates vascular inflammation in TNFα-stimulated vascular smooth muscle cells by reducing MAPK phosphorylation and inhibiting NF-κB activation (111).

Limited study has been conducted on the anti-inflammatory effects of catechin gallate, epigallocatechin, dihydrorobinetin, quercetin-3-arabinoside, quarcetin 3',4'-dimethyl ether 7-glucoside, isorhamnetin-3-glucoside, isorhamnetin 7-glucoside, isorhamnetin 3-O-α-L-rhamnoside, tamarixetin 3,7-bis-glucoside, 3,5,6-trihydroxy-7,4'-dimethoxy flavone, sitosterol β-glucoside, trifolin, vanillic acid-4-glucoside, syringetin-3-glucoside, L-sesamin, and 5-feruloyquinic acid.

4 Direct target of *Z. bungeanum* polyphenols

In the preceding sections, we primarily delineated the anti-inflammatory pharmacological activities of *Z. bungeanum* polyphenols, highlighting their modulation of inflammation through signaling pathways, including NF-κB, MAPK, Nrf2/keap1, and the NLRP3 inflammasome (Figure 3). However, to date, limited research has been conducted on the direct targeting of proteins or genes associated with inflammation by *Z. bungeanum* polyphenols. In this section, we consolidate and summarize the pertinent studies investigating the direct interactions of *Z. bungeanum* polyphenols with inflammatory-related proteins or genes (Table 2).

5 Clinical trials of *Z. bungeanum* polyphenols

Currently, several clinical trials have utilized *Z. bungeanum* polyphenols; however, their application in inflammatory conditions remains limited. Table 3 below summarizes completed clinical studies on *Z. bungeanum* polyphenols to date. Notably, no severe adverse reactions associated with these polyphenols have been reported across these clinical investigations, providing a certain degree of evidence supporting their safety profile.

6 Inflammatory diseases and other compositions in *Z. bungeanum*

6.1 Alkaloids

Alkaloids, such as hydroxy-alpha-sanshool (HAS), constitute the characteristic compounds in *Z. bungeanum*, contributing to the notable sensation of numbness in the mouth (124). In a rat model of type 2 diabetes mellitus (T2DM), Zanthoxylum alkylamides (ZA), a mixed extract containing hydroxyl-γ-sanshool, hydroxyl-β-sanshool, and hydroxyl-α-sanshool, demonstrated the ability to control inflammation and address protein metabolism disorders, consequently ameliorating T2DM. The PI3K/Akt/forkhead box O signaling pathway and the TNFα/NF-κB pathway are implicated in this process (125).

Among the alkaloids in *Z. bungeanum*, HAS has been extensively studied for its anti-inflammatory effects. HAS exhibits a neuroprotective effect on H2O2-stimulated PC12 cells without inducing cytotoxicity in normal PC12 cells. The suppression of apoptosis is achieved by regulating the PI3K/Akt signaling pathway (126). Oral administration of HAS markedly improves spontaneous locomotion, cognitive function, and histopathological injuries in a mouse model of Alzheimer's disease induced by D-galactose and AlCl3. The therapeutic effect of HAS involves the mitigation of oxidative stress damage and the activation of the Nrf2/HO-1 signaling pathway (127). As one of the main active ingredients in the herbal medicine TU-100, HAS enhances the production of antimicrobial defense molecules (ADM) by intestinal epithelial cells. TU-100, administered orally, prevents weight loss and colon ulceration in both TNBS-induced type-1 model colitis and OXN-induced type-2 model colitis. This suggests that HAS possesses anti-inflammatory properties and could potentially serve as a beneficial treatment agent for UC through the promotion of ADM production (128).

Zanthoxylin, another major alkaloid of *Z. bungeanum*, exhibits anti-inflammatory and pain-relieving effects in a variety of animal models. In mice, zanthoxylin alleviates pain in both general and formaldehyde-induced pain models. Its mechanism of action involves binding to the α7nAChR receptor and activating the JAK2/STAT3 signaling pathway, thereby inhibiting inflammation

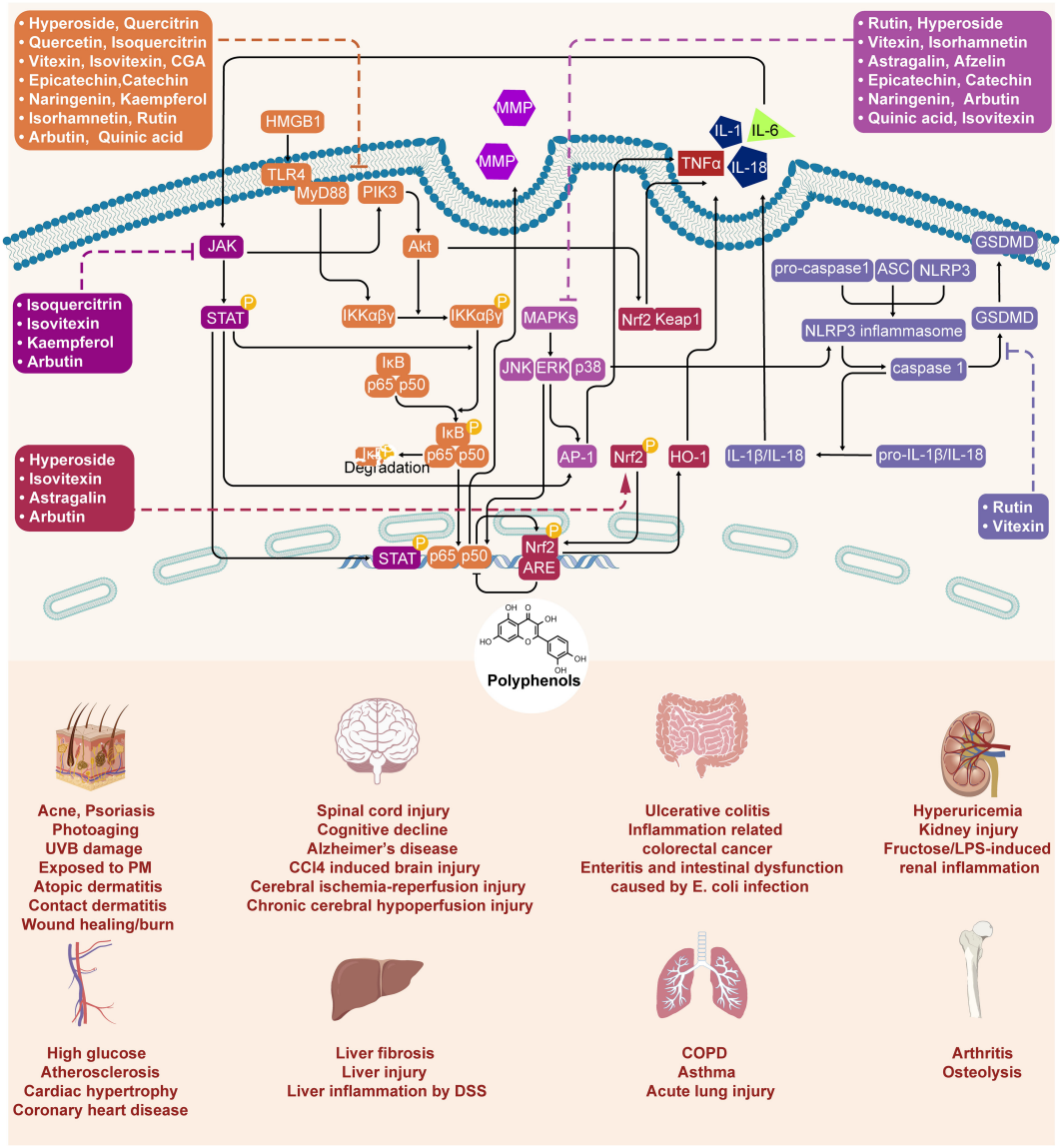

FIGURE 3
Molecule mechanism of polyphenols in *Z. bungeanum* and their anti-inflammatory effect.

and reducing the production of pro-inflammatory cytokines such as IL-6 and TNFα (129).

6.2 Fatty acid

Research on the anti-inflammatory properties of fatty acids in *Z. bungeanum* predominantly focuses on *Z. bungeanum* seed oil (ZBSO). The primary components of ZBSO include eicosoic acid, linolenic acid, linoleic acid, oleic acid, palmitic acid, arachidonic acid, stearic acid, eicosenoic acid, and docosahexenoic acid (130). In LPS-triggered lung epithelial cells, ZBSO effectively inhibits the production of pro-inflammatory cytokines and chemokines, including IL-6, IL-10, TNFα, PGE2, MMP2, MMP9, MCP1, and COX2. This inhibition is achieved by blocking the TLR4/MyD88/NF-κB signaling pathway. Additionally, ZBSO inhibits the nuclear translocation of NF-κB/p65 (131).

Zanthoxylum bungeanum Maxim seed (ZBMS), rich in oleic acid, linoleic acid, and α-linolenic acid, exhibits potential for treating asthma and stress-related disorders (132). ZBMS protects mice from histamine/acetylcholine-induced asthma, reduces citric acid-induced cough in guinea pigs, and increases swimming endurance and survival time in mice, indicating a positive anti-stress effect. In an OVA-induced airway inflammation mouse model, ZBMS treatment improved lung peak inspiratory airflow in a dose-dependent manner (132).

Another group examined ZBSO in an OVA-induced asthmatic mouse model, demonstrating its efficacy in alleviating airway inflammation, attenuates lung tissue injury and airway remodeling, and inhibits leukocytes and eosinophils infiltration into the airway.

TABLE 2 Direct target of *Z. bungeanum* polyphenols.

Polyphenols	Target	Verified by	Publication
Rutin	HMGB1	SPR	(112)
Quercetin	HMGB1	SPR	(112)
	PI3K1R	SPR	(113)
Kaempferol	HMGB1	SPR	(114)
	TNF- α	SPR	(115)
	CASP3 PARP1	SPR	(116)
	JAK3	IP	(117)

*SPR, Surface plasmon resonance;
BLI, Bio-Layer Interferometry;
IP, Immunoprecipitation;
ChIP, Chromatin immunoprecipitation.

ZBSO also reduces IL-5 and IL-4 in the bronchial airway, attenuates the induction of ICAM-1 and TNFα mRNA and protein expression levels, and alleviates ERK, JNK phosphorylation, c-fos and c-JUN induction in the lung tissue (133). ZBSO exhibits effective anti-inflammatory properties in the wound healing process. In SD rat models with deep second-degree burns, topical ZBSO application resulted in decreased levels of TNFα, IL-6, and IL-1β in serum, elevated IκBα, and reduced p-IκBα and p-NF-κB p65 expression (134). In copper comb-induced rat burn model, ZBSO can reduce the level of thiobarbituric acid reactant, IL-6, TNFα, increase GSH level and promote wound recovery (135). ZBSO also inhibits inflammation in bone-destroying diseases. In RAW264.7 cells stimulated with NF-κB ligand (RANKL), ZBSO decreases NF-κB, TNFα, NFATc1, and TRAP, leading to the inhibition of osteoclastogenesis. Among the fatty acids in ZBSO, alpha-linolenic acid (ALA) exhibits the strongest effect. In ovariectomized

TABLE 3 Completed clinical trials utilizing *Z. bungeanum* polyphenols.

Compound	ID	Study phase	Dose & Administraton	Condition	Publication
Quercetin and Rutin	NCT01847521	2	Quercetin 70 mg/10kg/d and Rutin 30 mg/10kg/d po	Autism Spectrum Disorders	/
Rutin	NCT03437902	2 & 3	180mg po	Type 2 Diabetes Mellitus	/
Quercetin	NCT00913081	4	500-2000 mg po one time	Flushing	/
	NCT01708278	1	500-2000 mg/d po	Chronic Obstructive Pulmonary Disease	(118)
	NCT02463357	4	1000mg/d po	Mountain Sickness	/
	NCT01722669	1	500mg po	Healthy	(119)
Epicatechin	NCT01856868	1 & 2	100mg/day, po	Becker Muscular Dystrophy	/
	NCT03236662	2	100mg/day, po	Becker Muscular Dystrophy	/
	NCT01691404	/	100mg/d po	Hypertension, Endothelial Dysfunction	(120–122)
Naringenin	NCT01091077	1	1000mg po	HCV Infection	/
	NCT04697355	/	900mg/d po	Energy Expenditure Safety Issues Glucose Metabolism	/
Kaempferol	NCT06060691	1	Topical	Female Sexual Dysfunction	/
Arbutin	NCT03868748	1	150-400mg/d po	Healthy Volunteers	/
Chlorogenic acid	NCT02245204	1	Injection*	Advanced Cancer	/
	NCT02136342	1	Injection*	Advanced Cancer	/
	NCT02728349	1	Injection*	Glioblastoma	/
	NCT02728349	1	Injection*	Glioblastoma	/
	NCT03758014	2 & 3	Injection* 3 mg/kg/d	Glioblastoma	/
	NCT02621060	2	1200 mg/d po	Impaired Glucose Tolerance	/
	NCT02929901	2 & 3	200mg/d po	Type 2 Diabetes Nonalcoholic Fatty Liver	(123)

osteoporotic rats, preventive and therapeutic interventions with ALA resulted in decreased levels of IL-1β, IL-6, TAK1, TRAP, NFATc1, and TNFα (136).

6.3 *Z. bungeanum* essential oil

Z. bungeanum essential oil (ZBEO) is the primary source of the distinctive flavor of Sichuan pepper, with terpenoids being a major component of ZBEO (2). ZBEO has demonstrated anti-inflammatory effects in various skin disease models. In a guinea pig model of psoriasis, ZBEO treatment significantly improved Baker scores and reduced inflammatory cell infiltration (137). In a mouse model of ultraviolet-induced skin photoaging, topical application of ZBEO improved photoaging damage, reduced skin thickening, and attenuated inflammatory cell infiltration. ZBEO also inhibits the levels of MMP9, MMP1, and MMP3 in skin tissue, enhance the activity of CAT, SOD, and GSH-Px/GPX, and reduced the production of the lipid peroxidation byproduct MDA. Furthermore, ZBEO effectively suppress the expression of TNFα, IL-6, IL-1β, and IL-1α (138). In a HaCaT cell inflammatory model induced by Propionibacterium acnes (P. acnes), pretreatment with ZBEO reduced the levels of TNFα, IL-1β, IL-8, and IL-6, as well as the mRNA levels of TLR2, IL-8, IL-6, and NF-κB (139).

ZBEO also shows therapeutic effects in gastrointestinal disorders due to its anti-inflammatory properties. ZBEO has demonstrated protective effects against DSS-induced colitis in mice. ZBEO doses of 20-80 mg/kg reduced myeloperoxidase activity, colonic pathological damage, colon length shortening, disease activity index, and DSS-induced weight loss (140, 141). Administration of ZBEO significantly reduced IL-1β, IL-12 (140), TNFα, VCAM-1, TLR8, and IL-11 (141) mRNA levels. ZBEO is reported to inhibit inflammation in colitis in mice by regulating the PPARγ and NF-κB pathways, and suppressing NLRP3 activation (140). Next-generation sequencing (NGS) verifies that ZBEO increases VCAM-1 and CYP, and suppresses CXCL and S100A8 to attenuate UC symptoms (141). *In vitro* studies also demonstrate that ZBEO can reverse the imbalanced expression of IL-1β, IL-6, IL-10, and TNFα in LPS-induced NCM460 colon epithelial cells (141).

ZBEO inhibited enteritis and intestinal dysfunction caused by E. coli infection in mice. Histopathological observations indicated that ZBEO significantly improved the impairment of intestinal tissue structure, which could be associated with its inhibitory effect on the gene expression of inflammatory cytokines such as IL-8, TNFα, TLR4, and TLR2 (142). Atomized inhalation of ZBEO protects mouse from inflammation related colorectal cancer by reducing inflammation and cancer transformation. Furthermore, a decrease in AChE activity, an increase in ChAT activity, an increase in α7nAChR expression, and a decrease in IL-6 mRNA levels are observed in ZBEO treated group (143).

6.4 Other extractions

Z. bungeanum-cake-separated moxibustion (ZBCS-moxi) is a traditional Chinese therapy that has been employed for centuries to treat rheumatoid arthritis. A recent study assessed the anti-inflammatory effects of ZBCS-moxi in a rat model of rheumatoid arthritis. The study found that rats treated with ZBCS-moxi for three weeks exhibited a significant reduction in paw volume, pannus formation, synovial hyperplasia of synovial membranes, and levels of TNFα and IL-1β in serum (144).

These findings suggest that Zanthoxylin and ZBCS-moxi may have therapeutic potential for the treatment of inflammation and pain. However, more research is needed to confirm these findings through clinical trials.

7 Discussion

Zanthoxylum bungeanum Maxim., or Chinese prickly ash, holds a rich history spanning over two millennia in traditional Chinese medicine (5). This herb has been extensively used orally and topically to address various ailments, including gastrointestinal discomfort, arthritis, and bruises (5, 7). Its significance extends beyond China, finding a place in traditional medical practices in countries such as India and Nepal (7). Additionally, the unique flavor and numbing taste of the dried fruit follicles of *Z. bungeanum* have made it a significant ingredient in Chinese cuisine (1). Over time, research on and applications of *Z. bungeanum* have expanded significantly. *Z. bungeanum* exhibits diverse pharmacological activities such as anti-inflammatory, analgesic, antibacterial, and anti-tumor properties, showcasing therapeutic effects on multiple organ systems, including the gastrointestinal tract, cardiovascular system, and nervous system (2). The plant contains over 140 compounds, including polyphenols, alkaloids, lignans, coumarin, fatty acids, and essential oils (2, 11, 12). Beyond its polyphenolic content, constituents like hydroxy-alpha-sanshool, a mixed extract of fatty acids, and essential oil extraction from *Z. bungeanum*, have proven anti-inflammatory efficacious in various systems, such as the nervous system (127) and digestive system (140, 141). As research progresses, the application of *Z. bungeanum* in both medical and daily contexts continues to broaden, promising potential benefits to human health.

Polyphenols from *Z. bungeanum* emerge as a promising class of natural compounds with potential health benefits, particularly in preventing and treating inflammatory diseases. Numerous studies have highlighted their anti-inflammatory and antioxidant properties through a variety of mechanisms, including:

- Inhibiting pro-inflammatory cytokine production, such as IL-1β, TNFα, and IL-6.
- Suppressing the NF-κB and MAPK signaling pathway, central to inflammation.
- Activating the Nrf2/HO-1 signaling pathway, protecting cells from oxidative damage.
- Modulating the immune response, promoting regulatory T cells and suppressing inflammatory T cells.

While much of the current research on polyphenols of *Z. bungeanum* has been conducted *in vitro* or in animal models, promising preclinical data suggest therapeutic potential for a range of inflammatory diseases in humans, including ulcerative

colitis, arthritis, asthma, chronic obstructive pulmonary disease, cardiovascular disease, and neurodegenerative diseases. In addition to their anti-inflammatory effects, polyphenols of *Z. bungeanum* have demonstrated other beneficial properties, such as anti-cancer, anti-diabetic, anti-bacterial, and neuroprotective effects.

Several clinical trials have tested *Z. bungeanum* polyphenols in non- inflammatory diseases, indicating the safety of these compound. Future research should prioritize human clinical trials to validate the clinical efficacy of polyphenols of *Z. bungeanum* on inflammatory diseases. Additionally, researchers should investigate:

- Optimal dosages and long-term safety of polyphenols of *Z. bungeanum*.
- Synergistic or antagonistic interactions of polyphenols of *Z. bungeanum* with other bioactive substances.
- Effects of polyphenols of *Z. bungeanum* on specific biomarkers of inflammation and disease activity.
- Mechanisms by which polyphenols of *Z. bungeanum* exert their beneficial effects.

Ultimately, research outcomes may contribute to the development of novel therapeutic interventions and dietary recommendations that harness the power of polyphenols of *Z. bungeanum* to improve human health and well-being. For example, polyphenols of *Z. bungeanum* could be used to develop:

- New drugs or dietary supplements for the prevention and treatment of inflammatory diseases.
- Functional foods or fortified beverages that promote overall health and well-being.
- Personalized nutrition plans that take into account individual genetic and environmental risk factors.

Overall, the polyphenols of *Z. bungeanum* are a promising class of natural compounds with the potential to play a significant role in human health and well-being. Further research is needed to fully elucidate their mechanisms of action and develop safe and effective therapies for human use.

Author contributions

GH: Funding acquisition, Investigation, Writing – review & editing. JQ: Conceptualization, Data curation, Writing – original draft. ZP: Validation, Visualization, Writing – original draft. XW: Resources, Validation, Visualization, Writing – original draft. NZ: Visualization, Writing – review & editing. XJ: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The relationship between dietary inflammatory index and metabolic syndrome and its components: a case study in Kashi urban, Xinjiang

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Introduction: This paper examines the association between the dietary inflammatory index (DII) and the risk of metabolic syndrome (MS) and its components among Uygur adults in Kashi, Xinjiang.

Methods: The study used the multi-stage random cluster sampling method to investigate the adult residents of Uighu aged over 18 years old in one county and one township/street of three cities in Kashi between May and June 2021. All dietary data collected were analyzed for energy and nutrient intake with a nutritional analysis software, followed by a calculation of DII. Logistic regression was used to estimate the association between DII and the risks of MS and its components.

Results: The maximum DII value across our 1,193 respondents was 4.570 to 4.058, with an average value of 0.256. When we analyzed the DII as a continuous variable, we determined the anti-inflammatory diet has been identified as a mitigating factor for metabolic syndrome (OR=0.586, 95% CI=0.395–0.870), obesity (OR=0.594, 95% CI=0.395–0.870), elevated fasting glucose levels (OR=0.422, 95% CI=0.267–0.668), and hypertension (OR=0.698, 95% CI=0.488–0.996). When the model was adjusted by sex, age, and occupation, we found a significant correlation between high- and low-density lipoproteinemia and DII (OR=1.55, 95% CI=1.040–2.323). The present study identified four distinct dietary patterns among the population under investigation. There was a linear trend in the incidence of MS and hypertension across low, middle, and high levels of fruits and milk dietary pattern model (p=0.027; p=0.033), within this dietary pattern may serve as protective factors against MS and hypertension, suggesting that fruits and milk within this dietary pattern may serve as protective factors against MS and hypertension. And the linear trend in the incidence of elevated fasting glucose and obesity across the low, medium, and high scores of meet and eggs dietary pattern (p=0.006; p<0.001), suggest that a diet rich in meat may potentially contribute to an increased risk of developing elevated fasting glucose levels and obesity. An observed linear trend in the incidence rate of high fasting blood glucose across low, moderate, and high scores of dried fruits and nuts dietary pattern (p=0.014), indicating that increased consumption of nuts acted as a protective factor against elevated fasting blood glucose levels and contributed to their reduction.

Discussion: The dietary inflammation index was integrated with the findings from the study on the dietary patterns of the sampled population, revealing that an anti-inflammatory diet demonstrated a protective effect against metabolic

syndrome, obesity, high fasting blood glucose, and hypertension in this specific population. laying the foundation for further research.

KEYWORDS

dietary inflammatory index (DII), metabolic syndrome, Xinjiang (China), Kashi urban, nation of Uygur, diet quality

1 Introduction

Metabolic syndrome (MS) is a cluster of conditions stemming from central obesity, including hypertension, dyslipidemia, impaired glucose tolerance, diabetes, and other metabolic abnormalities. MS involves a group of risk factors that are particularly important for the development of cardiovascular disease. Its prevalence in China has risen together with economic growth and subsequent changes in lifestyle. In 1992, the prevalence rate of MS was estimated at 13.3% by a cohort study covering 27,739 adults in 11 provinces and cities (1). The results of the cross-sectional survey of Chinese adults conducted by the Asian International Cardiovascular Disease Cooperation Group in 2000–2001 showed that the prevalence rate of MS in China had risen to 16.5% (2), and by 2013, Wang et al. (3) estimated it at 33.9% among urban residents.

Many epidemiological and clinical studies show that chronic and low-grade systemic inflammatory reaction may be the core point of MS pathogenesis and the connecting link between mutual transformation and interaction of various components, and is part of the initiation factor of MS-insulin resistance or hyperinsulinemia (4). At present, an increasing amount of evidence indicates that different dietary patterns, foods, and nutrients have anti-inflammatory or pro-inflammatory effects, suggesting that optimizing dietary structure can help improve chronic low-grade inflammation.

Therefore, some studies have used the population-based dietary inflammatory index (DII) to obtain the potential inflammatory factors in individual diets (5). The School of Public Health of the University of South Carolina summarizes all the literature and data relevant to the effect of common dietary ingredients/nutrients on serum inflammatory markers from 1950 to 2010, and calculates the inflammatory effect index of each dietary ingredient/nutrient (6, 7). The paper uses official data and relevant literature referring to 11 countries, to calculate (i) the average daily intake and standard deviation of the global average daily intake of common dietary ingredients/nutrients of the population, and (ii) their DII according to the daily dietary intake of the respondents, and provide an effective tool for accurate and quantitative evaluation of the level of dietary anti-inflammation/pro-inflammatory (6, 7). At present, many scholars use DII to evaluate cardiovascular disease, metabolic disease, cancer, and COPD (8–11), and think that the DII score can provide accurate insight into the potential of dietary inflammation and better explain the relationship between diet, inflammation, and cardiovascular metabolic disease.

Many population studies suggest that the dietary structure of the Uyghur population is questionable: with its high intake of calories, protein and fat and low intake of vitamins and trace elements, it is little wonder that the prevalence of MS has been estimated at 30–35% (12). Hitherto, no study has examined the impact of the overall anti-inflammatory/pro-inflammatory tendency on the MS of ethnic minorities in China. This study uses the DII developed by the School of Public Health of the University of South Carolina to investigate the overall dietary inflammation of Xinjiang Uygur residents and its contributing factors. The aim is to evaluate the dietary anti-inflammatory/pro-inflammatory tendency of MS and explore the relationship between DII and MS and its components in order to propose effective evaluation indicators for clinical prevention and treatment of MS, and provide a solid scientific reference for government departments to improve nutritional policy, dietary guidance, nutritional intervention, and research the pathogenic factors related to chronic diseases.

2 Object and method

2.1 Research object

The study was backed by empirical evidence and substantial financial support from the Xinjiang Uygur Autonomous Region Natural Science Foundation (the correlation between dietary patterns, TCF7L2 gene interaction, and diabetes mellitus in Xinjiang Uygur population, Project Number: 2016D01C242) and the Youth Research Voyage Project of the First Affiliated Hospital of Xinjiang Medical University (Study on the correlation between dietary inflammatory index and inflammatory factors of metabolic syndrome and glycolipid metabolism in Urumqi population, 2022YFY-QKQN-27). The subjects used the multi-stage stratified cluster random sampling method. Between May and June 2021, Kashgar City and Shule County were selected from the Kashgar region (one city and 11 counties) for this study, and then two townships (Haohan Township, Kashgar City, Tazihong Township, Shule County) and one street office (Chasa Street Office, Kashgar City) were randomly pickedfrom 28 townships/towns and streets, and three administrative villages and communities were selected from each township/town or street. A total of 1,193 Uighur adult residents≥18 years old in the survey site were investigated. We excluded pregnant women, nursing mothers, individuals on anti-stress medication, and others with purposefully differentiated dietary habits (such as fasting during Ramadan or attending weddings and services).

2.2 Data collection and methods

2.2.1 Data collection

(1) A questionnaire and dietary survey. The former collected basic information, such as sex, age, marital status, education level, occupation, and other demographic characteristics. The dietary questionnaire was mainly based on the *24-h dietary review*

questionnaire used in the 2002 survey of the dietary habits and health of Chinese residents (6), revised to consider the local characteristics of Xinjiang and the Uighur diet in Kashgar. We used a quasi-quantitative food frequency questionnaire (SQFFQ), modified to reflect the characteristics of the Uighur diet. The questionnaire took its final form after reliability and validity tests (13). During face-to-face interviews, the respondents were asked about the frequency and consumption of various foods in the past 12 months. Data sorting led to the creation of 12 food groups: grain, vegetables, fruits, beans and their products, milk and its products, meat, eggs, nuts and dried fruits, beverages, salt oil and other foods (excluding health food).

(2) Physical measurement and blood sample collection. Physical measurement was based on the 2002 survey standard of nutrition and health status of Chinese residents (6), and height (cm), weight (kg), waist circumference (WC, cm), hip circumference (HC, cm), blood pressure (SBP, mmHg), diastolic pressure (DBP, mmHg), and other indicators were collected; the body mass index (BMI = height (kg)/weight $(m)^2$) and waist to hip ratio (WHR = waist circumference (cm)/hip circumference (cm)) were calculated according to the corresponding formula. After collecting 5 mL of venous blood from the elbow of the subjects and centrifuging, the relevant biochemical indexes of the serum were determined, including fasting blood glucose (FPG, mmol/L), total cholesterol (TC, mmol/L), triglycerides (TG, mmol/L), high-density lipoprotein cholesterol (HDL-C, mmol/L), and low-density lipoprotein cholesterol (LDL-C, mmol/L).

2.2.2 Survey method

(1) A face-to-face questionnaire survey. The investigator explained the purpose of the survey to the respondents, obtained their consent in signed consent forms, then read through the questionnaire to respondents and filled in the responses. For the dietary survey, we used the continuous 3d-24 h retrospective inquiry method to collect the type and intake of all foods and cooking methods for each respondent in the previous three days to estimate the intake of edible salt and oils from the foods listed by each individual. In combination with the food weighing method, a random household survey was carried out in the families of some individuals in our sample to determine their families' consumption of edible salt and oil in the past month, which was distributed evenly to individuals according to the number of family population. Then we calculate the average daily food intake of each group.

(2) Physical examination in the township/town or street health centers where the survey took place. The investigators and medical staff who had received training in the physical examination standards conducted standardized measurements of the respondents' physical traits (as listed in 1.2.1 above). Height and weight were measured by a domestic height scale that was calibrated before use; respondents were asked to take off their hats, shoes, and clothes and assume a standing position of 30–40 degrees with their feet evenly distributed. Our team noted the average value after two consecutive measurements (the height measurement is accurate to 0.1 cm and the weight measurement to 1 kg) and calculated BMIs accordingly. The waist and hip circumference were measured with an inelastic soft leather ruler (with a minimum scale of 1 mm). Before taking measurements, we asked the respondents to wear thin underwear, and fully expose their abdomen and buttocks. Waist circumference was measured close to but not pressuring the skin through the middle point of the line between the lower edge of the 12th rib of the anterior superior iliac spine and the iliac crest in the midaxillary line. Hip circumference was measured at the maximum circumference of the hip (i.e., the most convex part of the pubic symphysis and the gluteus maximus). Our team noted the average value after two consecutive measurements (readings are accurate to 0.1 cm). We measured blood pressure with a domestic desktop sleeve mercury sphygmomanometer. After the subjects sat comfortably in a quiet room for 5 min, they were asked to assume a sitting position to measure the blood pressure of the right brachial artery. The first and fifth tones of Koch's were SBP and DBP, respectively. Their blood pressure was measured thrice (accurate to 1 mmHg; 1 mmHg = 0.133 kPa) with one-minute intervals between measurements. If the difference between any two measurements was more than 10 mmHg, we took a fourth measurement and used the average value of all four measurements.

(3) We collected 5 mL blood samples of elbow vein blood from all respondents, whom we asked to fast for more than 8 h, and centrifuged them at 4,500 r/min within 2 h after collection. All separated serum samples were measured in a Hitachi 7,600 Automatic Analyzer at the Laboratory of Kashgar People's Hospital for relevant biochemical indicators (FPG, TC, TG, HDL-C, LDL-C5). The equipment was operated by the same group of professional inspectors above the technician in charge, and the kit was provided by the Northern Institute of Biology. FPG was determined with the use of the GOP-POD method, serum TC and TG by terminal colorimetry, and serum HDL-C and LDL-C by selective melting.

2.2.3 Relevant definitions of MS

MS Research Collaboration Group of Diabetes Society of Chinese Medical Association, Recommendations on MS of Diabetes Society of Chinese Medical Association in 2004 (7), The individuals who satisfy all three criteria and possess all components are deemed to have metabolic syndrome: (1) overweight and/or obesity: BMI $\geq$ 25 kg/m^2; (2) Abnormal blood lipids: TG $\geq$ 1.7 mmol/L and/or HDL-C < 0.9 mmol/L (male) or < 1.0 mmol/L (female); (3) Hypertension: systolic/diastolic blood pressure $\geq$ 140/90 mmHg and/or confirmed as hypertension and treated; (4) Hyperglycemia: FPG $\geq$ 6.1 mmol/L and (or) 2 h postprandial blood glucose (2hPG)>7.8 mmol/L and (or) those who have been confirmed as type 2 diabetes and have been receiving treatment.

2.2.4 Calculation of the dietary anti-inflammatory index

The following DII formula developed by the University of South Carolina was used to calculate the DII of the respondents (6): DII of a certain dietary component/nutrient = (daily intake of this dietary component or nutrient—average daily intake of this dietary component

or nutrient *per capita* in the world)/standard deviation of daily intake of this dietary component or nutrient *per capita* in the world × Inflammatory effect index of this dietary component or nutrient. Then, we summarize the DII of various dietary components/nutrients in the diet, which is the total score of DII. The higher the positive value of DII, the stronger the tendency to promote inflammation; The higher the negative value of DII, the stronger the anti-inflammatory tendency.

The final dietary inflammation index (DII) comprised 45 dietary components or nutrients. Among these, 9 components (energy, carbohydrates, protein, fat, cholesterol, iron, vitamin B12, saturated fatty acids and trans fatty acids) were found to have pro-inflammatory properties while the remaining 36 components exhibited anti-inflammatory properties. The intake of these 45 dietary components was determined through dietary surveys. Subsequently, individual component intakes were integrated to assess the overall inflammatory potential of the diet. Due to limitations in our current version of nutrition calculator software used for this study, we calculated the daily intake of more than 20 common dietary components/nutrients in the respondents' diet and three-day data with the use of dietary nutrition analysis software, and measured for energy, protein, fat, carbohydrate, dietary fiber, iron, zinc, selenium, magnesium, VitA, VitC, VitE, VitB1, VitB2, niacin, cholesterol, folic acid, saturated fatty acid, monounsaturated fatty acid, and polyunsaturated fatty acid. We used the average daily intake of dietary ingredients/nutrients during the three-day period to calculate the DII value of the respondents using the DII formula. Then, according to the total quartile of DII, P25, P50 and P75 were divided into four groups. Q1 was the anti-inflammatory tendency group (DII < −2.029), Q2 (DII 2.029 ~ 0.374), Q3 (DII 0.374 ~ 2.514), and Q4 were the pro-inflammatory tendency group (DII>2.514).

2.3 Quality control

(1) Given the unique dietary habits of the Uygur residents in Kashgar, a preliminary survey and demonstration were conducted to tailor the content of our dietary survey to the actual diet of the respondents. Considering that the daily dietary structure of the individuals in our sample varies during the week, we adjusted the period to reflect both workdays and weekends. We collected the respondents' daily tableware and food as food models, using different food models to help them judge their food types and intake.

(2) We ensured that all personnel who conducted the physical examinations were relatively fixed, and all instruments and equipment were used only after appropriate calibration. The collection of blood samples was conducted by personnel assigned in strict accordance with quality control standards. All relevant operations were carried out by personnel assigned in strict accordance with the instructions and requirements of the experiment. All blood samples were subject to quality control in batches.

Data input was delegated to two persons, and the logical error correction function was set in the database to prompt in real time and correct in time. The obvious illogical outliers were eliminated, missing values within the allowable range were statistically processed, and possible confounding factors, such as age and sex, were controlled through hierarchical analysis.

2.4 Data processing method

Epidata3.0 software was used to input the original data, and each subject's daily intake of each nutrient was calculated using the nutrition calculator V2.65 standard version (prepared by the China Center for Disease Control and Prevention). We used MS Excel for data collation and the SPSS17.0 statistical software for data analysis. Measurement data consistent with the normal distribution were represented by the mean; the data of non-normal distribution were represented by the median (interquartile interval), and its natural logarithm was taken for statistical analysis. With the quartile level of the DII as the independent variable and MS and its components as the dependent variable, we analyzed the relationship between DII and MS and its components by using a two-class logistic regression model; we adjusted and controlled the confounding factors such as age, sex, occupation, and others, as covariates in the model fitting. The difference was statistically significant with $p < 0.05$.

3 Results

3.1 Sample parameters

A total of 1,193 people were surveyed, aged between 18 and 91, with an average 45.18 ± 15.010 years. The sample included 485 men (40.7%) with an average age 47.66 ± 15.737 years, and 708 women (59.3%) with an average age 43.48 ± 14.254 years. A total of 1,113 people (94.3%) were married or remarried; 56 were unmarried (4.7%), and 24 divorced or widowed (2%). 671 (56.2%) were farmers; 335 declared themselves unemployed (35.3%), 161 (13.5%) in employment and 26 (2.2%) retired. 1,027 (86.1%) had education below the high-school level, and 166 people (13.9%) had high-school level education or above. 448 respondents (37.6%) were city dwellers and 745 (62.4%) lived in rural areas (full presentation in Table 1). 429 adults with metabolic syndrome in Kashgar, Xinjiang, were investigated, with a

TABLE 1 Sample characteristics (*n*, % or $\bar{x} \pm s$).

Category	MS (*n* = 429)	Non-MS (*n* = 764)	*p*
Gender			
Male	114 (26.6)	289 (37.8)	<0.001**
Female	315 (73.4)	475 (62.2)	
Place of residence			
Urban	206 (48.0)	319 (41.8)	0.061
Rural areas	223 (52.0)	445 (58.2)	
Degree of education			
Below high school	390 (90.9)	665 (87.0)	0.056
High school and above	39 (9.1)	99 (13.0)	
Occupation			
Farmer	203 (47.3)	400 (52.4)	<0.001**
Other working or unemployed	126 (52.7)	364 (47.6)	
Age (average)	47.84 ± 10.94	43.39 ± 13.88	–

Chi-square test was used for counting data and t-test for measuring data. **$p < 0.001$, with significantly different results.

prevalence rate of 35.96%; 158 cases of high-altitude abdominal glucose with a prevalence rate of 13.24%; 195 cases of hypertension (prevalence rate: 16.35%); 766 cases of obesity (prevalence rate: 64.42%); 527 cases of hypertriglyceridemia (prevalence rate: 44.17%); 362 people with high-density lipoprotein disease (prevalence rate: 30.34%) Full data are illustrated in Table 2.

3.2 DII, MS, and its components

Our physical and laboratory examinations show that the DII in our sample varies from Q1-Q4 (anti-inflammatory grading to pro-inflammatory grading) to obesity ($\chi^2 = 9.825$, $p = 0.020$), high fasting blood glucose or not ($\chi^2 = 15.390$, $p = 0.002$), with or without metabolic syndrome ($\chi^2 = 15.626$, $p = 0.001$) was statistically significant. We found no statistically significant difference between the DII components with or without hypertriglyceridemia, with or without low high-density lipoprotein, and with or without hypertension (see Table 3).

TABLE 2 Physical examination and DII of the respondents (n, % or $\bar{x} \pm s$).

Category	MS (n = 429)	Non-MS (n = 764)	p
BMI(kg/m^2)	30.57 ± 4.30	26.02 ± 4.89	<0.001**
WHR	0.95 ± 0.07	0.89 ± 0.08	<0.001**
TG (mmol/L)	2.48 ± 1.25	1.44 ± 0.88	<0.001**
HDL (mmol/L)	1.07 ± 0.22	1.14 ± 0.24	<0.001**
SBP (mmHg)	139.97 ± 20.51	113.56 ± 18.92	<0.001**
DBP (mmHg)	90.31 ± 13.60	74.11 ± 12.51	<0.001**
FPG (mmol/L)	7.40 ± 3.09	5.44 ± 1.72	<0.001**
DII	0.403 ± 3.49	(−0.386) ± 3.972	0.003**

BMI is the body mass index; WHR is the Waist-to-Hip Ratio; TG is the triglyceride; HDL is high density lipoprotein; SBP and DBP are the systolic and diastolic blood pressure; FPG is the fasting blood glucose; DII indicates the dietary inflammatory index. **$p < 0.001$; results are significantly different.

3.3 OR value of DII and risk of MS and its components

Our research shows that the maximum DII value across our 1,193 respondents was 4.570 to 4.058, with an average value of 0.256. When we analyzed the DII as a continuous variable, we revealed the anti-inflammatory diet has been identified as a mitigating factor for metabolic syndrome (OR = 0.586, 95% CI = 0.395–0.870), obesity (OR = 0.594, 95% CI = 0.395–0.870), elevated fasting glucose levels (OR = 0.422, 95% CI = 0.267–0.668), and hypertension (OR = 0.698, 95% CI = 0.488–0.996). When the model was adjusted by sex, age, and occupation, we found a significant correlation between high-and low-density lipoproteinemia and DII (OR = 1.55, 95% CI = 1.040–2.323; as in Table 4).

3.4 Dietary patterns, MS and its components

A total of four factors with eigenvalues greater than 1 were extracted through factor analysis, resulting in a cumulative contribution rate of 65.93%. This suggests that the extracted common factors effectively capture the variability within each food group. The four derived models from this analysis include: a grain and vegetable-based dietary model, a fruit and milk-based dietary model, a meat and egg-based dietary model, a dried fruit and nut-based dietary model. The results of the multiple logistic regression analysis indicated no significant association between the grain-vegetable dietary pattern and MS or its components. The consumption of fruit and milk was inversely associated with the prevalence of multiple

TABLE 3 DII grades, MS, and its components in our sample of Uygur adults (n, %).

MS and its components		DII classification				Total	χ^2	p
		Q1 (<-2.029)	Q2 (−2.029–0.374)	Q3 (0.374–2.514)	Q4 (>2.514)			
Obesity	Y	125 (29.3)	110 (25.8)	101 (23.7)	91 (21.3)	427	9.825	0.020*
	N	171 (22.3)	189 (24.7)	197 (25.7)	209 (27.3)	766		
Hypertriglyceridemia	Y	178 (26.7)	164 (24.6)	159 (23.9)	165 (24.8)	666	4.569	0.206
	N	114 (21.6)	136 (25.8)	143 (27.1)	134 (25.4)	527		
Hypohigh-density lipoprotein	Y	198 (23.8)	213 (25.6)	209 (25.2)	211 (25.4)	831	3.381	0.337
	N	104 (28.7)	85 (23.5)	89 (24.6)	84 (23.2)	362		
High fasting blood glucose	Y	283 (27.3)	260 (25.1)	243 (23.5)	249 (24.1)	1,035	15.390	0.002*
	N	22 (13.9)	39 (24.7)	51 (32.3)	46 (29.1)	158		
hypertension	Y	261 (26.2)	254 (25.5)	244 (24.4)	239 (23.9)	998	3.115	0.374
	N	42 (21.5)	46 (23.6)	52 (26.7)	55 (28.2)	195		
MS	Y	204 (26.7)	195 (25.5)	180 (23.6)	185 (24.2)	764	15.626	0.001**
	N	78 (18.2)	100 (23.3)	131 (30.5)	120 (28.0)	429		

*$p < 0.05$, **$p < 0.001$, and results are significantly different. In this chart, "Y" represents the presence of metabolic syndrome (MS), while "N" indicates its absence. Revised sentence: Please refer to Section 2.2.3 for the diagnostic criteria of MS.

TABLE 4 DII and OR values of MS and its components.

Group	Case (*n*)	OR[a]	95%CI	OR[b]	95%CI
	MS				
Q1	78	1		1	
Q2	100	**0.586**	**0.395–0.870**	0.966	0.617–1.512
Q3	131	0.787	0.541–1.144	1.018	0.688–1.507
Q4	120	1.118	0.783–1.596	1.379	0.954–1.994
	Obesity				
Q1	171	1		1	
Q2	189	**0.594**	**0.432–0.817**	1.121	0.776–1.619
Q3	197	0.749	0.543–1.033	1.049	0.746–1.474
Q4	209	0.842	0.610–1.163	1.057	0.756–1.477
	Hypertriglyceridemia				
Q1	114	1		1	
Q2	136	0.784	0.568–1.083	1.184	0.819–1.710
Q3	143	1.021	0.745–1.401	1.269	0.911–1.768
Q4	134	1.117	0.816–1.530	1.319	0.954–1.824
	Hypohigh-density lipoprotein				
Q1	104	1		1	
Q2	85	1.316	0.924–1.873	**1.554**	**1.040–2.323**
Q3	89	0.996	0.692–1.434	1.073	0.736–1.566
Q4	84	1.066	0.743–1.530	1.067	0.737–1.543
	High fasting blood glucose				
Q1	22	1		1	
Q2	39	**0.422**	**0.267–0.668**	0.695	0.415–1.164
Q3	51	0.83	0.559–1.233	1.082	0.712–1.644
Q4	46	1.154	0.792–1.681	1.47	0.992–2.178
	Hypertension				
Q1	42	1		1	
Q2	46	**0.698**	**0.488–0.996**	1.103	0.722–1.684
Q3	52	0.785	0.553–1.114	0.991	0.678–1.449
Q4	55	0.937	0.665–1.319	1.216	0.844–1.752

[a]Indicates the establishment of a logistic regression model with DII index.
[b]Signifies a logistic regression model based on gender, age, occupation, and DII index.
The statistically significant results are indicated by the bold part with $p<0.05$. An odds ratio (OR) less than 1 and a 95% confidence interval (CI) ranging from 0 to 1 (excluding 1) suggest a potential protective factor for the corresponding disease, while an OR greater than 1 and a CI greater than 1 (excluding 1) indicate a possible risk factor for the corresponding disease.

sclerosis (MS) and hypertension, with individuals having a high intake showing 0.41 times and 0.33 times higher risk for MS and hypertension compared to those with low intake (95% CI 0.22–0.87, 95% CI 0.25–0.92). Furthermore, there was a linear trend in the incidence of MS and hypertension across low, middle, and high levels of this dietary pattern model ($p=0.027$; $p=0.033$), suggesting that fruits and milk within this dietary pattern may serve as protective factors against MS and hypertension. The meat-egg dietary pattern did not exhibit a significant association with multiple sclerosis (MS); however, it demonstrated a positive correlation with elevated fasting glucose levels and obesity. Individuals with high consumption had 1.35 times higher odds of having elevated fasting glucose (95% CI 0.89–2.84) and 3.26 times higher odds of being obese (95% CI 2.21–5.71), compared to those with low consumption levels. Furthermore, there was a linear trend in the incidence of elevated fasting glucose and obesity across the low, medium, and high scores of this dietary pattern ($p=0.006$; $p<0.001$). These findings suggest that a diet rich in meat may potentially contribute to an increased risk of developing elevated fasting glucose levels and obesity. The dried fruits and nuts dietary pattern demonstrated a significant inverse association with elevated fasting blood glucose levels. Specifically, individuals consuming a higher quantity of nuts had a 0.20 times lower prevalence of high fasting blood glucose compared to those with low nut intake (95%CI 0.12–0.75). Furthermore, there was an observed linear trend in the incidence rate of high fasting blood glucose across low, moderate, and high scores of this dietary pattern ($p=0.014$), indicating that increased consumption of nuts acted as a protective factor against elevated fasting blood glucose levels and contributed to their reduction. The specifics are presented in Table 5.

4 Discussion

The Uygurs generally like to eat red meat, preferably beef and mutton (smoked or stewed), which abound in Xinjiang, whereas their white meat and seafood intake is generally low. Our analysis of their dietary habits shows that nuts and dried fruits with high factor load values remain part of their weekly diet. Our study confirms higher values for the factor load of salt and oil food groups, which were included in the first matrix of the Uygur dietary pattern. Some studies have shown that fruits are rich in dietary fiber, minerals, antioxidant substances, and vitamins. In addition, a reasonable daily fruit intake can reduce the risk of diabetes and cardiovascular disease (14). However, in view of the sample size and other factors, the study found no difference in fruit intake between individuals with more than three items and their related components and individuals without MS and its related components abnormalities (15). Moreover, milk and dairy products are rich sources of calcium. Some studies have found that they may reduce blood pressure and obesity, while Khan et al. (16) found that the intake of milk and its products are negatively correlated with waist circumference, hypertension, and MS. Back to our study, after adjusting the calcium intake, we say the OR value dropped, but the difference remained statistically significant ($p<0.05$). The Fifth National Health and Nutrition Survey in South Korea showed that (17) the prevalence of MS in individuals with a high intake of milk or yoghurt was significantly lower than that with low intake ($p<0.001$).

Our study also determined a positive correlation between the dietary pattern of meat-and-eggs type and high fasting blood glucose and obesity. The high blood sugar of meat eaters is almost ubiquitous, which may be related to the fact that a high meat intake tends to reduce glucose tolerance. Cantero et al. (18) conducted a multifactorial analysis of the dietary risk factors of obesity among 871 Hispanic and 1,599 non-Hispanic white women in the United States, and found that the prevalence of obesity was increased due to the western dietary pattern, rich in saturated fatty acid and energy intake, dominated by red meat and refined grain intake. However, a different

TABLE 5 The relationship between various dietary patterns and metabolic syndrome and its components.

Dietary pattern	The level of intake	MS and its components					
		MS	High fasting blood glucose	Hypertension	Obesity	Hypertriglyceridemia	High-density lipoprotein anemia
Type of grain and vegetable	Q_1[a]	1.00	1.00	1.00	1.00	1.00	1.00
	Q_2	1.36 (0.72–2.98)	0.73 (0.46–1.76)	1.05 (0.62–2.49)	1.08 (0.64–2.32)	1.16 (0.61–2.51)	1.01 (0.52–2.16)
	Q_3	1.39 (0.78–3.05)	0.69 (0.53–1.25)	1.45 (0.67–2.55)	1.25 (0.71–2.63)	1.38 (0.85–2.82)	1.45 (0.91–2.98)
	P	0.306	0.237	0.408	0.649	0.239	0.216
Type of fruit and milk	Q_1	1.00	1.00	1.00	1.00	1.00	1.00
	Q_2	0.47 (0.25–0.91)	0.63 (0.37–1.59)	0.56 (0.31–0.98)	0.97 (0.58–1.67)	0.89 (0.47–1.65)	1.64 (0.75–2.08)
	Q_3	0.41 (0.25–0.91)	0.52 (0.25–0.91)	0.45 (0.25–0.91)	0.83 (0.25–0.91)	0.61 (0.25–0.91)	2.28 (0.25–0.91)
	P	0.027*	0.081	0.033*	0.432	0.187	0.279
Type of meet and eggs	Q_1	1.00	1.00	1.00	1.00	1.00	1.00
	Q_2	1.07 (0.61–2.33)	1.23 (1.85–2.49)	1.45 (0.92–3.42)	2.54 (1.54–4.25)	1.26 (0.89–2.95)	1.14 (0.73–2.15)
	Q_3	1.58 (0.98–3.02)	1.35 (1.89–2.84)	1.63 (0.98–3.54)	3.26 (2.21–5.71)	1.38 (0.93–3.12)	1.34 (0.95–2.73)
	P	0.256	0.006**	0.384	<0.001**	0.349	0.385
Type of dried fruits and nuts	Q_1	1.00	1.00	1.00	1.00	1.00	1.00
	Q_2	0.74 (0.41–1.58)	0.33 (0.19–0.81)	0.45 (0.31–1.58)	0.73 (0.49–1.67)	0.61 (0.32–1.53)	1.82 (0.91–3.25)
	Q_3	0.65 (0.29–1.36)	0.20 (0.12–0.75)	0.36 (0.18–1.22)	0.67 (0.42–1.55)	0.49 (0.36–1.35)	2.28 (0.98–3.58)
	P	0.093	0.014*	0.071	0.474	0.432	0.487

* indicates statistical significance at $p \leq 0.05$; ** indicates statistical significance at $p \leq 0.01$.
[a]Indicates the dietary patterns were categorized into low, medium, and high intake levels, with Q1 serving as the reference group.

diet pattern with a higher intake of vegetables, fruits, low-fat milk, and whole grains, and a lower intake of meat and beverages, can reduce obesity. Abe et al. (19) Among the male population, a multi-food and beverage dietary pattern containing medium-energy cheese and fat meat increases the risk of hyperglycemia and central obesity, while a fiber/bread dietary pattern rich in high-energy fiber reduces the risk. For the female population, a diet rich in white bread increases the risk of hyperinsulinemia, whereas a diet rich in milk and fat reduces the risk of hyperinsulinemia (20). Luo Tao et al. (21) found that high DII values correlate with an increased risk of cardiovascular metabolic diseases in Xinjiang's multi-ethnic population. The key to controlling the impact of DII on cardiovascular metabolic diseases is to help a population group maintain moderate levels of waist circumference, blood pressure, and LDL. This study provides a pilot reference for future inflammatory investigations on local Uygurs population incorporating the genomics and metageomics data.

The dietary inflammation index was integrated with the findings from the study on the dietary patterns of the sampled population, revealing that an anti-inflammatory diet demonstrated a protective effect against metabolic syndrome, obesity, high fasting blood glucose, and hypertension in this specific population. Analysis of dietary patterns suggested that fruits and dairy products may possess anti-inflammatory properties for individuals with metabolic syndrome and hypertension, while nuts were identified as potential anti-inflammatory foods for those with high fasting blood glucose. Conversely, meat was found to be a pro-inflammatory food source for individuals with high fasting blood glucose and obesity. In a comprehensive survey on dietary habits and metabolic syndrome in China, it was discovered that adherence to the traditional diet rich in fruits, vegetables, and aquatic products plays a crucial role in preventing and managing metabolic syndrome (22). The findings of a meta-analysis examining the association between dairy consumption and the incidence of metabolic syndrome suggest that an increased intake of dairy products may potentially mitigate the risk of developing metabolic syndrome (23). A certain finding suggested that of the 6 common types of milk consumed, semi-skimmed and soya milk products were protective against essential hypertension, whereas skim milk had the opposite effect (24). The study conducted in Urumqi, Xinjiang on middle-aged and elderly individuals revealed that the appropriate consumption of nuts exhibited a protective effect against fasting blood glucose abnormalities (25). Numerous studies have consistently demonstrated that excessive consumption of meat, particularly red meat (such as lamb, beef, and pork), is associated with an increased risk of obesity, hypertension, hyperlipidemia, and abnormal blood sugar levels (26–28).

Some limitations of this study must be acknowledged. First, to create our sample we relied on voluntary participants willing to undergo a physical examination and provide ample dietary data. Second, fieldwork took place during the spring and summer, but dietary intake may be affected by seasonal changes; moreover, our 24-h dietary regression method for three consecutive days cannot fully represent long-term dietary habits. Finally, the survey primarily targets the general population who willingly participate in community or town physical examinations, spanning from 18 to 90 years of age, with notable variations in demographic characteristics. Following age stratification, the sample size within each stratum is relatively small,

potentially impacting the representativeness of the samples. Subsequent research will delve into a comprehensive analysis of the multi-ethnic population and explore factors such as ethnicity, age, and other variables during stratification.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author or the primary author.

Ethics statement

The studies involving humans were approved by the First Affiliated Hospital of Xinjiang Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

YaZ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Software, Supervision, Writing – original draft. XL: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Software, Supervision, Validation, Writing – original draft. YS: Conceptualization, Data curation, Formal analysis, Investigation, Software, Writing – original draft. YJ: Conceptualization, Investigation, Methodology, Project administration, Writing – original draft. JuC: Formal analysis, Methodology, Supervision, Writing – original draft. XY: Conceptualization, Formal analysis, Methodology, Writing – review & editing. YuZ: Conceptualization, Formal analysis, Supervision, Validation, Writing – original draft. JiC: Funding acquisition, Methodology, Validation, Writing – review & editing. XZ: Methodology, Project administration, Writing – original draft. HX: Conceptualization, Data curation, Formal analysis, Funding acquisition, Software, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Turmeric: from spice to cure. A review of the anti-cancer, radioprotective and anti-inflammatory effects of turmeric sourced compounds

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Turmeric (*Curcuma longa*) has been extensively studied for its diverse pharmacological properties, including its potential role as an anticancer agent, antioxidant, and radioprotector. This review provides an overview of the chemical composition of turmeric, focusing on its main bioactive compounds, such as curcuminoids and volatile oils. Curcumin, the most abundant curcuminoid in turmeric, has been widely investigated for its various biological activities, including anti-inflammatory, antioxidant, and anticancer effects. Numerous *in vitro* and *in vivo* studies have demonstrated the ability of curcumin to modulate multiple signaling pathways involved in carcinogenesis, leading to inhibition of cancer cell proliferation, induction of apoptosis, and suppression of metastasis. Furthermore, curcumin has shown promising potential as a radioprotective agent by mitigating radiation-induced oxidative stress and DNA damage. Additionally, turmeric extracts containing curcuminoids have been reported to exhibit potent antioxidant activity, scavenging free radicals and protecting cells from oxidative damage. The multifaceted pharmacological properties of turmeric make it a promising candidate for the development of novel therapeutic strategies for cancer prevention and treatment, as well as for the management of oxidative stress-related disorders. However, further research is warranted to elucidate the underlying mechanisms of action and to evaluate the clinical efficacy and safety of turmeric and its bioactive constituents in cancer therapy and radioprotection. This review consolidates the most recent relevant data on turmeric's chemical composition and its therapeutic applications, providing a comprehensive overview of its potential in cancer prevention and treatment, as well as in radioprotection.

KEYWORDS

turmeric, chemical composition, antioxidant properties, antioxidant, radioprotectant

1 Introduction

Turmeric (*Curcuma longa*) (Figure 1), originating from India, is a curry spice that has garnered significant attention in recent decades due to its composition of bioactive curcuminoids—curcumin, demethoxycurcumin, and bisdemethoxycurcumin.

Curcumin (Figure 2), known chemically as 1,7-bis-(4-hydroxy-3-methoxyphenyl)-hepta-1,6-diene-3,5-dione, is a lipophilic polyphenol believed to exhibit anticancer, antibiotic, anti-inflammatory, and anti-aging properties, as indicated by various *in vitro*, *in vivo* studies, and clinical trials. Despite its potential, the therapeutic application of curcumin is hindered by challenges such as poor aqueous solubility, limited bioavailability, and unfavorable pharmacokinetic profiles. To overcome these issues, numerous formulations of curcumin have been developed (1, 2).

However, suboptimal sample preparation and analysis methodologies often impede accurate assessments of bioactivities and clinical efficacy. This review provides a summary of recent research on the biological, pharmaceutical, and analytical aspects of curcumin, covering various formulation techniques and discussing associated clinical trials and *in vivo* outcomes.

Turmeric, a member of the *Zingiberaceae* family, is a perennial plant that reaches a height of up to one meter, featuring oblong or cylindrical rhizomes. Externally, these rhizomes are brown and include an egg-shaped primary rhizome known as the "tuber" and multiple branched secondary rhizomes referred to as the "rhizome."

FIGURE 1
The turmeric plant.

Internally, the rhizomes exhibit colors ranging from yellow to yellow-orange, attributed to pigments called curcuminoids, with diverse pharmacological activities (3–5).

Chemically, curcuminoids, specifically diarylheptanoids, consist of two aryl groups connected by a chain with seven carbons. Among these, curcumin (diferuloylmethane) stands out as the most significant bioactive curcuminoid, alongside others like desmethoxycurcumin and bisdesmethoxycurcumin found in turmeric rhizomes (6).

Extensive research, encompassing preliminary, preclinical, and clinical studies, underscores the pharmacological significance of curcuminoids, the yellow pigment in turmeric. Its versatile properties include anti-inflammatory, immunomodulatory, antioxidant, hypolipidaemic, antimicrobial, anticarcinogenic, antitumor, radioprotective, neuroprotective, hepato-protective, nephroprotective, cardio-protective, and vasoprotective activities (7, 8). Curcumin's impact extends to various biochemical pathways, influencing molecular targets such as cytokines, transcription factors, kinases, growth factors, and microRNAs (9).

Turmeric, also known as Indian saffron, boasts a rich history of use as an herbal medicine, spice, and coloring agent. Records dating back to 600 BC in an Assyrian herbal book, references by the renowned Greek physician Dioscorides, and mentions in Islamic traditional medicine (ITM) contribute to its historical significance. Turmeric is integral to Chinese traditional medicine (TCM), Ayurveda, and various folk medicines worldwide, with traditional uses ranging from topical treatment for skin disorders to internal remedies for poor digestion and liver function. Recognizing the valuable insights from traditional medicine in guiding natural product-based drug discovery, researchers explore the medicinal applications of turmeric across different traditional systems and investigate the modern pharmacological activities of curcumin, bridging the knowledge from ancient practices to current clinical trials (10, 11).

2 Methods

Comprehensive literature searches were conducted across various databases, including Pubmed, Scifinder, ScienceDirect, Medline, Embase, Google Scholar, and Web of Science. The key terms employed for the search encompassed topics such as turmeric, *Curcuma longa*, curcuminoids, curcumin, bioavailability, bioactive compounds, pharmacokinetic, pharmacological effects. Additionally, a thorough examination of articles published in peer-reviewed journals was performed through a library search.

3 Biological activities

Turmeric displays a rich chemical diversity, with around 235 compounds identified so far, predominantly comprising phenolic compounds and terpenoids (Figure 3). The non-curcumin compounds exhibit diverse chemical structures, including 22 diarylheptanoids, diarylp entanoids, 8 phenylpropenes, various phenolic compounds, 68 monoterpenes, 109 sesquiterpenes, 5 diterpenes, 3 triterpenoids, 4 sterols, 2 alkaloids, and 14 other compounds (12, 13).

Turmeric is a plant with a diverse chemical profile (Figure 4 and Table 1), its extracts are obtained using ethanol, methanol, water, or ethyl acetate, and they are both water-soluble and water-insoluble. The

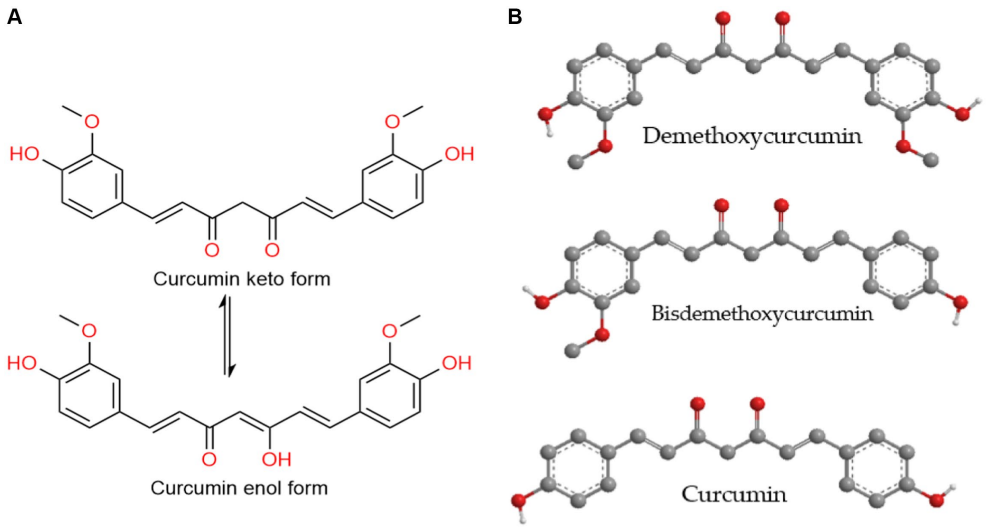

FIGURE 2
(A) The tautomerization of the curcumin molecule. **(B)** Curcuminoids – the main yellow pigments found in turmeric.

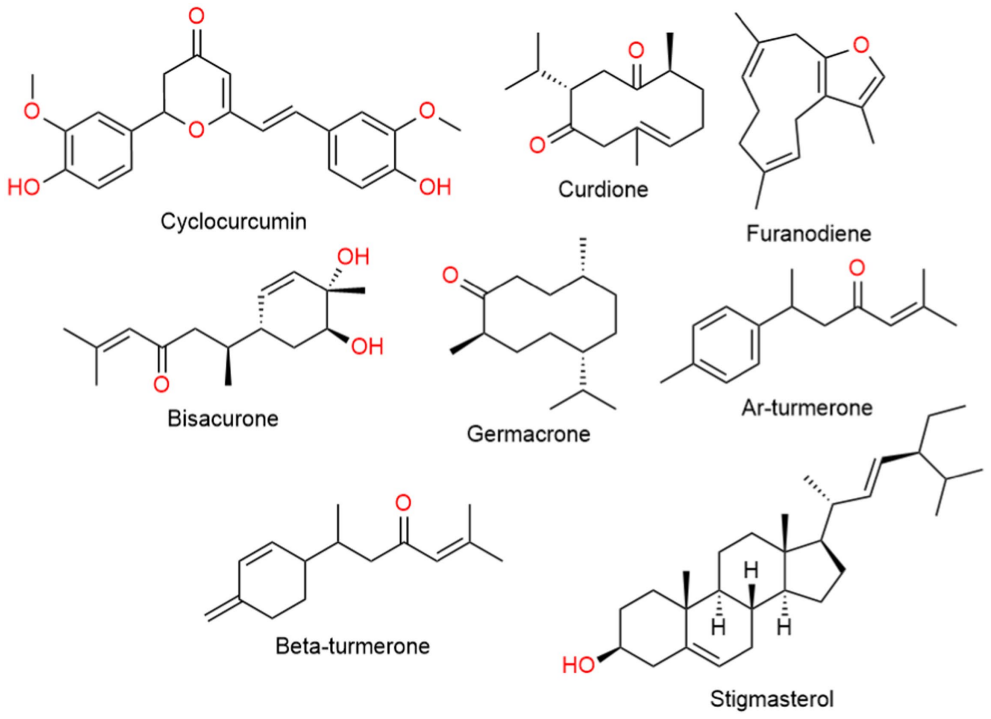

FIGURE 3
Chemical structure of the common components of turmeric, other than curcumin.

water-insoluble fraction comprises turmeric oil and polyphenols, mainly diarylheptanoids (curcuminoids), with curcumin constituting 80%, demethoxycurcumin 18%, and bisdemethoxycurcumin 2%. While 70% ethanol is the preferred solvent for extracting curcuminoids from turmeric, hydrodistillation followed by hexane extraction is the chosen method for separating essential oils. Curcumin, demethoxycurcumin, and bisdemethoxycurcumin collectively may make up over 30% of the ethanol extract of turmeric. Additionally, a distinctive component exclusive to *C. longa* is cyclocurcumin (15, 16).

3.1 Anti-cancer effects

Curcumin the primary constituents of turmeric has demonstrated efficacy across various stages of cancer progression, exerting inhibitory

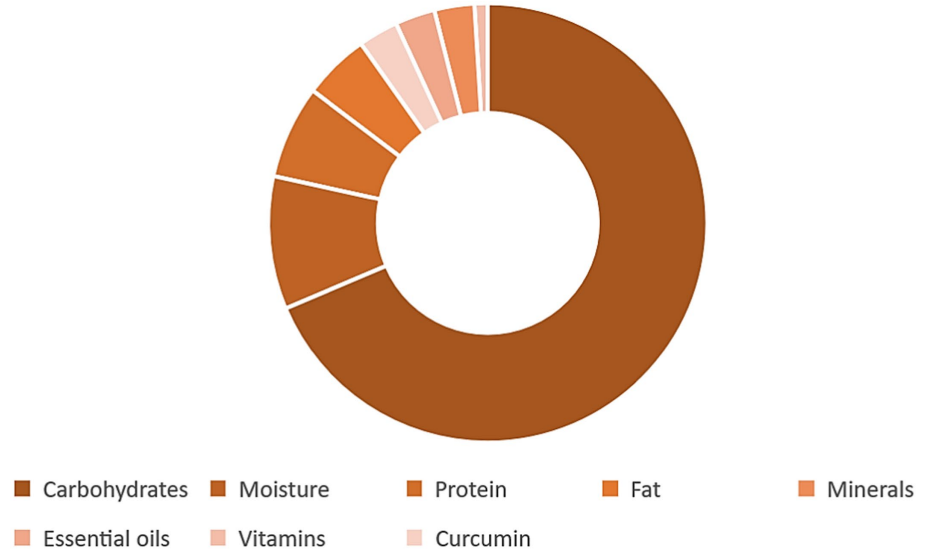

FIGURE 4
The chemical profile of the turmeric plant.

TABLE 1 The percentage by weight of the compounds found in the turmeric plant (14).

Constituent	Percentage by weight (%)
Curcuminoids	1–6
Volatile oils	3–7
Fiber	2–7
Mineral matter	3–7
Protein	6–8
Fat	5–10
Moisture	6–13
Carbohydrates	60–70

effects on the transformation, initiation, development, and invasion of tumors, as well as angiogenesis and metastasis. It has been identified as a suppressor of tumor cell growth through modulation of key cellular pathways, including the cell proliferation pathway involving cyclin D1 and c-myc, the cell survival pathway targeting Bcl-2, Bcl-xL, cFLIP, XIAP, and cIAP1, the caspase activation pathway encompassing caspase-8, caspase-3, and caspase-9, the tumor suppressor pathway involving p53 and p21, the death receptor pathway through DR4 and DR5, and various cell signaling pathways, including protein kinase pathways such as c-Jun N-terminal kinases (JNK), protein kinase B (PKB or Akt), and 5′ adenosine monophosphate-activated protein kinase (AMPK) (17).

Curcuminoids exhibit a diverse range of biological activities (Figure 5). In the context of MCF-7 human breast tumor cells, the impact of curcuminoids and cyclocurcumin was investigated. DMC displayed superior inhibitory effects compared to CUR and BDMC, attributed to the presence of phenolic hydroxyl groups, methoxyl groups, and the diketone moiety. Notably, cyclocurcumin did not influence MCF-7 cell proliferation, indicating that the diketone system within curcuminoids likely contributes to their antiproliferative effects (18).

Semsri et al. explored the influence of pure CUR on the expression of the Wilm's tumor 1 (WT1) gene in leukemic K562 cell lines. The study revealed that CUR's effects were mediated through PKCa signaling upstream of the WT1 transcription factor's auto-regulatory function. Pure CUR impacted WT1 protein-promoter binding, reduced WT1-mRNA levels, and decreased protein levels in K562 cells, contributing to its anti-proliferative effects. This suggests the potential therapeutic utility of CUR in the development of approaches for treating leukemia (19).

Jiang et al. identified the antitumor constituents in curcuminoids from *C. longa* (Figure 6) on He La cells, demonstrating a significant correlation between curcuminoids and antitumor activity. The inhibitory role of CUR in lipolysis was investigated in 3T3-L1 adipocytes, revealing its potential to attenuate TNF-α-mediated lipolysis. This antilipolytic effect could underlie CUR's ability to reduce plasma free fatty acid levels and improve insulin sensitivity (20).

CUR emerged as a potent tight binding inhibitor of human carbonyl reductase 1 (CBR1), inhibiting daunorubicinol formation. This inhibition could enhance the therapeutic effectiveness of daunorubicin by preventing heart tissue damage (21).

Yodkeeree et al. (22) conducted a study to compare the impact of CUR, DMC, and BDMC on the expression levels of urokinase plasminogen activator, metalloproteinases (MMPs), membrane type 1 (MT1-MMP), tissue inhibitor of MMPs, and the *in vitro* invasiveness of human fibrosarcoma cells. The order of potency in inhibiting cancer cell invasion was found to be BDMC > DMC > CUR. Zymography analysis revealed that, in a dose-dependent manner, CUR, DMC, and BDMC significantly reduced urokinase plasminogen activator and active MMPs from the cells. Notably, BDMC and DMC exhibited greater potency in this regard compared to CUR. All three forms of curcuminoids significantly inhibited collagenase and MMPs. DMC and BDMC demonstrated higher antimetastatic efficacy than CUR, attributable to their differential down-regulation of extracellular matrix (ECM) degradation enzymes (23).

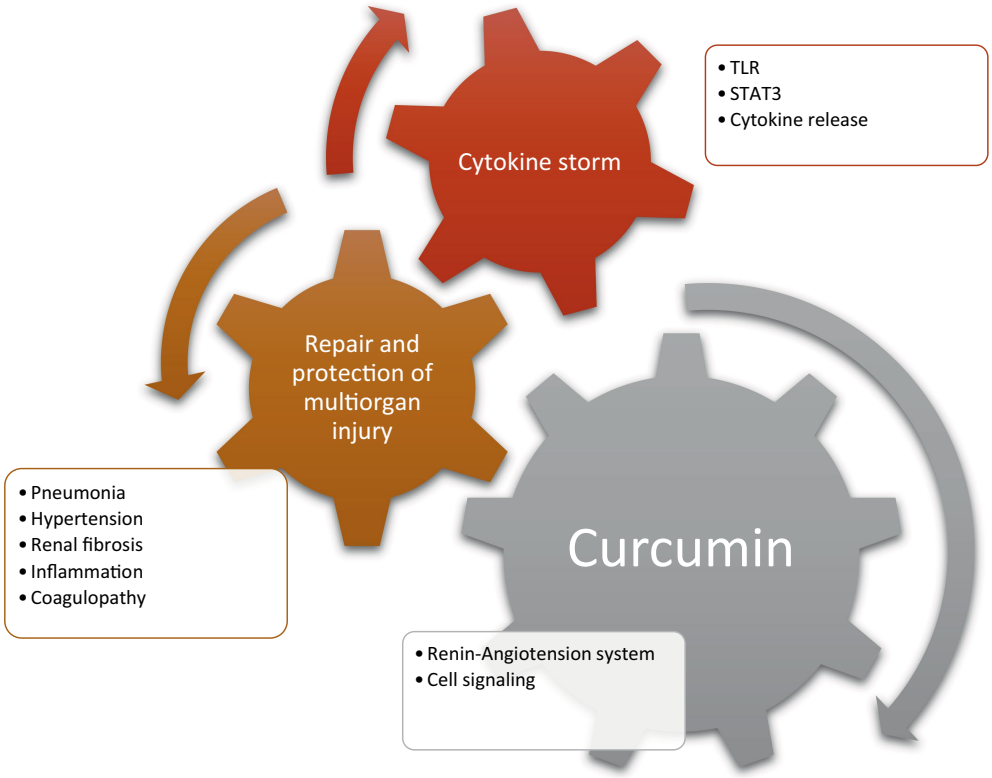

FIGURE 5
The biological activity of curcumin on different ailments.

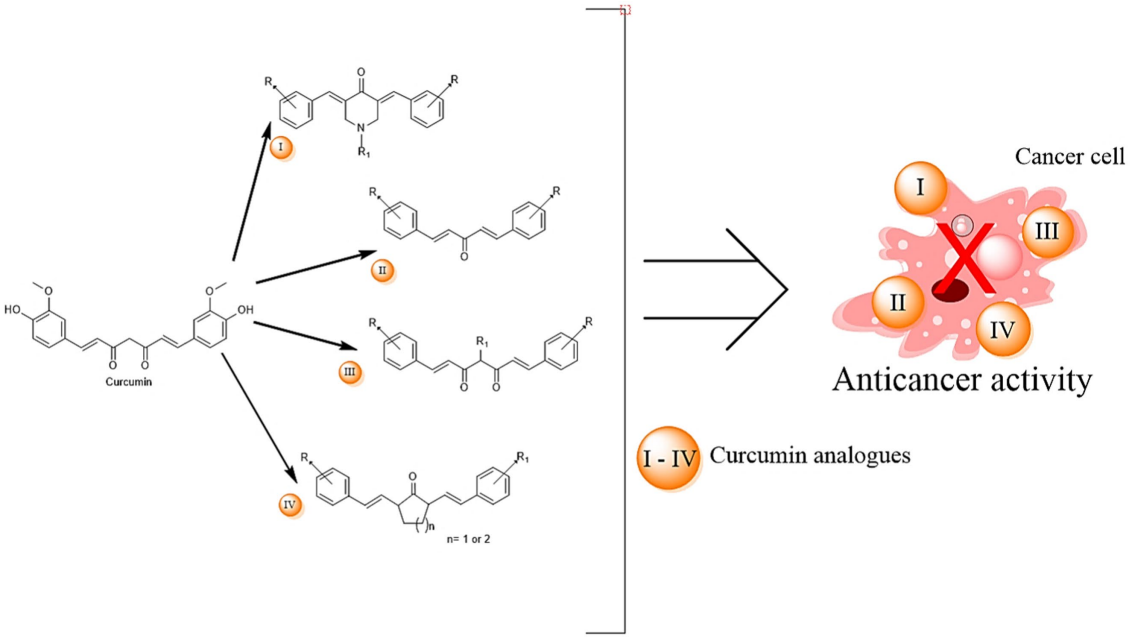

FIGURE 6
Curcumin analogues and their use in cancer treatment.

The administration of DMC resulted in the inhibition of nuclear factor-kappa B's DNA binding activity, a factor that orchestrates the expression of MMPs, urokinase plasminogen, intercellular adhesion molecule-1, and chemokine receptor 4. The anti-invasive effect of DMC appears to be primarily mediated through the modulation of the expression of proteins associated with invasion, potentially by targeting

nuclear factor-kappa B in MDA-MB-231 cells (24). Moreover, curcuminoids-mediated photodynamic therapy (PDT) exhibited a substantial suppression of cell viability in breast cancer cell lines, with DMC-PDT demonstrating the most pronounced anti-proliferative effect. The potential of DMC as a novel photosensitizer in PDT for cancer treatment was substantiated by its ability to reverse cell viability, reduce LC3 conversion, and inhibit PARP cleavage, all of which were attenuated by pre-treatment with a singlet oxygen scavenger or JNK inhibitor in the context of DMC-PDT. Notably, DMC-PDT displayed superior efficacy compared to DMC alone in curtailing cell viability in breast cancer cell lines, suggesting its promising role as a potential photosensitizer in cancer therapy (25, 26).

The metabolic profile of *Rhizoma paridis* saponins combined with turmeric intervention in H22 hepatocarcinoma mice showed promising anticancer effects by suppressing levels of amino acids, lipid compounds, and carbohydrates in tumor tissues (27).

In a research investigation focused on the monocarbonyl analogue of B63, derived through chemical modifications of curcumin's structure, this compound demonstrated a heightened antiproliferative impact compared to curcumin specifically on colon cancer cells. Furthermore, utilizing a lower dosage of B63 (50 mg/kg B63 versus 100 mg/kg curcumin) still resulted in the suppression of tumor growth, akin to the effects observed with curcumin (28).

3.2 Radioprotective effects

Curcuminoids, as potent antioxidant polyphenols, exhibit radiomodulatory properties by conferring radioprotection to non-cancerous cells while sensitizing tumor cells to radiation. In a study conducted by Lopez-Jornet et al. (29) the potential protective effects of lycopene and CUR on the parotid glands of female Sprague Dawley rats during radiotherapy were explored. Morphological and histopathological analyses revealed reduced cell necrosis in the CUR-treated group compared to other groups. Pre-administration of lycopene and CUR 24 h before irradiation contributed to mitigating structural damage to the salivary glands. Sebastià et al. (30) reported a dual action of polyphenols present in CUR, manifesting as both radioprotective and radiosensitive effects. The observed radiosensitization was attributed to compromised G2-checkpoint functionality, diminishing its capacity to effectively halt damaged cells in the G2-phase and resulting in a significant increase in radiation-induced chromatid breaks. The simultaneous dual-mode action of these polyphenols suggests that the overall net effect—whether radioprotective or radiosensitizing—depends on the cell-cycle status of the cells at the time of irradiation (31, 32).

Belcaro et al. (33) conducted a clinical investigation evaluating a specialized lecithin delivery system of CUR (Meriva) in 160 cancer patients undergoing chemotherapy and radiotherapy. The study findings led the authors to conclude that the formulated CUR has the potential to reduce the pain-related side effects associated with cancer therapy (33).

In another clinical trial involving 30 breast cancer patients, the protective effects of Curcumin C3 Complex® (6 g/day) against radiodermatitis were assessed. Parameters such as moist desquamation, pain level, redness, and severity of radiation dermatitis were measured. The curcumin group exhibited a significant reduction in moist desquamation and the severity of radiation dermatitis compared to the placebo group (35). Another study investigated the effectiveness of Vicco® turmeric cream (Vicco Laboratories, Parel, India), containing sandalwood and turmeric oil, in alleviating radiodermatitis induced by radiotherapy in 50 patients with head and neck cancer. The cream was applied daily (five times a day) from the first day and continued until 2 weeks after treatment completion. Acute skin reactions were monitored twice a week. Results indicated a notable decrease in dermatitis grades among patients using Vicco® turmeric cream at all evaluated time points (36, 37).

3.3 Anti-inflammatory effects

Inflammation represents a fundamental and noteworthy defensive mechanism employed by organisms in response to tissue damage. This reaction is elicited by various factors, including ischemic injury resulting from insufficient blood supply to a tissue or organ, physical trauma, exposure to toxins, infection, or other forms of trauma (38). It is imperative to effectively curtail the inflammatory response once its necessity diminishes to prevent undesirable tissue damages and cellular destruction, potentially leading to chronic inflammation (39).

The inflammatory process involves the participation of leukocytes or inflammatory cells, namely neutrophils, lymphocytes, and macrophages. Subsequent to the inflammatory cascade, leukocytes release specific elements such as eicosanoids, vasoactive peptides and amines, cytokines, and acute-phase proteins. These factors act in concert to mediate the inflammatory procedure, thereby averting further tissue damage and ultimately facilitating the healing and restoration of tissue function (40).

Curcumin, a component extensively utilized in Eastern medicine, has demonstrated therapeutic efficacy in treating various chronic diseases and inflammatory disorders, including airborne diseases. Attributed to its phenolic composition, curcumin exhibits antioxidant properties, preventing apoptosis by promoting the growth of inhibited cells. Turmeric, containing curcumin, enhances safety in food by preventing peroxide formation and surpasses vitamin E in effectively preventing lipid oxidation. Components extracted from *Curcuma longa* display significant antioxidant effects, playing a crucial role in preventing lipid oxidation (41, 42).

Traditionally, turmeric has been topically applied for skin diseases, insect bites, and chickenpox in India, serving as an alternative medical support for wound healing (Figure 7). Curcumin treatment accelerates wound contraction, increases fibronectin and collagen expression in myofibroblasts, and enhances granulation tissue formation and neovascularization in mouse-wound models with diabetes and hydrocortisone (43). Curcumin reduces injuries induced by hydrogen peroxide in yellow keratinocytes and fibroblasts, significantly decreasing wound healing time. In mouse models, curcumin exhibits antiulcer effects, reducing lipid peroxidation and protein oxidation, and promoting re-epithelialization to reverse gastric epithelial cell damage (44).

Numerous common skin disorders are associated with the dysregulation of the inflammatory response. Curcumin has demonstrated the ability to down-regulate various inflammatory targets, including lipoxygenase, cyclooxygenase-2, and inducible nitric oxide synthase. Additionally, it acts as an inhibitor of several inflammatory cytokines, such as TNF-α, interleukin-1, −2, −6, −8,

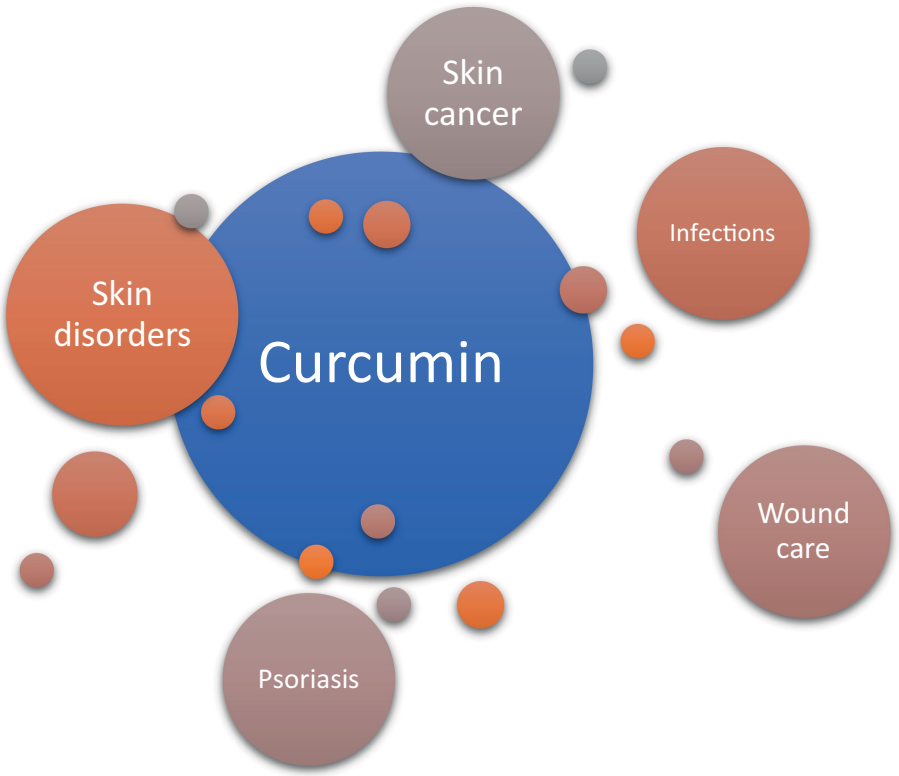

FIGURE 7
The effects of applying curcumin products to skin lesions and pathologies.

and−12 (45). The transcription factor nuclear factor kappa B (NF-κB), which governs cyclooxygenase-2 and inducible nitric oxide synthase and regulates cellular proliferation, is proposed to be suppressed by curcumin (46). TNF-α, implicated in psoriasis and atopic dermatitis, triggers proinflammatory cytokines and activates NF-κB (47). Hence, the potential reduction of NF-κB by curcumin could contribute to its therapeutic efficacy in managing inflammatory skin diseases (48). Mohanty et al. applied a curcumin-loaded oleic acid-based polymeric bandage (COP) topically on the backs of wounded rats and observed a downregulation in the expression of various kinases in the PI3K/AKT/NF-κB pathway. The application of the COP bandage resulted in the downregulation of P13K and pAKT kinases, leading to reduced activation of the NF-κB gene and decreased inflammation. Additionally, an upregulation in I-κB-α protein, which inhibits the NF-κB pathway, was observed. Therefore, Mohanty et al. demonstrated that curcumin effectively reduces inflammation at wounded sites by modulating the NF-κB pathway (49). In contrast to Mohanty's findings, an *in vivo* study reported an increase in inflammatory cell infiltration in burn wounds on rats treated with curcumin compared to untreated groups (50). However, the study did not specify the type of inflammatory cells measured, necessitating further investigations to elucidate the proinflammatory effects of curcumin on wounds. Interestingly, curcumin was also found to enhance nitric oxide (NO) production in excision wounds of mice exposed to gamma radiation (51). Increased NO production has been shown to promote wound healing in patients by enhancing inflammation (35). Although Jagetia and Rajanikant proposed that the increase in NO contributed to improved wound healing with curcumin treatment, the majority of studies provide evidence that curcumin indeed reduces inflammation. By mitigating the inflammatory response, damaged skin can more efficiently progress to later stages of healing, such as proliferation and remodeling. Uncontrolled and prolonged inflammation may delay these subsequent stages and impede the overall wound healing process (52). Despite its potent modulative effects on wound healing, curcumin faces challenges related to low bioavailability, rapid metabolism, inadequate solubility, and sensitivity. Exploring new formulations, such as nanoparticles, is crucial to overcoming these limitations and harnessing the full potential of curcumin (53).

Curcumin's also influence extends to inhibiting platelet production, removing mitogens that stimulate the rapid growth of mononuclear blood cells, and partially inhibiting the protein kinase enzyme (54). Given the well-established role of oxidative stress in the pathogenesis of various diseases (e.g., myocardial ischemia, ischemia–reperfusion, bleeding, shock, nerve cell damage, and cancer), curcumin's anti-inflammatory and antioxidant properties are substantiated. It eliminates various forms of reactive oxygen species (ROS), including hydroxyl radicals and nitrogen dioxide radicals. The antioxidant capacity of curcuminoids has been reported to be equivalent to that of ascorbic acid (55).

The inflammation and the oxidative stress and its associated alterations in neuroplasticity play pivotal roles in the development of this neurodevelopmental disorder. Recent studies have suggested a potential role for curcumin in the treatment of depression and bipolar disorder. Adding curcumin to antidepressant drugs has demonstrated significant reductions in depressive symptoms compared to a placebo

supplement. Furthermore, a recent meta-analysis has provided support for the effectiveness of adjunctive curcumin in managing depression and anxiety disorders. Importantly, curcumin has been shown to be well-tolerated and safe in various randomized clinical trials involving humans (56).

Functioning as a potent hydroxyl radical scavenger and superoxide radical capturer (Figure 8), curcumin protects DNA from oxidative injury by retaining free radicals. Following oral intake, it undergoes hydrogenation in the intestines, transforming into tetrahydrocurcumin, and is subsequently absorbed, distributed into the blood and tissues, and excreted in the bile. Curcumin supplementation has been shown to reduce muscle damage induced by eccentric exercise in rats (57).

Multifaceted anticancer effects of turmeric encompasses various aspects, including the modulation of key cellular pathways, the impact on specific cancer cell lines, the inhibition of metastasis, and the exploration of potential therapeutic applications. Curcumin, a primary constituent of turmeric, has exhibited remarkable efficacy throughout multiple stages of cancer progression. Its inhibitory effects extend to the transformation, initiation, development, and invasion of tumors, as well as angiogenesis and metastasis. This broad spectrum of action positions curcuminoids as potent suppressors of tumor cell growth.

3.3.1 Mechanistic insights

The discussion delves into the intricate molecular mechanisms underlying curcuminoids' anticancer effects. The modulation of crucial cellular pathways, including the cell proliferation, cell survival, caspase activation, tumor suppressor, and death receptor pathways, elucidates the diverse strategies employed by curcuminoids in targeting cancer cells. These pathways involve key regulators such as cyclin D1, c-myc, Bcl-2, Bcl-xL, caspases, p53, p21, DR4, and DR5 (58).

3.3.2 Cell-line specific effects

Studies on specific cancer cell lines, such as MCF-7 human breast tumor cells, highlight the differential potency of curcuminoids. For instance, DMC demonstrates superior inhibitory effects compared to CUR and BDMC, emphasizing the importance of structural elements like phenolic hydroxyl groups, methoxyl groups, and the diketone moiety.

3.4 Potential therapeutic applications

The research extends its focus to potential therapeutic applications. In leukemia treatment, pure CUR shows promise in modulating the expression of the WT1 gene, indicating its potential utility in leukemia therapy. Furthermore, the exploration of curcuminoids in lipolysis inhibition suggests a potential avenue for reducing plasma free fatty acid levels and improving insulin sensitivity. The order of potency in inhibiting cancer cell invasion is identified as BDMC > DMC > CUR, and their ability to significantly reduce urokinase plasminogen activator and active MMPs underscores their potential in inhibiting invasion and metastasis (59). The discussion introduces the application of curcuminoids in photodynamic therapy (PDT), revealing their substantial suppression of cell viability in breast cancer cell lines. The heightened anti-proliferative effect observed with DMC-PDT, coupled with its potential as a novel photosensitizer, suggests a promising avenue for cancer therapy. Additional studies explore the metabolic profile of curcuminoids in combination with other agents, showcasing their anticancer effects by suppressing levels of amino acids, lipid compounds, and carbohydrates in tumor tissues. The discussion also touches upon the enhanced antiproliferative impact of a monocarbonyl analogue of B63 compared to curcumin, specifically in colon cancer cells (60).

Regarding radiomodulatory properties of curcuminoids, there are a collection of studies exploring turmeric bioactive compounds particularly curcumin, and their potential applications in the context of cancer therapy. These studies both preclinical and clinical investigations, shedding light on the multifaceted effects of curcuminoids in the presence of radiation.

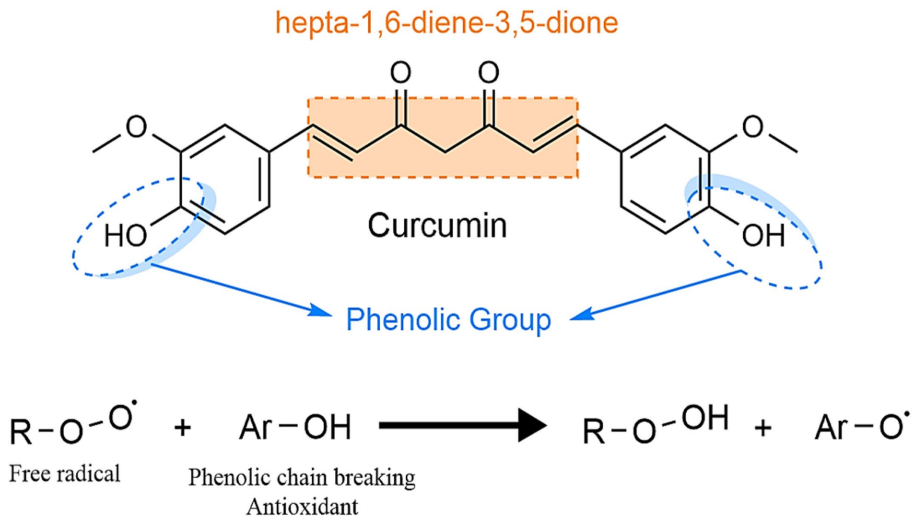

FIGURE 8
The antioxidant action of curcumin.

Studies show a dual nature of curcuminoids, acting as both radioprotectors for normal cells and radiosensitizers for tumor cells. The study by Lopez-Jornet et al. (29) on female Sprague Dawley rats during radiotherapy elucidates the potential protective effects of curcumin on parotid glands. Morphological and histopathological analyses revealed reduced cell necrosis in the CUR-treated group, indicating a radioprotective effect. Furthermore, the pre-administration of lycopene and CUR contributed to mitigating structural damage to the salivary glands. (30) reported a dual action of polyphenols in CUR, manifesting both radioprotective and radiosensitive effects. The radiosensitization was attributed to compromised G2-checkpoint functionality, leading to increased radiation-induced chromatid breaks (61).

Inflammation, oxidative stress, and neuroplasticity-related changes play key roles in the development of this neurodevelopmental disorder. Recent studies have suggested a potential role for curcumin in the treatment of depression and bipolar disorder. Adding curcumin to antidepressant medications has shown significant reductions in depressive symptoms compared to a placebo supplement (62).

Curcumin (Figure 9) has the complexity of a long carbon chain that bonds at the ends with two benzenes. Along this chain there can be found both carbonyl and hydroxyl oxygens which provide curcumin its specific character, besides these the double bonds also have a role in the bioavailability and polymerization of multiple molecules and the formation of bonds with other diverse componds.

The diketo moiety can also act as a potent metal chelator, coordinationg metal ions and forming with them a complex salt. The phenolic hydroxyl groups also act as potent antioxidants by donating their hydrogens to free radicals, minimizing the formation of reactive oxygen species and inhibiting oxidative stress.

In summary, the cumulative evidence presented in the text underscores the multifaceted and promising anticancer properties of curcuminoids. From elucidating molecular mechanisms to exploring specific applications, the diverse range of studies contributes valuable insights into the potential of curcuminoids as effective agents in cancer therapy.

This review highlights curcumin's inhibitory effects on various stages of cancer progression, including transformation, initiation, development, invasion, angiogenesis, and metastasis. This broad spectrum of action positions curcuminoids as potent suppressors of tumor cell growth. The involvement of key regulators such as cyclin D1, c-myc, Bcl-2, caspases, p53, p21, DR4, and DR5 is explored, providing a mechanistic understanding of the diverse strategies employed by curcuminoids in targeting cancer cells. Potential therapeutic applications, including leukemia treatment, lipolysis inhibition, and photodynamic therapy, are explored, showcasing the versatility of curcuminoids in diverse cancer-related contexts (63, 64).

In the context of cancer therapy, we find a dual nature of curcuminoids, acting as both radioprotectors for normal cells and radiosensitizers for tumor cells. Insights from preclinical and clinical investigations shed light on the potential of curcuminoids in minimizing radiation-induced damage to normal tissues while enhancing the sensitivity of tumor cells to radiation. The findings collectively suggest that curcuminoids have promising applications in cancer therapy, acting both as protectants for healthy cells and sensitizers for cancer cells. Despite valuable insights, the article acknowledges potential limitations and calls for future research to standardize methodologies, explore long-term effects, and elucidate molecular mechanisms. Randomized controlled trials are proposed to strengthen the scientific basis for integrating curcuminoids into cancer treatment regimens (65, 66).

4 Conclusion

In this review we find the role of curcumin in modulating inflammatory responses and promoting wound healing. Through its antioxidant properties and the down-regulation of inflammatory targets, curcumin emerges as a promising agent in managing inflammatory skin diseases. Discrepancies in findings prompt further investigations, while the acknowledgment of challenges underscores the need for innovative formulations like nanoparticles to maximize curcumin's therapeutic potential in wound healing.

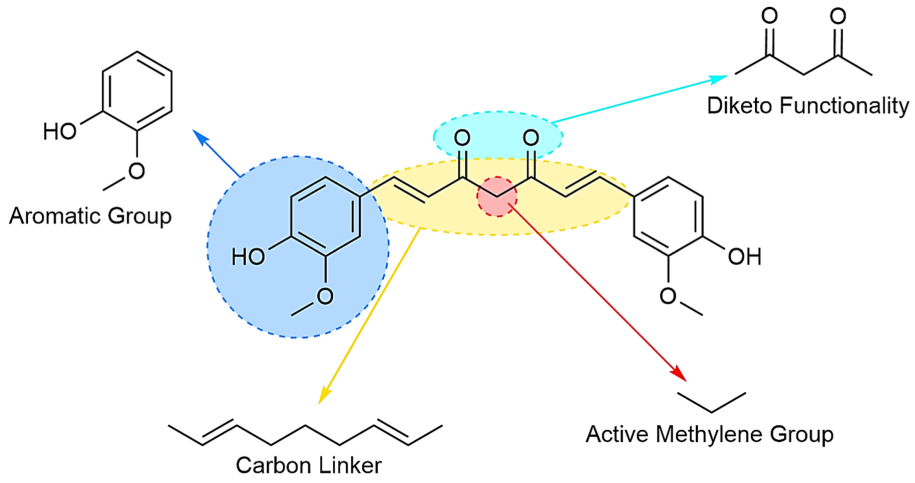

FIGURE 9
The building blocks of the curcumin molecule and its multifaceted uses.

The present article provides a comprehensive overview of curcumin's multifaceted roles in cancer therapy, inflammation, and wound healing. The diverse applications of curcuminoids in cancer treatment, coupled with their immunomodulatory properties, present exciting prospects for future research and clinical applications. The nuanced understanding of curcumin's mechanisms of action contributes to its potential integration into mainstream cancer therapies and wound care, offering a natural and versatile approach to disease management.

Novel aspects underlined in the review paper are related to the versatility of curcuminoids in cancer-related contexts, showcasing potential therapeutic applications beyond traditional chemotherapy. In the realm of cancer therapy, we uncover a dualistic property of curcuminoids, wherein they serve as both radioprotective agents for healthy cells and radiosensitizing agents for tumor cells. This distinctive attribute carries implications for mitigating radiation-induced harm to normal tissues while concurrently augmenting the susceptibility of tumor cells to radiation therapy.

While acknowledging the promising therapeutic potential of curcuminoids, we have uncovered potential limitations such as the low bioavailabiliy of the molecule and its fast metabolism, althought these present as obstacles, they can be used to our advantage for making different curcumin formulations that are easily metabolized and do not leave traces behind. Besides these, the bioavailabily of curcumin can be potentially increased by combining it with different bioavailable molecules. Curcumin can also be used as a chelator for different metal ions, for adminestering or removing them from the body, this property seems to be useful in different supplements.

In summary, our review has uncovered the multifaceted potential of curcumin both as an immunomodulator, as a radioprotective, anticancer medication and so much more. Curcumin boasts multiple benefits and presents itself as an interesting subject for future research.

Author contributions

MC: Writing – original draft, Writing – review & editing. IIL: Writing – original draft, Writing – review & editing. CrG: Writing – original draft, Writing – review & editing. AS: Writing – original draft, Writing – review & editing. LDD: Writing – original draft, Conceptualization, Software. BDS: Writing – original draft, Writing – review & editing. DD: Writing – original draft, Writing – review & editing. GC: Writing – original draft, Writing – review & editing. ERBG: Writing – original draft, Writing – review & editing. CaG: Writing – original draft, Writing – review & editing. MB: Writing – original draft, Writing – review & editing.

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Dietary supplementation with mulberry leaf flavonoids and carnosic acid complex enhances the growth performance and antioxidant capacity via regulating the p38 MAPK/Nrf2 pathway

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Introduction: This study aimed to investigate the regulatory effects of mulberry leaf flavonoids and carnosic acid complex (MCC) on the growth performance, intestinal morphology, antioxidant, and p38 MAPK/Nrf2 pathway in broilers.

Methods: A total of 256 healthy 8-day-old female yellow-feathered broilers were randomly divided into 4 equal groups: a control group (CON) fed a basal diet, an antibiotic group (CTC) supplemented with 50 mg/kg chlortetracycline, and two experimental groups (MCC75, MCC150) fed basal diets with 75 mg/kg and 150 mg/kg of MCC, respectively. The experiment lasted for 56 days, with days 1–28 designated as the initial phase and days 29–56 as the growth phase.

Results: The results on the growth performance showed that diets supplemented with MCC and CTC decreased the feed-to-gain ratio (F/G), diarrhea rate, and death rate, while significantly increasing the average daily weight gain (ADG) ($p < 0.05$). Specifically, the MCC150 group enhanced intestinal health, indicated by reduced crypt depth and increased villus height-to-crypt depth ratio (V/C) as well as amylase activity in the jejunum. Both the MCC and CTC groups exhibited increased villus height and V/C ratio in the ileal ($p < 0.05$). Additionally, all treated groups showed elevated serum total antioxidant capacity (T-AOC), and significant increases in catalase (CAT) and glutathione peroxidase (GSH-Px) activities were observed in both the MCC150 and CTC groups. Molecular analysis revealed an upregulation of the jejunal mRNA expression levels of PGC-1α, Nrf2, and Keap1 in the MCC and CTC groups, as well as an upregulation of ileum mRNA expression levels of P38, PGC-1α, Nrf2, and Keap1 in the MCC150 group, suggesting activation of the p38-MAPK/Nrf2 pathway.

Discussion: These findings indicate that dietary supplementation with MCC, particularly at a dosage of 150 mg/kg, may serve as a viable antibiotic alternative, enhancing growth performance, intestinal health, and antioxidant capacity in broilers by regulating the p38-MAPK/Nrf2 pathway.

KEYWORDS

mulberry leaf flavonoids, carnosic acid, broiler, growth performance, antioxidant

1 Introduction

The expansion of chicken farming is associated with substantial challenges, including environmental stressors, prevalent diseases, and substandard feeding practices, which significantly compromise the immune health and growth performance of broilers (1). While antibiotics have been employed to enhance disease resistance and boost production metrics, their use has been marred by considerable drawbacks, notably the specter of antibiotic residues persisting in poultry products (2). Antimicrobial resistance has escalated into a critical global health emergency, spurred by the overutilization and incorrect application of antibiotics within the realm of animal husbandry, which catalyzes the emergence of resistant microbial strains (3). Specifically, the poultry industry has been implicated as a major source of this problem, wherein the routine use of antibiotics for growth promotion has elicited heightened concern over potential impacts on human health and environmental safety (4). Therefore, it is urgent to explore alternative strategies to promote poultry growth and disease prevention. Plant extracts, which have been shown to possess antimicrobial, antioxidant, and immune-stimulating properties, emerge as a promising avenue for diminishing the reliance on antibiotics in poultry farming (5, 6).

Mulberry leaf flavonoids are one of the significant active components of mulberry plants, mainly including quercetin, kaempferol, rutin, morin and its derivatives (1). Extensive research indicates that these flavonoids can effectively improve the antioxidant properties and oxidative stress resilience of broilers, alongside bolstering their immunity and disease resistance (7, 8). Moreover, these compounds have been found to facilitate the growth and development of broilers, suggesting their promising utility in broiler production practices (1). Carnosic acid is a phenolic diterpenoid primarily extracted from rosemary and other Lamiaceae plants, and its content in air-dried rosemary leaves can range from 3 to 10% (9). There are currently few studies on the application of carnosic acid, which is a natural fat-soluble antioxidant with demonstrated antibacterial, anti-inflammatory, and antioxidant effects (9, 10). Although these two natural chemicals have been the subject of extensive research in recent years due to their health benefits, their complexes have received comparatively little attention. Based on the extensive research on these two natural substances, we hypothesize that their complex has growth-promoting and antioxidant effects on livestock and poultry, potentially serving as a substitute for antibiotics. Therefore, the major objectives of this study were to investigate the effects of dietary supplementation with mulberry leaf flavonoids and carnosic acid complexes (MCC) on broiler performance, nutrient digestibility, intestinal digestive enzymes, intestinal morphology, and antioxidant properties, to evaluate the potential of MCC as a green feed additive.

Abbreviations: MCC, Mulberry leaf flavone and carnosic acid complexes; ADFI, Average daily feed intake; DM, Dry matter; EE, Ether extract; T-AOC, Total antioxidant capacity; GSH-PX, Glutathione peroxidase; MDA, Malondialdehyde; β-actin, Beta-actin (loading control); TNF-α, Tumor necrosis factor; p38 MAPK, P38 mitogen-activated protein kinase; PGC-1α, Peroxisome proliferator-activated receptorγcoactivetor-1α; ADG, Average daily gain; F/G, Feed/gain; CP, Crude protein; CF, Crude fiber; SOD, Superoxide dismutase; CAT, Catalase; Nrf2, Nuclear factor erythroid 2-related factor 2; JNK, C-Jun N-terminal kinase; IL-6, Interleukin-6; IL-1β, Interleukin-1β.

2 Materials and methods

2.1 Animal ethics statement

To ensure animal welfare, all experiments and methods are designed to minimize animal suffering. All experiments and sample collection procedures were performed according to the Chinese guidelines for animal welfare and were approved by the Institutional Animal Care and Use Committee of Hunan Agricultural University (Permit Number: CACAHU 2020-0821).

2.2 Animals and experimental treatments

A total of 256 healthy 8-day-old female yellow-feathered broilers, with an average initial body weight of 101.0 ± 2.0 g, were randomly divided into four groups. Each group had 8 replicates of 8 birds each. The four groups were denoted as the CON group (basal diet), the CTC group (basal diet with 50 mg/kg chlortetracycline), the MCC75 group (basal diet supplemented with 75 mg/kg MCC), and the MCC150 group (basal diet supplemented with 150 mg/kg MCC). The addition dosage of MCC was determined based on the preliminary experiments of our research team. The experiment included a 7-day pretest period and a subsequent 56-day trial period. According to the standard nutritional requirement of broilers (Agricultural industry standard of the people's Republic of China—chicken breeding standard NY/T33-2004), the basic diet formula of the formal trial period was divided into two periods (d 1–28 and d 29–56), and its nutrient profile is shown in Table 1.

The MCC used in the experiment was sourced from Hunan Geneham Pharmaceutical Co., Ltd. It consisted primarily of 25% mulberry leaf flavone, 25% carnosic acid, and the remaining components served as carriers. Of which, mulberry leaf flavonoids were obtained by dissolving mulberry leaves in water, followed by two rounds of reflux, and subsequently concentrating and spray drying the filtrate. Carnosic acid was extracted from *Salvia miltiorrhiza* using alcohol, followed by high-pressure filtration and spray drying.

The experiment was conducted at the breeding base of Hunan Agricultural University. Prior to the test, the floor walls of the chicken house and the chicken cage underwent a cleaning, disinfection, and ventilation process for 3 days. The test chickens were raised in four-layer fully enclosed chicken cages (60 cm width × 120 cm length × 50 cm height) with artificial lighting throughout the test. The humidity was controlled at 50–70%, and the temperature was maintained at 33–35°C from d 1–7 and at 27–31°C from d 8–14, gradually reducing to approximately 22°C until d 28. All birds were fed twice a day at 08:00 h and 16:00 h. Water and feed (crumbled) were provided *ad libitum*, the chicken house was regularly cleaned, and immunizations were administered as per standard protocols.

2.3 Sample collection

At the end of the trial, one broiler close to the average weight of each replicate was chosen, weighed, and data were collected (8 birds per group, respectively). Subsequently, blood samples from the jugular vein were collected in 10 mL centrifuge tubes, centrifuged at 3,500 r/min for 10 min at 4°C, and the obtained serum samples were stored at

TABLE 1 Ingredients and nutrients composition of the basal experimental diet (air-dry basis, %).

Ingredients	1–28 days	29–56 days	Nutrient level[b]	1–28 days	29–56 days
Corn	62.20	67.50	ME (MJ/kg)	12.40	12.54
Soybean meal	28.00	28.00	CP	20.49	18.80
Puffed soybeans	6.00	0.00	Lysine	1.13	1.00
Soybean oil	0.10	1.20	Methionine	0.46	0.40
DL-Methionine	0.10	0.10	Cystine + Methionine	0.83	0.74
Dicalcium phosphate	1.50	1.30	Ca	1.00	0.90
Stone powder	1.20	1.10	AP	0.45	0.40
Choline	0.10	0.00			
NaCl	0.30	0.30			
Premix[a]	0.50	0.50			
Total	100.00	100.00			

[a]Provided per kilogram of diet: 50 mg of Cu; 50 mg of Fe; 50 mg of Mn; 60 mg of Zn; 1 mg of I; 0.5 mg of Se; 50,000 IU of vitamin A; 15,000 IU of vitamin D3; 130 IU of vitamin E; 10 mg of vitamin K3; 20 mg of vitamin B1; 0.5 mg of vitamin B2; 0.5 mg of vitamin B6; 75 mg of vitamin B12; 0.4 mg of biotin; 30 mg of pantothenic acid; 6 mg of folic acid; 160 mg of nicotinic acid.
[b]Calculated values.

−20°C. The birds were euthanized by cervical dislocation. The spleen, thymus, and bursa of Fabricius were removed and weighed. The digesta from the middle jejunum was transferred to a 1.5 mL centrifuge tube, temporarily stored in liquid nitrogen, and then stored at −80°C. Additionally, samples for morphological analysis were collected from the middle jejunum and middle ileum (1–2 cm). The mucosa of jejunum and ileum was collected, rapidly frozen with liquid nitrogen, and stored at −80°C.

2.4 Growth performance

The broilers were weighed on the 0th, 28th, and 56th days of the experiment, and the feed intake was recorded during the experiment to calculate the average daily feed intake (ADFI), average daily gain (ADG), feed conversion ratio (F/G), diarrhea rate, and death rate of broilers in the early, late, and overall stages. The diarrhea rate was calculated as follows: Diarrhea rate (%) = number of diarrhea broilers/(total number of broilers × total experimental days) × 100. Diarrhea was defined as watery manure with an irregular shape.

2.5 Immune organ index

The immune organ index ($n = 8$) was calculated by dividing the fresh weight (g) of the immune organs (thymus, spleen and bursa of Fabricius) by the pre-slaughter weight (g) of the chickens (6).

$$\text{Immune organ indexes}(\%) = 100 \times \text{Immune organ weight}(\text{g}) / \text{body weight}(\text{g}).$$

2.6 Apparent digestibility of nutrients

During the final 7 days of the experiment (d 50–56), 0.3% titanium dioxide (TiO_2) was added to the diet as an exogenous indicator, and feed samples were randomly collected from each group and stored for testing. Fecal samples were collected on the last 4 days of the test period (d 53–56), and approximately 50 g of representative fecal samples were collected daily in the fecal pan under each cage using a five-point method to remove debris such as feathers and dander in the feces, and 10 mL of 10% dilute sulfuric acid was added to each 100 g of feces for nitrogen fixation and stored in a freezer at 20°C. The fecal samples were then properly combined, primary dried for approximately 6 h at 65°C, and weighed before being kept for testing. The crude extract content of ether in feces and feed was determined by the Soxhlet extraction method, the crude ash content of feces and feed was determined by high temperature ignition at 550°C, the crude protein content was determined by the Kjeldahl method, and the crude fiber content was determined by a semi-automatic fiber detector. The apparent digestibility of nutrients in the diet was calculated according to the following formula (11):

$$\text{AD}(\%) = \left[1 - (\text{G1} \times \text{F2}) / (\text{G2} \times \text{F1})\right] \times 100.$$

AD, apparent digestibility of nutrients in the diet; G1, content of titanium in the diet; F1, content of a nutrient in the diet; G2, content of titanium in feces; F2, content of a nutrient in feces.

2.7 Intestinal digestive enzyme activity

The amylase (AMY, Kit Serial No: C016), lipase (LIP, Kit Serial No: A054), and trypsin (TP, Kit Serial No: A080) levels in jejunal contents ($n = 8$) were determined following the protocols of commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions.

2.8 Morphological structure of intestinal tract

Briefly, intestinal samples were dehydrated and embedded in paraffin (Thermo Fisher Scientific, Kalamazoo, MI, United States), and then sectioned into 4-μm thick histological slices for hematoxylin and eosin staining (HE). Representative fields were chosen for

photography from a large number of randomly selected non-continuous fields. The ratio of villus height to crypt depth was calculated by measuring intestinal villus height and crypt depth with IMAGEEX, an image analysis program included in the YLE-21DY microscopic imaging system (Leica, Germany).

2.9 Serum antioxidant

The activities of total superoxide dismutase (T-SOD, Kit Serial No: A001), glutathione peroxidase (GSH-Px, Kit Serial No: A005), catalase (CAT, Kit Serial No: A007), total antioxidative capacity (T-AOC, Kit Serial No: A015), and the content of malondialdehyde (MDA, Kit Serial No: A003) were assayed ($n = 8$) using colorimetric methods with a Microplate Reader (Infinite M200 PRO, TECAN, Switzerland). The assays were conducted using the commercial kits purchased from Nanjing Jiancheng Biotechnology Co., Ltd. (Nanjing, China) and following their corresponding procedures.

2.10 Expression of antioxidation-related gene in the intestinal p38-MAPK/Nrf2 pathway

Total RNA ($n = 8$) was extracted from the jejunum and ileum mucosa by using the Trizol method (R401-01, Vazyme, Nanjing, China), and then reverse transcription and real-time quantitative PCR were performed using the reverse transcription kit (R223-01, Vazyme, Nanjing, China) and fluorescence quantitative kit (Q711-02, Vazyme, Nanjing, China), respectively. The quality and quantity of extracted RNA were determined using a Nanodrop Spectrophotometers (IMPLEN, CA, United States) and a Qubit Fluorometer (Life Technologies, CA, United States). The primers for the target gene were synthesized by Tsingke Biotechnology Co., Ltd. (Table 2). Real-time PCR analysis of the gene expression was performed using SYBR Green (Thermo Fisher Scientific, MA) on an ABI 6 flex real-time PCR instrument (Thermo Fisher Scientific). The reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 1 min. Melt curve analysis was performed to confirm the PCR amplification specificity (12). The target gene relative expression was calculated according to the $2^{-\Delta\Delta Ct}$ method and the housekeeping gene *β-actin* was chosen as an internal reference gene.

2.11 Statistical analysis

SPSS 22.0 statistical software (SPSS Inc., Chicago, IL, United States) was used for general linear model (univariate) analysis. The Duncan's multiple comparison method was employed for significant difference analysis, with $p < 0.05$ serving as the criterion for significant difference, and $p < 0.01$ indicating extremely significant difference. All results were expressed as mean ± standard deviation (SD).

3 Results

3.1 Growth performance

The effects of dietary supplementation of MCC on growth performance are presented in Table 3. In the 1–28 d period, the F/G

TABLE 2 Sequence of primers for real-time PCR.

Target gene	Accession no.	Nucleotide sequence of primers (5′-3′)	Product size (bp)
β-actin	NM_205518.2	F: ATGATGATATTGCTGCGCTCGT	139
		R: CCCATACCAACCATCACACCCT	
JNK	NM_205095.1	F: TGACCGAGTGAGGAGACGAT	211
		R: ACTGTATCGAACGCAGCACA	
TNF-α	NM_204267	F: TGTGTATGTGCAGCAACCCGTAGT	229
		R: GGCATTGCAATTTGGACAGAAGT	
IL-6	NM_204628.1	F: AAATCCCTCCTCGCCAATCT	106
		R: CCCTCACGGTCTTCTCCATAAA	
p38 MAPK	NM_001353939.1	F: GCGAGTCCCTAATGCCTACG	199
		R: ACAACTGTTGAGCCACACTCA	
IL-1β	NM_204524.1	F: ACTGGGCATCAAGGGCTACA	142
		R: GCTGTCCAGGCGGTAGAAGA	
PGC-1α	XM_015285697.2	F: CCAAAGGACACGCTCTAGATCA	76
		R: TCTCGATCGGGAATATGGAGAA	
Nrf2	XM_025152148.1	F: ATCACGAGCCCTGAAACCAA	143
		R: GGCTGCAAAATGCTGGAAAA	
Keap1	XM_025145847.1	F: GTACCAGATCGACAGCGTGG	197
		R: GGCAGTGGGACAGGTTGAAG	

JNK, c-Jun N-terminal kinase; TNF-α, tumor necrosis factor-α; IL, interleukin; p38 MAPK, p38 mitogen-activated protein kinase; PGC-1α, peroxisome proliferator-activated receptor γ coactive-tor-1α; Nrf2, Nuclear factor erythroid 2-related factor 2; Keap1, kelch like ECH associated protein 1.

TABLE 3 Effect of mulberry leaf flavonoids and carnosic acid complex (MCC) on growth performance of broilers.

Items		Groups				*p*-value
		CON	CTC	MCC75	MCC150	
d 1–28	ADFI (g/d)	45.75 ± 0.56	44.83 ± 0.56	44.35 ± 0.56	44.83 ± 0.56	0.333
	ADG (g/d)	19.20 ± 0.39	19.82 ± 0.39	20.30 ± 0.39	19.32 ± 0.45	0.215
	F/G	2.39 ± 0.05^{a}	2.26 ± 0.05^{ab}	2.19 ± 0.05^{b}	2.31 ± 0.05^{ab}	0.033
d 29–56	ADFI (g/d)	91.88 ± 1.55	90.72 ± 1.55	88.66 ± 1.55	87.60 ± 1.79	0.267
	ADG (g/d)	29.77 ± 0.55^{b}	32.09 ± 0.55^{a}	30.77 ± 0.55^{ab}	31.68 ± 0.63^{a}	0.032
	F/G	3.09 ± 0.07^{a}	2.84 ± 0.07^{b}	2.88 ± 0.07^{ab}	2.77 ± 0.08^{b}	0.027
d 1–56	Initial BW (g)	102.89 ± 1.07	100.98 ± 0.93	99.75 ± 0.93	100.98 ± 0.93	0.193
	Final BW (g)	1475.14 ± 17.08^{b}	1554.46 ± 17.08^{a}	1529.64 ± 17.08^{ab}	1512.21 ± 17.08^{ab}	0.021
	ADFI (g/d)	67.97 ± 0.78	67.69 ± 0.78	66.50 ± 0.78	66.03 ± 0.90	0.309
	ADG (g/d)	24.49 ± 0.30^{b}	25.96 ± 0.30^{a}	25.53 ± 0.30^{a}	25.50 ± 0.34^{ab}	0.012
	F/G	2.78 ± 0.05^{a}	2.61 ± 0.05^{b}	2.61 ± 0.05^{b}	2.59 ± 0.05^{b}	0.024
	Diarrhea rate (%)	7.84 ± 0.43^{a}	2.16 ± 0.43^{b}	1.65 ± 0.43^{b}	0.77 ± 0.43^{b}	<0.001
	Death rate (%)	9.38 ± 1.69^{a}	1.56 ± 1.69^{b}	1.56 ± 1.69^{b}	1.56 ± 1.69^{b}	<0.001

Data are presented as mean ± SD ($n = 8$). In the same row, values with no letter or the same letter superscripts mean no significant difference ($p > 0.05$), while with different small letter superscripts indicate a significant difference ($p < 0.05$). CON, basal diet; CTC, basal diet with 50 mg/kg chlortetracycline; MCC75, basal diet supplemented with 75 mg/kg MCC; MCC150, basal diet supplemented with 150 mg/kg MCC; BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; F/G, feed-to-gain ratio.

TABLE 4 Effect of mulberry leaf flavonoids and carnosic acid complex (MCC) on indices immune organs of broilers (%).

Items	Groups				*p*-value
	CON	CTC	MCC75	MCC150	
Spleen index, %	0.17 ± 0.02^{b}	0.17 ± 0.02^{b}	0.23 ± 0.02^{a}	0.20 ± 0.02^{ab}	0.021
Thymus index, %	0.24 ± 0.02	0.23 ± 0.02	0.18 ± 0.02	0.25 ± 0.02	0.136
Bursa index, %	0.21 ± 0.02	0.22 ± 0.02	0.18 ± 0.02	0.18 ± 0.02	0.553

Data are presented as mean ± SD ($n = 8$). In the same row, values with no letter or the same letter superscripts mean no significant difference ($p > 0.05$), while with different small letter superscripts indicate a significant difference ($p < 0.05$). CON, basal diet; CTC, basal diet with 50 mg/kg chlortetracycline; MCC75, basal diet supplemented with 75 mg/kg MCC; MCC150, basal diet supplemented with 150 mg/kg MCC.

ratio was significantly lower ($p < 0.05$) in MCC75 group compared to the in the CON group, while no significant difference ($p > 0.05$) was observed in ADFI and ADG among the 4 groups. In the 29–56 d period, the MCC150 and CTC groups exhibited significantly higher ADG ($p < 0.05$) and lower F/G ratio ($p < 0.05$) compared to the CON group. There was no significantly difference ($p > 0.05$) observed in ADFI among the 4 groups. In the whole period of the experiment, the F/G ratio, the diarrhea rate, and the dead panning rate were lower ($p < 0.05$) in the CTC, MCC75, and MCC150 groups compared to the CON group. Additionally, compared to the CON group, both the MCC75 and CTC groups exhibited significantly greater ADG ($p < 0.05$).

3.2 Immune organ indexes

Table 4 showed the effect of MCC on the immune organ indexes of broilers. The spleen index of the MCC 75 group was markedly higher ($p < 0.05$) than that of the CON and CTC groups. However, there were no significant differences in the thymus index and bursa index among the 4 groups ($p > 0.05$).

3.3 Apparent digestibility

The apparent digestibility of dry matter (DM) was significantly higher ($p < 0.05$) in the MCC150 group than in the CON group (Table 5). Furthermore, ether extract (EE) digestibility markedly improved by 7.66, 7.19, and 6.66% ($p < 0.05$) in the CTC, MCC75, and MCC150 groups, respectively, compared to the CON group. Additionally, the content of crude protein (CP) was significantly greater ($p < 0.05$) in the MCC75 and MCC150 groups compared to the CON and CTC groups, while no significant difference was observed among crude fiber (CF) and ash digestibility ($p > 0.05$).

3.4 Intestinal digestive enzyme activity

The digestive enzyme activity of amylase, lipase, and trypsin in the jejunum of broilers was presented in Table 6. Compared with the control group, the amylase activity in the jejunum was significantly increased in the MCC and CTC groups ($p < 0.05$). However, no significantly differences were observed in the activities of jejunal lipase and trypsin among the 4 groups ($p > 0.05$).

TABLE 5 Effect of mulberry leaf flavonoids and carnosic acid complex (MCC) on nutrient apparent digestibility of broilers (%).

Items	Groups				*p*-value
	CON	CTC	MCC75	MCC150	
DM	94.82 ± 0.24^{b}	95.25 ± 0.24^{ab}	95.52 ± 0.26^{ab}	95.95 ± 0.24^{a}	0.019
CP	72.45 ± 1.28^{b}	73.28 ± 1.28^{b}	77.27 ± 1.28^{a}	77.41 ± 1.28^{a}	0.019
EE	82.15 ± 1.46^{b}	88.44 ± 1.46^{a}	88.06 ± 1.46^{a}	87.62 ± 1.46^{a}	0.015
CF	39.99 ± 4.51	38.64 ± 4.03	40.32 ± 4.03	43.62 ± 4.51	0.870
Ash	22.18 ± 2.23	25.23 ± 1.93	26.80 ± 1.93	24.80 ± 2.07	0.491

Data are presented as mean ± SD ($n = 8$). In the same row, values with no letter or the same letter superscripts mean no significant difference ($p > 0.05$), while with different small letter superscripts indicate a significant difference ($p < 0.05$). CON, basal diet; CTC, basal diet with 50 mg/kg chlortetracycline; MCC75, basal diet supplemented with 75 mg/kg MCC; MCC150, basal diet supplemented with 150 mg/kg MCC; DM, dry matter; CP, crude protein; EE, ether extract; CF, crude fiber.

TABLE 6 Effect of mulberry leaf flavonoids and carnosic acid complex (MCC) on digestive enzyme activity of jejunum in broilers.

Items	Groups				*p*-value
	CON	CTC	MCC75	MCC150	
α-Amylase (U/mgprot)	170.34 ± 96.05^{c}	766.99 ± 96.05^{a}	394.85 ± 88.93^{bc}	538.28 ± 83.18^{ab}	0.005
Lipase (U/mgprot)	244.52 ± 97.45	622.76 ± 97.45	362.44 ± 97.45	440.84 ± 97.45	0.071
Trypsin (U/mgprot)	19173.67 ± 8230.13	29111.33 ± 7361.25	18689.53 ± 8230.13	19395.61 ± 7361.25	0.366

Data are presented as mean ± SD ($n = 8$). In the same row, values with no letter or the same letter superscripts mean no significant difference ($p > 0.05$), while with different small letter superscripts (a, b, and c) indicate a significant difference ($p < 0.05$). CON, basal diet; CTC, basal diet with 50 mg/kg chlortetracycline; MCC75, basal diet supplemented with 75 mg/kg MCC; MCC150, basal diet supplemented with 150 mg/kg MCC.

TABLE 7 Effect of mulberry leaf flavonoids and carnosic acid complex (MCC) on intestinal tissue morphology in broilers.

Items		Groups				*p*-value
		CON	CTC	MCC75	MCC150	
Jejunum	Villus height, μm	716.28 ± 40.52	784.16 ± 40.52	830.97 ± 36.99	814.21 ± 40.52	0.218
	Crypt depth, μm	139.04 ± 3.74^{a}	134.45 ± 4.10^{a}	138.28 ± 4.10^{a}	122.45 ± 4.10^{b}	0.035
	V/C ratio	5.15 ± 0.36^{b}	5.82 ± 0.40^{b}	6.14 ± 0.36^{ab}	6.65 ± 0.40^{a}	0.024
Ileum	Villus height, μm	496.42 ± 49.24^{b}	749.69 ± 55.04^{a}	696.46 ± 55.04^{a}	707.37 ± 60.30^{a}	0.006
	Crypt depth, μm	155.31 ± 3.12^{a}	148.77 ± 3.12^{ab}	146.2 ± 3.12^{ab}	141.01 ± 3.12^{b}	0.031
	V/C ratio	3.24 ± 0.31^{b}	5.03 ± 0.25^{a}	4.76 ± 0.25^{a}	5.01 ± 0.28^{a}	0.001

Data are presented as mean ± SD ($n = 8$). In the same row, values with no letter or the same letter superscripts mean no significant difference ($p > 0.05$), while with different small letter superscripts indicate a significant difference ($p < 0.05$). CON, basal diet; CTC, basal diet with 50 mg/kg chlortetracycline; MCC75, basal diet supplemented with 75 mg/kg MCC; MCC150, basal diet supplemented with 150 mg/kg MCC; V/C ratio, villus height-to-crypt depth (V/C) ratio.

3.5 Histomorphology of intestinal tract

Dietary MCC150 decreased the crypt depth ($p < 0.05$) and increased the V/C ratio value ($p < 0.05$) in the jejunal (Table 7; Figure 1). In the ileal, the villus height and villus height-to-crypt depth (V/C) ratio value were increased ($p < 0.01$) in the CTC, MCC75, and MCC150 groups. Furthermore, the MCC150 group decreased the crypt depth in the ileal compared to the CON group ($p < 0.05$).

3.6 Serum antioxidation

According to the data presented in Table 8, it can be observed that dietary supplementation with MCC75, MCC150, and CTC significantly increased the T-AOC values in serum ($p < 0.01$). Additionally, the CAT activities were significantly increased ($p < 0.05$) in the CTC and MCC150 groups compared to the CON group. It is worth noting that dietary supplementation with MCC150 also led to an increase in GSH-Px levels ($p < 0.05$). However, there were no significant differences ($p > 0.05$) in T-SOD and MDA levels among the 4 groups.

3.7 Expression of antioxidant related genes in intestinal p38-MAPK/Nrf2 pathway

In the jejunum, dietary supplementation with CTC, MCC75, and MCC150 decreased ($p < 0.05$) the relative mRNA expression abundance of C-Jun N-terminal kinase (JNK), while increasing the mRNA levels of Peroxisome proliferator-activated receptorγcoactivetor-1α (PGC-1) and nuclear factor erythroid 2-related factor 2 (Nrf2) ($p < 0.05$) (Figure 2).

In the ileum, dietary supplementation with MCC150 increased the relative mRNA expression abundance of P38 mitogen-activated

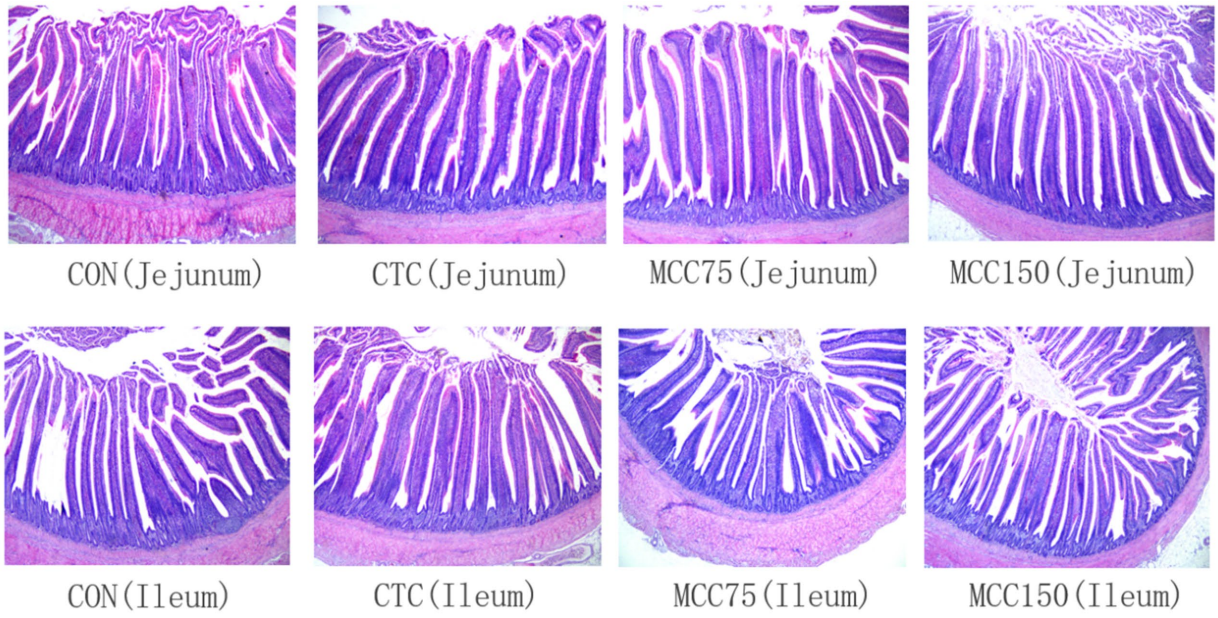

FIGURE 1
Morphological structure of jejunum and ileum in yellow-feathered broilers (HE staining, 40×). CON, basal diet; CTC, basal diet with 50 mg/kg chlortetracycline; MCC75, basal diet supplemented with 75 mg/kg MCC; MCC150, basal diet supplemented with 150 mg/kg MCC.

TABLE 8 Effect of mulberry leaf flavonoids and carnosic acid complex (MCC) on serum antioxidant indexes of broilers.

Items	Groups				*p*-value
	CON	CTC	MCC75	MCC150	
T-AOC (U/mL)	0.97 ± 0.11^{c}	1.82 ± 0.11^{a}	1.30 ± 0.10^{b}	1.54 ± 0.10^{ab}	<0.001
SOD (U/mL)	773.95 ± 45.99	866.15 ± 45.99	811.54 ± 65.03	692.42 ± 49.16	0.105
GSH-Px (U/mL)	1588.80 ± 41.30^{b}	1709.68 ± 41.30^{ab}	1611.58 ± 41.30^{ab}	1729.74 ± 38.64^{a}	0.049
CAT (U/mL)	13.69 ± 1.67^{b}	20.21 ± 1.56^{a}	17.92 ± 1.67^{ab}	19.75 ± 1.67^{a}	0.038
MDA (nmol/mL)	12.25 ± 1.40	12.49 ± 1.52	11.83 ± 1.52	12.52 ± 1.52	0.987

Data are presented as mean ± SD ($n = 8$). In the same row, values with no letter or the same letter superscripts mean no significant difference ($p > 0.05$), while with different small letter superscripts (a, b, and c) indicate a significant difference ($p < 0.05$). CON, basal diet; CTC, basal diet with 50 mg/kg chlortetracycline; MCC75, basal diet supplemented with 75 mg/kg MCC; MCC150, basal diet supplemented with 150 mg/kg MCC; T-AOC, total antioxidant capacity; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; MDA, malondialdehyde.

protein kinase (P38), PGC-1, Nrf2, and Kelch like ECH associated protein 1 (Keap1) ($p < 0.05$) (Figure 3). Notably, the mRNA expression levels of Nrf2 were markedly higher ($p < 0.05$) in the MCC150 group than in the CTC group.

4 Discussion

Flavonoids, which are anti-inflammatory and antioxidant chemicals, can influence animal immunity and growth performance by regulating lipid metabolism, immunological function, and growth axis function (1, 7). Simultaneously, carnosic acid, a plant phenolic acid molecule, exhibits antibacterial, anti-inflammatory, antioxidant, hypoglycemic, and hypolipidemic properties (9, 10). It can function as an antibiotic replacement by modulating lipid metabolism and blocking cholinesterase, thereby improving animal growth (13). A previous study found that dietary supplementary with 200 mg/kg, 400 mg/kg, and 800 mg/kg of flavonoid (quercetin) could increase ADG in broilers (14). In the present study, the ADG increased and the F/G ratio, diarrhea rate, and dead panning rate decreased when supplemented with MCC or chlortetracycline, indicating that the synergistic effect of MCC could promote broiler growth and development with comparable antibiotic efficacy. Nutrient metabolism can influence animal growth, and it has been demonstrated that mulberry leaf flavonoids increase the rate of metabolism in calves after weaning (1). Moreover, because flavonoids have a structure similar to estradiol, they can interact with the hypothalamus and pituitary gland, helping to regulate hormone levels and promote growth in animals. Qi et al. (15) also revealed that *Allium* flavones could boost serum hormone and insulin-like growth factor-1 levels, thereby promoting broiler chicken growth. Therefore, the promotive effect of MCC on growth performance is likely closely associated with the presence of mulberry leaf flavonoids. These results suggest that supplementing an appropriate amount of MCC into the diets of broilers is feasible.

The immune organ index is commonly used to measure the immune system function and overall health status of animals. There

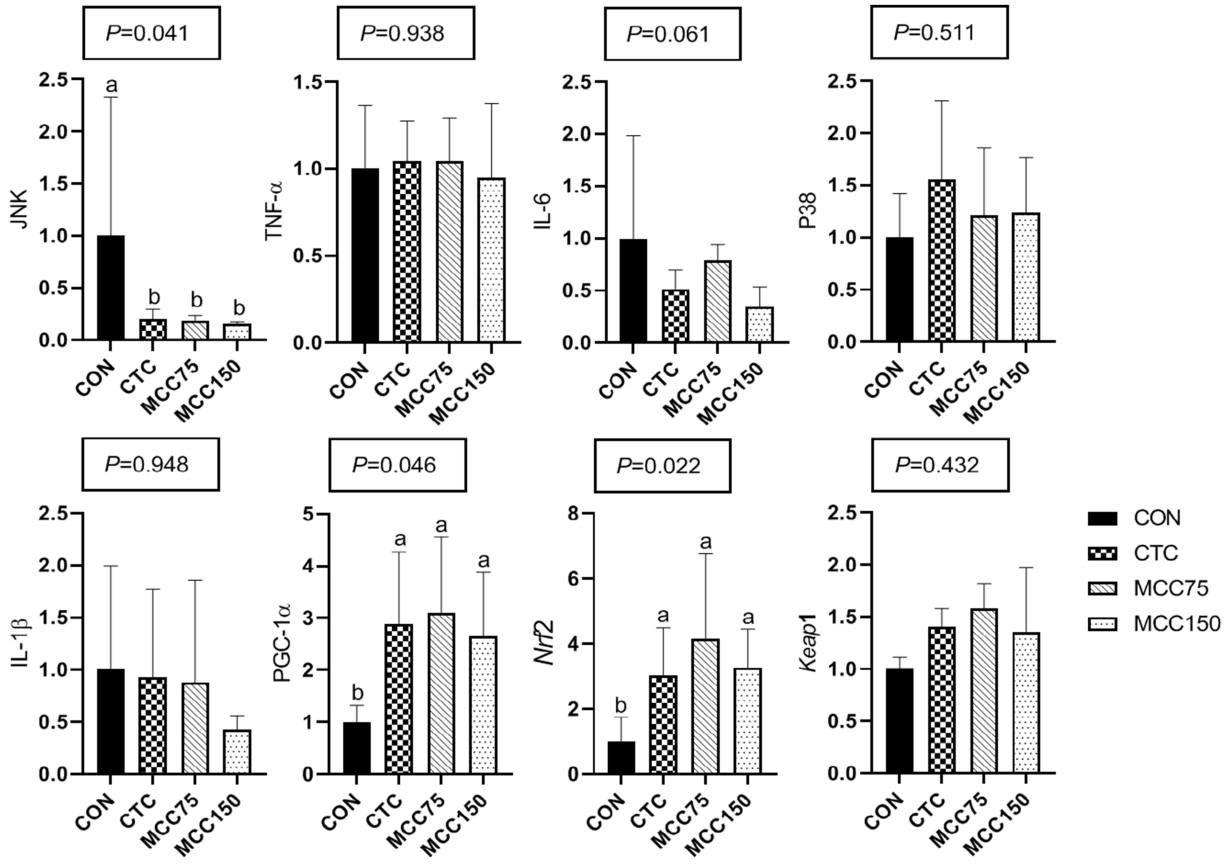

FIGURE 2
Effect of MCC on antioxidation-related gene expression in p38-MAPK/Nrf2 pathway in jejunum. Bars represent the means ± SD ($n = 8$), bars with different letters on top represent statistically significant results ($p < 0.05$). CON, basal diet; CTC, basal diet with 50 mg/kg chlortetracycline; MCC75, basal diet supplemented with 75 mg/kg MCC; MCC150, basal diet supplemented with 150 mg/kg MCC; JNK, c-Jun N-terminal kinase; TNF-α, tumor necrosis factor-α; IL, interleukin; p38 MAPK, p38 mitogen-activated protein kinase; PGC-1α, peroxisome proliferator-activated receptor γ coactive-tor-1α; Nrf2, nuclear respiratory factor 2; Keap1, kelch like ECH associated protein 1.

is a positive correlation between the immunological organ index and immune organ development, and immune function rises with immune organ index (16). The thymus, spleen, and Fabricius bursa play crucial roles as immune organs in birds, and their indices serve as valuable indicators for assessing the organism's immune status (8, 17). The thymus functions as a central immune organ that secretes T lymphocytes and also plays a significant role in the neuroendocrine network (8). The spleen, as an important peripheral immune organ, directly affects broiler immunity (6, 17). Furthermore, previous research has demonstrated that plant flavonoids can promote the development of immune organs and enhance animal immunity (8). For example, the supplementation of alfalfa flavonoids in the feed can enhance the growth performance, spleen and bursa weights, as well as aspartate transaminase activity of meat geese (8). The results of this experiment and the above conclusion show some similarities, as MCC was observed to increase the spleen index and facilitate the development of immune organs. This effect could potentially be attributed to the action of mulberry leaf flavonoids in increasing protein synthesis and secretion while fully promoting the immune mechanisms of the spleen.

Animal growth and development are related to apparent digestibility of nutrients, which can directly reflect the digest and absorb ability of the animal body (18). Recent studies have demonstrated the positive effects of rosemary extract on enhancing nutrient digestibility in weaned piglets (19). Additionally, supplementation of 1,000 mg/kg quercetin has been found to increase the apparent digestibility of DM and nitrogen in growing pigs (20). Moreover, flavonoids derived from mulberry leaves have shown the potential to improve the digestibility of DM, CF, and metabolizable energy in broilers (21). In the present study, our results showed that the MCC increased the apparent digestibility of DM, CP, and CF in broilers. This suggested that MCC could effectively improve the apparent digestibility of nutrients in broilers, with a similar effect to that of chlortetracycline. The rate of nutrient digestibility and absorption in animals has a favorable correlation with animal growth, which further supports the aforementioned finding that MCC could enhance broiler growth performance. Gut microbiota plays a crucial role in digestive processes and possess a diverse metabolic repertoire closely associated with food metabolism (22). Furthermore, a study has demonstrated that flavonoids and carnosic acid can induce changes in gut microbiota composition (23). Flavonoids can increase nutrient digestion and utilization by promoting the growth of probiotics in the intestine while inhibiting the growth of harmful bacteria (24). Therefore, it is hypothesized that the MCC could improve nutrient digestibility in broilers via influencing intestinal bacteria in broilers. Unfortunately, the intestinal bacteria were not

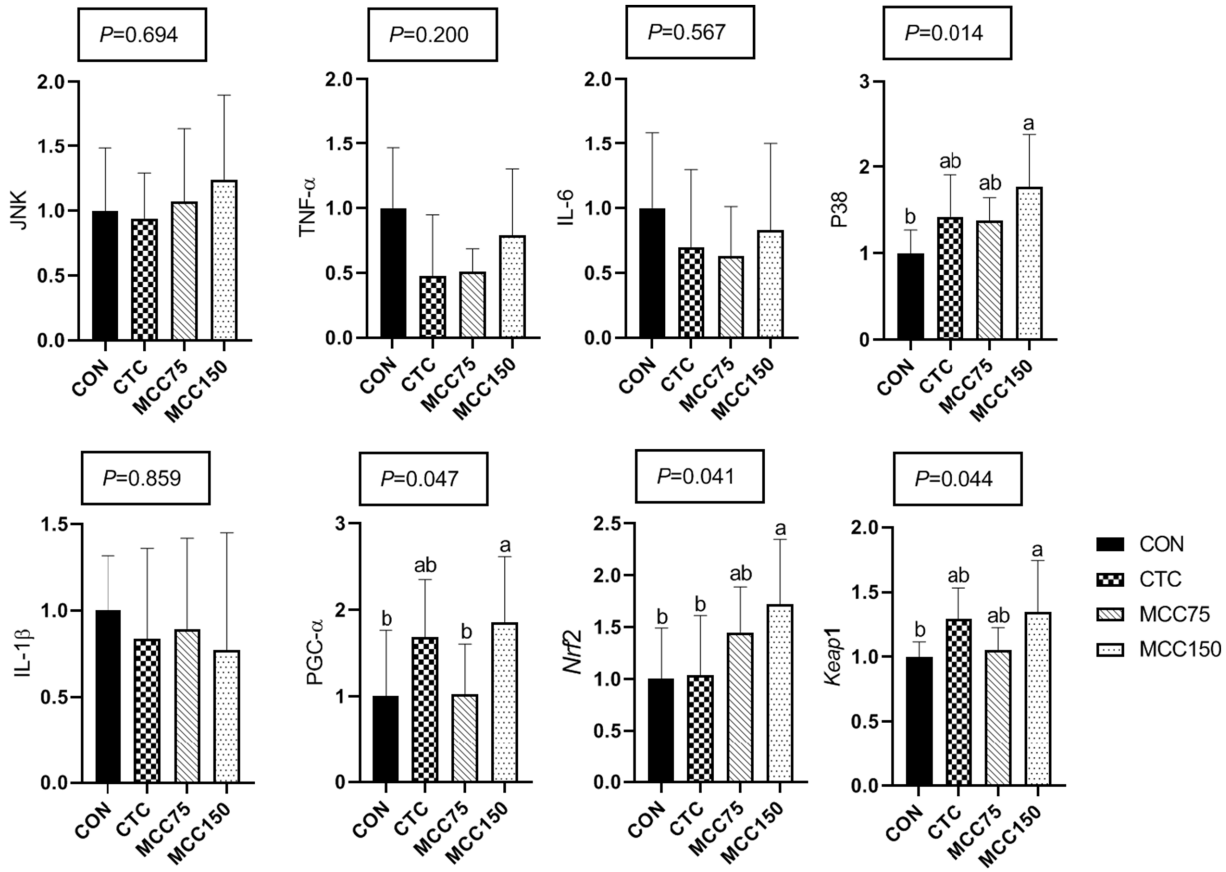

FIGURE 3
Effect of MCC on antioxidation-related gene expression in p38-MAPK/Nrf2 pathway in ileum. Bars represent the means ± SD ($n = 8$), bars with different letters on top represent statistically significant results ($p < 0.05$). CON, basal diet; CTC, basal diet with 50 mg/kg chlortetracycline; MCC75, basal diet supplemented with 75 mg/kg MCC; MCC150, basal diet supplemented with 150 mg/kg MCC; JNK, c-Jun N-terminal kinase; TNF-α, tumor necrosis factor-α; IL, interleukin; p38 MAPK, p38 mitogen-activated protein kinase; PGC-1α, peroxisome proliferator-activated receptor γ coactive-tor-1α; Nrf2, nuclear respiratory factor 2; Keap1, kelch like ECH associated protein 1.

detected in our study. Mulberry leaf flavonoids could also improve nutritional digestion and absorption in broilers by boosting intestinal villus formation, expanding the area of intestinal digestion and absorption, and enhancing the activity and production of intestinal digestive enzymes (25).

The enzymes in the intestine of chickens, namely amylase, lipase, and trypsin, play a vital role in the digestion and breakdown of nutrients. Flavonoids have been proven to increase the activity of digestive enzymes. Ding et al. (1) observed that mulberry leaf flavonoids might enhance the growth of intestinal villi in broilers and dramatically increase digestive enzyme activity. Figueroa-Perez et al. (26) demonstrated that flavonoids could boost the growth of good bacteria in the colon, limit the proliferation of dangerous bacteria, and enhance intestinal trypsin and amylase activity. Moreover, relevant studies have demonstrated that MCC could affect the composition and activity of the microbiota, improving the growth of intestinal epithelial cells and exerting beneficial effects on intestinal barrier function and gastrointestinal inflammation (27, 28). Our results showed that combining chlortetracycline with 150 mg/kg MCC could significantly increase the activity of jejunum-amylase in broilers, indicating that MCC could increase the activity of intestinal digestive enzymes in broilers. This may be related to the modulation of microbial metabolism in the intestine by MCC, thereby promoting the secretion of digestive enzymes and enhancing the activity of relevant digestive enzymes (28).

The small intestine is the primary site of nutritional absorption in animals, and the morphological structure of the gut plays an essential role in the digestion and absorption of numerous nutrients (29). Under normal conditions, intestinal villi can significantly increase the surface area for nutrients digestion and absorption. Additionally, the crypt depth is inversely correlated with the ability of intestinal epithelial cells to secrete digestive juices, whereas a larger V/C ratio corresponds to a higher digestive and absorptive capacity in the intestine (30). By enhancing the alkaline phosphatase activity of intestinal epithelial cells, hawthorn flavone compounds can encourage epithelial cell growth and proliferation, enhance the intestinal epithelial barrier, and promote the development of intestinal villous tissue (31). Flavonoids have been shown to increase the height of the ileal villus in broilers (32). Additionally, carnosic acid improved intestinal crypt architecture and goblet cell loss, according to research conducted by Yang et al. (33). In mice with colitis, rosemary extract supplemented with carnosic acid was able to enhance intestinal barrier integrity (28). In this study, 150 mg/kg MCC could significantly increase the villus height and V/C value of

the jejunum and ileum while decreasing the crypt depth in broilers. These results were in agreement with the previous research (34). Broilers' intestinal tracts may benefit from the MCC because flavonoids lower oxidative stress by inhibiting inflammatory factors, down-regulating the expression of NADPH oxidase, and up-regulating the intestinal hormone glucagon-like peptide (GLP)-2, which strengthens the intestinal barrier (34). As a result, the MCC complex can promote the secretion of jejunal amylase, the development of intestinal villi, and effectively improve the morphological structure of the intestinal tract. This leads to a greatly increase in the digestion and absorption area of nutrients in the intestinal tract, and improves the apparent digestibility of nutrients in broilers, thereby improving broiler growth performance.

The strength of antioxidant performance is a key indicator of physical health, reflecting the level of the body's antioxidant defense system and its ability to scavenge free radicals (35). SOD plays a vital role in scavenging superoxide anion radicals in the body and maintaining a balance between oxidation and anti-oxidation process (36). GSH-Px, as an essential peroxidase in the body, catalyzes the conversion of GSH into oxidized glutathione and efficiently eliminating hydrogen peroxide (37). SOD and GSH-Px can effectively eliminate excessive free radicals and prevent peroxides from damaging the structure and function of the cell membrane. Additionally, CAT decomposes hydrogen peroxide in the body and acts as an important antioxidant enzyme (36). The MDA is one of the byproducts of lipid peroxide metabolism in the body, created by the action of oxygen free radicals on the membrane. The MDA level is inversely connected with the level of cellular oxidative damage and can serve as an indirect indicator (38). Carnosic acid dramatically enhanced the levels of GSH and SOD, and decreased the level of MDA caused by DSS, according to Yang et al. (33); this indicates that carnosic acid could be important in the development of treatments for inflammatory disorders linked to oxidative stress. By controlling prooxidants and antioxidant enzymes, carnosic acid can increase broilers' antioxidant capacity (39). It has been demonstrated that the bioavailability of flavonoids could limited when supplemented in animal diets and may be less likely to directly exert antioxidant capacity (40). The mulberry leaf flavonoids are able to incorporate substantial antioxidant activity by scavenging free radicals and chelating metals (41). The antioxidant capacity increased and plasma MDA levels decreased when dietary supplementation of total flavonoids from *Artemisia annua* in Wenchang hens, as shown in a study by Guo et al. (7). Similarly, alfalfa flavonoids were found to enhance plasma T-AOC activity as well as the gene expression of antioxidant enzymes in broilers (42). Chen et al. (43) also demonstrated that the flavonoid quercetin could alleviate changes in CAT and SOD activities in oxidatively injured cells. In the present study, our results showed that MCC and chlortetracycline could increase serum T-AOC levels and the activities of CAT and GSH-Px, indicating that MCC could effectively increase the antioxidant level of broilers, which was in agreement with a previous study in broilers (42).

To elucidate how MCC might enhance the antioxidant capacity of broilers, we examined the role of the p38 mitogen-activated protein kinase (p38 MAPK) signaling pathway. The p38 MAPK signaling pathway is a common mechanism for intracellular information transmission, primarily involved in gene transcription, stress response, inflammatory response, and cellular immune regulation (44). The p38 MAPK pathway is critical in regulating the expression of several antioxidant enzyme genes (45). Moreover, the p38 signaling pathway stimulates the production of the transcription factor Nrf2 (46), which further enhances the cellular antioxidant defense system. Peroxisome proliferator receptor gamma coactivator 1 (PGC-1) is a transcriptional regulator that plays an important function in the anti-oxidative stress system and can enhance the body's antioxidant capacity by promoting the production of cellular antioxidant enzymes (47). The Nrf2-Keap1 signaling pathway is one of critical pathway for cellular protection against oxidative stress. Under normal physiological conditions, Nrf2 binds to Keap1, forming a complex that is recognized and degraded by the proteasome via polyubiquitinated markers. However, when the Nrf2-Keap1 pathway is activated, the complex dissociates, allowing Nrf2 to translocate into the nucleus. Once in the nucleus, Nrf2 binds to the antioxidant response element (ARE) and stimulates the transcription of genes involved in antioxidant enzymes, thereby enhancing cellular antioxidant capacity and tolerance to oxidative stress (48). Our results revealed that MCC and chlortetracycline could reduce JNK mRNA expression while increasing PGC-1 and Nrf2 mRNA expression in broiler jejunal mucosa. The combination of 150 mg/kg MCC increased the expression of p38, PGC-1, Nrf2, and Keap1 mRNA in broiler ileal mucosa. Flavonoids containing carnosic acid have been shown to possess robust antioxidant properties by activating the Nrf2-Keap1 pathway. Previous studies have reported that flavonoids facilitate the transcription of Nrf2 to the nucleus, where it binds to antioxidant response element (ARE) and stimulates the transcription of antioxidant proteins, phase II detoxifying enzymes, and other genes (49). Flavonoids may also interact with AhR (aryl hydrocarbon receptor), leading to the dissociation of the Keap1/Nrf2 complex and facilitating Nrf2 translocation into the nucleus, thereby enhancing the transcription of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and catalase (50). Furthermore, Lee and Jang (51) demonstrated that carnosic acid could promote Nrf2 nuclear displacement, effectively reducing the generation of harmful ROS (reactive oxygen species) and promoting the translation of phase II antioxidant enzymes. Carnosic acid may also protect against DSS-induced decreases in Nrf2 protein levels by interfering with the interaction of Cullin3 and Keap1 (33). The results of this study were basically consistent with the above studies, suggesting that MCC could influence gene expression in the intestinal p38-MAPK/Nrf2 signaling pathway and improve the ability of broilers to resist oxidative stress, and thus promote growth. However, further investigations are needed to elucidate the underlying mechanisms of p38-MAPK/Nrf2 activation by MCC. Furthermore, it is widely recognized that antioxidants play a pivotal role in protecting against inflammatory diseases, as oxidative stress and inflammation are intricately interconnected (52, 53, 54). In light of this, it is plausible to hypothesize that MCC may also contribute to reducing inflammation in broilers, thus warranting further exploration.

5 Conclusion

Collectively, these results demonstrate that dietary supplementation with MCC could effectively improve growth performance, intestinal morphology, nutrient absorption, and antioxidant capacity in broilers, which may be related to regulation of the MAPK/Nrf2 signaling

pathway. These findings provide valuable insights into the potential benefits of MCC for broiler performance. Thus, it is feasible and beneficial to use MCC at a dosage of 150 mg/kg as an antibiotic alternative in the diet of broilers. Future research will focus on elucidating the mechanisms of MCC's effects on the MAPK/Nrf2 pathway and assessing its long-term impacts and economic feasibility in commercial broiler production.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved and conducted in strict accordance with the guidelines recommended and ethically approved by the Institutional Animal Care and Use Committee of Hunan Agricultural University (Permit Number: CACAHU 2020-0821). The study was conducted in accordance with local legislation and institutional requirements.

Author contributions

CL: Investigation, Writing – original draft. HH: Data curation, Methodology, Validation, Writing – original draft. YC: Supervision, Writing – original draft, Writing – review & editing. YZ: Investigation, Writing – review & editing. TM: Methodology, Writing – review & editing, Software. BT: Investigation, Writing – review & editing. WH: Conceptualization, Formal analysis, Methodology, Writing – review & editing. XF: Data curation, Formal analysis, Methodology, Writing – review & editing. DX: Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

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Conflict of interest

YZ was employed by Geneham Pharmaceutical Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1428577/full#supplementary-material

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Valorization of animal waste proteins for agricultural, food production, and medicinal applications

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Introduction: Animal waste proteins have been increasing in the past decade, along with consumer demands. Their huge volume and the environmental issues caused by improper treatment probably pose a massive threat to human health. These animal waste proteins contain many valuable bioactive peptides and can be used not only as nutrient substances but also as primary functional ingredients in many industries, including agriculture, food, and pharmaceuticals. However, the advancement of the value-added application of animal waste proteins within the past 10 years has not been elucidated yet. In this regard, this paper scrutinized the studies on the applications of hydrolysates and peptides from animal waste proteins throughout the last decade, hoping to display a whole picture of their value-adding applications.

Methods: The Web of Science and Google Scholar were searched from January 1, 2013, to December 12, 2023. This review included field trials, *in vitro* and *in vivo* assays, and *in silico* analysis based on literature surveys or proteolysis simulation. The quality of the included studies was evaluated by Journal Citation Reports, and the rationality of the discussion of studies included.

Results: Numerous studies were performed on the application potential of hydrolysates and peptides of animal waste proteins in agricultural, food, and medicinal industries. Particularly, due to the nutritional value, safety, and especially competitive effects, the peptide with antioxidant, antimicrobial, antihypertensive, antidiabetic, or antithrombotic activities can be used as a primary functional ingredient in food and pharmaceuticals.

Discussion: These value-added applications of animal waste proteins could be a step towards sustainable animal by-products management, and simultaneously, open new avenues in the rapid development of nutraceuticals and pharmaceuticals. However, further studies on the bioavailability and structure-activity relationship are required to verify their therapeutic effects.

KEYWORDS

animal waste protein, valorization, bioactive peptide, functional ingredient, agriculture, food, pharmaceutical

1 Introduction

Over the past few years, there has been a significant surge in the global intake of high-protein foods (Peydayesh et al., 2022). When the meat industry and slaughterhouses yield a tremendous amount of meat products, a copious supply of protein-rich by-products is also produced. These animal by-products are frequently considered to be low-value and therefore discarded (Chakrabarti et al., 2018; Etemadian et al., 2021; Yao et al., 2022; Yang et al., 2023). However, it is worth noting that these by-products contain a diverse range of highly valuable bioactive compounds. Recently, there has been a significant upswing in the research of bioactive peptides derived from animal waste proteins. These peptides have been discovered to possess distinctive properties and intricate compositions with exceptional potential in diverse industries, including but not limited to agriculture, nutraceuticals, pharmaceuticals, and cosmetics (Korhonen and Pihlanto, 2006; Giordano et al., 2018; Karami and Akbari-Adergani, 2019; Phadke et al., 2021). As a result, researchers are keenly exploring their applications in various fields, intending to meet the continuous need for protein and unlock their full potential (Owji et al., 2018; Chavez and Uchanski, 2021; Lee S. Y. et al., 2021; Lee S. et al., 2021; Madhu et al., 2022).

Animal waste proteins can be one of the best sources of bioactive peptides, which are crucial molecules and may exert physiological effects in life (Wadhwa and Bakshi, 2016; Peydayesh et al., 2022; Timorshina et al., 2022). Animal waste proteins are mainly obtained from by-products or unused parts of animals from slaughterhouses after their primary processing for food production, such as skin, bones, cartilage, tendons, organs, trimmings, and other components that are not used for human consumption (Mora et al., 2014; Zhao et al., 2021). Nowadays, engineers and researchers are working hard to give value to these waste proteins by converting them into functional ingredients and new valuable products with high potential human health value and, at the same time, to reduce environmental pollution caused by them (dos Santos et al., 2021; Li et al., 2021; Martinez-Burgos et al., 2021; Norouzi et al., 2022). The hydrolysates and potential benefits of these animal proteins are immense and far-reaching for humanity and sustainability (Wadhwa and Bakshi, 2016; Peydayesh et al., 2022). Therefore, it is crucial to explore and implement ways in which animal waste proteins can be utilized to the fullest extent to support sustainable development and improve our overall quality of life (Cheung et al., 2015).

Several methods were used to generate the desired proteins and peptides, including direct extraction, chemical methods, enzymatic hydrolysis, and microbial fermentation (Pagán et al., 2021; Wen et al., 2023b). However, the choice of the method for the hydrolysis of proteins usually depends on their sources. The enzymatic hydrolysis and microbial fermentation methods were demonstrated to improve the solubility, viscosity, emulsification, and gelation propriety of peptides generated. These methods improved the peptide's nutritional quality, which may hold significant advantages for human health by reducing any associated factors that affect their applications (Marciniak et al., 2018; Zhu et al., 2022). The peptides unlocked from parent proteins can boost the immune system, improve digestion and adsorption of food, reduce inflammation, and promote the regeneration of cells and tissues (like skin and hair), remarkably improving our quality of life (Ullah et al., 2018; Wang B. et al., 2021). Furthermore, these peptides can be used as food additives, like natural preservatives and nutrition enhancers (Chi et al., 2015a; Nielsen et al., 2017).

To date, due to the strengthening of environmental protection policies, resource scarcity, and food security, livestock and aquaculture industries are meeting the harmless treatment and resource application problems of animal waste proteins, which attract the most interest of scientists to find better ways to solve them. Nowadays, much research has improved the enzymatic hydrolysis or microbial fermentation methods to produce various protein or peptide-based products, including food for both humans and animals, medicine, fertilizers, and antibiotics (Korhonen and Pihlanto, 2006; Dai et al., 2016; Minj and Anand, 2020). To show a whole picture of the application potential of animal waste proteins and the bioactive peptides derived from them, this study focuses on the production method for bioactive peptides derived from protein-rich animal wastes and their applications in agriculture, food industry, and medicine (Figure 1).

2 Methods

2.1 Literature search

For the purpose of the review, Web of Science and Google Scholar were searched for all published studies. During searching, the following topics were used for each section: "livestock," "meat by-product," "aquatic by-product," "fish waste," "food processing waste," "animal waste protein" or "waste animal protein" for sources of animal waste proteins; "animal waste protein" or "waste animal protein," "burning" or "combustion," "burying" or "burial," "rendering," and "compost" or "composting" for the traditional treatment of animal waste proteins; "microbial fermentation" or "fermentation" and "enzymatic hydrolysis" or "hydrolysis" for biotechnological methods for releasing peptides from animal waste proteins; "agricultural application," "plant growth promotion," "abiotic stress tolerance" or "heat stress" or "salinity stress" or "drought stress," "biotic stress tolerance" or "resistance to microorganism" or "resistance to fungi/bacteria/virus," and "animal waste proteins" for agricultural application; "food addictive," "functional food," "enzyme in gastrointestinal system," and "animal waste proteins" for food application; "medicinal application" or "pharmaceuticals," "bioactivity" or "antihypertensive" or "antioxidant" or "antimicrobial" or "antidiabetic" or "antithrombotic," "peptides," and "animal waste protein" for medicinal application. The data range was restricted in the past decade (January 1, 2013 to December 12, 2023). Furthermore, a backward citation search was performed for the searched articles.

2.2 Study selection

The searched articles were all imported into Endnote 20 (Clarivate Analytics, United States). After removing the duplicate records, two investigators independently screened the titles and the abstracts of all the retained articles, according to the inclusion and exclusion criteria. After excluding irrelevant articles in the initial screening, the same two investigators carefully read the relevant sections of the retained articles and extracted the useful information. Any disagreements were resolved by consulting other authors.

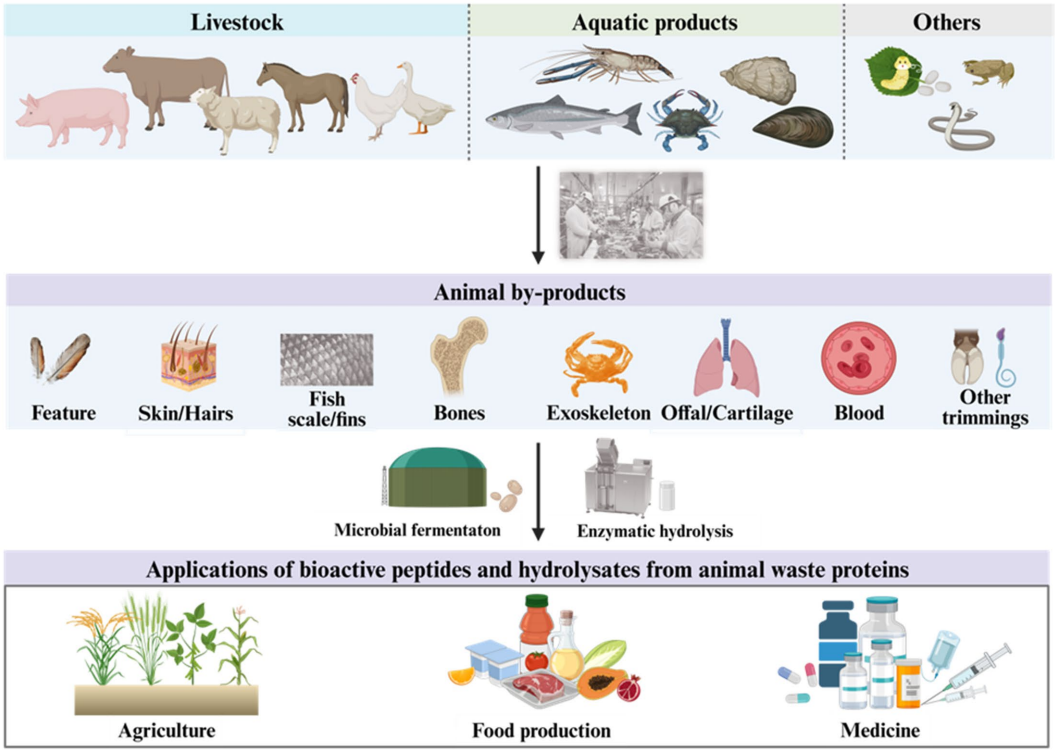

FIGURE 1
Schematic of the valorization of animal waste proteins. Created with BioRender.com.

Studies were included if they: (1) involved the source, treatment, or application of animal waste proteins; (2) involved the direct use of peptides or other components from animal waste proteins in field studies, *in vitro* studies, and *in vivo* studies, but had no description of the treatment of animal waste proteins; (3) were the statistical analysis based on database or computer simulation using a software; (4) were the latest reviews that can provide a part of data to this review or the relevant reports from influential governments and international organizations. Studies were excluded if they: (1) were related to this review, but the argument of the relevant section is not tenable. (2) investigated the application of the hydrolysates and peptides from animal waste proteins other than in agriculture, food, and medicinal industries; (3) investigated the health-promoting effects of the hydrolysates and peptides from animal waste proteins other than antihypertension, antioxidant, antimicrobial, hypoglycemic, and antithrombosis. The statistical data of the number of articles used for this study is shown in Figure 2.

3 Sources of animal waste proteins

Animal waste proteins are mainly from the by-products of meat and aquatic products processing industries. According to the National Bureau of Statistics of China (National Bureau of Statistics of China, 2023), in 2020, the raw meat yield (including pork, beef, mutton, and poultry meat) was 7.639 million tons. The aquatic product yield in 2020 was 6.549 million tons (Ministry of Agriculture and Rural Affairs of the People's Republic of China, 2020). The production of raw meat and aquatic food was accompanied by large amounts of animal wastes and fish wastes, which caused burdensome disposal problems and environmental concerns. For instance, the meat yield percentage for pork is around 72–80%, while for beef it is 50–60%. Since disposal costs and efficiency are previously prioritized, these wastes are directly burned or buried. Later, people realized that the lipids in the animal and fish products processing wastes could be recovered for animal feeds, cosmetics, etc., and thus used as a raw material for the rendering system. Besides, agriculturists found that the animal and fish products processing wastes could be converted to small-molecular organic compounds, like humus, through microbial metabolism under favorable conditions. Therefore, agriculturists compost the animal and fish products processing wastes.

3.1 Meat source

A huge amount of waste is generated during meat product processing due to the rapid growth of meat consumption throughout the world. These wastes can be classified into two groups: liquid blood and solid bones and trimmings. The blood consists of plasma and blood cells and is rich in proteins. After separation and hydrolysis or extraction, the protein hydrolysate, bioactive peptides, and especially thrombin, fibrinogen, heme iron peptide, and globin, can be obtained (Figure 3). These extracted proteins or peptides are beneficial for human health as dietary supplements or pharmaceuticals.

The trimmings and bones are rich in fat and proteins. Mature beef cattle or pigs have skeletal muscles that contain roughly 70% protein on a dry-matter basis (Sun et al., 2016; Bravo et al., 2023). The trimmings include skin, hair/bristle, feathers, horns, hooves, tails,

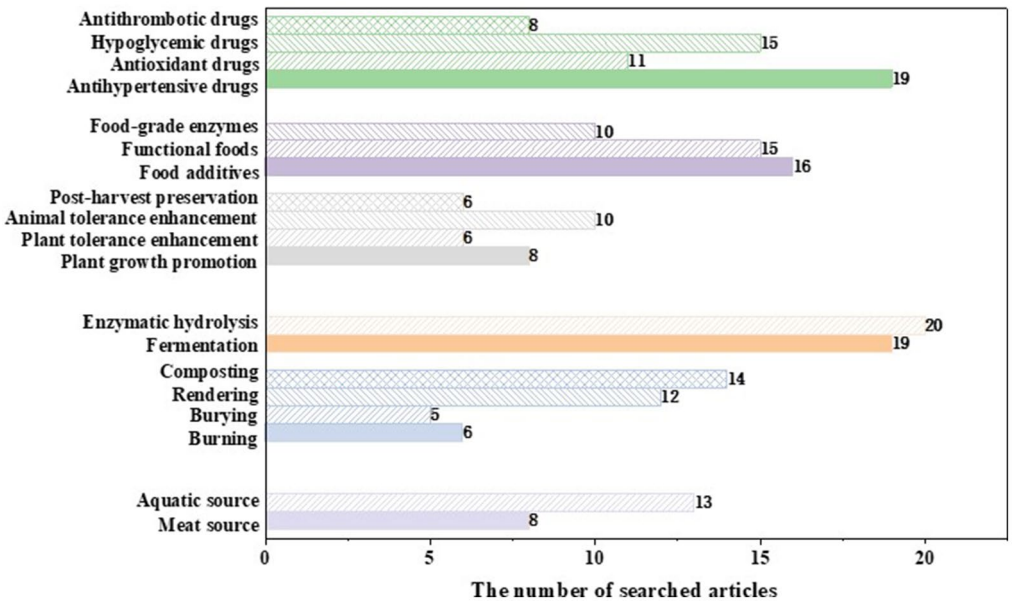

FIGURE 2
The number of searched articles.

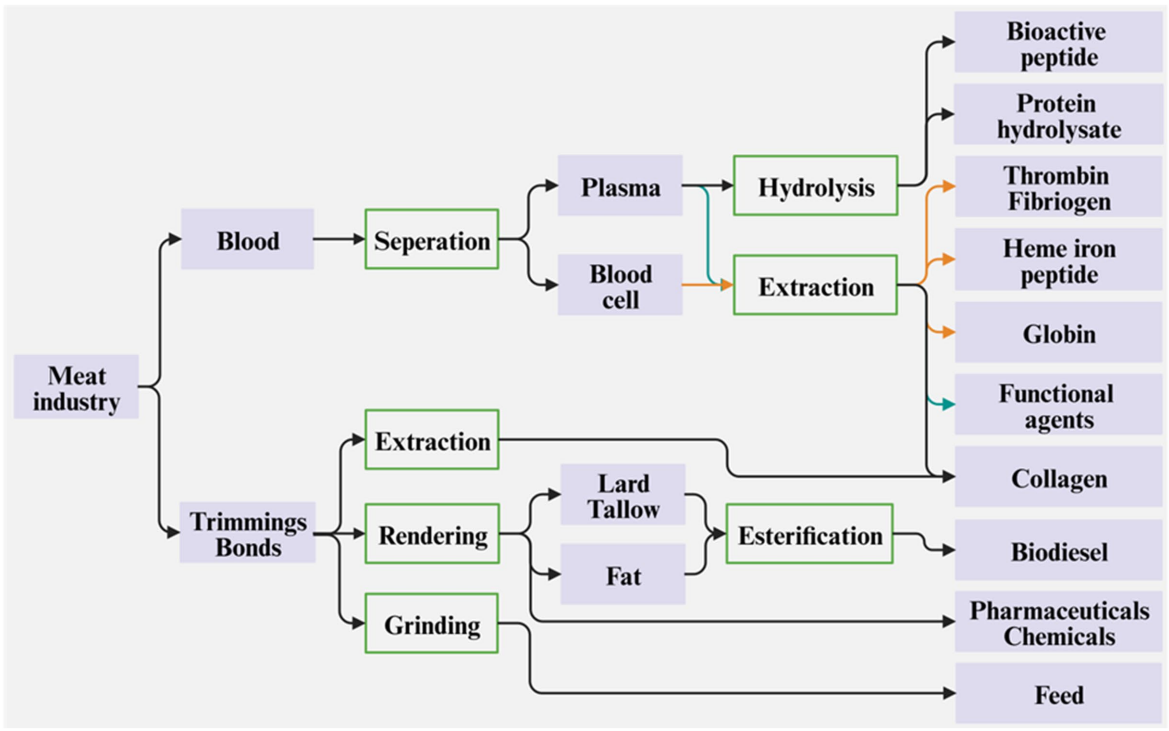

FIGURE 3
Route for valorizing meat processing by-products.

viscera/cartilage, and deboning residues (Wadhwa and Bakshi, 2016; Marciniak et al., 2018). Through the rendering technique, the lard/tallow, fat, chemicals, pharmaceuticals, and animal feeds can be produced using trimmings. The lard/tallow and fat can be further processed into biodiesel through an esterification reaction, which helps to alleviate not only the environmental concerns but also the energy crisis. Due to being rich in proteins and other nutrients [like minerals (Tran et al., 2020)], the trimmings are one of the best raw materials to extract collagens and the bones can be ground into powder to produce animal feeds.

3.2 Aquatic source

The fish processing industry has a meat yield percentage of about 60%. The fish products processing waste includes skin, scale/fin, head, viscera (like liver), roes, bones, exoskeletons, shells, and carcasses (Ahn et al., 2014; Sila et al., 2014; Silva et al., 2014; Chi et al., 2015c). These wastes often contain protein-rich materials, which are typically processed into the animals' dietary supplements and feeds, fish meal, and fertilizers (Subhan et al., 2021). However, these products do not make full use of the value of the fish products processing wastes because some proteins and peptides derived from these wastes through enzymatic hydrolysis can play a huge role in treating chronic diseases (Lee and Hur, 2017; Phadke et al., 2021; Ucak et al., 2021). For example, the protein hydrolysate obtained by hydrolyzing the stomach and intestine of smooth hound sharks by Purafect, Esperase, and Neutrase, exhibited a good therapeutic effect on hypertension, cancer, and infections (Abdelhedi et al., 2016). Compared to the proteins and peptides derived from meat processing sources, those derived from aquatic sources characterized by short chain length, the presence of lysine or arginine at the C-terminal, and possessing more hydrophobic amino acids, exhibit a high capability of regulating blood pressure and immune system and killing microorganisms (Ngo et al., 2016). For instance, the gelatin obtained by using Alcalase to hydrolyze giant squid (*Dosidicus gigas*) exhibited an extremely high angiotensin-converting enzyme inhibitory (ACE-I) ability ($IC_{50} = 0.34$ mg/mL) and those obtained by using Esperase exhibited an extremely high cytotoxic effect on cancer cells ($IC_{50} = 0.13$ mg/mL for human breast carcinoma and $IC_{50} = 0.10$ mg/mL for glioma cell lines). These two gelations were mostly composed of peptides with molecular weights of 500–1,400 Da.

4 Traditional treatment methods for animal waste proteins

4.1 Burning process

Burning or incineration reduces the volume of animal products processing wastes and animal carcasses volume by converting them to ash, which is very beneficial concerning limited waste disposal space. The volume of solid wastes can be reduced by over 90% by burning (Yamamoto et al., 2018; Velusamy et al., 2020). Besides, burning has two other great advantages. The one is that high temperatures destroy pathogens (e.g., *Escherichia coli* and *Salmonella* sp.), mitigating disease transmission risks (Franke-Whittle and Insam, 2013; Mozhiarasi and Natarajan, 2022). It is because the temperature during burning is usually maintained at 850–1,200°C to thermally decompose the animal by-products and carcasses completely. The other is that burning generates heat energy, which can be used to supply heat directly or generate electricity by steam turbines to satisfy the heating and electricity demands of livestock cultivation, aquaculture and even surrounding. However, burning has a disadvantage of air pollutants release. The pollutants produced during burning animal products processing wastes include particulate matter, noxious gases (sulfur dioxide and nitrogen oxides), odors, and potentially toxic substances, depending on the composition of the wastes. Therefore, incinerators should guarantee the purification of exhaust gases to reduce secondary environmental pollution and comply with local environmental regulations and emission control policies. Considering burning animal by-products and carcasses can raise local public concerns about air pollution, community engagement, transparency, and emission control technologies are essential for its public acceptance. Besides, animal by-products and carcasses contain many valuable substances with high commercial application potential, like protein and fat. Thus, Burning is not a particularly good method of disposal.

4.2 Burying process

Burying animal by-products and carcasses in the soil allows them to decompose naturally. This method emits less greenhouse gas and particulate matter compared to burning. The process involves digging a hole, placing the waste by-products or carcasses inside, and covering it with soil. The time it takes for buried objects to decompose varies depending on several factors, including the type of buried objects, burial depth, and burial site conditions. Burying offers several advantages, including quick disposal, reduced costs and logistical challenges, and enhanced soil structure and organic matter content (Yuan et al., 2013). However, the natural decomposition is slow and thus the resultant low release of nutrients will limit the agronomic benefits of buried animal by-products and carcasses as a nutrient source for crops or soil improvement. It also has potential disadvantages of land occupation, groundwater contamination, odor issues, disease transmission, and regulatory compliance requirements (Kim and Kim, 2017). Burying animal by-products and carcasses is banned in the European Union and some states of the United States. Although burying is permitted in some countries, strict regulations are implemented, like Scotland (Agriculture and Rural Economy Directorate of Scotland, 2023). Proper techniques are crucial for burying animal by-products and carcasses to avoid environmental contamination and disease spread. The proper techniques involve selecting appropriate burial sites, ensuring proper ventilation, monitoring the site regularly for potential environmental contamination, and handling and disposing of protective gear properly during the burial process to prevent disease spread.

4.3 Rendering process

Burning and burying are two simple and fast methods for disposing of animal by-products and carcasses, but not ideal methods because these wastes and mortalities contain various valuable components, for instance, proteins and fat (McGauran et al., 2021; Pagán et al., 2021). In contrast, the rendering process is favorable for recovering these valuable components for value-added applications. The basic process of rendering is shown in Figure 4. This process can be classified into wet rendering and dry rendering, which both include heating (or cooking), pressing, separation, and drying stages (Adewale et al., 2015). The difference between the two kinds is that hot water or hot steam is used to pressurize feedstocks to separate fats in wet rendering, while direct heating is used in dry rendering (Shi and Ge, 2020). Dry rendering is the most commonly used method to convert animal by-products and mortalities to useful industrial, agricultural, and pharmaceutical materials. After the heating and pressing stages, the proteins and lipids are mostly separated. The obtained lipids (including fats and oils) include tallow, grease, poultry fast, and lard,

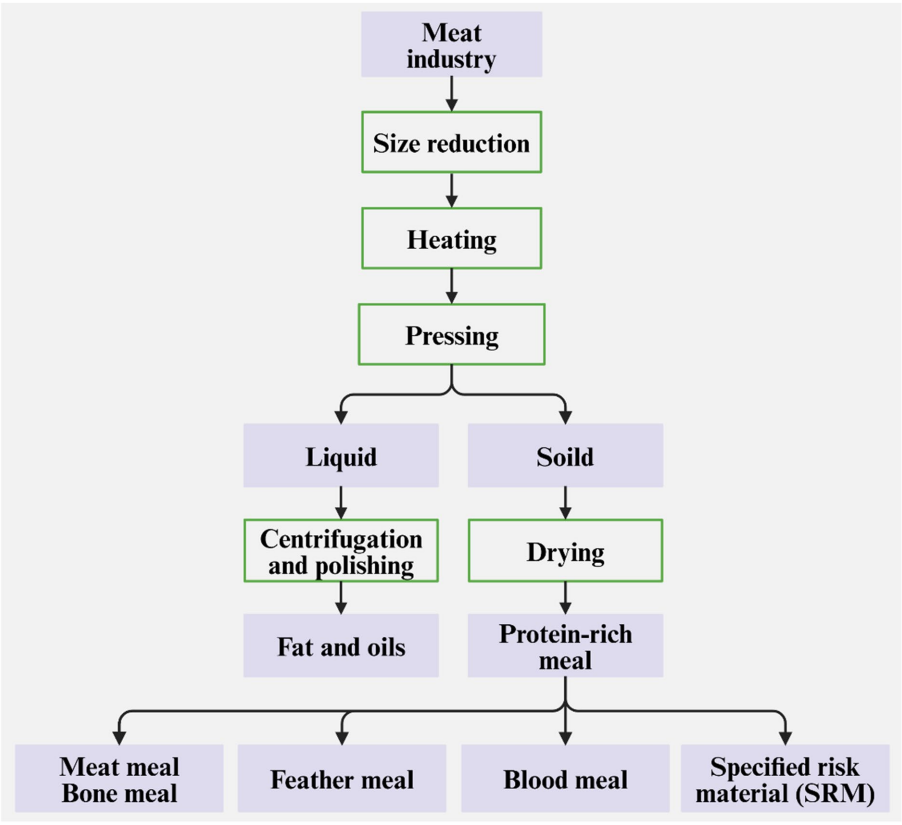

FIGURE 4
Basic process flow of rendering to recover fats, oils, and protein-rich meal products (Mekonnen et al., 2016).

and are good raw materials for the oleochemical industry to produce animal feed, soap and cosmetics, pet food, and more recently to produce biofuels (Mekonnen et al., 2016). The use of them to produce biodiesel can be realized through microemulsions, pyrolysis (Ben Hassen-Trabelsi et al., 2014), and transesterification reactions (Emiroglu et al., 2018; Keskin, 2018). The obtained proteins can be used to produce bio-based plastics, wood adhesives, surfactants, firefighting foams, and flocculants (Thakur et al., 2023). Therefore, the rendering process can be considered as a sustainable strategy of resource utilization, not only mitigating the environmental contamination caused by animal by-products and mortalities but also fully utilizing these discards. Due to the high temperature (up to 100°C), animal-borne pathogenic microbes [like *Listeria monocytogenes* and *Salmonella species* (Karyotis et al., 2017)] can be killed during rendering, but prion proteins that can cause transmissible spongiform encephalopathies cannot be destroyed. Consequently, certain cattle tissues, like the brain and spinal cord, denoted as the specified risk materials (SRM) due to the highest possibility of carrying prion proteins, are banned from rendering industries to produce protein-and fat-rich materials (Mekonnen et al., 2016).

4.4 Composting process

Composting is a sustainable and natural processing method to convert organic materials, including animal by-products and carcasses, food scraps, yard waste, and biogas residues, into soil amendment or fertilizers by passive or active methods (Gooding and Meeker, 2016; Lim et al., 2017). The passive methods include static piles and turned windrows, while the active ones include aerated static pile systems and in-vessel systems. The regular turning of windrows is one of the most important management techniques for composting, aimed at supplying enough oxygen for aerobic microorganisms (Hong et al., 2014). Regular turning can also expedite water evaporation and maintain proper temperature ranges for compost piles, promoting the maturity of compost piles and making the humus crumblier. Since the anaerobic fermentation inside piles is avoided by turning, the odors and potential nuisances for nearby communities are minimized. However, the regular turning of windrows is laborious and time-consuming (Wan et al., 2022). The time required ranges from months to a year, depending on materials, pile size, and desired decomposition. Thus, it requires sufficient space for piles, which may be a challenge for facilities with limited land availability.

During composting, animal by-products and carcasses are decomposed into simple or dissolved inorganic materials under aerobic microorganisms [mainly bacteria like *Actinomycetes* and *Bacteroidetes* (Huhe et al., 2017)] and finally converted into stabilized organic matter (compost or humus), which is a dark, crumbly substance rich in organic matter and essential nutrients like nitrogen, phosphorus, and potassium (Thomson et al., 2022). Therefore, it is a great soil amendment to ameliorate the fertility, structure, and moisture-holding capacity of soil and promote crop growth. To meet the needs of microorganisms' metabolism and promote the maturity

of compost piles, other organic materials, like sawdust (Michalopoulos et al., 2019), are usually mixed with animal by-products and mortalities for the carbon-nitrogen (C/N) ratio adjustment. Microorganism agents are also inoculated into compost piles (Gaind, 2014). With the use of humus, chemical fertilizers can be minimized, contributing to maintaining favorable physicochemical properties [like pH (Fleming et al., 2013)], nutrition, and structure for soil and sustainable agriculture practices. Moreover, by diverting from landfills or open-air storage, composting helps reduce the emissions of odors and volatile compounds during the breaking of organic matter, avoiding waterbody eutrophication and mitigating air pollution. As with the rendering, a properly managed composting process can effectively inactivate most pathogens, parasites, and weed seeds present in the wastes to be processed, for example, *Escherichia coli*, Ascaris eggs, cockspur grass seeds (Khadra et al., 2021; Rai et al., 2021), due to elevated temperature and pH (Lepesteur, 2022).

5 Biotechnological methods for extracting peptides from animal waste proteins

Although fat, proteins, minerals, and other organic matter are recovered or used based on rendering or composting processes, the values of these substances have not been fully reflected in the obtained products, especially the proteins. Numerous studies indicated that the protein hydrolysates of animal by-products contain various bioactive peptides, like antihypertensive peptides and antioxidant peptides, which are most beneficial for disease treatment (Vázquez et al., 2020; Ramakrishnan et al., 2023). Thus, using animal waste proteins as a source of bioactive peptides is a way to further expand their application range, transforming them into more valuable and profitable products than meat meals. Currently, two biotechniques, enzymatic hydrolysis, and microbial fermentation, have been considered to be the most valuable for decoding bioactive peptides from precursor proteins (Cruz-Casas et al., 2021). Their applications in valorizing animal waste proteins are detailed below (see Table 1).

5.1 Microbial fermentation

Microbial fermentation is the other typical biotechnological method to release bioactive peptides from animal waste proteins. It utilizes the proteolytic enzymes synthesized by indigenous or inoculating microorganisms during their metabolism to break down proteins into small molecules and release the peptides and amino acids (Nasri et al., 2022; Wen et al., 2023b). Therefore, the use and control of microorganisms is one of the most important factors for realizing complete hydrolysis of proteins and obtaining peptides with high bioactivity.

The microorganisms involved in fermentation are bacteria and fungi. Among the bacteria, the lactic acid bacteria are the most beneficial because of their safety, high proteolytic ability, and high adaptability (Cruz-Casas et al., 2021). Lactic acid bacteria are found not only in nature but also in people's digestive systems, for example, *Lactobacillus acidophilus* and *Lactobacillus Casei* (Krasaekoopt and Watcharapoka, 2014). It has been recognized as "generally recognized as safe" (GRAS) by the U.S. Food and Drug Administration (FDA) and has been used in food industries since the 1940s. The commonly used lactic acid bacteria genera include *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, and *Weissella* in food fermentations. The protein hydrolysis by lactic acid bacteria is generally divided into three steps: first, casein is broken into oligopeptides by cell envelope protease. Second, oligopeptides are transported into lactic acid bacteria cells by transporters, including permeases, ABC transporters, and antiports (Lorca et al., 2015). Finally, oligopeptides are hydrolyzed into small peptides or free amino acids by intracellular endopeptidases (e.g., calpins and cathespins) and exopeptidases (e.g., aminopeptidases) (Zou et al., 2023). Some lactic acid bacteria are widely employed in research due to their efficient hydrolysis performance, and they are *Lactococcus lactis*, *Lactobacillus helveticus*, and *Lactobacillus delbrueckii* ssp. *Bulgaricus*. For instance, *L. lactis* RQ1066 had a degree of hydrolysis of mung bean milk of 16.62 ± 0.75% after 24 h fermentation and 18.45 ± 0.29% after 48 h fermentation at room temperature (Liang et al., 2022). Lactic acid bacteria can adapt to various environments and change their metabolism accordingly. It was reported that a typical lactic acid bacteria is aerotolerant, acid-tolerant, organotrophic, and a strictly fermentative rod or coccus (König and Fröhlich, 2017). Compared to bacteria, fungi also have been used to ferment animal by-products but not so common due to the limited source and proteolytic activity (Sadh et al., 2018; Cruz-Casas et al., 2021).

The fermentation conditions and time should be carefully controlled to realize high protein hydrolysis of animal waste proteins and obtain peptides with high bioactivities. This is because fermentation conditions significantly affect the metabolism of microorganisms, and microorganisms will continue to break bioactive peptides for growth if fermentation time exceeds the optimal time length. The condition generally involves temperature, pH, moisture, nutrients, etc. (Melini et al., 2019). For instance, the prevailing anaerobic condition, low initial pH, and higher salt and sugar facilitate the growth of lactic acid bacteria. However, in some unsmoked meat products that should have a sour taste, the amount of the added lactic acid bacteria, sugar level, and water activity are carefully controlled (Kumar et al., 2017).

Compared to enzymatic hydrolysis, microbial fermentation is a more inexpensive biotechnique to extract bioactive peptides from animal waste proteins. This is because the microorganisms used and their culture processes are not costly (Akbarian et al., 2022). Besides, the microorganisms secrete an entire set of proteases, instead of one or several, which makes proteins in substrates adequate and shortens the production cycle of peptide-based products (Song et al., 2023). Additionally, if lactic acid bacteria are employed, the proteases secreted by them will expressed in the cell membrane, simplifying the subsequent purification of peptides (Agyei and Danquah, 2011). However, microbial fermentation has disadvantages of the generation of undesirable substances [like live bacteria, bacteria debris, exopolysaccharides, and organic acids (Mora-Villalobos et al., 2020)] and the implementation of optimal fermentation conditions. In a study, to recover proteins from monkfish by-products (heads and viscera), the effect of temperature, pH, and protease concentration was first investigated using a mixture of monkfish and water [ratio = 1:1 (w/v)], based on which the optimal fermentation conditions were obtained and were 57.4°C, pH 8.31, alcalase with a concentration of 0.05% (v/w), and 3 h for hydrolysis.

TABLE 1 Some application examples in the food industry of bioactive peptides and hydrolysates from animal waste proteins.

Animal source	By-product	Extraction method	Reaction condition	Effect	Method to oberve the effect	Application	References
Fish	Heads, skins, and skeletons of carp fish	Enzymatic hydrolysis with alcalase	pH 8.0, 55°C, and 3 h	Antioxidant	*In vitro* study: evaluating DPPH radical scavenging activity, hydroxyl radical scavenging activity, and ferric reducing power	Functional food ingredients and pharmaceutical products	González-Serrano et al. (2022)
	Waste meat of anchovy (*Coilia mystus*)	Homogenization and enzymatic hydrolysis with a mixture of alcalase, papain, and pancreatin	pH 6.8, 55°C, and 3 h	Memory improvement	*In vitro* study: conducting mouse behavioral trial and inhibition of acetylcholinesterase *In vivo* study: conducting H_2O_2-stressed PC12 cell assay and inhibition of acetylcholinesterase	Therapeutic potential for memory deficits	Su et al. (2016)
	Muscles of Gadidae	Enzymatic hydrolysis with pepsin	pH 3.0, 37°C, and 8 h	Antioxidant	*In vitro* study: evaluating DPPH radical scavenging activity	Safe food preservatives and functional food ingredients	Maky and Zendo (2021)
	Skin of unicorn leatherjacket	Autolysis and enzymatic hydrolysis with glycyl endopeptidase extracted from papaya	40°C and 1 h	Immunomodulation	*In vitro* study: determining the pro-inflammatory cytokine and NO production of RAW264.7 cells	Functional food ingredients	Karnjanapratum et al. (2016)
Pig	Liver	Enzymatic hydrolysis with papain, bromelain, alcalase, and flavourzyme	Papain: pH 6.0 and 37°C; bromelain: pH 6.0 and 40°C; alcalase: pH 8.0 and 50°C; flavourzyme: pH 5.5 and 50°C; 7 h	Antioxidant	*In vitro* study: evaluating DPPH radical scavenging activity, ABTS Radical scavenging activity, ferric reducing antioxidant power, and oxygen radical absorbance capacity	Functional food	López-Pedrouso et al. (2020)
Cattle	Skeletal muscles	Enzymatic hydrolysis with pepsin	pH 3.0, 37°C, 8 h	Antimicrobial activity		Safe food preservatives and functional food	Maky and Zendo (2021)
Chicken	Liver	Ultrasonic-assisted alkaline extraction	40°C; a pulsed on-time of 2 s and off-time of 3 s; 24 kHz and a maximum power of 300 W	Better surface hydrophobicity, water/oil holding capacity, and emulsifying properties	Fluorescence spectroscopy for determining surface hydrophobicity Suspending protein and water/oil in a centrifuge tube. Then vortexing and centrifuging samples for calculating water/oil holding capacity Absorbance measurement for calculating emulsifying activity and emulsion stability indexes	Food preservatives	Zou et al. (2017)

(Continued)

TABLE 1 (Continued)

Animal source	By-product	Extraction method	Reaction condition	Effect	Method to oberve the effect	Application	References
Goat	Deboning meat of Kacang goat (*Capra aegagrus* hircus)	Homogenization and enzymatic hydrolysis with flavourzyme and protamex	Step 1: protamex: pH 7.0, 50°C, and 1 h Step 2: Flavourzyme: pH 7.0, 50°C, and 1, 3, and 5 h	ACE inhibition and antihypertensive activity	*In vitro* study: measuring absorbances of sample, blank, and control solutions to calculating ACE inhibitory activity *In vivo* study: using spontaneous hypertensive rats (SHR) and performing oral administration for determining antihypertensive activity	Primary or supplement ingredients of functional food	Mirdhayati et al. (2016)

5.2 Enzymatic hydrolysis

Enzymatic hydrolysis is a way of using enzymes to cleave peptide bonds to liberate the encrypted peptides and has been widely used to extract bioactive peptides from animal waste proteins on an industrial scale (Figure 5). The often-used enzymes include pepsin, bromelain, trypsin, neutrase, chymotrypsin, alcalase, papain, flavourzyme, and protamex. The source of the often-used enzyme is usually well-known in the bioengineering field. For example, the papain is from papayas, and the trypsin is from the pancreas of pigs, cows, or sheep. Each enzyme has two crucial functions in catalyzing protein hydrolysis: binding affinity and catalytic performance. The protease contains one or several active sites with catalysis. The amino acid residue on the catalytic site can recognize and bind to a special peptide bond in the substrate, based on which protease can easily combine with the substrate and then for the enzyme-substrate complex. Besides, the amino acid residue on the catalytic site can supply the acidic or alkalic environment or functional groups required by catalytic reaction, based on which proteases promote the break of peptide bonds. After protease finishes hydrolyzing substrates, it will release products, return to the active state, and come into the next protein hydrolysis (Jovanović et al., 2016).

The protease used for hydrolyzing animal waste proteins had a wide source, including animal, plant, and microorganisms. The most used proteases are pepsin, trypsin, chymotrypsin, papain, bromelain, neutrase, alcalase (e.g., As1398 and protease K), flavourzyme, and protamex (Dey and Dora, 2014; Gajanan et al., 2016; Teshnizi et al., 2020; Tacias-Pascacio et al., 2021). For instance, the pepsin was usually obtained from porcine gastric mucosa and was used to hydrolyze the by-products of marine fishes, like the skin and bone of Spanish mackerel (Li et al., 2013) and the spines and skulls of skipjack tuna (Yu et al., 2014), to obtain collagen and peptides. Like microbial fermentation, appropriate conditions, including time, pH, temperature, enzyme specificity, and substrate/enzyme ratio, should be guaranteed to maximize the catalytic activity of proteases and the efficiency of enzymatic hydrolysis. A study found that the alcalase had the highest degree of hydrolysis (DH) for shrimp waste proteins (mainly consisting of head and shell of *Penaeus monodon*) and DH increased with temperature (50–60°C) during alcalase hydrolysis. The response surface graphs revealed that the optimal hydrolysis conditions were 59.37°C, pH 8.25, 1.84%, and 84.42 min (Dey and Dora, 2014). Another study observed that the porcine gastric mucin could not be hydrolyzed by pepsin at neutral pH because of the inactivity of stomach-derived pepsin at pH 7 (Schömig et al., 2016).

The enzymatic hydrolysis for animal waste proteins is characterized by mild reaction conditions and selectivity. The rational temperature range for most proteases is 50–60°C, and the higher one for some proteases does not exceed 70°C. The pH used for hydrolysis using microorganism-derived protease, which is more often used in hydrolyzing animal waste proteins on the industrial scale, is located at 5.5–8.0 (Dey and Dora, 2014; Razzaq et al., 2019). Therefore, this biotechnology has no high requirement of energy, facilities, and control, saving cost and simplifying management. In addition, due to the substrate specificity of protease, the enzymatic hydrolysis has remarkable regioselectivity, for instance, the preferential cleaving hydrophobic amino acid residues, especially the aromatic residues, of pepsin (Tavano, 2013). This substrate specificity offers an excellent suggestion to determine the protease for the given substrate, resulting in a high DH and hydrolysates with desirable compositions and properties. Moreover, no secondary products are generally generated during the enzymatic hydrolysis. The production of desirable amino acid sequences with secondary products renders the enzymatic hydrolysis ecologically sound.

However, enzymatic hydrolysis is plagued with low yield and high cost at an industrial scale. To improve DH, pretreatment is carried out, like thermal and acid treatment, but it has the risk of destroying peptides (Fauzi et al., 2016; Feng et al., 2017). The higher cost compared with microbial fermentation is attributed to the high price of protease (Aspevik et al., 2016). To solve the two problems, some technologies are incorporated into enzymatic hydrolysis, including microwave heating, ultrasound, high voltage electrical treatments (including pulsed electric field and electrical arc), and high hydrostatic pressure (Mikhaylin et al., 2017; Thoresen et al., 2020; López-Pedrouso et al., 2023b).

6 Applications of bioactive peptides and hydrolysates from animal waste proteins

6.1 Agricultural application of bioactive peptides and hydrolysates from animal waste proteins

Peptides derived from animal waste proteins have potential applications in agriculture. They can be used as natural fertilizers, biostimulants, and biopesticides, improving soil health, promoting

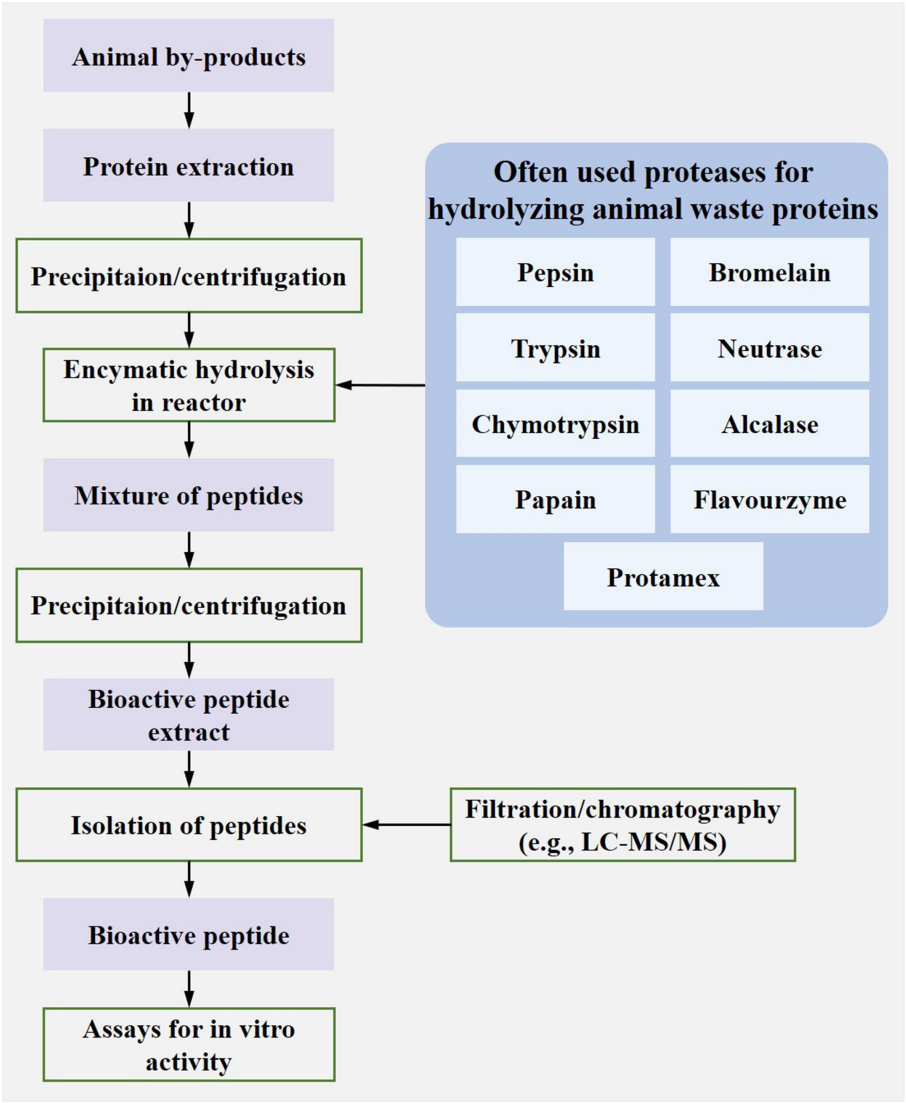

FIGURE 5
Schematic of the typical industrial production of bioactive peptides from animal waste proteins [modified from Mora et al. (2014)].

plant growth and yield, enhancing plant stress tolerance, and providing protection against pests and diseases (Da Silva, 2018).

6.1.1 Plant growth promotion

The ability to promote plant growth of animal waste proteins can be attributed to two aspects: ameliorating soil quality and directly enhancing the physiological processes. For one thing, the free amino acids, soluble proteins, and peptides that occur in hydrolyzed animal waste proteins can directly adjust the C/N ratio (Bhari et al., 2021). These nitrogen-containing nutrients also can increase the total count of heterotrophic bacteria in soil (e.g., nitrogen fixers and phosphate solubilizers), which is an important indicator for soil fertility, and thus change the rhizosphere microorganisms indirectly promote plant growth (Paul et al., 2013; Bhange et al., 2016). For another, amino acids, peptides, and proteins are the essential nutrients of plants and are required by a series of metabolic activities, including synthesizing nucleic acids, proteins, chlorophyll, vitamins, alkaloids, terpenoids, and forming vegetable tissues and organs. Thus, these materials from animal waste proteins can effectively promote plant growth in each stage, from seed germination to early root and shoot growth, and finally to blossom and fruition (Figures 6A,B) (Nurdiawati et al., 2019; Jagadeesan et al., 2023).

6.1.2 Abiotic stress tolerance enhancement of plants

Research demonstrates that the peptides and amino acids in animal waste protein hydrolysates can induce plant defense responses to some unfavorable conditions, including heat/cold, salinity, drought, and acidity, and thus enhance their tolerance to these abiotic stresses (Figure 6C) (Colla et al., 2015). A study used commercial animal-originated protein hydrolysates, neutralized with calcium salts, to treat *Diospyros kaki L.* cv. "Rojo Brillante" grafted on *Diospyros lotus L.* to explore their effects on the tolerance to soil affinity of *Diospyros lotus L. Diospyros lotus L.* is highly sensitive to salinity, especially chloride. The tree treated with protein hydrolysates had a lower leaf chloride uptake, stem water potential, and leaf necrosis than the untreated trees, indicating the used animal-originated protein hydrolysates

enabled an improved tolerance to salinity for persimmon trees. The improvement of tolerance to salinity was due to two complementary mechanisms of salts: first, Ca^{2+} enhanced the tree's ability to exclude chloride; second, the Ca^{2+}, proline, glycine, glutamate, and glycine betaine in the protein hydrolysates stimulated the tree's mechanism to increase compatible solutes proline and glycine betaine, which was indirectly demonstrated by the lower stem water potential (Visconti et al., 2015). The beneficial effect of proline on enhancing plant tolerances to abiotic stresses has also been observed in another study (Lucini et al., 2015), where a remarkable increase of proline occurred in lettuce under saline conditions. The enhanced tolerance to heat stress of the plant treated by animal-originated protein hydrolysates was observed in research. In a study, the lettuce (*Lactuca sativa L.* var. *capitata*) treated by Terra-Sorb Foliar, an animal-derived protein hydrolysate obtained by enzymatic hydrolysis, had a higher total fresh weight (root and aerial part) and stomatal conductance in three controlled cold environments, i.e., diurnal cold (4°C/20°C), nocturnal cold (22°C/2°C), and radicular cold (6°C at root zone and 4°C/20°C in air), than controlled environment (22°C/20°C). Besides, the heat stress tolerance of perennial ryegrass (*Lolium perenne L.*) under three temperatures (20°C, 28°C, and 36°C) was evaluated. The results showed that the ryegrass treated with Terra-Sorb falior had a higher photosynthetic efficiency and higher levels of photosynthetic pigments (chlorophylls and carotenoids). The comparison between Terra-Sorb falior treatment and the other three treatments (Terra-Sorb falior + nutrient solution, nutrient solution, and nutrient solution matching to Terra-Sorb falior) revealed that it was the biostimulant effect exerted by the amino acids in Terra-Sorb falior, instead of its nitrogen fertility effect, that enhanced the plant's tolerance to heat stress.

6.1.3 Biotic stress tolerance enhancement of livestock and fish

The huge demand for meat and fish brings about the continual development of an intensive culture of livestock and fish. In intensive livestock and fish farms, the animals have a high risk of infectious diseases caused by pathogens. The use of antibiotics is a quick and powerful method to control these diseases. However, it suffers from several adverse effects, including the development of drug resistance in animals and antibiotic residues in both animals and the environment, which pose a threat to food quality, environmental protection, and human health. The search for alternative strategies is important. In this sense, research found that some protein hydrolysates of animals could improve the disease resistance of farmed livestock and fish, and thus higher yield and healthier food was obtained.

The mechanisms that animal-originated protein hydrolysates improve animals' disease resistance involve immune stimulation, pathogen destruction, oxidative radical clearance, or stress and satiety adjustment. A study found that juvenile red seabream (*Pagrus major*) fed with the diet prepared by using about 5% protein hydrolysates (krill hydrolysates, shrimp hydrolysates, or tilapia hydrolysate) to

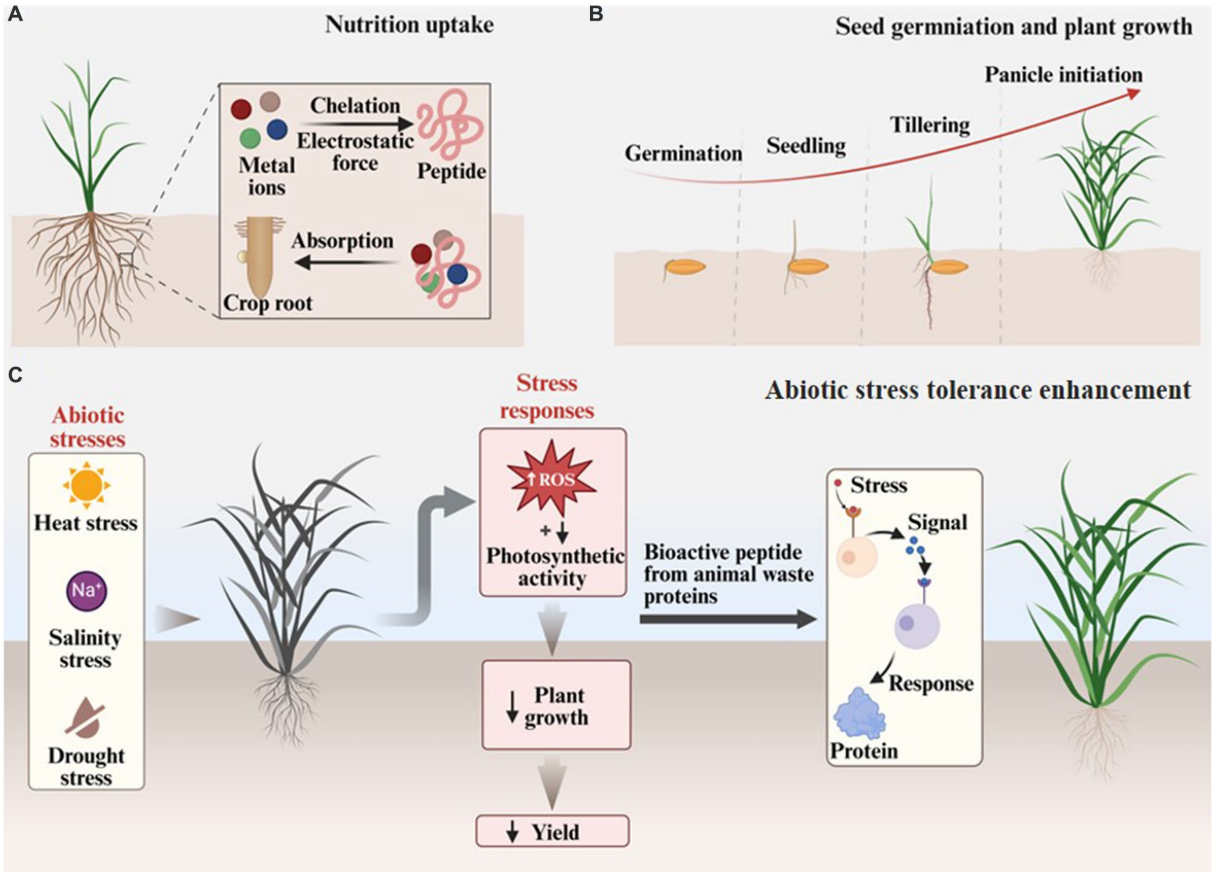

FIGURE 6
Agricultural application of animal waste proteins. **(A)** Nutrition uptake. **(B)** Seed germination and plant growth. **(C)** Abiotic stress tolerance enhancement.

replace 10% fish meal had increased antiprotease and superoxide dismutase activities and enhanced total immunoglobulin level than the fish fed with basal fish meal. Moreover, the juvenile red seabream fed with the prepared diet exhibited a higher resistance against *Edwardsiella tarda* (Bui et al., 2014). Another study also showed that the abalone (*Haliotis midae*) fed with a commercial protein hydrolysate at a low inclusion level (ACTIPAL HP 1, 6%) showed increased cellular immunity because the phagocytic activities of their hemocytes were improved by 18% compared to the control diet (Goosen et al., 2014). Some protein hydrolysates from animal by-products have exhibited antimicrobial effects against pathogenic species, like bovine hemoglobin hydrolysates (the main component of bovine cruor by-products), seafood skin hydrolysates, beef sarcoplasmic protein hydrolysates, and fish collagen (Anal et al., 2013; Zamorano-Apodaca et al., 2020; Beaubier et al., 2021). Compared with the antibiotics on the market, the peptides from animal waste proteins have a broader spectrum and faster action, despite they are not as powerful as antibiotics. Specifically, a study showed a peptide faction from collagen hydrolysates obtained by mixed by-products (skins, heads, and skeletons) of various fish species (pompano dolphinfish, seabass, squid, ray, snapper, weakfish, guitarfish, mullet, and different sharks) exhibited antimicrobial and antioxidant activities, which could be as a potential ingredient in both agricultural and pharmaceutical industries (Zamorano-Apodaca et al., 2020). In addition, the opioid-like peptides have been extracted from fish and bovine hemoglobin and can affect the nervous system, adjusting pain, sleep, and behavior, showing interesting applications as anti-stress agents (Lafarga and Hayes, 2014; Mora et al., 2014). They can also regulate the digestion and ingestion of food and can be used as satiety agents for animal obesity control (Iwaniak et al., 2018; Tyagi et al., 2020).

6.1.4 Post-harvest preservation

Postharvest storage is an equally important stage as seedling and growth for agriculture because the harvested products are perishable, especially the fresh vegetables and fruits. For instance, strawberries are susceptible to postharvest decay mainly due to gray mold and rhizopus rot caused by *Botrytis cinerea* (Pers.) and *Rhizopus stolonifer* (Ehrenb.) (Romanazzi et al., 2013). Traditionally, chemosynthetic fungicides are applied to retain the freshness of vegetables and fruits during the periods of storage and transportation. However, they are not permitted in the context of sustainable and organic agriculture because of environmental and health issues. In this regard, alternatives are required. Among these, resistant inducers can increase plant disease defenses and also can exert their antimicrobial activities, with the potential for large-scale application. Among the natural materials, protein hydrolysates produced with animal or plant extracts have gained scientific interest. A study found that six hydrolysates of casein, soybean, pea, lupin, malt, and yeast, with a concentration of 1.6 mg/mL, could significantly reduce the disease incidence and severity of wounded citrus fruit caused by *Penicillium digitatum*, the main postharvest pathogen of citrus fruit. Among the six hydrolysates, casein, lupin, and soybean exhibited the most powerful introduction of resistance. This indicated that these protein hydrolysates could be used as resistant inducers to extend the storage duration (Lachhab et al., 2015). Similarly, another study showed that when used in the field during grape growth, casein hydrolysates enable a gray mold incidence reduction of 94%. When used *in vivo* trials, the protein hydrolysates of casein could reduce gray mold by 54% at a concentration of 0.8 g/L. When simultaneously used before and after harvest, they enabled a storage rot reduction of 40% (Lachhab et al., 2016). These studies indicate protein hydrolysates enable an extension of the postharvest storage period, with a low risk of pesticide-resistant strains and a better satisfaction of increasingly high requirements of food safety (Albert, 2013). In addition, considering that the microbial infection of fruits may happen at the flowering phase (Romanazzi et al., 2016), a combination of preharvest and postharvest can further extend the storage periods of vegetables and fruits because the latent infection and pathogen inoculum in the field are decreased more compared the only use of postharvest (Lachhab et al., 2016).

6.2 Food application of bioactive peptides and hydrolysates from animal waste proteins

Protein hydrolysates are the best use form of protein concerning nutritional value with variety and balance of amino acids and high solubility. Through enzymatic hydrolysis (Figure 5), animal waste proteins can be decomposed into free amino acids and peptides, which are high-value substances used to develop new healthy foods as additives and functional ingredients and produce food-grade enzymes (Figure 7) (Sila and Bougatef, 2016; Zou et al., 2019).

6.2.1 Food additives

Plentiful studies have reported that the protein and its hydrolysates derived from animal by-products possess various favorable characteristics for food processing, such as antioxidant and antimicrobial activities and good abilities of foaming, emulsion, and fat adsorption, and have the potential use as additives (Lafarga et al., 2015; Lorenzo et al., 2018; Fang et al., 2020; Zhang et al., 2023b). The antioxidant peptides, used as food additives, can delay the irreversible decay of the food matrix from a few hours to several months and even years when proper strategies are implemented. For instance, a study extracted the α137–141 fragment (Thr-Ser-Lys-Tyr-Arg), a small (653 Da) and hydrophilic peptide, from bovine cruor, which contained mainly hemoglobin. The study found that the α137–141 (0.5%, w/w) reduced the lipid oxidation of ground beef by 60%, delaying its rancidity. Moreover, the α137–141 inhibited the growths of microbes, including coliform, mold, yeast, and lactic acid bacteria. These results indicated that the α137–141 possessed antioxidant and antimicrobial activities and could be used as functional ingredients for food preservatives (Przybylski et al., 2016). Interestingly, another study showed that four peptide fractions of collagen hydrolysates of common carp by-products (skeletons, skins, and heads) exhibited antioxidant activity and emulsifying and foaming properties. Among these peptide fractions, the fraction (<3 kDa) exhibited the strongest hydroxyl radical (95.4%, 10 mg/mL) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (87%, 1 mg/mL) scavenging activities and reducing power (0.34, 10 mg/mL), while the faction (>30 kDa) exhibited the greatest emulsifying activity index, foaming activity, and foaming stability, but the lowest emulsion stability (González-Serrano et al., 2022). The good foaming and emulsifying properties of proteins hydrolysates derived from fish by-products (skeletons, heads, and skins) (Zamorano-Apodaca et al., 2020) and porcine livers (Verma et al., 2019) have also been demonstrated.

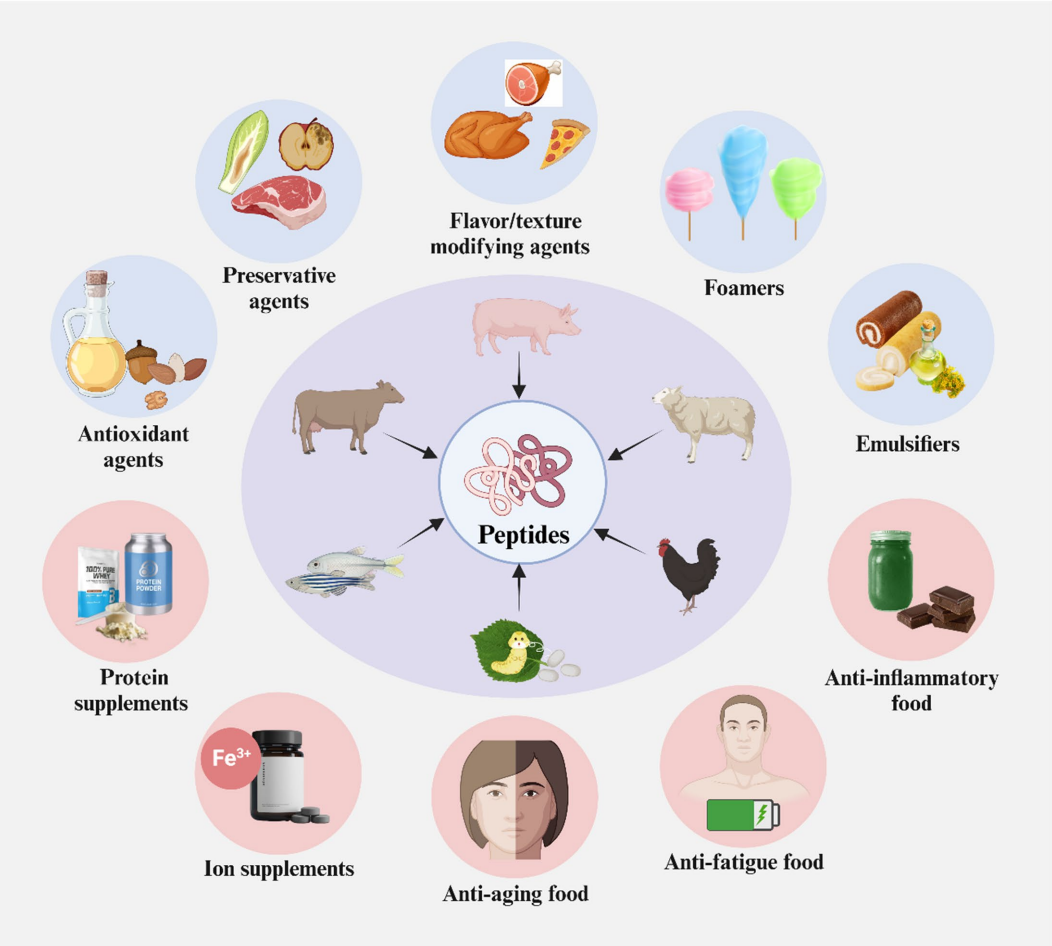

FIGURE 7
Food application of proteins and peptides derived from animal products processing wastes. Created with BioRender.com.

6.2.2 Functional foods

Meat and fish contain not only the complete set of essential amino acids but also based on these amino acids, contain plentiful bioactive peptides. Thus, the peptides or hydrolysates of animal waste proteins show potential use as functional ingredients for foods besides as basic nutritive sources. These functions involve antioxidants, iron supplements, fatigue resistance, and anti-inflammation (Zou et al., 2021). In a study, the porcine liver was hydrolyzed with different enzymes, time lengths, and membrane pore sizes. The hydrolysates obtained using alcalase at 8 h exhibited the strongest antioxidant activity: the fraction (>30 kDa) obtained using alcalase exhibited the best DPPH (562 μg/Trolox/g), ferric reducing antioxidant power (FRAP) (82.9 μmol Fe^{2+}/100 g), and oxygen radical absorbance capacity (ORAC) (53.2 mg Trolox/g) activities. The fraction (>30 kDa) obtained using bromelain at 4 h exhibited the strongest antimicrobial activity with a *Brochothrix* inhibition of 91.7% (Borrajo et al., 2020). More surprisingly, a study first extracted a peptide, AJHbα, with strong antimicrobial activities from the hemoglobin alpha chain in the liver of a Japanese eel (*Anguilla japonica*), finding that the AJHbα, with a molecular weight of 2,388.05 Da, could kill 8.64% ± 3.91% of *E. tarda* (Zhang et al., 2013). Similarly, a study observed the peptides and amino acids with antioxidant and anti-fatigue effects in monkfish hydrolysates using both *in vitro* and *in vivo* assays. The *in vivo* assay showed mice administrated with monkfish liver hydrolysates had a longer climbing period than the control group, and in their hepatic and kidney homogenate, a higher level of superoxide dismutase was detected (Xu et al., 2017). Another study extracted a tripeptide (Pro-Ala-Tyr) from salmon pectoral fin hydrolysates and found it could significantly inhibit the NO (63.80%), prostaglandin E2 (45.33%), and three pro-inflammatory cytokines syntheses in RAW264.7 cells because of its inhibitory effect on inducible NO synthesis protein and cyclooxygenase-2 (Ahn et al., 2015).

However, studies on peptides or protein hydrolysates of animal by-products used as ingredients of functional foods are limited, especially in clinical studies. Therefore, further studies are warranted to develop their uses in the food industry and bioavailability. Besides, some animal wastes, like the brain and spinal cord of cattle denoted as the specified risk materials (SRM), cannot be used to produce food ingredients due to the highest possibility of carrying prion proteins (Mekonnen et al., 2016). Moreover, different guidelines and safety assessments have been established by regulatory agencies, such as FDA [U.S. Food and Drug Administration (FDA), 2022] and the European Food Safety Authority (EFSA) [Madende and Hayes, 2020; European Food Safety Authority (EFSA), 2023], for the food use of extracts from animal by-products. Thus, thorough testing and evaluation are required before approval, though it has certain economic, environmental, and social benefits.

6.2.3 Food-grade enzymes

Multiple enzymes have been extracted from animal by-products and become one of the important ingredients in food processing. These enzymes generally are gastrointestinal proteases and include pepsin, trypsin, and chymotrypsin (Udenigwe and Howard, 2013). The pepsin can be extracted from the stomachs of pigs, cattle, and sheep. An excellent review found that pepsin was more often used to hydrolyze eggs to release ACE inhibitory peptides compared to the enzymes extracted from microorganisms (Lee and Hur, 2017). The trypsin and chymotrypsin are found in the small intestine and secreted by the duodenum (Sauer and Merchant, 2018). The three food-grade enzymes have been excessively used in hydrolyzing animal waste proteins, for instance, using pepsin to explore novel bioactive peptides in fish and beef skeletal muscles (Maky and Zendo, 2021) and to release antihypertensive peptides from bovine lactoferrin (Fernández-Musoles et al., 2014) and using trypsin to hydrolyze rice bran to obtain antioxidant and ACE inhibitory peptides (Wang X. et al., 2017). These food-grade enzymes can also be used together to enhance the release of encrypted peptides from parental proteins. For instance, the deer skin hydrolysates prepared by the combination of pepsin and trypsin to exhibited a much more potent DPP-IV inhibitory activity than that prepared by pepsin (Jin et al., 2015). Similarly, pepsin and pancreatin, an agent containing trypsin, were used together to treat trout frame proteins (Ketnawa et al., 2018). The discovery of these food-grade enzymes in animal waste proteins and the commercial versions based on them have played a huge role in the extraction of bioactive peptides from natural sources (Lee and Hur, 2017).

6.3 Medicinal application of bioactive peptides and hydrolysates from animal waste proteins

The peptides extracted from animal waste proteins exhibit various bioactivities. Besides, due to the cost-efficiency and the smaller possibilities of drug resistance and side effects, these peptides show a promising application prospect in the medicinal industry, on which much research has been done (Mahgoub et al., 2021; Wen et al., 2023b). Among these investigations, the antihypertensive, antioxidant, antimicrobial, antidiabetic, and antithrombotic activities of peptides extracted from animal waste protein have occupied much attention, and are summarized in the study.

6.3.1 Antihypertensive drugs

These years have witnessed a growth of hypertension due to the changes in diet and work styles, leading to an increasing demand for cost-effective and safe hypertensive therapy (Zaky et al., 2022). Numerous studies have found that naturally occurring peptides showed antihypertensive activity through different action mechanisms and could be an effective ingredient for hypotension (Khiari et al., 2014; Meinert et al., 2016; Mahdi and Ojagh, 2017; Pujiastuti et al., 2019; Bravo et al., 2023).

These action mechanisms can be classified into two types: renin-angiotensin system and kinin-arginine-nitric oxide system (Figure 8). In the renin-angiotensin system, some peptides can inhibit the release renin, an enzyme catalyzing the conversion of angiotensinogen to angiotensin I (Harnedy and FitzGerald, 2013), while some peptides can inactivate ACE, an enzyme that can catalyze the conversion of angiotensin I to angiotensin II and also the degradation of bradykinin, an enzyme that can relax blood vessels, to inactive peptide fragments in kinin-arginine-nitric oxide system (Siltari et al., 2016; Wang X. et al., 2017). Besides, some peptides act as angiotensin II receptor blockers, inhibiting angiotensin II-mediated vasoconstriction and releases of antidiuretic hormone and aldosterone, all of which can induce blood pressure (Fernández-Musoles et al., 2014). In the kinin-arginine-nitric oxide system, apart from the effect of inactivation ACE, the peptides rich in arginine contribute to synthesizing more nitric oxide, a substance that enables vasodilation, lowering blood pressure (Mas-Capdevila et al., 2019). A study showed found many peptides isolated from the fibrinogen hydrolysates of bovine slaughterhouse blood had ACE and renin inhibition. Among these peptides, a tripeptide SLR had ACE and renin inhibitory IC_{50} values of 0.17 and 7.29 mM and a peptide RR was resistant to gastrointestinal digestion (Lafarga et al., 2015). Similarly, the peptide fraction (<1 kDa) of the skin gelatin hydrolysate and bone gelatin hydrolysate of pangasius catfish (*Pangasius sutchi*) had ACE inhibitory IC_{50} values of 3.2 and 1.3 μg/mL respectively, higher than untreated gelatins and the other two fraction (>10 kDa and 3–10 kDa), but all three fractions showed resistance to gastrointestinal digestion. Besides, the fraction (<1 kDa) was rich in hydrophobic amino acids, like glycine and proline (Mahmoodani et al., 2014). A further study of Lafarga et al. (2015) identified three peptides (His-Phe, Tyr-Arg, and His-Arg) with both ACE and renin-inhibitory activities and one peptide (His-Leu-Pro) with ACE inhibitory activity in the bovine hemoglobin hydrolyzed by papain. His-Arg had ACE and renin-inhibitory IC_{50} values of 0.19 and 7.09 mM, respectively (Lafarga et al., 2016). These studies demonstrated that the peptide fractions could have a role in improving human health as a functional ingredient of drugs or nutraceuticals, but also reflect that there is a relationship between the antihypertensive activity and amino acid sequence and molecular weight and the bioavailability of peptide after administration should be considered, which necessitate the more studies.

6.3.2 Antioxidant drugs

When many more free radicals [like reactive oxygen/nitrogen species (ROS and NOS)] are produced in human bodies, causing the amount to exceed the scavenging capability of antioxidant enzymes and other antioxidants (like glutathione and vitamins), the free radicals that are not scavenged will oxidate nucleic acids, proteins, and lipids, leading to serious damage of cells and tissues. This phenomenon is called oxidative stress. If oxidative stress is not controlled, various illnesses might happen, such as tumors, aging, Parkinson's disease, and Alzheimer's disease (Forman and Zhang, 2021; Zhang et al., 2023a). In this sense, the study of antioxidants with high efficiency, safety, and low cost is significant for human well-being.

Many antioxidant peptides have been identified in protein hydrolysates of animal by-products, most of which consist of 4–16 amino acids and have a small molecular weight [e.g., a range of 0.4–2 kDa claimed by two studies (Khiari et al., 2014; Zaky et al., 2022)] (López-Pedrouso et al., 2020; Akbarian et al., 2022; González-Serrano et al., 2022; López-Pedrouso et al., 2023a). Although the mechanisms of antioxidant peptides to mitigate oxidative stress have not been clear yet, studies have found that the antioxidant effects of peptides are based on donating proton or electron to free radicals, chelating metals to prevent the production of free radicals, and trapping lipid peroxyl radicals and are related to the size,

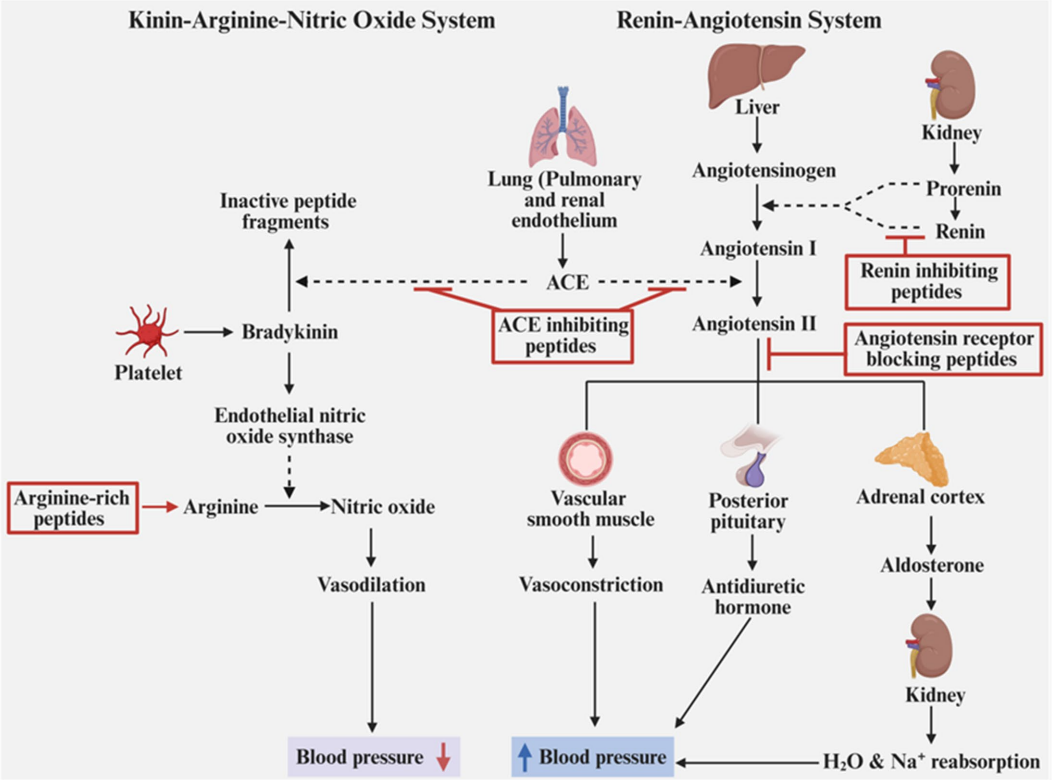

FIGURE 8
Mechanisms of the action of antihypotensive peptides. Created with BioRender.com.

hydrophobicity, and amino acid composition (Zaky et al., 2022). A study extracted three novel peptides with strong antioxidant activities from protein hydrolysate of bluefin leatherjacket skin. They are Gly-Ser-Gly-Gly-Leu, Phe-Ile-Gly-Pro, and Gly-Pro-Gly-Gly-Phe-Ile, with molecular weights of 389.41, 432.52, and 546.63 Da, respectively. Their antioxidant activities were evaluated by scavenging capabilities of free radicals, including DPPH•, HO•, and O_2^{-}•. These strong antioxidant activities were supposed to be due to the small size and the presence of hydrophobic and aromatic amino acid residues (Chi et al., 2015b). Smaller peptides are thought to have easier access to free radicals compared to larger ones and a higher possibility of crossing the intestinal barrier and exert antioxidant functions. Hydrophobic amino acids containing non-polar aliphatic groups, including leucine, isoleucine, proline, alanine, tryptophan, tyrosine, and valine, have a high reactivity to polyunsaturated fatty acids. Among aromatic amino acids, His, can donate protons to inactive free radicals, while tyrosine, tryptophan, and phenylalanine can donate electrons to free radicals to convert them into stable substances. Apart from free radical scavenging capabilities, some studies also demonstrated that the antioxidant activity of extracted peptides from animal waste proteins based on high FRAP values, for instance, a porcine liver hydrolysate (0.09%, w/w) with a FRAP value of 21.50 ± 0.78 (Verma et al., 2019) and the peptide LGEHNIDVLEGNEQFINAAK extracted from porcine liver hydrolysates with a positive correlation with FRAP (0.592) (López-Pedrouso et al., 2020). The ferric ion is a pro-oxidant metal, playing an important role in lipid peroxidation. Therefore, the conversion of ferric form to ferrous form enables a mitigation of lipid peroxidation. Some amino acids with reducibility, like Tyr and Trp, can chelate with ferric ions at their functional groups with lone pairs of electrons, like-NH2, contributing to the reduction of ferric ions.

6.3.3 Antimicrobial drugs

The antimicrobial effect exhibited by peptides of hydrolysates derived from animal waste proteins intrigues scientists, doctors, and health workers because it provides a safe alternative to antibiotics, which are deeply plagued with its induction of microorganism resistance in human bodies, farming animals, and even nature (Wang et al., 2016; Maky and Zendo, 2021).

Over 75% of antimicrobial peptides (AMPs) originated from animals, according to a statistic (as of September 2017) (Kumar et al., 2018). Based on the structure, antimicrobials can be classified into three categories: α-helical peptides, β-sheet peptides, and extended/flexible peptides. The AMPs originated from common animal (like bovines and pigs) waste proteins have all three structures, for instance, bovine myeloid AMP (BMAP)-27 and porcine myeloid AMP (PMAP)-36 both have α-helical structures (Lv et al., 2014; Yang et al., 2019). In terms of subcategory, most of these AMPs belong to cathelicidins, one of the most diverse vertebrate AMPs with 12–80 amino acids (Valdez-Miramontes et al., 2021). For instance, PMAP-36 has a sequence of GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGCG-NH_2. Despite that AMPs from different sources have different sequences and structures, they share several common points, including a net positive charge with a range of +2 to +13 (even to +14), hydrophobicity, and amphipathicity (Lv et al., 2014; Kumar et al., 2018). Many AMPs contain positively charged amino acids,

including leucine, arginine, or histidine, for instance, the AMP GLSRLFTALK derived from Anchovy (*Engraulis japonicus*) cooking wastewater (Tang et al., 2015). In AMPs, hydrophobic amino acids typically occupy 50% of the total. Hydrophobicity is a very important property for AMPs because it determines the extent to which AMPs partition into the membrane lipid bilayer when AMPs interact with microorganisms. Amphipathicity can be considered as a result of the balance between the cationic and hydrophobic residues at both the primary sequence level and the two-dimensional or three-dimensional structure of AMPs. The antimicrobial activity of peptides is closely related to their hydrophobicity, net charge, and hydrophobicity (Hollmann et al., 2016; Wang C.-K. et al., 2017).

These AMPs originated from animal waste proteins and are characterized by a wider spectrum of activity than those derived from microorganisms (Bhat et al., 2015). They can be used as the primary functional ingredient of antiviral, antibacterial, antifungal, and antiparasitic agents and exert their antimicrobial effect by acting on microorganisms or hosts (Figure 9) (Schmidt and Wong, 2013; Mahdi and Ojagh, 2017; Bechaux et al., 2019; Maestri et al., 2019; Yang et al., 2019). They can kill the microorganism by inhibiting the synthesis of nucleic acid (DNA and RNA) and proteins, proteins from functioning, cell wall formation, intercalating DNA, disrupting cell membranes, and activating autolysis. When acting on the host of pathogens, they can protect the host by binding or neutralizing microbial products, promoting the translation, stability, and processing of inflammatory cytokines, inhibiting Nuclear factor κB (NF-κB) movement, blocking the signal pathway of protein kinase, and activating immunocytes. For example, the leakage of unbroken cytoplasm and DNA fiber and the loss of cell integrity were observed in the treated *E. coli* by an AMP faction derived from camel whey hydrolysate, indicating the peptide exerted an antimicrobial effect through the inhibition of cell wall formation and disruption cell membrane (Abdel-Hamid et al., 2016).

6.3.4 Hypoglycemic drugs

Diabetes is a serious chronic disease and mainly includes Type 1 diabetes and Type 2 diabetes. Compared to Type 1 diabetes, Type 2 diabetes is much more common, occupying 90–95% of the total diabetes cases (Patil et al., 2015). It occurs when the body cannot generate or use insulin and thus blood glucose rises to a higher level. The generation of insulin is primarily modulated by two important peptide incretin hormones, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). However, these two incretin hormones can be rapidly cleaved by a metabolic enzyme, dipeptidyl peptidase-IV (DPP-IV) (Figure 10A) (Kęska et al., 2019). Therefore, the peptide with DPP-IV inhibitory activity has the potential for Type 2 diabetes treatment.

Many antidiabetic peptides have been identified in animal waste proteins, such as the peptides Gly-Pro-Phe-Pro-Leu-Pro-Asp and Gly-Ala-Thr-Phe-Gly-Phe-Phe-Tyr-Leu identified in porcine skin gelatin hydrolysate (Huang et al., 2014). Most of these peptides consist of no more than eight amino acids and have molecular weights of 200–2,000 Da. They generally have a sequence of X-Pro or X-Ala at the N-terminal, where X is a hydrophobic amino acid and probably has a small size. Considering that DPP-IV has specificity for cleaving X-Pro or X-Ala fragments from the N-terminal of peptides and proteins, antidiabetic peptides with a sequence of X-Pro or X-Ala at the N-terminal may act as substrate-type inhibitors (FitzGerald et al., 2014; Nongonierma et al., 2014; Jin et al., 2015). Differently, a study acquired 45 peptides with antidiabetic activity from the amphibian innated immune system through bioinformatic analysis, summarizing their proposed action mechanisms and recognizing their consensus amino acids, including alanine, glycine, lysine, and leucine (Figure 10B) (Soltaninejad et al., 2021). These proposed action mechanisms and main amino acids are not the same as those of the antidiabetic peptides mentioned above.

The antidiabetic effect of peptides has been thought to pertain to their sequence length, charge, and hydrophobicity (Kuo-Chiang et al., 2013), but there is no consensus in terms of the antidiabetic peptides derived from animal waste proteins. For instance, the tripeptide Gly-Pro-Hyp and tetrapeptide Gly-Pro-Ala-Gly, derived from a porcine skin hydrolysate fraction, exhibited DPP-IV inhibitory activity (IC_{50} = 49.6 and 41.9 μM, respectively) (Kuo-Chiang et al., 2013) similar to the pentapeptide Ile-Pro-Ala-Val-Phe derived from porcine skin hydrolysate (IC_{50} = 44.7 μM) (Silveira et al., 2013). Besides, the peptides Gly-Pro-Val-Gly-Hyp-Ala-Gly-Pro-Pro-Gly-Lys and Gly-Pro-Val-Gly-Pro-Ser-Gly-Pro-Hyp-Gly-Lys, derived from deer skin hydrolysate, exhibited similar DPP-IV inhibitory activity (IC_{50} = 83.3 and 93.7 μM, respectively) (Jin et al., 2015). Likely, the peptides Arg-Ala-Ser-Asp-Pro-Leu-Leu-Ser-val, Arg-Asn-Asp-Asp-Leu-Asn-Tyr-Ile-Gln, and Leu-Ala-Pro-Ser-Leu-Pro-Gly-Lys-Pro-Lys-Pro-Asp, derived from an egg-yolk protein by-product exhibited a similar DPP-IV inhibitory activity (IC_{50} ranging from 361.50 to 426.25 μM) (Zambrowicz et al., 2015).

These comparisons seem to mean that the antidiabetic peptides with similar sequence lengths have similar activity. However, the peptide Gly-Pro-Val-Gly-Pro-Ser-Gly-Pro-Hyp-Gly-Lys, also derived from deer skin hydrolysate, exhibited an antidiabetic activity (IC_{50} = 318.1 μM) much lower than the other two peptides consisting of 11 amino acids mentioned above (Jin et al., 2015). Additionally, based on the statistical analysis of 45 antidiabetic peptides from the amphibian innate immune system, a study supposed that the antidiabetic peptides with a higher net positive charge and weaker hydrophobicity exhibited a stronger insulinotropic effect (Soltaninejad et al., 2021). However, this relationship has not been observed in animal-derived peptides (Rivero-Pino et al., 2020; He et al., 2023; Li et al., 2023). Specifically, the study (Nasri et al., 2015) showed that two protein hydrolysates derived from goby fish using *Bacillus mojavensis* A21 protease fraction (HFFD + GPH-A) and triggerfish protease fraction (HFFD + GPH-TF), respectively, could reduce blood glucose level and hepatic glycogen and protect the kidney of high-fat-high-fructose diet (HFFD)-fed rats by reversing the HFFD-induced uric acid reduction and creatinine level increase in serum and preventing some HFFD-induced changes in the kidney (including tubular dilatation, glomerular space, vacuolization, and epithelial cells necrosis of the proximal tubule) (Figure 10C). However, the amino acid composition analysis showed higher percentages of hydrophobic amino acids (41.33 and 38.42%) in both HFFD + GPH-A and HFFD + GPH-TF.

6.3.5 Antithrombotic drugs

Apart from hypertension, thrombosis (i.e., the blood clotting inside the vessels) is another major cause of cardiovascular disease and can lead to several serious results, including paralysis, myocardial

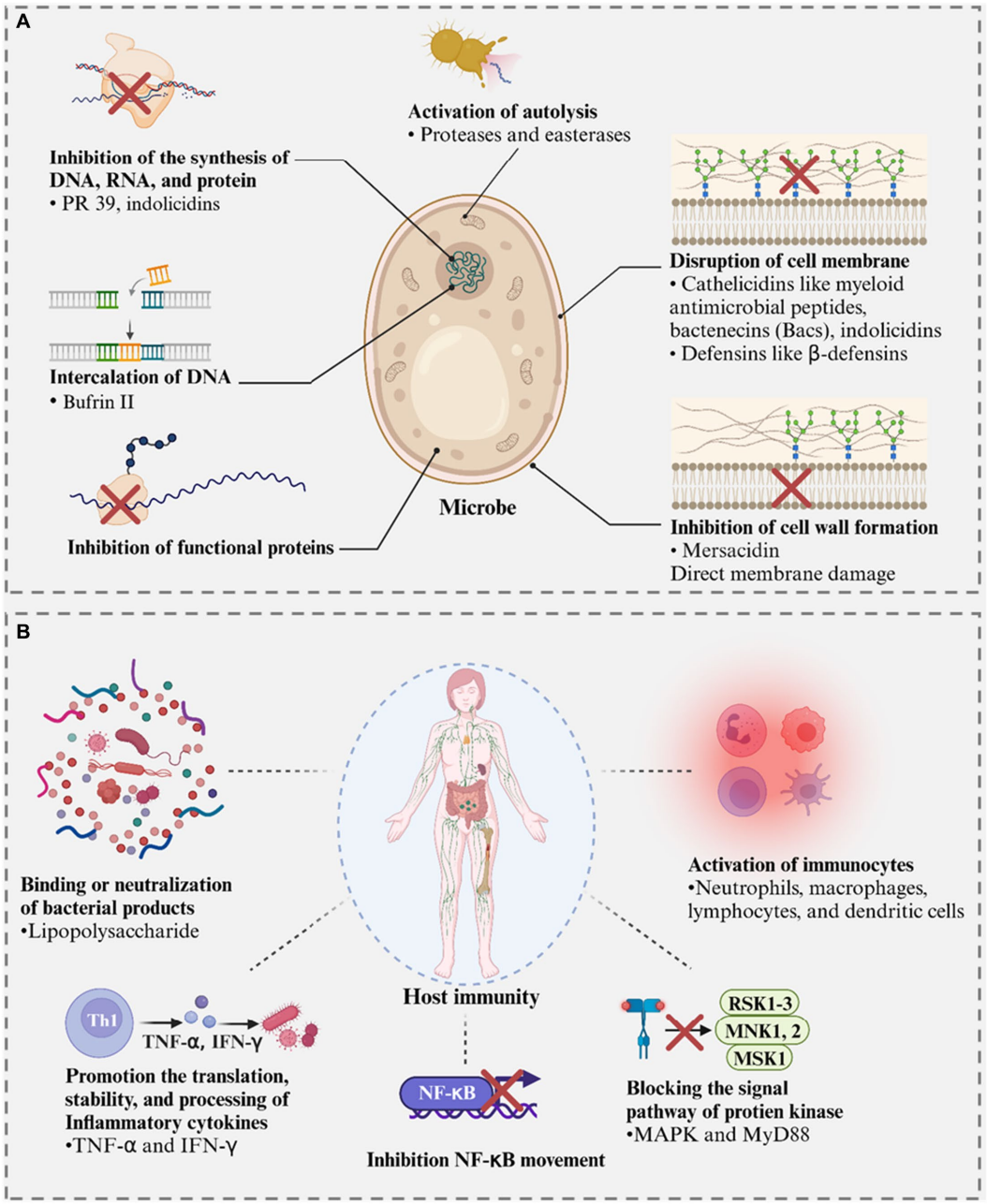

FIGURE 9
Mechanisms of the action of antimicrobial peptides (AMPs). **(A)** Inhibiting the growth of microbes and killing microbes. **(B)** Activating immune response and reducing the impact of microbial products. Created with BioRender.com.

infarction, and vascular diseases. Due to the safety and comparable antithrombotic effect to synthetic drugs [like aspirin and heparin (Indumathi and Mehta, 2016; Cheng et al., 2018)], antithrombotic peptides derived from animal waste proteins are considered to be a good alternative to them and have continuously gained attention in the past 10 years, though not as much as the aforementioned four kinds of bioactive peptides (Madhu et al., 2022; Wen et al., 2023a). For instance, a study found that the mackerel skin gelatine hydrolysate exhibited high antithrombotic activity, probably owing to the presence of the peptide tripeptide Phe-Gly-Asn with a molecular weight of 337 Da (Khiari et al., 2014).

Anticoagulants are one of the main therapeutic drugs for antithrombic diseases. Fortunately, research has identified anticoagulant peptides in animal meat and by-products (Kong et al., 2014; Cheng et al., 2018; Qiao et al., 2018; Bezerra et al., 2019; Ucak et al., 2021). The anticoagulant effect of these peptides is often evaluated by the extension of activated partial thromboplastin time, thrombin time, and prothrombin time. The study (Bezerra et al., 2019) proved that the peptides extracted from the hydrolysate of a mixture of chicken combs and wattles were anticoagulant and very ACE-inhibitory. The anticoagulant effect was achieved by activating partial thromboplastin time.

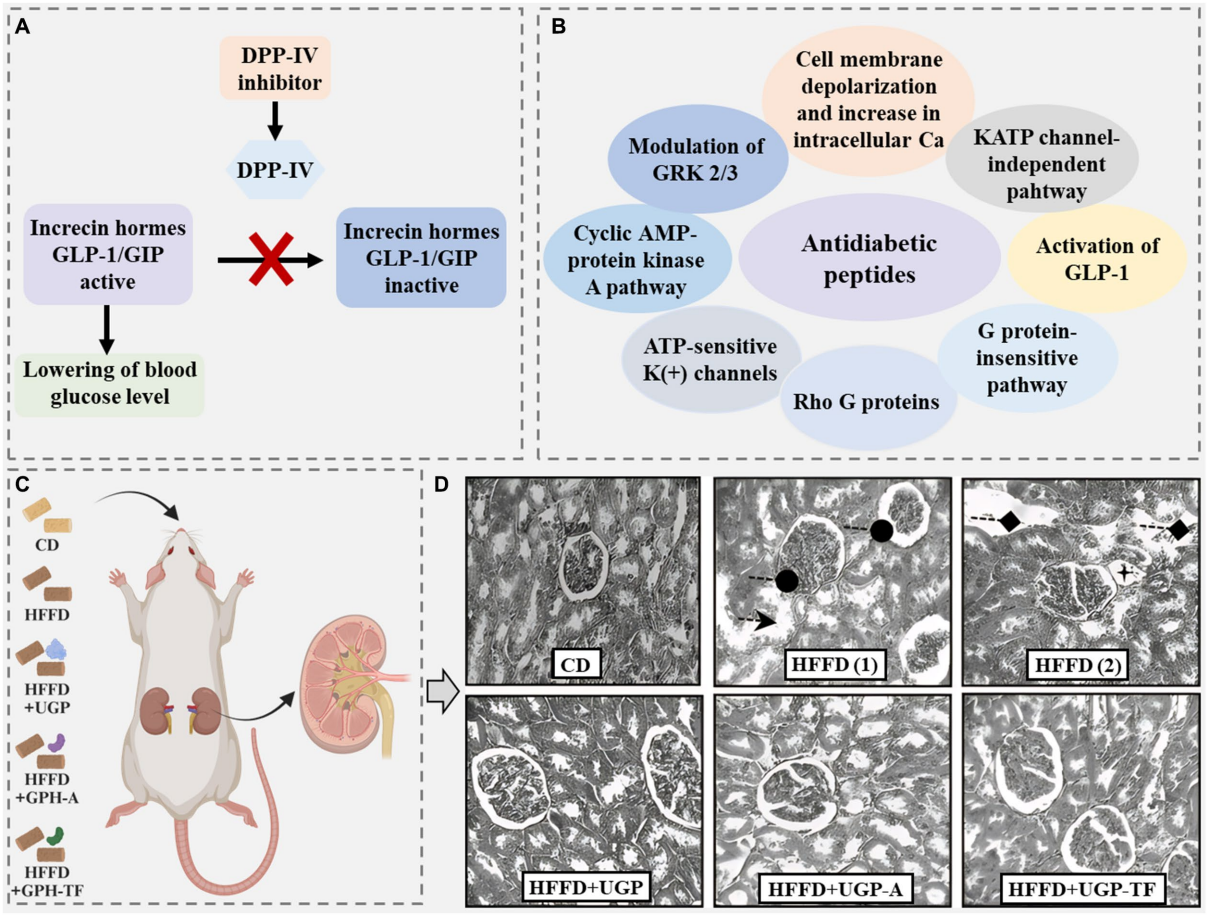

FIGURE 10
(A) Proposed mechanisms of action for antidiabetic peptides (Soltaninejad et al., 2021). **(B)** Scheme of the activity of the dipeptidyl peptidase IV (DPP-IV) inhibitor. GLP-1, glucagon-like peptide-1; GIP, glucose-dependent insulinotropic peptide (Kęska et al., 2019). **(C)** Wistar rat-feeding assay by different diets: CD, control diet; HFFD, high-fat-high-fructose diet; HFFD + UGP, HFFD + undigested goby fish muscle proteins; HFFD + GPH-A, HFFD + goby fish muscle protein hydrolysate obtained with the *Bacillus mojavensis* A21 protease fraction; HFFD + GPH-TF, HFFD + goby fish muscle protein hydrolysate obtained with the triggerfish protease fraction. **(D)** Histopathology of kidney tissues from CD group, high-fat-high-fructose diet (HFFD) group [(1) and (2)], HFFD + undigested goby fish muscle proteins (HFFD + UGP) group, HFFD + (HFFD+GPH-A) group, and HFFD + (HFFD + GPH-TF) group. Photomicrographs were taken by optic microscopy: ×200. - - ➤: tubular dilatation; - - ●: glomerular space; - - ◆: vacualization; ✦: epithelial cells necrosis of the proximal tubules. Modified from Nasri et al. (2015).

7 Challenges

Animal waste proteins will continue to grow worldwide, along with consumer demands. Their adding-value applications through advanced biotechnological methods in agricultural, food processing, and medical fields not only mitigate the environmental pressure brought by their discard but also bring new opportunities for the progress of agriculture, food processing, and medicine. However, these adding-value applications also face some challenges, including bioavailability or stability during gastrointestinal digestion and the relationship between the bioactivity and properties of peptides.

7.1 Stability and bioavailability of bioactive peptides from animal waste proteins

The stability and bioavailability of bioactive peptides are extremely important to exert their activities in functional food and pharmaceuticals. After oral administration, peptides may be decomposed into smaller molecules during gastrointestinal digestion, resulting in the reduction and even loss of their activities (Ketnawa et al., 2018; Wang K. et al., 2021; Cai et al., 2022; Zhang et al., 2023b). For example, a study identified many peptides with ACE, renin, and DPP-IV inhibitory activities in bovine fibrinogen fraction. However, the computer simulation of gastrointestinal digestion predicted some peptides were cleaved by pepsin, trypsin, and chymotrypsin into amino acids (Lafarga et al., 2015). Therefore, the resistance of peptides to gastrointestinal digestion should be considered when assessing their effects on human bodies and animals. Recently, the computer simulation of proteolysis has been used for predicting the decomposition of proteins in the gastrointestinal system, like the ExPASy PeptideCutter used in Lafarga et al. (2015) and the BIOPEP-UWM database used in Kęska et al. (2019). However, in the gastrointestinal system of humans and animals, the digestion and adsorption of proteins is a more complicated process than the simulated cleavage of proteins *in silico* analysis, due to the effect of multiple factors, including intestinal motility and body temperature fluctuation (Li et al., 2020; Sensoy, 2021).

Therefore, *in vivo* study and the subsequent clinic trial are essential to explore the absolute bioavailability and effect of bioactive peptides after being oral administration. The combination of *in silico* analysis and *in vivo* study may open new avenues in the rapid development of functional food and pharmaceuticals with bioactive peptides as primary ingredients (Ketnawa et al., 2018).

7.2 Action mechanisms of bioactive peptides from animal waste proteins

The comprehension of the action mechanisms of bioactive peptides is fundamental for them to be used as functional ingredients in industrial products. The action mechanisms of bioactive peptides are associated with their amino acid composition, structure, hydrophobicity, and charge. The comprehension of these associations will be beneficial to the identification of novel bioactive peptides and the synthesis of bioactive peptides. However, the association of some bioactivities of peptides with their feathers has not been clear yet. As mentioned above, the peptides with X-Pro or X-Ala (X represents a hydrophobic amino acid) at the N-terminal derived from livestock or aquatic product protein hydrolysates are competitive with GLP-1 GIP as the substrate of DPP-IV, based on which these peptides enable the reduction of blood glucose level. However, a database analysis of amphibian-originated antidiabetic peptides concluded that these peptides exert the antidiabetic through eight different mechanisms and have four consensus amino acids (alanine, glycine, lysine, and leucine), an average sequence length of 22.24, and an average net charge of 3.50 (Soltaninejad et al., 2021), quite different from features of those derived from livestock and aquatic sources, including DPP-IV inhibition, no more than eight amino acids, and richness in proline.

8 Conclusion

This paper reviewed the advancement of the value-added application of animal waste proteins in the past decade. Microbial fermentation and enzymatic hydrolysis are the most favorable biotechnological methods to treat animal waste proteins to decode bioactive peptides from parental proteins, especially enzymatic hydrolysis, which is more efficient at producing the peptide with a specific activity and more used in finding novel bioactive peptides in animal waste proteins, than microbial fermentation. The bioactive peptides produced enable the promotion of whole-life growth of plants, enhancement of both abiotic and biotic stress tolerance, and prolongation of post-harvest preservation of agricultural products. In the food industry, these peptides have been used as additives and primary functional ingredients. Besides, animal waste proteins are also good sources of food-grade enzymes. In addition, these bioactive peptides show a prospective application in medicine as a functional ingredient, including antihypertension, antioxidant, hypotensive, and antithrombosis. These value-added applications of animal waste proteins may be a step towards sustainable animal by-products management and circular bioeconomy and, simultaneously, open new avenues in the rapid development of nutraceuticals and pharmaceuticals.

Author contributions

SB: Writing – review & editing, Writing – original draft. QW: Writing – review & editing, Writing – original draft. LZ: Writing – review & editing, Project administration, Conceptualization. YC: Writing – review & editing. JY: Writing – review & editing. PC: Writing – review & editing. YS: Writing – review & editing, Supervision. TZ: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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Conflict of interest

YC was employed by Jiangsu Weiguang Biotechnology Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Glossary

ACE-I	Angiotensin-converting enzyme inhibitory
AMP	Antimicrobial peptide
BMAP	Bovine myeloid antimicrobial peptide
C/N ratio	Carbon-nitrogen ratio
DH	Degree of hydrolysis
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPP-IV	Dipeptidyl peptidase-IV
EFSA	European Food Safety Authority
FDA	The U.S. Food and Drug Administrating
FRAP	Ferric reducing antioxidant power
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide 1
GPH	Goby fish muscle proteins
GRAS	Generally recognized as safe
HFFD	High-fat-high-fructose diet
HFFD + GPH-A	HFFD + goby fish muscle protein hydrolysate obtained with the *Bacillus mojavensis* A21 protease fraction
HFFD + GPH-TF	HFFD + goby fish muscle protein hydrolysate obtained with the triggerfish protease fraction
IC_{50}	Half maximal inhibitory concentration
INF-γ	Interferon γ
MAPK	Mitogen-activated protein kinase
MyD88	Myeloid differentiation 88
NF-κB	Nuclear factor κB
NOS	Reactive nitrogen species
ORAC	Oxygen radical absorbance capacity
PMAP	Porcine myeloid antimicrobial peptide
ROS	Reactive oxygen species
SRM	Specified risk material
TNF-α	Tumor necrosis factor α
UGP	Undigested goby fish muscle proteins

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Neurodegenerative diseases and catechins: (–)-epigallocatechin-3-gallate is a modulator of chronic neuroinflammation and oxidative stress

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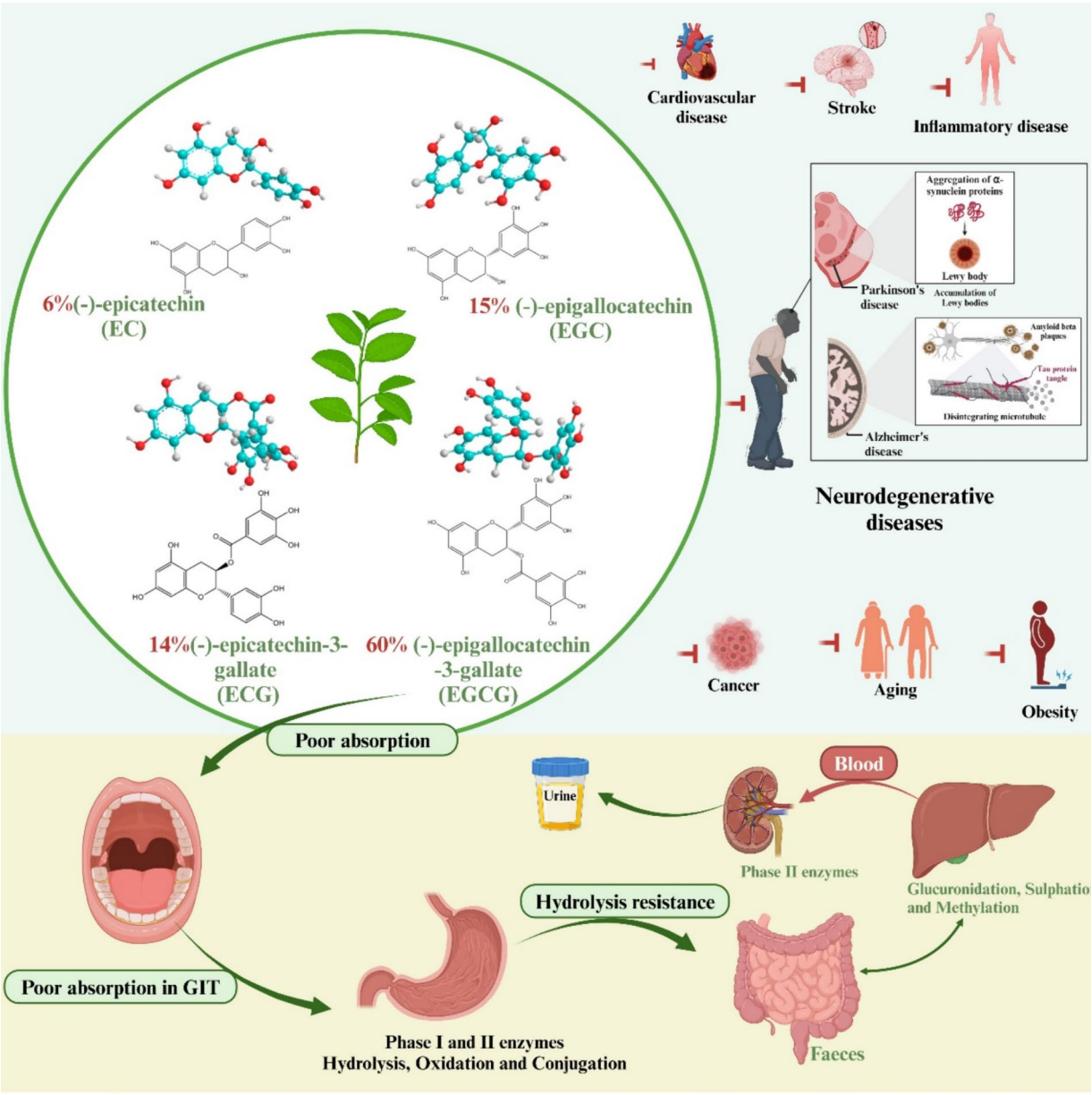
Catechins, a class of phytochemicals found in various fruits and tea leaves, have garnered attention for their diverse health-promoting properties, including their potential in combating neurodegenerative diseases. Among these catechins, (–)-epigallocatechin-3-gallate (EGCG), the most abundant polyphenol in green tea, has emerged as a promising therapeutic agent due to its potent antioxidant and anti-inflammatory effects. Chronic neuroinflammation and oxidative stress are key pathological mechanisms in neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). EGCG has neuroprotective efficacy due to scavenging free radicals, reducing oxidative stress and attenuating neuroinflammatory processes. This review discusses the molecular mechanisms of EGCG's anti-oxidative stress and chronic neuroinflammation, emphasizing its effects on autoimmune responses, neuroimmune system interactions, and focusing on the related effects on AD and PD. By elucidating EGCG's mechanisms of action and its impact on neurodegenerative processes, this review underscores the potential of EGCG as a therapeutic intervention for AD, PD, and possibly other neurodegenerative diseases. Overall, EGCG emerges as a promising natural compound for combating chronic neuroinflammation and oxidative stress, offering novel avenues for neuroprotective strategies in the treatment of neurodegenerative disorders.

KEYWORDS

catechins, (–)-epigallocatechin-3-gallate, neurodegenerative diseases, Alzheimer's disease, Parkinson's disease

1 Introduction

Catechins, a class of physiologically active phytochemicals, are commonly found in the fruits and leaves of various plants, including tea, apricots, cherries, peaches, blackberries, strawberries, blueberries, raspberries, and cocoa (1). Research indicates that catechins possess numerous health-promoting properties, notably benefiting cardiovascular disease, metabolic syndrome, diabetes, cancer, stroke, and neurodegenerative diseases (Figure 1) (2–9). As predominant polyphenols in tea, constituting approximately 30% of the dry mass of tea leaves, catechins serve as key functional components. Major green tea polyphenols encompass (–)-epicatechin (EC), (–)-epicatechin gallate (ECG), (–)-epigallocatechin



	Molecular formula	Molecular weight	Number of phenoile OH groups
EGCG	$C_{22}H_{18}O_{11}$	458.38	8
EGC	$C_{15}H_{14}O_7$	306.27	5
ECG	$C_{22}H_{18}O_{10}$	442.37	7
EC	$C_{15}H_{14}O_6$	290.3	4

Green tea catechin profile	EGCG (10–15%) > EGC (6–10%) > ECG (2–3%) > EC (2%)
Anti-inflammatory	EGCG > EGC > ECG > EC
Radical scavengers	EGCG > ECG > EGC > EC
Reduced efficiency of lipid peroxidation	EGCG > ECG > EGC > EC

FIGURE 1
The chemical structures of four common green tea catechins are depicted. Their potential as therapeutic agents for common diseases is discussed. Additionally, the absorption and metabolism of green tea catechins are explored, accompanied by diagrams illustrating the absorption process across various organs of the body. Molecular formula, molecular weight, number of phenoile OH groups of four common catechins. Comparison of the four common catechins in green tea catechin profiles, anti-inflammatory, radical scavengers, and reduced efficiency of lipid peroxidation effects.

(EGC), and (−)-epigallocatechin gallate (EGCG) (Figure 1) (10, 11). EGCG, the most abundant among green tea catechins at 60%, garners significant interest due to its broad spectrum of benefits elucidated in clinical trials, animal studies, and cell culture research (12). The molecular weight of EGCG is 442.37. Mechanisms underlying EGCG's multifaceted health effects include antioxidant properties, anti-inflammatory activity, interactions with plasma membrane proteins, activation of second messenger and signaling pathways, modulation of metabolic enzymes, and promotion of autophagy (13–15).

Neurodegenerative diseases manifest through the gradual and progressive degeneration of nerve cells in defined regions of the brain and spinal cord, leading to functional impairment. Prominent examples encompass Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) (16–18). Although the specific cellular and molecular mechanisms vary across these diseases, common features include oxidative stress, mitochondrial dysfunction, DNA damage, protein aggregation, and neuroinflammation (18, 19). Notably, chronic neuroinflammation and oxidative damage represent shared pathological hallmarks among all neurodegenerative diseases (20, 21). Neuroinflammation serves as a common defense mechanism to protect the brain by removing or inhibiting various pathogens (22). This inflammatory response plays a crucial role in facilitating tissue repair and preserving tissue homeostasis (23). Typically, neuroinflammation abates upon successful tissue repair or pathogen clearance (22, 24). However, when the inflammatory stimulus persists, chronic neuroinflammation ensues (22, 25). Various factors contribute to sustained inflammatory responses, including protein aggregation, systemic infections, gut microbiota dysbiosis, aging, and genetic mutations. Prolonged activation of microglia and astrocytes, key players in neuroinflammation, can precipitate neurodegenerative diseases (26–28). Furthermore, neurons exhibit heightened susceptibility to oxidative damage, attributed to their elevated content of unsaturated fatty acids, rendering them susceptible to free radical attack and peroxidation. Additionally, increased levels of iron in specific brain regions further augment neuronal vulnerability to oxidative stress (29). Consequently, interventions targeting anti-neuroinflammatory and antioxidant pathways hold particular significance in the context of neurodegenerative diseases.

EGCG, a natural polyphenol abundant in green tea, exhibits promising neuroprotective properties attributed to its potent anti-inflammatory and antioxidant activities (12). Accumulating evidence underscores its therapeutic potential in the prevention and treatment of neuroinflammatory and neurodegenerative disorders (30). EGCG demonstrates notable neuroprotective efficacy by modulating signals implicated in autoimmune responses, enhancing interplay between the nervous and immune systems, and effectively attenuating inflammatory processes. Furthermore, EGCG exhibits iron chelation capabilities, scavenges free radicals, and exerts significant antioxidant effects, as evidenced by pertinent studies (31). Therefore, this review comprehensively explores the role of EGCG in various neurodegenerative conditions, particularly AD and PD, with a focus on elucidating its molecular mechanisms underlying anti-neuroinflammatory and antioxidant actions.

2 Antioxidant and anti-inflammatory effects of EGCG

Multiple investigations have substantiated the beneficial impact of green tea on neurodegenerative disorders. For instance, Shinichi Kuriyama et al. studied 1,003 elderly individuals aged over 70 years to assess the influence of green tea intake on cognitive function (32). Their findings revealed that subjects consuming more than 100 mL of green tea twice daily exhibited reduced susceptibility to neurodegenerative diseases (32). Similarly, Hu et al. conducted a 13-year longitudinal study involving nearly 30,000 Finnish adults, demonstrating that individuals consistently consuming over 600 mL of green tea daily exhibited a diminished risk of developing PD (33). These observations underscore the association between green tea consumption and a lowered incidence of neurodegenerative conditions.

The health-promoting bioactive components of green tea catechins include a wide range of isomers, the most representative of which are mainly four (EGCG, ECG, EGC and EC), with EGCG accounting for the vast majority of green tea research (34, 35). The biological action of the molecule will be determined by its chemical structure. EGCG ($C_{22}H_{18}O_{11}$) is a catechin flavanol, specifically a gallate ester formed by the condensation of gallic acid with the (3R)-hydroxyl group of (−)-epigallocatechin, labeled A, B, C, and D (Figure 2) (36). The pentacosanoyl group esterification on Carbon −3 of the C-ring, along with hydroxyl groups on Carbon −3′, −4′, and −5′ of the B-ring, underlie EGCG's robust antioxidant activity compared to other catechins. The D- and B-rings contribute to its reactive oxygen species (ROS) neutralizing properties, with the D-ring further enhancing its anticancer and anti-inflammatory attributes. EGCG has seven hydroxyl groups in its aromatic ring. The location and number of hydroxyl groups on the ring determines its biological activity, giving EGCG greater antioxidant properties than EGC or EC, as well as water solubility, making EGCG highly permeable to the blood–brain barrier (BBB) (37). EGCG has been reported to cross the BBB within 0.5 h. Moreover, EGCG features two structures—the ortho-3′,4′-dihydroxy moiety and the 4-keto, 3-hydroxyl, or 4-keto, and 5-hydroxyl moiety—that can chelate metal ions, thereby neutralizing their activity. In essence, EGCG's distinctive chemical structure and composition confer potent antioxidant and anti-inflammatory properties, suggesting potential benefits in select neurodegenerative disorders (38).

Following oral administration, EGCG undergoes limited absorption by the intestines, resulting in minimal entry into the bloodstream and tissues (39). The constrained bioavailability of orally administered EGCG arises from factors including extreme pH conditions, digestive enzymes, and EGCG's restricted membrane permeability within the intestinal wall (9). Within the body, EGCG undergoes extensive biotransformation via sulfonation, glucuronidation, and methylation reactions (39). Its half-life is approximately 3.9 h, with complete metabolism occurring within 24 h (40). Furthermore, the biological effects of EGCG are contingent on concentration levels. Plasma concentrations ≤10 μM elicit antioxidant, anti-inflammatory, and insulin-sensitizing effects. Conversely, plasma EGCG levels exceeding 10 μM may induce pro-oxidant activity, augmenting autophagy and cell death, and are commonly employed in tumor therapy (41).

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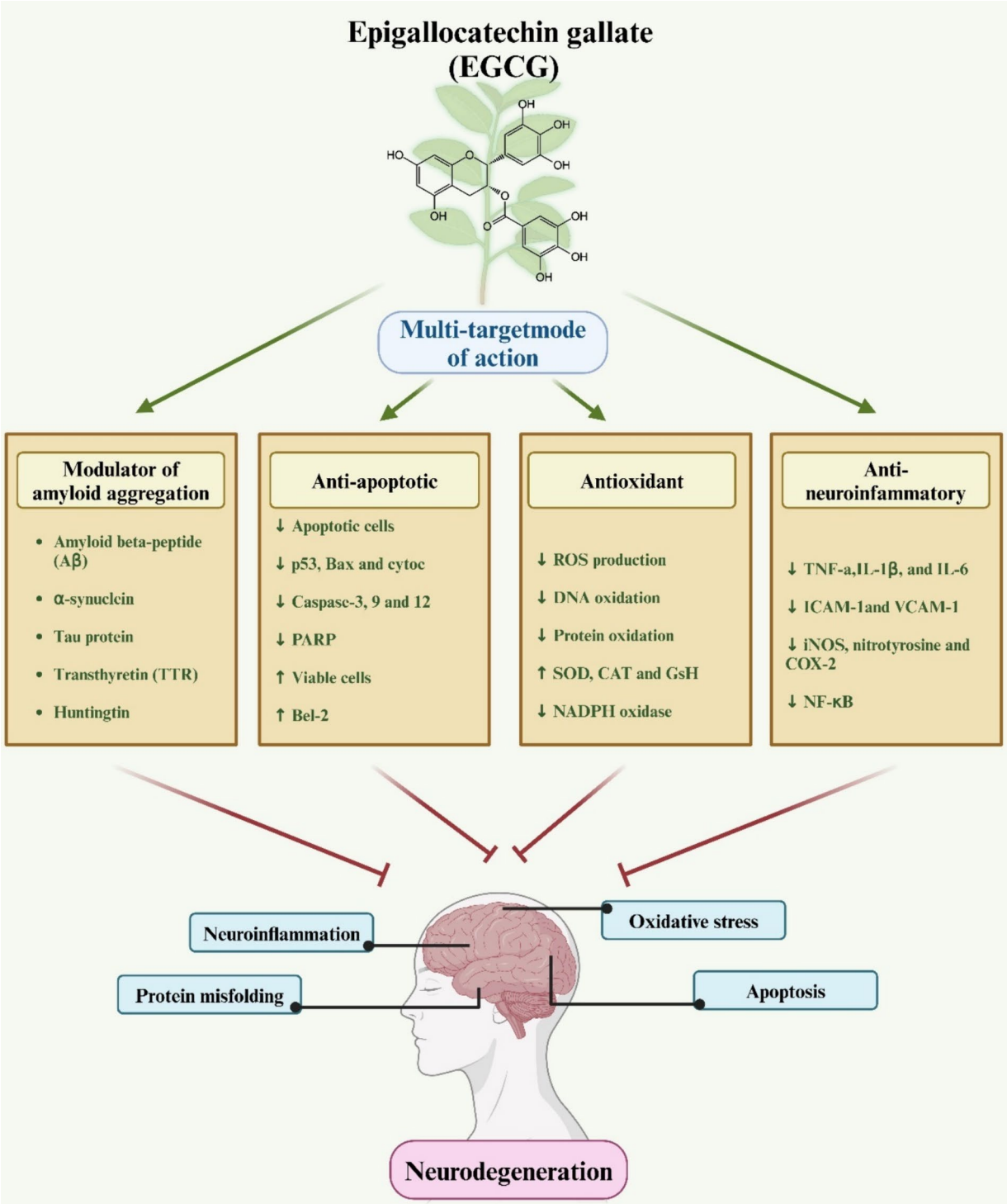

FIGURE 2
A schematic representation elucidates the role of EGCG in neuroprotection. The diagram illustrates how EGCG exerts antioxidant, anti-inflammatory, and anti-apoptotic effects via various molecular mechanisms, thereby conferring protection against neurodegenerative diseases.

2.1 Anti-chronic neuroinflammatory effects of EGCG

Neuroinflammation serves as a protective mechanism within the nervous or central nervous system (CNS) against various threats including infections, toxic metabolites, autoimmunity, and traumatic brain injury, with the aim of eliminating harmful substances and damaged tissues (42). This process entails the activation of glial cells, which serve as neuroprotective agents by removing endogenous and exogenous substances while safeguarding themselves from ROS (43). Notably, microglia, as ubiquitous innate immune cells in the CNS, are pivotal contributors to neuroinflammation, participating in both

anti-inflammatory and pro-inflammatory responses (44). The anti-neuroinflammatory properties of EGCG primarily involve the inhibition of microglial activation and the modulation of pro-inflammatory cytokine expression (45). The pro-inflammatory or neuroprotective functions of microglia are contingent upon their activation status (46). Pathogens or cellular debris induce heightened expression of pro-inflammatory cytokines such as IFNs and LPS, prompting microglial activation from a resting state (47). Activated microglia upregulate pro-inflammatory mediators including IL-1β, IL-23, TNF-α, IL-6, NO, and SOC3 via NF-κB and STAT1 pathways (48). In neuroinflammation, activated microglia sustain the release of pro-inflammatory cytokines, perpetuating chronic inflammation and generating cytotoxic molecules such as ROS and RNS (49). Extensive scientific evidence underscores the role of persistent inflammation in promoting neurodegenerative disorders. Conversely, neuroprotective microglia activated by IL-13, IL-10, and IL-4 secrete various factors associated with neuroprotection and tissue repair, including TGF-β, Chi3l3, Arginase 1, Ym1, IGF-1, and Fzd1 (48).

The effects of EGCG on microglia encompass: (1) Modulation of microglial activation under inflammatory conditions, primarily within the M1/M2 spectrum (50). M1 microglia release neurotoxic and inflammatory factors such as IL-6, IL-1β, and TNF-α, contributing to neuronal damage and death, while M2 microglia secrete neurotrophic factors including BDNF, IL-4, and IL-10, fostering neuronal growth and protection (51). EGCG downregulates M1 markers (IL-6, TNF-α, and IL-1β) and upregulates M2 markers (IL-10 and NQO1) in microglia, thereby modulating the M1/M2 ratio and mitigating neurotoxicity and neuronal damage arising from microglial hyperactivity (13). (2) EGCG induces M1 polarization via various signaling pathways including TLR4/NF-κB, JAK2/STAT3, TLR2, TLR4, JNK/P38, thereby suppressing the activation of inflammatory vesicles and reducing microglial inflammation and neurotoxicity (13). (3) Voltage-gated proton channels play a pivotal role in microglial NADPH oxidase-dependent ROS generation (52). EGCG impedes proton channel function in microglia without affecting channel gating processes. This inhibition of proton channels constitutes a significant mechanism through which EGCG suppresses microglial activation and neurotoxicity (53). (4) Neuronal injury or neuroinflammation triggers microglial activation, leading to NO production. NO reacts with cysteine thiols, resulting in protein S-nitrosylation, which regulates various cell signaling and protein activities, including protein misfolding and mitochondrial apoptosis. EGCG attenuates protein S-nitrosylation in activated microglia (54). In summary, EGCG mitigates excessive inflammatory responses and neurotoxicity induced by inflammation by inhibiting inducible NO synthase activity, reducing oxidative stress levels, and modulating the M1/M2 ratio in microglia.

2.2 Antioxidant effects of EGCG

EGCG, a significant natural antioxidant, demonstrates efficacy in neutralizing ROS like hydrogen peroxide, superoxide anions, and hydroxyl radicals (55). Its antioxidant properties stem from the polyhydroxyl structure and gallic acid moiety, which facilitate free radical scavenging, while the presence of phenolic moieties can lead to quinone generation via oxidative sensitivity (56). EGCG exerts antioxidant effects through diverse mechanisms, including hydrogen atom transfer (HAT), electron transfer, and catalytic metal chelation (Figure 2) (57). ROS are metabolically generated by organelles such as mitochondria, peroxisomes, and the endoplasmic reticulum (58). Normally, the antioxidant system efficiently eliminates ROS. However, oxidative stress prompts a shift in signaling pathways, fostering inflammation via pathways like NF-κB, PKC, MAPK, Nrf-2, and PI3K/Akt (59). EGCG mitigates oxidative stress by modulating these pathways (38, 60).

Moreover, studies have indicated that EGCG exerts a direct antioxidant effect by chelating free transition metals such as iron and copper (61). EGCG functions as a free radical scavenger, acting through two mechanisms: HAT and single electron transfer reaction (SET), in relation to its one-electron reduction potential (62). Additionally, EGCG enhances the activity of phase II enzymes and detoxification enzymes, including catalase, glutathione peroxidase (GPX), superoxide dismutase (SOD), and glutathione S-transferase (63). The regulation of these enzymes is primarily governed by Nrf2, which binds to cis-acting regulatory elements to initiate the gene expression of antioxidant enzymes (64). Furthermore, EGCG attenuates excessive levels of NO generated by inducible nitric oxide synthase (iNOS) (65). NO plays a crucial role in various physiological processes at appropriate concentrations. However, under oxidative stress, NO can act as a pro-inflammatory mediator, generating reactive nitrogen species (RNS) such as peroxynitrite (66). Studies have demonstrated that EGCG inhibits iNOS activity, thereby enhancing the bioavailability of NO levels (67). Additionally, EGCG effectively suppresses the activity of xanthine oxidase, an enzyme involved in purine catabolism and uric acid formation, thereby mitigating the associated increase in ROS (68). Moreover, EGCG inhibits the expression of cyclooxygenase-2 (COX-2), an enzyme crucial for fatty acid metabolism that is upregulated during inflammation, particularly in activated macrophages (69).

3 Neuroprotective role of EGCG in the context of neurodegenerative diseases

Neurodegenerative disease is a common and growing cause of mortality and morbidity worldwide (70), with 152 million people expected to receive the effects of the disease by 2060 (71), including AD, PD, HD, ALS, and prion diseases (72). Among various forms of dementia, AD exhibits the highest prevalence, accounting for 62%, followed by PD (73). The pathology of AD is characterized by the accumulation of extracellular amyloid β (Aβ) plaques and the formation of intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein (38). Clinical manifestations encompass memory loss, cognitive impairment, personality changes, and in severe cases, hallucinations and seizures (74). PD onset is marked by progressive degeneration of dopaminergic neurons within the substantia nigra, leading to diminished levels of striatal dopamine and its metabolites in the adult brain (75). Clinical features include motor dysfunction, bradykinesia, tremors, gait and balance disturbances, cognitive decline, and disorientation (76). ALS, commonly known as Lou Gehrig's disease, represents an adult-onset progressive neurodegenerative disorder characterized by selective motor neuron degeneration (77). This degeneration progressively affects both upper and lower motor neurons within the brain and

spinal cord. The etiology of ALS remains largely elusive in the majority of cases, with fewer than 10% attributed to specific genetic mutations involving genes such as SOD1, C9orf72, TDP43, and FUS (78). HD arises from an unstable polyglutamine repeat expansion within the first exon of the IT-15 gene, which encodes the 350 kDa huntingtin protein (79). The aggregation propensity of huntingtin fibers contributes to the progressive degeneration of cortical and striatal neurons, alongside the formation of neuronal inclusions containing aggregated huntingtin. Clinical manifestations encompass movement disorders and psychiatric symptoms including chorea, coordination deficits, depression, psychosis, and obsessive-compulsive disorder (80).

While the pathological and clinical presentations of neurodegenerative diseases vary, they share common features including specific pathological alterations within distinct brain regions and the degeneration of various neuronal subtypes. Key factors contributing to neurodegenerative processes encompass the dysregulation of pro-apoptotic proteins, oxidative stress damage, immune-mediated inflammation, mitochondrial dysfunction, and reduced expression of trophic factors (81–83). Here we focus on the crosstalk between EGCG and neurodegenerative diseases in terms of EGCG anti-neuroinflammation and oxidative stress. Neuroinflammation and oxidative stress are intertwined, as inflammation amplifies ROS production while ROS, in turn, exacerbate inflammation (84). ROS can directly activate the NF-kB transcription factor pathway, promoting the synthesis of inflammatory cytokines (85). Given the multifactorial nature of neurodegenerative pathologies, the emergence of novel therapeutic strategies is imperative. The antioxidant properties and neuroprotective effects of EGCG have garnered significant attention from researchers worldwide, positioning it as a promising treatment for neurological disorders and a cytoprotective agent. In this section, we delve into the role of EGCG in mitigating oxidative stress and chronic neuroinflammation in two prevalent neurodegenerative diseases: AD and PD.

3.1 Alzheimer's disease

Neurodegenerative disease affects an estimated 24 million individuals globally, with AD being the most prevalent disease (86). In developed Western nations, individuals aged over 85 exhibit an AD prevalence ranging from 24 to 33%, a figure that escalates with advancing age (87). Given the global aging demographic, AD is poised to become a substantial public health concern over the next two decades and has been identified as a research priority (86). The pathogenic mechanisms underlying AD encompass microglia-induced inflammation, elevated intracellular calcium levels, disruption of antioxidant defense systems, cholinergic dysfunction, overactivation of glutamate receptors, and amplification of the inflammatory response (88). Despite the availability of various medications for managing AD, a definitive treatment remains elusive (89), underscoring the pressing need for research into novel therapeutic approaches and adjunctive therapies. Optimal antioxidant levels in the body have been associated with cognitive preservation, and several studies have demonstrated the neuroprotective effects of catechins, highlighting their potential as adjunctive therapy in select neurodegenerative diseases. These effects rely on the anti-inflammatory and antioxidant properties of catechins (90). Moreover, multiple studies have established a correlation between tea consumption, reduced risk of severe cognitive impairment, and a lower prevalence of AD.

3.1.1 Observational epidemiologic study of green tea consumption and risk of AD

Moeko Noguchi-Shinohara et al. conducted a 2-year follow-up survey of 490 subjects over 60 years of age with cognitive performance and blood tests. Even after correcting for potential confounders, drinking green tea was found to significantly reduce the chance of cognitive deterioration (91). In a questionnaire-based study of 1,003 Japanese participants aged 70 or older, Shinichi Kuriyama et al. discovered a correlation between higher green tea drinking and a lower prevalence of cognitive impairment (32). A brief analysis of tea consumption and prevalence of AD in different country regions by Fernando et al. revealed that countries with higher intake of tea, such as Japan, China, and India, had lower prevalence of AD, whereas European and American countries with lower intake of tea had higher prevalence of AD (92). Although epidemiological data favorably show a negative relationship between drinking tea and the preponderance of AD in that part of the country, any correlation between tea consumption and AD prevalence should be evaluated with caution because the effects of racial differences, dietary preferences, and lifestyle cannot be excluded (92). Yang Yuhuan et al. conducted a questionnaire survey to gauge the cognitive function of seniors 60 years of age and older in the Huangshi community in order to better understand the prevalence of mild cognitive impairment (MCI) and its influencing factors (93). The survey data were tested by chi-square test and it was concluded that the prevalence of MCI was lower in occasional tea drinkers, which may be related to the caffeine and catechins contained in tea, caffeine can reduce the level of Aβ in the brain, which is beneficial for improving cognitive function, while catechins have strong antioxidant capacity, but the study did not prove the relationship between tea drinking and AD prevalence. Wang, Ziqi et al. performed the Mini-Mental State Examination (MMSE) for the assessment of cognitive function in 870 people aged 90 years or older, and cardinality testing of the collected data revealed that the mild cognitive index was significantly different from normal in those who regularly consumed animal oils and legumes (94). In contrast, no significant differences were found for the other 10 foods, including tea, in both the unadjusted and adjusted models (94). Numerous studies have demonstrated the potential of tea consumption to mitigate cognitive decline in older adults; however, experimental evidence supporting its efficacy in AD is lacking (95). Controlled studies examining AD cases have not yielded significant findings regarding tea consumption, thus limiting the inference of beneficial effects of green tea catechins solely based on AD pathogenesis and *in vitro* studies (96). Despite this, the observed efficacy of green tea in AD surpasses initial expectations, warranting further investigation into the specific role of catechins in AD patients.

3.1.2 Experimental studies and mechanisms of AD

Given that Aβ aggregation is recognized as a pivotal factor in the pathogenesis of AD and its impact on the human nervous system, Mahsa Amirpour et al. investigated the neuroprotective potential of green tea in a streptozotocin (STZ)-induced AD model. Their study examined the effects of green tea on cognitive decline, inflammation, and oxidative stress (97). The findings demonstrated that the active

compounds present in green tea could mitigate cognitive impairment and ameliorate learning and memory deficits associated with STZ injection (81). Furthermore, green tea may reduce the risk of AD through antioxidative and anti-inflammatory pathways, thus positioning it as a potential preventive intervention (90) (Table 1).

Tingting Chen et al. used mice as a model to demonstrate that the polyphenolic compounds EGC and ECG effectively alleviated Aβ40 aggregation and protofibrillar toxicity by chelating Cu^{2+} and Zn^{2+} and reduced ROS production, thereby mitigating Cu^{2+}-Aβ40 and Zn^{2+}-Aβ40induced neuronal toxicity (111). The results showed that tea polyphenols had significant beneficial effects on different aspects of AD pathology (112). Among them, catechin ECG had the most significant effect due to the therapeutic effect of ECG through the BBB, reducing Aβ plaques in the brains of APP/PS1 mice and thus protecting neurons from damage (111). Therefore, the potential of catechins to prevent or improve AD symptoms was laterally demonstrated (111). Lee JW et al. found that EGCG reduced Aβ1-42-induced memory dysfunction by altering the secretion of α-secretase, in addition to EGCG inhibiting Aβ1-42-induced apoptosis (113). These findings imply that EGCG may be a useful tool for delaying the start or progression of AD (Figure 3).

3.1.3 EGCG anti-neuroinflammatory activity in AD

Neuroinflammation as a pathogenesis of AD has been confirmed by numerous studies. It has been found that cerebrospinal fluid levels of pro-inflammatory factors such as IL-1β, IL-6, and TNF-α are high in AD patients and increase with disease progression (114, 115). In addition, microglia, which play an important role in chronic neuroinflammation, are also involved in this process. Microglia resist the onset and progression of AD by degrading Aβ and tau. However, Aβ in turn activates microglia through TLRs to release pro-neuroinflammatory mediators. In the early stages of AD development, neuroprotective phenotypic microglia appear around Aβ plaques (116, 117). However, in late AD pathogenesis, elevated expression of proinflammatory factors will result in the emergence of microglia with a proinflammatory phenotype and a decrease in their phagocytic activity (118, 119). Pro-inflammatory microglia drive tau proliferation and toxicity by promoting neuroinflammation, such as activation of NLRP3 inflammasomes or induction of NF-kB signaling (23). Defective microglial autophagy leads to dysregulation of lipid metabolism, which increases the pathology of tau within neurons further exacerbating AD (23).

Numerous studies have shown that EGCG treatment of AD is associated with chronic neuroinflammation induced by microglia of anti-inflammatory phenotype (105). Wei et al. conducted *in vitro* experiments demonstrating that EGCG effectively suppressed the expression of TNFα, IL-1β, IL-6, and iNOS while concurrently restoring intracellular antioxidant levels, including Nrf2 and HO-1. These actions counteracted the pro-inflammatory effects of microglia (120). Furthermore, EGCG inhibited the secretion of pro-inflammatory factors from Aβ-induced pro-inflammatory microglia phenotypes and attenuated microglial neurotoxicity (121). Importantly, EGCG also mitigated Aβ-induced cytotoxicity by attenuating ROS-mediated NF-κB activation and MAPK signaling pathways, including JNK and p38 signaling (121). *In vitro* investigations have demonstrated that Aβ deposition significantly diminishes following intraperitoneal injection of EGCG at a dose of 20 mg/kg or oral administration of EGCG at 50 mg/kg in drinking water (109, 122). Similarly, Li et al. observed a substantial reduction in Aβ deposition in the frontal cortex (60%) and hippocampus (52%) following oral administration of EGCG at a dose of 20 mg/kg/day for 3 months in an AD mouse model (123). Furthermore, recent findings by Lee et al. revealed that EGCG attenuated LPS-induced memory impairment and neuronal apoptosis, concomitant with a reduction in the expression of inflammatory cytokines TNF-α, IL-1β, and IL-6 (105). These results align with *in vitro* observations, suggesting that EGCG holds promise as a therapeutic agent for neuroinflammation-associated AD.

3.1.4 EGCG antioxidant activity in AD

The brain is particularly vulnerable to oxidative damage due to its high content of easily oxidizable lipids, elevated oxygen consumption rates, and limited antioxidant defense mechanisms. Age-related increases in brain oxidation contribute to the recognized risk of AD (124). Under normal physiological conditions, SOD catalyzes the conversion of superoxide anions to hydrogen peroxide, thereby safeguarding cells against free radical assault. However, in the presence of elevated levels of certain metal ions such as Fe and Cu, SOD can convert hydrogen peroxide to the more hazardous hydroxyl radical (125). Notably, AD patients exhibit heightened SOD activity, diminished glutamine synthetase activity, and elevated lipid peroxidation, collectively resulting in heightened oxidative stress and accumulation of free radicals. Free radicals inflict damage upon biofilms, disrupting the intracellular milieu and precipitating cellular senescence and demise (126). Peroxidation of impaired lipids results in ribonucleic acid inactivation, prompting DNA and RNA cross-linking and instigating DNA mutations (127). Decomposition of peroxidized lipids yields aldehydes, such as acrolein, which react with phosphoric acid and proteins to generate lipofuscin (128). Accumulation of lipofuscin in the brain contributes to cognitive impairment (129). Furthermore, mitochondrial dysfunction and oxidative stress in AD patients are intricately intertwined, with evidence indicating mutual exacerbation, culminating in AD pathogenesis (130).

Numerous studies have delineated the involvement of increased oxidative stress in AD pathogenesis, and highlighted the potential of EGCG's antioxidant properties in mitigating this process (131, 132). Abdul M. Haque et al. observed that long-term administration of green tea catechins to AD model mice significantly ameliorated cognitive impairment, accompanied by reduced ROS levels and enhanced antioxidant capacity in the hippocampus and cortex (133). Similarly, Regina Biasibetti et al. investigated the effects of oral EGCG administration (10 mg/kg/day) for 1 month in a rat model of dementia, revealing cognitive deficits reversal and notable reductions in ROS levels and NO production (110). Catechins exert their antioxidative effects by scavenging free radicals and chelating metal ions such as Fe and Cu, thereby reducing ROS production. This dual action mitigates oxidative stress in both peripheral and brain tissues, thereby inhibiting further deterioration of cognitive deficits-associated behaviors (134). Mitochondrial dysfunction enhances ROS generation via the NADPH oxidase pathway (135). EGCG reinstates mitochondrial respiration rate, ATP levels, ROS levels, and membrane potential (102). Its antioxidant properties scavenge ROS production and safeguard against mitochondrial damage (136). Furthermore, EGCG treatment mitigates neuronal apoptosis triggered by endoplasmic reticulum stress subsequent to Aβ exposure. The

TABLE 1 Specific benefits and mechanisms of action of EGCG in AD.

Animal model	EGCG administration	Outcome measures	Neuroprotective mechanisms	Publication
Aβ 25-35-induced AD rat model.	EGCG (100, 250 or 600 mg/kg/d) by gavage for 9 weeks.	Decreased Tau hyperphosphorylation in the hippocampus; inhibited BACE1 expression and activity as well as Aβ1-42 expression; increased Ach by reducing AchE activity.	Antioxidative stress.	(98)
APP/PS1 transgenic mice (AD model).	EGCG (50 mg/kg) by gavage for 4 months.	Reduced cognitive deficits in AD model mice; improved brain dendritic integrity and synaptic protein expression levels; inhibited microglia activation and reduced pro-inflammatory cytokines (IL-1β); reduced β-amyloid (Aβ) plaques in the hippocampus.	Anti-inflammatory; neuroprotective; anti-amyloidogenic.	(99)
SAMR1 and SAMP8 mice.	EGCG (5 or 15 mg/kg/d) by gavage for 60 days.	Alleviates deterioration of cognitive function; reduced brain NEP levels and decreased accumulation of Aβ.	N/A	(100)
APP/PS1 mice.	EGCG (40 mg/kg/d) orally for 3 months.	Reduces synaptic deficits; reduces neuroinflammation and Aβ plaque accumulation; enhances learning ability and spatial memory.	N/A	(101)
APP/PS1 mice.	EGCG-containing (10 mg/mL) drinking water for 5.5 months.	Restoration of mitochondrial respiration rate, MMP, ROS production, and ATP levels; reduction in toxic levels of brain Aβ.	Antioxidant; reduces mitochondrial dysfunction.	(102)
APP/PS1 mice	EGCG (30 mg/kg/d) by gavage for 90 days.	Reduced brain parenchyma and cerebrovascular Aβ deposition; increased expression of nonamyloidogenic soluble APP-α and α-secretase candidate proteins, as well as decreased expression of amyloidogenic soluble APP-β and β-secretase proteins; alleviated synaptic toxicity, neuroinflammation and oxidative stress.	Anti-neuroinflammatory; antioxidant stress.	(103)
Aβ injection induces AD rat model.	Intraperitoneal injections of EGCG (10 mg / kg) were administered for 3 weeks (every other day).	Reduces Aβ accumulation; restores motor coordination and memory.	N/A	(104)
LPS-induced neuroinflammation and memory impairment in mice.	EGCG (1.5 mg/kg or 3 mg/kg) was administered orally for 3 weeks.	Prevented memory damage and neuronal apoptosis; inhibited elevated Aβ levels and APP and β-site APP cleavage enzyme 1 expression; prevented astrocyte activation; decreased levels of cytokines (TNF-α, IL-1β, GM-CSF, ICAM-1, and IL-16); reduced iNOS and COX-2 expression.	Anti-neuroinflammatory; antioxidant stress.	(105)
SAMP8 mice	EGCG (5 or 15 mg/kg/d) orally for 8 weeks.	Improves spatial learning ability and memory impairment; reduces levels of Aβ1-42 and BACE-1; prevents hyperphosphorylation of tau.	N/A	(106)
APP/PS1 mice	EGCG (2 mg/kg/d) orally for 4 weeks.	Improved cognitive impairment; reduced Aβ and APP expression and inhibited neuronal apoptosis; activation of TrkA signaling and inhibition of p75NTR signaling.	Adjust the TrkA/p75NTR signal balance.	(107)
APP/PS1 mice	EGCG (2 or 6 mg/kg/d) orally for 4 weeks.	Improves learning and memory deficits; decreases hippocampal levels of IRS-1pS636 and Aβ42; inhibits TNF-α/JNK signaling; increases Akt and glycogen synthase kinase-3β phosphorylation in the hippocampus.	Attenuates central insulin resistance.	(108)
Tg APPsw transgenic mice	Intraperitoneal injection of EGCG (20 mg / kg/d) for 60 days.	Promotes APP for nonamyloidogenic processing; reduces cerebral amyloidosis.	N/A	(109)
STZ-induced AD mouse model.	EGCG (10 mg/kg/d) by gavage for 4 weeks.	Reduces cognitive impairment; reverses AChE activity, GPX activity, NO metabolites, and ROS levels.	Antioxidant stress.	(110)

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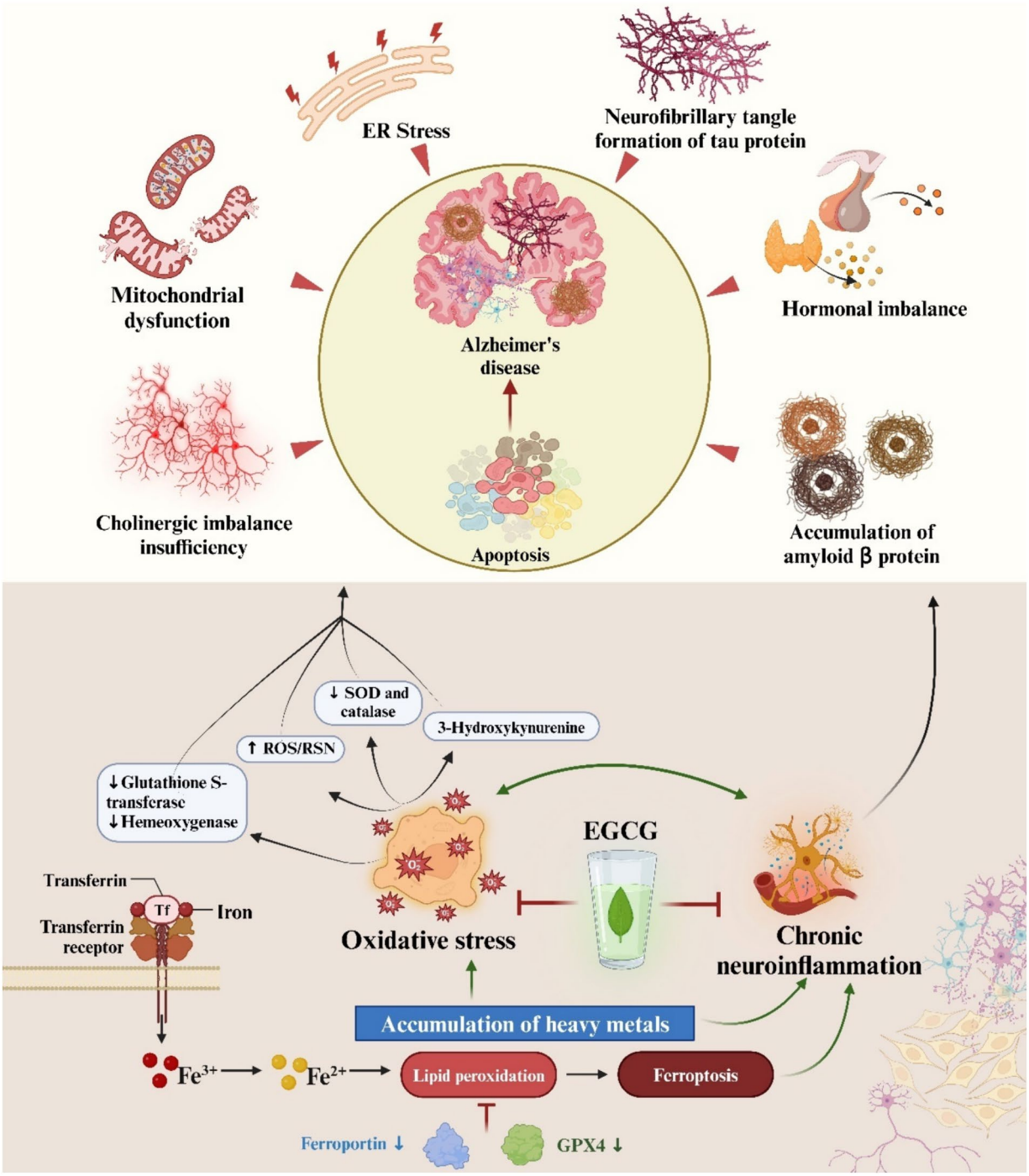

FIGURE 3
The multifactorial pathophysiology of Alzheimer's disease is depicted in an illustration. Furthermore, epigallocatechin-3-gallate is highlighted as a potential therapeutic intervention for AD, attributed to its ability to counteract oxidative stress and chronic neuroinflammation.

inflammatory response to neuronal injury induced by various stimuli culminates in the release of pro-inflammatory cytokines and cytotoxins, further exacerbating oxidative stress (137). Numerous studies have demonstrated EGCG's protective effects against lipopolysaccharide-induced memory impairment and inflammatory responses (105, 138). Through mechanisms associated with protein kinase C (PKC), which facilitates the generation of nontoxic soluble peptide APPβ (sAPPβ) and cell survival, catechins may exert an influence on AD (139, 140). Levites et al. reported that EGCG (1–5 μM) enhances sAPPβ production from PC12 and human neuroblastoma cells (141).

3.2 Parkinson's disease

PD follows AD as the second most prevalent neurodegenerative disorder affecting middle-aged and elderly individuals. While PD is uncommon before the age of 50, its incidence escalates markedly with

advancing age, peaking between 70 and 85 years, afflicting 7 to 10 million individuals worldwide (142, 143). Pathologically, PD is characterized by the degeneration and loss of dopaminergic neurons within the substantia nigra pars compacta, accompanied by the formation of eosinophilic inclusion bodies known as Lewy bodies within the residual neurons. These alterations disrupt the balance between dopamine and cholinergic neurotransmitters, culminating in aberrant motor function within the basal ganglia. The resultant motor and non-motor symptoms include postural reflex deficits, bradykinesia, muscular rigidity, gait disturbances, and resting tremor (144, 145). The episodic nature of PD in most cases suggests a multifactorial etiology involving genetic susceptibility and environmental influences. While the precise pathogenesis remains elusive, current hypotheses implicate abnormal aggregation of α-synuclein, mitochondrial dysfunction, calcium dyshomeostasis, oxidative stress, and neuroinflammation (146).

3.2.1 Observational epidemiologic study of green tea consumption and risk of PD

In order to determine the relationship between PD incidence and tea consumption, Quintana et al. examined a total of 12 studies from 1981 to 2003, comprising 2,215 cases and 145,578 controls. Their analysis revealed that tea consumption can prevent PD and that this protective effect is more pronounced in the Chinese population (147). In order to study the non-hereditary factors associated with PD, Hosseini Tabatabaei N. et al. used a sample of 150 people, including 75 PD patients and 75 people as controls, and showed that tea intake was protective against PD and that adherence to daily tea consumption reduced the risk of PD by 80% (148). A case–control study was conducted by Harvey Checkoway et al. By studying and counting PD cases ($n = 210$) and controls ($n = 347$), it was found that people who drank two or more cups of green tea per day had a reduced incidence of PD compared to those who did not drink green tea (149). According to research by E-K Tan and colleagues, drinking one unit of tea (3 cups per day for 10 years) would result in a 28% decrease in the incidence of PD (150). The effects of tea consumption on 60 patients with idiopathic PD were examined by Chahra CD et al. According to the study's findings, PD patients who drank tea in addition to traditional medication experienced improvements in their non-motor symptoms and depression (143). Boris Kandinov et al. also demonstrated that drinking tea and smoking delayed the age of PD attacks, while drinking coffee may have the opposite effect (151). Observational epidemiological studies in PD have more experimental data demonstrating a protective effect of green tea compared to AD, and even though epidemiological findings support the beneficial effects of tea consumption, some have not yet provided clear evidence. Therefore, more research is required to determine the connection between drinking tea and the risk of PD.

3.2.2 Experimental studies and mechanisms of PD

Pathological accumulation of metal ions or a rapid increase in monoamine oxidase B (MAO-B) activity can induce endogenous dopamine (DA) oxidation, leading to α-synuclein aggregation, mitochondrial dysfunction, and other factors contributing to the heightened incidence of PD. Consequently, mitigation strategies involve the use of ROS scavengers, DA oxidation inhibitors, MAO-B inhibitors, and DA quenchers (152). Zhou et al. demonstrated that catechins can impede DA oxidation by inhibiting enzymes and metal ions. Furthermore, they inhibit MAO-B activity, detoxify ROS, DA quenchers, and harmful DA oxidation byproducts, while regulating the Nrf2-Keap1 and PGC-1 pathways. These findings underscore the inhibitory effects of tea polyphenols on DA-related toxicity (153). In a study by Shyh-Mirnin Ph.D. et al., the influence of EGCG on MAO-B enzyme activity in the adult rat brain was investigated, revealing a decrease in MAO-B enzyme activity (154).

PD primarily affects dopaminergic neurons in the substantia nigra pars compacta (SNpc) region of the brain (155). The neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6-OHDA) specifically damage this brain region, resulting in the loss of dopaminergic neurons (156). This neuronal loss leads to disrupted neural firing patterns and impaired motor control (157). Weinreb et al. investigated the impact of pretreatment with tea extract (0.51 mg/kg) and the tea polyphenol EGCG (2.10 mg/kg) on dopamine neurogenesis loss in the substantia nigra of MPTP-induced PD mouse models (158). Their study revealed a considerable mitigation of neurogenesis loss (158). Siddique Y. H. et al. examined the effects of EGCG in an α-synuclein (h-αS) transgenic Drosophila model of PD, analyzing statistical data and markers of changes in climbing capacity, lipid peroxidation, and apoptosis (159). Their findings demonstrated that various concentrations of EGCG (0.25, 0.50, and 1.0 g/mL) substantially delayed the loss of climbing ability in Drosophila, while reducing oxidative stress and apoptosis (159).

In a study by Tingting Zhou et al., a PD mice model induced by MPTP was utilized to investigate the potential therapeutic effects of EGCG for PD. The results demonstrated that EGCG administration ameliorated impaired locomotion behavior in MPTP-treated mice and protected tyrosine hydroxylase-positive cells in the substantia nigra pars compacta from MPTP-induced toxicity (160). Additionally, following EGCG treatment, flow cytometric analysis revealed an increase in the CD3 + CD4+ to CD3 + CD8+ T cell ratio in peripheral blood of MPTP-treated mice. Furthermore, EGCG appeared to downregulate the expression of inflammatory mediators such as TNF and IL-6 in serum (160). These findings suggest that EGCG may confer neuroprotective effects in MPTP-induced PD mice models, potentially by modulating peripheral immune responses.

Current understanding of PD pathogenesis implicates neurofilaments, synaptic vesicle proteins, and ubiquitinated α-synuclein as primary contributors to the disease pathology (161). Additionally, Lewy bodies may exacerbate the release of free radicals, excessive nitric oxide synthesis, microglia-mediated inflammation, and disruption of protein degradation pathways, further exacerbating the pathophysiology (162). Specific beneficial effects and mechanisms of action of EGCG in PD are summarized in Table 2. In conclusion, EGCG exhibit diverse pharmacological activities in PD by modulating gene expression and interfering with signaling pathways (172). Despite substantial experimental evidence supporting this notion, challenges such as low solubility, limited bioavailability, and BBB impermeability hinder efficient delivery of EGCG to the brain and impede clinical translation (173). Overcoming these obstacles necessitates cross-sectional studies aimed at elucidating chemical modification strategies and optimizing drug delivery mechanisms to enhance their therapeutic efficacy.

3.2.3 EGCG anti-neuroinflammatory activity in PD

Numerous studies have established neuroinflammation as a significant etiological factor in PD, playing a pivotal role in its early

TABLE 2 Specific benefits and mechanisms of action of EGCG in PD.

Animal model	EGCG administration	Outcome measures	Neuroprotective mechanisms	Publication
MPTP-induced PD mouse model.	EGCG (50 mg/kg/day) gavage administration for 20 days.	PD mice recovered motor behavior; increased the CD3CD4 to CD3CD8 T-lymphocyte ratio in the peripheral blood; and decreased the inflammatory factor (TNF-α and IL-6) expression in the serum.	Anti-neuroinflammatory.	(160)
LPS (substantia nigra injection)-induced PD rat model.	EGCG-Loaded Liposomes 2 μL/d (12.5 μM) was administered for 14 days.	Recovery of dyskinesia in PD rats; reduction of TNF-α production in the brain substantia nigra region; prevention of BV-2 activation.	Anti-neuroinflammatory.	(163)
Paraquat-induced TH > dj-1-β-RNAi/+ *Drosophila melanogaster* flies (PD Drosophila model)	Feed 0.5 mM EGCG for 15 days.	Drosophila restored lifespan and locomotor activity, with decreased lipid peroxidation and neurodegeneration.	Antioxidative stress.	(164)
Rotidone (ROT)-induced PD rat model.	Intravenous EGCG (100 or 300 mg/kg/d) for 21 days.	NO levels and lipid peroxidation were reduced; SDH, ATPase, and ETCase activities, and catecholamine levels were elevated; and levels of neuroinflammatory and apoptotic markers were reduced.	Antioxidant effects; prevention of mitochondrial dysfunction; anti-neuroinflammatory effects; anti-apoptotic effects.	(165)
MPTP-induced PD mouse model.	EGCG (2 and 10 mg/kg/day) gavage administration for 10 days.	Prevention of nigrostriatal dopamine neuron death; restoration of striatal dopamine and tyrosine hydroxylase protein levels; elevation of striatal antioxidant enzymes SOD and catalase activity.	Antioxidant; iron chelate.	(158)
MPTP-induced PD mouse model.	EGCG (2 and 10 mg/kg/day) gavage administration for 10 days.	Reduced neurotoxicity in PD mice; restored rotational latency; increased striatal dopamine concentration and nigral ferritin expression.	Antioxidative stress.	(166)
α-Synuclein preformed fibers (α-syn-PFFs)-induced PD mouse model.	Intraperitoneal injection of EGCG (10 mg/kg/day) for 7 days.	Reduces anxiety-like behavior and dyskinesia in mice; reduces neuronal degeneration and accumulation of p-α-syn in Lewy bodies and Lewy neurons; reduces expression of pro-inflammatory cytokines (IL-6, IL-1, and TNF-α) while promoting expression of anti-inflammatory cytokines (TGF-β, IL-10, and IL-4).	Anti-neuroinflammatory.	(167)
MPTP-induced PD mouse model.	EGCG (25 mg/kg) was administered by gavage for 1, 2, 4 and 7 days.	Prevents loss of TH-positive cells in the SN and loss of TH activity in the striatum; maintains HVA levels in the striatum; decreases nNOS expression in neurons.	Antioxidative stress.	(168)
MPTP-induced PD mouse model.	Intraperitoneal injection of EGCG (10 mg/kg or 50 mg/kg per day) for 14 days.	Reduced neuronal death rate and iNOS expression.	Antioxidative stress.	(169)
MPTP-induced PD mouse model.	EGCG (25 mg/kg/day) gavage administration for 7 days	Increased rotational latency; elevated striatal dopamine concentration; and higher substantia nigra ferritin expression.	Reduction of oxidative stress; iron-export protein ferroportin in substantia nigra.	(166)
LPS -induced PD rat model.	Intraperitoneal injection of EGCG (10 mg/kg/d) for 7 days.	Decreased expression of TNF-α and NO; increased levels of dopamine neurons.	Anti-neuroinflammatory; anti-oxidative stress.	(170)
MPTP-induced PD mouse model.	EGCG (25 mg/kg/day) gavage administration for 6 days	Protected tyrosine hydroxylase (TH)-positive cells in the substantia nigra (SN) and TH activity in the striatum; reduced nNOS expression in the substantia nigra and neuronal nNOS expression.	Antioxidative stress.	(168)
L-DOPA and carbidopa-induced PD rat model.	Only one oral dose of EGCG (25 mg/kg).	Restores striatal dopamine accumulation; reduces glutamate-induced oxidative cytotoxicity by inactivating the NF-kB signaling pathway; reduces neuronal death.	Antioxidative stress; COMT inhibiton.	(171)

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pathogenesis. Notably, activated microglia make substantial contributions to this process. Evidence supporting the involvement of activated microglia-mediated chronic neuroinflammation in PD includes: (1) Pro-inflammatory effects are often observed in activated microglia surrounding dopaminergic neurons, with the degree of microglial activation correlating with dopaminergic endings loss in PD (174, 175). (2) Injured neurons release excessive α-synuclein, activating proinflammatory factors like TNF-α, NO, and IL-1β produced by microglia, thereby modulating chronic neuroinflammation in PD (176, 177). (3) Jmjd3, critical for microglial cell phenotype expression, when inhibited, leads to overactivation of pro-inflammatory microglial responses, exacerbating neuroinflammation and neuronal cell death (178). Additionally, it has been proposed that α-synuclein aggregates exert toxicity on neurons only in the presence of microglia (179, 180). In PD patients, misfolded α-synuclein is released from injured neurons into the extracellular fluid, where it binds to Toll-like receptors (TLRs), Fcγ receptors (FcγR), or nucleotide-binding oligomerization domain-like receptors (NLRPs), further activating microglia (181–184). The proinflammatory cytokines released by activated microglia subsequently activate protein kinase R (PKR), leading to phosphorylation of α-synuclein at Ser129, a process considered of significant pathological importance, particularly in Lewy bodies of PD patients. Moreover, microglia are involved in the clearance of protein deposits, including α-synuclein and Aβ, from astrocytes (185–187). Activation of microglia upregulates MHC I expression on neurons, promoting neuronal presentation of α-synuclein antigen. Subsequently, these neurons are targeted and eliminated by α-synuclein-reactive T cells (187). The emergence of α-synuclein pathology follows microglial activation, suggesting α-synuclein's pivotal role in PD progression, albeit not as an initiator. Similarly, mounting evidence suggests that the immune response contributes to neuronal death as a cause rather than a consequence (22).

A growing body of evidence suggests that EGCG may impede or postpone the progression of PD by targeting chronic neuroinflammation. EGCG exhibits potent anti-inflammatory activity both *in vitro* and *in vivo*, primarily attributed to its ability to inhibit microglia-induced cytotoxicity (120). *In vitro* studies have demonstrated that EGCG suppresses the secretion of pro-inflammatory factors from LPS-activated microglia by downregulating the expression of iNOS and TNF-α (188). Furthermore, EGCG has been shown to inhibit microglial activation and reduce neuronal damage in SH-SY5Y and rat mesencephalic cultures (188). Gülşen Özduran et al. reported that EGCG restored viability in PD model cells, inhibited apoptosis, and enhanced survival by attenuating 6-OHDA-induced expression of TNF-α and IL-1β in SK-N-AS cells (189). The findings from the *in vivo* study corroborate those observed *in vitro*, further substantiating the potential of EGCG to mitigate the inflammatory response associated with microglia-mediated damage to dopaminergic neurons. Al-Amri et al. demonstrated that EGCG significantly increased the number of TH-immunoreactive neurons in the midbrain of PD model rats by reducing the production of TNF-α and NO (170). Similarly, EGCG liposomes alleviated symptoms in a PD rat model by suppressing the expression of NO and TNF-α in microglia exhibiting an LPS-induced inflammatory phenotype (165). In summary, EGCG shows promise as a therapeutic and prophylactic agent for PD, exerting neuroprotective effects both *in vivo* and *in vitro* through the inhibition of neuroinflammation (Figure 4).

3.2.4 EGCG antioxidant activity in PD

PD patients commonly exhibit reduced mitochondrial complex I activity and increased ROS production (190). This diminished function of proton pumps on mitochondria, coupled with decreased membrane voltage and the opening of permeability channels, initiates the apoptotic process. Deficiency in mitochondrial complex I can result in oxidative stress, heightening neuronal susceptibility to excitotoxic injury. The densely packed substantia nigra is particularly vulnerable to elevated oxidative stress compared to other brain regions. Under normal conditions, H_2O_2 generated by dopamine toxicity is neutralized by reduced glutathione, mitigating potential harm. However, in the remaining dopamine neurons of PD patients, ineffective scavenging of H_2O_2 may occur due to compensatory mechanisms, including accelerated toxicity production in dopamine metabolism, heightened monoamine oxidase (MAO)-B activity, and reduced glutathione levels (191). Excessive H_2O_2 reacts with Fe^{2+} via Fenton chemistry, yielding highly toxic hydroxyl radicals, culminating in lipid peroxidation and apoptosis of nigral neurons. This oxidative stress and mitochondrial dysfunction form a reciprocal relationship, perpetuating a vicious cycle.

The neuroprotective effects of EGCG, attributed to its antioxidant properties, have been observed in PD (Figure 4). Typically, α-synuclein localizes to the mitochondria-associated membrane, and its presence may disrupt mitochondrial function by promoting the formation of the mitochondrial permeability transition pore (mPTP), leading to mitochondrial membrane potential (MMP) loss, subsequent mitochondrial degradation, and ultimately cell death (192). Compounds capable of preserving mitochondrial activity are therefore deemed invaluable in combating PD. EGCG has been shown to safeguard mitochondrial function by preventing Ca2+ influx through voltage-gated calcium channels and mitochondrial Ca2+ uptake via the mitochondrial Ca2+ uniporter (159, 193). Furthermore, *in vivo* studies have demonstrated EGCG's ability to reduce oxidative stress by decreasing serum protein carbonyls and mitigating neurotoxicity in the MPTP-induced mouse model of PD (166). Similarly, Pinto et al. reported that EGCG improved cognitive dysfunction induced by 6-OHDA in male Wistar rats. 6-OHDA is known to induce ROS generation. EGCG treatment reversed striatal oxidative stress and attenuated immunohistochemical alterations (194). In conclusion, EGCG has been shown to alleviate PD by inhibiting neurotoxin-induced oxidative stress injury both *in vitro* and *in vivo*.

4 EGCG bioavailability, toxicity, and safe dose

When evaluating EGCG for clinical therapeutic applications, significant concerns arise regarding its safety, toxicity, and optimal dosage post-treatment. While numerous studies have highlighted EGCG's beneficial impact on neurodegenerative diseases due to its antioxidant and anti-neurotoxic properties, others have reported adverse effects such as heightened oxidative stress and the generation of toxic EGCG metabolites (195–197). Hence, there is a critical need for systematic investigations into EGCG's bioavailability, toxicity profile, and appropriate dosing regimens.

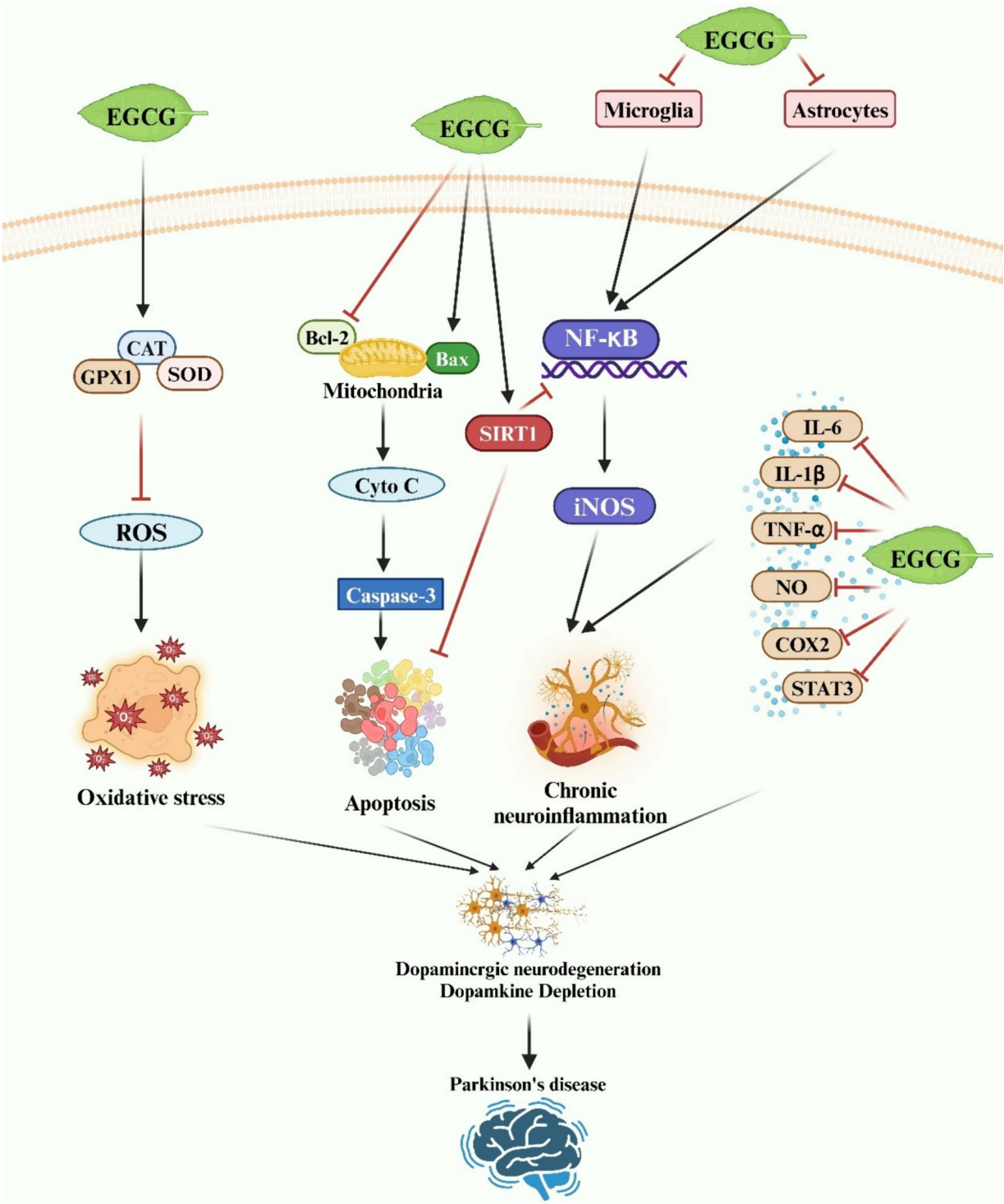

FIGURE 4
A schematic representation illustrates the neuroprotective effects of EGCG in Parkinson's disease. EGCG exerts neuroprotection by inhibiting oxidative stress, neuronal apoptosis, and neuroinflammatory responses via diverse molecular mechanisms.

4.1 Bioavailability of EGCG

EGCG has been extensively investigated for its notable health-promoting effects, with particular focus on its neuroprotective properties. Despite these benefits, the bioavailability of EGCG is limited, posing a challenge for its clinical application in treating neurodegenerative diseases. Following oral administration, EGCG exhibits a mean peak plasma concentration between 1.3 and 2.2 h, a half-life ranging from 1.9 to 4.6 h, and is almost completely metabolized within 24 h (198). Pharmacokinetic studies reveal that merely 0.1% of the ingested EGCG dose reaches detectable levels in the bloodstream at its peak concentration time (Tmax) in

healthy individuals (39, 199). This minimal absorption occurs primarily through passive diffusion (paracellular and transcellular diffusion) in the small intestine, while the remaining EGCG reaches the colon to be degraded by intestinal microbial enzymes (198, 200, 201). EGCG undergoes phase II metabolism in enterocytes and hepatocytes following ingestion (199, 202). The polyphenolic hydroxyl structure of EGCG facilitates binding reactions such as methylation, glucuronidation, sulfation, and cysteine binding, contributing to its limited bioavailability (203). Upon entering the colon, EGCG encounters rapid hydrolysis of conjugate groups like glucuronides and sulfates by colonic microbiota. Subsequently, glycosides are released and further catabolized into ring cleavage products and low molecular weight phenolic acids (198, 204). While absorption studies traditionally focus on the small intestine, phenolic acid metabolites degraded by colonic microorganisms constitute approximately 40% of the ingested EGCG, underscoring the significant role of colonic metabolism in EGCG bioavailability.

Keiko Unno et al. demonstrated that EGCG can penetrate the BBB to access the brain parenchyma, influencing neuronal cell proliferation and neurogenesis, thus potentially mitigating neurodegenerative diseases (34). There are two common views on the impact of EGCG bioavailability on neuroprotection. We know that only a small fraction of oral EGCG is absorbed into the circulation. In addition, Shimizu et al. found that oral EGCG accumulates primarily in the gut (50%), with less than 0.01% distributed in the liver, blood, and brain (205). Upon comparison of the distribution of EGCG in mice following oral and intravenous administration, it was observed that the majority of orally administered EGCG entered the bloodstream in its glucuronidated form. Additionally, a significant portion of EGCG accumulated in the small intestine and colon (206). Intravenously injected EGCG was rapidly distributed in an uncoupled state in other tissues such as brain, liver, and lung (207, 208). These findings underscore the notion that while intravenous EGCG achieves rapid tissue penetration, oral administration necessitates absorption through the intestine followed by redistribution to tissues and organs. Thus, intestinal absorption emerges as a critical factor limiting EGCG bioavailability and its potential neuroprotective effects across the BBB.

An alternative perspective posits that the gut harbors a substantial population of immune cells and neural networks, and EGCG has the potential to modulate signaling and functional disruptions in intestinal neuroimmune communication via the brain-gut axis (13, 209–211). This theory underscores the gut-brain axis as pivotal in brain injury, neuroinflammation, and related diseases, with microbiota signaling pathways playing a crucial role in neuroprotection (212, 213). It is known that gut microbes can metabolize EGCG into fission products that are more bioavailable and easier to pass through the BBB to exert neuroprotective effects (214). Moreover, evidence supporting EGCG's neuroprotective effects via BBB-mediated anti-neuroinflammation and reduction of oxidative stress includes: (i) EGCG enhances dopamine neuron activity in the gut, decreases serotonin levels in the colon, and increases hippocampal 5-hydroxytryptamine levels by enhancing intestinal permeability (215–217). (ii) EGCG alleviates intestinal inflammation and repairs the intestinal barrier by altering the gut microbiome. The alteration of the gut microbiome ultimately results in the alleviation of neuroinflammation and neurodegenerative diseases by affecting physiological processes such as immune cell development and migration, amyloid deposition, BDNF and NMDA signaling (218–220). (iii) EGCG also impacts the metabolome of gut microbes, influencing short-chain fatty acids, secondary bile acids, and tryptophan-related metabolites (221, 222). These metabolites traverse the BBB and modulate the host's nervous system.

4.2 Toxicity of EGCG

Despite its limited oral bioavailability, EGCG can induce toxicity, particularly when administered in fasted states or at high doses. Numerous studies have questioned whether EGCG has a clinical therapeutic role, as well as concerns about EGCG toxicity during treatment of various neurodegenerative diseases. Multiple system atrophy (MSA) is a rare neurodegenerative disorder characterized by neuronal loss and gliosis in various regions of the CNS, including the striatum, olivocerebellum, and central autonomic structures (223). A histopathological hallmark of MSA is the presence of oligodendrocyte cytoplasmic inclusions containing misfolded and aggregated α-synuclein (223, 224). EGCG has been shown to inhibit α-synuclein aggregation and mitigate associated toxicity. Johannes Levin et al. conducted a randomized, double-blind, parallel-group, placebo-controlled clinical trial, which demonstrated that 48 weeks of EGCG treatment did not alter disease progression or provide clinical benefit in MSA (225). Two patients discontinued EGCG therapy due to severe hepatotoxicity during the trial (225). The study concluded that elevated transaminase concentrations at therapeutic doses greater than 1,200 mg would cause hepatotoxicity (225). However, the study affirms that EGCG is generally well tolerated in humans and supports the idea that EGCG therapy acting on the α-synuclein oligomer formation may be an effective target for the treatment of neurodegenerative diseases (225). Additionally, numerous animal studies have highlighted adverse effects of EGCG, particularly affecting the liver and kidneys (226–228). We focus on the hepatotoxicity and nephrotoxicity of EGCG and briefly summarize the other adverse effects of EGCG (gastrointestinal toxicity).

4.2.1 Hepatotoxicity of EGCG

The liver is known to be the major drug metabolizing organ in the human body. Initially, K Nakagawa et al. examined the distribution of EGCG (500 mg/kg body weight) in the body after 1 h of oral administration in rats (227). They observed that EGCG concentrations were highest in the intestine, followed by the liver, with plasma levels approximately one-fourth of those in the liver and notably lower concentrations in the brain (227). Autopsy findings further confirmed EGCG induced hepatotoxicity, correlating the extent of liver damage with dosage, route, and duration of EGCG administration (228). Studies on oral EGCG toxicity have documented varying degrees of hepatotoxicity, ranging from mild elevation in liver enzymes (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) to severe hepatocellular necrosis and bile duct hyperplasia as therapeutic doses increased (229). Thus, it is evident that the liver is a significant target organ for EGCG toxicity.

Animal studies have shown that the severity of liver injury produced by EGCG treatment is related to dose, administration route, and treatment duration. Balaji Ramachandran et al. investigated the

relationship between the adverse effects of EGCG treatment with dose and administration route by giving EGCG (108, 67.8, 21.1, and 6.6 mg/kg/d) orally or intraperitoneally to mice (230). Subcutaneous injection of 108 mg/kg EGCG resulted in severe hepatic parenchymal congestion, hepatocellular balloon-like degeneration, kupffer cell hyperplasia, and calcification (acute hepatitis); serum levels of bilirubin, AST, ALT, and ALP were markedly elevated, leading to mortality by the 8th day of treatment (230). Mice injected with 67.8 mg/kg EGCG subcutaneously exhibited moderate hepatic peritoneal and mild lobular inflammation; elevated serum AST and ALT levels were observed, with mortality occurring by day 16 of the experiment (230). In comparison to subcutaneous injection, oral administration of EGCG resulted in lower hepatotoxicity, with significant liver damage observed only in mice receiving 108 mg/kg EGCG orally. Notably, increasing EGCG doses correlated exclusively with hepatic toxicity, ranging from mild periportal inflammation to severe hepatitis (230). Similarly, Dongxu Wang et al. investigated the dose-dependent hepatotoxic effects of subcutaneously injected EGCG (55, 70, and 125 mg/kg/day) in mice (229). Their findings revealed that all mice injected with 125 mg/kg or 70 mg/kg EGCG succumbed within 2 days, showing severe hepatotoxicity characterized by elevated serum levels of ALT, AST, and 4-HNE, along with increased expression of Nrf2 target genes in the liver. Mice injected with 55 mg/kg EGCG exhibited hepatotoxic effects but survived the duration of the study (229). It has also been demonstrated that subcutaneous injection of 45 mg/kg/day of EGCG represents the maximum tolerated dose in mice, with long-term administration at this dose showing no impact on the body's oxidative defense mechanisms (231). However, injections of 55 or 75 mg/kg/day of EGCG induced hepatotoxicity in mice, accompanied by inhibition of hepatic antioxidant enzymes and increased nuclear distribution of Nrf2 (231). Furthermore, repeated injections of 75 mg/kg/day of EGCG altered the oxidative defense mechanism, significantly reducing levels of SOD, catalase, and GPX (231). Subcutaneous injection of EGCG in mice at doses exceeding 100 mg/kg/day induces severe hepatotoxicity and dose-dependent mortality, with higher concentrations leading to accelerated death. This treatment also inhibits Nrf2 target gene expression and diminishes antioxidant defense capacity (231). Similarly, gavage administration of EGCG yielded comparable results: mice exhibited hepatic congestion and a slight elevation in ALT levels after receiving 750 mg/kg/day of EGCG for 5 consecutive days (228). Following gavage of 750 mg/kg/day of EGCG for 7 consecutive days, mice exhibited a significant increase in ALT, MDA, MT, and γH2AX levels in the liver, along with hepatocyte degeneration, resulting in a mortality rate of 75% (232). single gavage of 1,500 mg/kg of EGCG led to a 108-fold increase in ALT levels and an 85% mortality rate among mice (232). Metabolites EGCG-2′-cysteine and EGCG-2″-cysteine were detected in urine following high-dose gavage of EGCG (233). Notably, EGCG administered via diet was well tolerated and demonstrated reduced hepatotoxicity compared to gavage administration in animals (233). Studies administering EGCG to Beagles indicated that fasting increased the likelihood of hepatotoxicity compared to animals that were fed prior to treatment (234). These findings underscore the influence of dose, route of administration, treatment duration, and nutritional status on EGCG-induced hepatotoxicity.

Animal experiments have shown that EGCG induced hepatotoxicity correlates with changes in several oxidative stress markers in the body, including MDA, 4-HNE, MT, γH2AX, and Nrf2 (228, 229, 231, 235). MDA and 4-HNE are products of lipid peroxidation and serve as biochemical indicators of oxidative stress (228). MT and γH2AX are molecular markers associated with oxidative stress. All these biomarkers suggest that hepatotoxicity induced by EGCG treatment is largely induced by oxidative stress (201). Nrf2 functions as a crucial transcription factor in antioxidant defense. Under normal physiological conditions, Nrf2 is sequestered by Keap1; however, during oxidative stress, Nrf2 dissociates from Keap1 and translocates to the nucleus where it binds to antioxidant response elements. This activation of the Nrf2-ARE signaling pathway upregulates the expression of various antioxidant genes such as HO-1, GST, and NADP (H): NQO1 (231). The Nrf2-ARE signaling pathway activates and enhances the expression of downstream antioxidant enzymes, serving as a critical cellular defense mechanism against oxidative stress (236). This pathway, particularly in the liver, is pivotal in mitigating EGCG-induced hepatotoxicity (236). Animal studies have shown that subcutaneous injection of EGCG at 45 mg/kg/day in mice does not impair major hepatic antioxidant defenses but modestly increases hepatic expression of Nrf2 target genes (231). Conversely, injection of 75 mg/kg/day of EGCG inhibits major hepatic antioxidant enzymes while significantly elevating Nrf2 expression and its target genes (231). Injection of 100 mg/kg/day of EGCG notably suppresses the hepatic Nrf2 pathway (231). These findings indicate a biphasic response of Nrf2 to different EGCG doses. In summary, EGCG-induced hepatotoxicity involves the inhibition of major antioxidant enzymes, with the Nrf2 salvage pathway playing a crucial role in mitigating toxicity. However, this pathway becomes inhibited at higher concentrations of EGCG.

4.2.2 Other toxicities of EGCG

Nora O. Abdel Rasheed et al. investigated potential nephrotoxic effects of EGCG treatment in diabetic mice, a crucial concern due to the kidney's vulnerability in diabetes (237). Diabetic mice injected with 100 mg/kg EGCG daily for 4 days exhibited decreased resistance to oxidative stress, as indicated by elevated NADPH oxidase levels and reduced expression of Nrf2, HO-1, and HSP90 (237). Serum levels of CYS-C and NGAL were significantly elevated, and histopathological analysis confirmed EGCG-induced renal injury in diabetic mice (237). Similarly, another study demonstrated nephrotoxicity in colitis mice treated with green tea extract containing 35% EGCG, evidenced by increased serum creatinine levels (a nephropathy biomarker), and elevated expression of antioxidant enzymes (HO-1 and NQO1) and HSP 90 (238). These findings collectively underscore the potential nephrotoxic effects of EGCG treatment, exacerbated by oxidative stress implicated in diabetes and its complications (239). Thus, caution is advised when considering EGCG supplements for diabetic patients, particularly at high doses.

In addition to nephrotoxicity and hepatotoxicity, numerous studies have documented gastrointestinal toxicity associated with EGCG administration, whether by gavage or in diet, in animal models (240–242). The severity of gastrointestinal effects varied with dosage, ranging from mild gastric erosion and vomiting to severe ulceration, hemorrhage, and epithelial necrosis. Notably, gastrointestinal toxicity was more pronounced in animals administered EGCG via gavage or when fasted, whereas administration via diet, water, or capsule resulted in milder effects (234, 243). In conclusion, treatment with EGCG at high doses or for prolonged duration may have adverse effects, and the above data suggest that the boundary between protective and toxic doses of EGCG may be narrow.

4.3 Safe dose of EGCG

Another critical issue is establishing safe dosage levels of EGCG to optimize therapeutic efficacy while minimizing adverse effects. Current clinical studies on EGCG dosages vary widely, and extrapolation from animal dosages to humans is virtually impossible (244–255). Safety data from human studies indicate distinct toxicity thresholds for EGCG consumed as a beverage compared to capsules or tablets, necessitating separate consideration of safe intake levels. Studies have shown that ingestion of up to 676 mg of EGCG in capsules or tablets did not result in significant adverse effects in healthy adults or patients with various conditions (256). In addition, liver toxicity has been documented with the intake of 800 mg or 1,200 mg of EGCG (225, 253). However, considering that the pro-health benefits of EGCG are similar to those of nutrients. Jiang Hu et al. used an approach similar to the Institute of Medicine (IOM) nutrient risk assessment to determine the safe intake of EGCG (257). The results indicated that the safe intake of EGCG in capsules or tablets for adults is 338 mg/day (257). This safe dose is consistent with the dose derived from animal data (322 mg/day) and is consistent with recent doses proposed by Yates et al. (258) and Dekant et al. (195). Regarding the toxicity threshold for EGCG intake in the form of beverages, the highest reported intake level of EGCG was 704 mg/day with no apparent adverse effects (245). For the current study, it is still uncertain what the standardized safe intake level of EGCG is, as the data currently available from human clinical studies may vary in terms of design, duration, and subject populations. However, the results of the current analyses suggest that diluting and/or slowing the rate of systemic administration of EGCG often appears to be better tolerated by the body. Even so, careful calculation of daily EGCG intake is important when EGCG is used as a dietary supplement. When other EGCG sources are available, EGCG intake may require health-based guidance. The use of EGCG as a clinical agent for neurodegenerative diseases still requires further evaluation of toxicity and dosage.

5 Conclusion

In conclusion, this review highlights the significant potential of EGCG, a prominent catechin abundant in green tea, as a therapeutic agent for neurodegenerative diseases. By targeting chronic neuroinflammation and oxidative stress, EGCG demonstrates promising neuroprotective effects in conditions such as AD and PD. Through its antioxidant properties and anti-inflammatory activities, EGCG shows efficacy in mitigating key pathological mechanisms associated with neurodegeneration. The comprehensive exploration of EGCG's molecular mechanisms, including its modulation of autoimmune responses, nervous-immune system interactions, and inflammatory pathways, underscores its therapeutic relevance in AD and PD. Observational epidemiological studies and experimental investigations provide compelling evidence for EGCG's neuroprotective effects, supporting its potential as a therapeutic intervention. Furthermore, EGCG's ability to scavenge free radicals, chelate iron, and attenuate neuroinflammatory processes highlights its multifaceted mechanisms of action. Overall, EGCG emerges as a promising natural compound with the capacity to combat chronic neuroinflammation and oxidative stress, offering novel avenues for the development of neuroprotective strategies in the treatment of neurodegenerative disorders. Further research into EGCG's therapeutic potential, including clinical trials and mechanistic studies, is warranted to fully elucidate its efficacy and safety profile in neurodegenerative diseases.

Author contributions

SL: Writing – original draft, Writing – review & editing. ZW: Writing – review & editing. GL: Supervision, Writing – review & editing. MC: Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Advances in the application and mechanism of bioactive peptides in the treatment of inflammation

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Inflammation is a normal immune response in organisms, but it often triggers chronic diseases such as colitis and arthritis. Currently, the most widely used anti-inflammatory drugs are non-steroidal anti-inflammatory drugs, albeit they are accompanied by various adverse effects such as hypertension and renal dysfunction. Bioactive peptides (BAPs) provide therapeutic benefits for inflammation and mitigate side effects. Herein, this review focuses on the therapeutic effects of various BAPs on inflammation in different body parts. Emphasis is placed on the immunomodulatory mechanisms of BAPs in treating inflammation, such as regulating the release of inflammatory mediators, modulating MAPK and NF-κB signaling pathways, and reducing oxidative stress reactions for immunomodulation. This review aims to provide a reference for the function, application, and anti-inflammation mechanisms of BAPs.

KEYWORDS

bioactive peptides, inflammation, immunomodulation, inflammatory mediators, pathways

1 Introduction

Inflammation is a normal immune response of the body's innate and adaptive immune systems to infections (1), which can protect the body from damage caused by external toxins and stimuli (2). It is a way to self-heal, repair damaged tissues, and combat pathogens (3). However, the attack of inflammatory factors will result in cellular necrosis and the reduction of metabolic and immune functions, eventually leading to tissue damage and organ dysfunction. The duration of inflammation is different, which could be divided into acute and chronic inflammation (4). Many chronic diseases are associated with inflammation, including arthritis, inflammatory bowel disease (5), cardiovascular diseases (6), osteoporosis (7), cancer (8), and obesity (9). Therefore, combating inflammatory damage is one of the major health challenges of the 21st century. Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and ibuprofen, are a class of chemically synthesized anti-inflammatory drugs that do not contain steroid structures (10). They are the most widely

used anti-inflammatory drugs. However, numerous studies have shown that NSAIDs have various side effects on the host, including hypertension, nephrotic syndrome, cardiovascular toxicity, acute renal failure, and gastrointestinal complications (1). Additionally, antibiotics can be used to treat inflammation, but they can induce to the emergence of antibiotic-resistant superbugs. Therefore, there is an urgent need to explore new strategies for anti-inflammation. Since the first antimicrobial peptide Cecropins was discovered in 1981, the antibacterial and anti-inflammatory activity of peptides has attracted more and more attention from academia (11). Research has described that the peptide GPETAFLR possessed anti-inflammatory activity, effectively inhibiting neuroinflammation and maintaining stability in the central nervous system (12).

BAPs refer to short-chain amino acid sequences with active biological functions within organisms, typically consisting of 2 to 20 amino acid residues interconnected by peptide or amide bonds (13). The arrangement and combination of these amino acid residues are different and can form linear or cyclic structures (13). The sources of BAPs are diverse, mainly including animals, plants, microorganisms, marine organisms, soy products, milk, and fermented products (14). When BAPs remain inactive within parent proteins, they can become active upon enzymatic release through peptide cleavage (15). Apart from being generated through the hydrolysis of parent proteins, BAPs can also be produced via microbial fermentation. In order to obtain BAPs with specific activity, specific proteases with a wide range of functions are usually used for hydrolysis (16).

Peptides offer several advantages over traditional drugs in disease treatment (17). For example, their low molecular weight allows them to penetrate membranes effectively (18, 19), making them more potent (20). Furthermore, bioactive peptides (BAPs) have the potential for targeted therapy with minimal or negligible toxicity, even at low concentrations (21). Inflammation occurs after the activation of inflammatory pathways by triggering factors, leading to the release of inflammatory agents (22). Concurrently, the anti-inflammatory characteristics of BAPs may be influenced by molecular weight, amino acid composition (hydrophobic amino acids, positively charged amino acids, specific amino acids), and amino acid position (3).

This review provides a detailed overview of the research status of BAPs in the treatment of skin inflammation, intestinal inflammation, pulmonary inflammatory disease, arthritis, and ocular inflammation. Subsequently, it delves into the immunomodulatory mechanisms employed by BAPs in the treatment of inflammation, such as regulating the release of inflammatory mediators, modulating mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB) signaling pathways, and reducing oxidative stress response for immunomodulation. The aim is to seek new strategies for inflammation treatment and provide references for the development and application of anti-inflammatory peptides.

2 The functions of BAPs

BAPs exhibit a wide array of functions including antimicrobial, antioxidative, anti-inflammatory, memory-enhancing, antithrombotic and antihypertensive activities, regulation of gastrointestinal absorption, appetite suppression, opioid modulation, immune modulation, and cell regulation. According to different functions, BAPs are mainly divided into anti-inflammatory peptides, antimicrobial peptides (AMPs), antioxidant active peptides, anticancer active peptides, antihypertensive peptides, and neuropeptides (Table 1). Anti-inflammatory peptides can modulate immune responses and alleviate inflammation. They can suppress the production of pro-inflammatory cytokines and the activation of inflammatory pathways, or directly interact with immune cells. BAPs with antibacterial activity are called AMPs.The activity of AMPs may be attributed to their ability to effectively disrupt bacterial cell walls or membranes with a strong negative charge, exerting their action with cations and their hydrophobic effect (15). They may also attack microbial membranes or cytoplasmic components, altering their cellular functions and leading to cell death (23). AMPs can inhibit the synthesis of cell walls, nucleic acids, and proteins by engaging various enzymes within target cells (23). AMPs possess minimal to provoke resistance (24), thereby conferring a natural advantage over antibiotics for combating microbial infections. Han et al. (25) discovered that AMPs containing tryptophan can downregulate the expression of DNA replication initiation genes in cells, consequently demonstrating efficacy in combating multidrug-resistant *Pseudomonas aeruginosa*.

The antioxidant effect of BAPs can slow down or prevent cellular damage (26). With the disturbance of the prevailing environment, oxidative stress reactions occur, resulting in the release of free radicals, which may contribute to health issues, including cancer, cardiovascular, and other diseases (27). These peptides primarily consist of 5-16 hydrophobic amino acids (27). They typically include tyrosine, whose phenolic side chain serves as an important scavenger of free radicals (28). Hydrophobic amino acids can increase the penetration rate of peptides to cell membranes, and enhance the ability of peptides to reach mitochondria, which is one of the main sites of free radical production (29, 30). An important feature of the antioxidant activity of BAPs is their hydrophobicity. It helps protect the polyunsaturated fatty acids and other lipophilic targets from oxidation (29, 30). Teng et al. (31) reported that jellyfish peptides (JPHT-2) were effective antioxidants which could scavenge free radicals. The peptides enhanced the levels of superoxide dismutase (SOD) and inhibited oxidative damage by H_2O_2. Gao et al. (32) reported a new anti-inflammatory peptide from sturgeon muscle, and found that it can effectively inhibit the release of NO, IL-6 and IL-1β, increase the SOD activity in the LPS-induced RAW264.7 cells, and down-regulate MAPK pathway. Zhou et al. (33) described that milk casein-derived peptide OEPVL could regulate the release of nitric oxide (NO) and the production of cytokines IL-4, IL-10, IFN-γ, and TNF-α *in vivo*, thereby achieving the purpose of inhibiting LPS-induced inflammation.

3 Anti-inflammation of BAPs

As infection affects or damages different organs within the body, an inflammatory response occurs to combat infection, address injury, and facilitate self-repair. However, inflammatory factors

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TABLE 1 The names/sequences and source of BAPs with different functions.

Species	Peptide names/sequences	Source	Reference
Anti-inflammatory peptides	GPETAFLR	*Lupinus angustifolius* L.	(34)
	DAPAPPSQLEHIRAA, AADGPMKGILGY	*Lateolabrax maculatus*	(35)
	SSEDIKE	Amaranth proteins	(36)
	Lectin	Red algae *Amansia multifida*	(37)
	VHYAGTVDY	Sturgeon muscle	(32)
	PRRTRMMNGGR	Juice of cooked tuna	(38)
	KQSESHFVDAQPEQQQR	Simulated gastrointestinal digestion of extruded adzuki bean protein	(39)
	MSCP	*Chanos chanos*	(40)
	VVNEGEAHVELVGPKGNKETLEYES, AMPVNNPQIHDFFL	Beans (*Phaseolus vulgaris* var. pint)	(41)
	WNLNP	OPEH (*Crassostrea hongkongensis*)	(42)
Antimicrobial peptides	Turgencin A	Arctic sea squirt *Synoicum turgens*	(43)
	Myticusin-beta	Mytilus coruscus	(44)
	Temporin-1CEh	Rana chensinensis	(45)
	EQLTK	Bovine α-L A	(46)
	ISGLIYEETR, IGNGGELPR, ILVLQSNQIR	*Saccharina longicruris*	(47)
	cNK-2(RRQRSICKQLLKKLRQQLSDALQNNDD)	Chicken NK-lysin	(48)
	Clavanin-MO (FLPIIVFQFLGKIIHHVGNFVHGFSHVF-NH_2)	Hemocytes of marine tunicates	(48)
	Phylloseptin-PV1	*Phyllomedusa vaillantii*	(49)
	GDVIAIR	Chia seed	(50)
	TSKYR, STVLTSKYR, TSKYR	Human hemoglobin: active peptide α137-141	(51)
	AGLAPYKLKPIA	Ovotransferrin	(52)
	YPWTQR, ITMIAPSAF, DSYEHGGEP, VVSGPYIVY	Egg yolk	(53)
Antioxidant active peptides	GGAW	Octopus	(54)
	JPHT-2	Jellyfish	(31)
	WSVPQPK	Human β-CN	(55)
	VPP, IPP	Whey protein concentrate (WPC)	(56)
	GAPGPQMV	Skipjack tuna (*K. pelamis*) bones	(57)
	GPGGFI	*N. septentrionalis* skin	(58)
	SMRKPPG	Peony (*P. suffruticos*) seed	(59)
	YFPH	*Limanda aspera*	(60)
	GFPGRLDHWCASE	Flaxseed (*Linum usitatissimum*)	(61)
		Finger millet (*Eleusine coracanac*) protein hydrolysate	(62)

(Continued)

TABLE 1 Continued

Species	Peptide names/sequences	Source	Reference
	TSSSLNMAVRGGLTR, STTVGLGISMRSASVR		
	VECYGPNRPQF	Algae (*Chlorella vulgaris*) protein waste	(63)
	IDHY, VVER	Water-soluble protein (*Gracilariopsis chorda*)	(64)
	VLPVPQK	Milk	(65)
Anticancer active peptides	Callyaerins A-F, Callyaerins H	*Callyspongia aerizusa*	(66)
	Bowman-Birk-type PI	*Phaseolus acutifolius*	(67)
	Homophymine A	Marine sponge *Homophymia* sp.	(68)
	FIMGPY	Skate (*Raja porosa*) cartilage protein hydrolysate	(69)
Antihypertensive peptides	IVDR, WYK, VASVI	Paralichthys olivaceus	(70)
	VHVV	Soybean	(71)
	ERYPIL, VFKGL, WEKAFKDED, QAMPFRVTEQE	Egg white hydrolysate	(72)
	DGVVYY	Seed meal of tomato	(73)
	BCH, BCH-III	Chicken blood	(74)
	PPL, PAP, AAP	Iberian dry-cured ham	(75)
Neuroactive peptides	Doppelganger-related peptides	Cone snail toxins	(76)
	Arginine vasopressin	Hypothalamus	(77)
	Glucagon-like peptide-1	Proglucagon derived peptide	(78)
	Human urotensin-II	Central nervous system	(79)

can attack cells, leading to cell death, reduced cellular metabolism, and compromised immune function (3). BAPs can treat skin, intestine, lung, joint and eye inflammation, etc (Figure 1). BAPs can regulate the inflammatory pathways, the levels of cytokines or gut microbiota, and alleviate oxidative stress (Table 2).

3.1 Skin inflammation

The skin serves as a physical barrier between internal and external environments (107). Various factors can induce inflammatory responses in the skin, primarily due to immune dysregulation caused by internal diseases, infections, and allergic reactions. Skin inflammation is a primary manifestation of chronic autoimmune inflammatory diseases such as psoriasis, atopic dermatitis (AD), and lupus erythematosus (108). Approximately, 60 million people suffer from psoriasis, a chronic, systemic, immune-mediated inflammatory skin disease (109). As previously described, the synthetic peptide LKEKK (150-500 μg) combined with Aldara cream containing 5% imiquimod was applied to the ears of the imiquimod-induced psoriasis mouse model (80). After 6 days of treatment, the thickness of mouse ears was significantly reduced, indicating that the development of inflammation was effectively inhibited. Traditional medications for AD often yield unsatisfactory results. Lee et al. (83) reported a short peptide TPS240 and investigated its therapeutic effect in a DNCB-induced AD mouse model. The control group was treated with the same concentration of dexamethasone. Finally, it was found that the symptoms of AD in the TPS240 group were alleviated, and the skin damage was significantly restored by using 5 mg/kg TPS240. The body weight of mice treated with 5 mg/kg dexamethasone decreased and the organs contracted abnormally. TPS240 exerts its anti-AD effect by inhibiting the activation of NF-κB and STAT3, which is similar to dexamethasone and has no side effects. These results indicated that TPS240 would be a safe and effective drug for AD. Systemic lupus erythematosus (SLE) is an autoimmune disease that can promote chronic inflammation (110). It has been reported that the artificial peptide pConsensus, which blocks the PD-1/PD-1 ligand 1 pathway

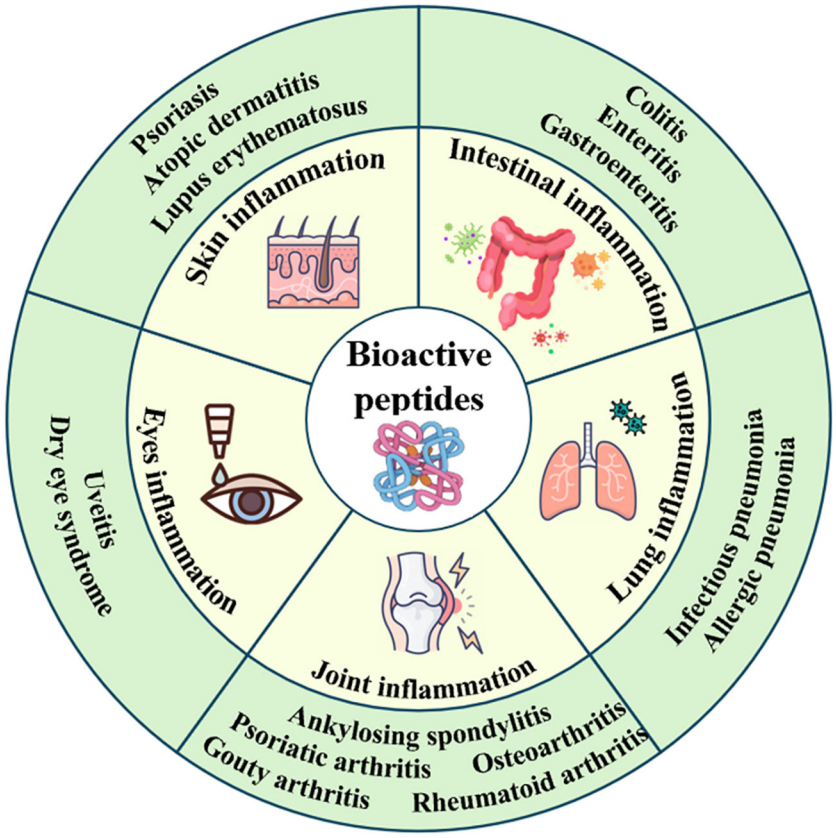

FIGURE 1
Scheme of the treatment of organ inflammation by BAPs.

in untreated mice, promotes tolerance and inhibits SLE (111). Schall et al. (112) reported the peptide P140 could clear harmful T and B cells, and normalize the immune response in lupus-susceptible mice.

Additionally, due to the skin's susceptibility to various injuries, wounds disrupt its environmental barrier, leading to a cascade of inflammatory responses. Controlling inflammation is crucial for maintaining skin health. Li et al. (113) demonstrated that the peptide OA-RD17 extracted from *Odorrana-andersonii* skin tissue could activate MAPK to promote macrophage proliferation and migration, block inflammation and propel wound healing by inhibiting NF-κB. OA-RD17 could accelerate the regeneration of full-thickness skin wounds in mice, showing that the repair rate of skin wounds was nearly 100%. At the same time, it had a certain repair effect on deep second-degree burns and isolated skin wounds of diabetic patients. OA-RD17 could up-regulate the expression of miR-632 and promote the regeneration of full-thickness skin wounds in rats, and the repair rate reached 92.4%. Therefore, BAPs with their antimicrobial and immune-modulating functions offer efficacious therapeutic approaches for wound healing and skin inflammation.

3.2 Intestinal inflammation

The intestine plays a crucial role in human health, serving as a site for digestion and nutrient absorption, and the largest organ of the immune system (114). The intestinal barrier is essential for separating the external environment from the host's internal environment. As the intestine is exposed to pathogens or other toxic substances, inflammatory responses occur under the influence of harmful stimuli (115). Enteritis is a prevalent inflammatory bowel disease. So far, the main methods used clinically for enteritis treatment include drug therapy, dietary interventions and surgical treatment. However, the treatment outcomes are often unsatisfactory. Therefore, it is very important to find a better and safer treatment method. BAPs have immunomodulatory and anti-inflammatory effects, making it possible to effectively treat intestinal inflammation and provide a new treatment for enteritis. Zhi et al. (116) reported that walnut-derived peptide leucine-proline-phenylalanine (LPF) could promote the repair of the intestinal epithelial barrier, reduce levels of pro-inflammatory cytokines, and exert protective and restorative effects on DSS-induced colitis in mice. It was found that the number of apoptotic cells in the treatment group was significantly less than that in the DSS group. The percentages of reduction in the three groups of DSS + 50 mg/kg LPF, DSS + 100 mg/kg LPF, and DSS + 200 mg/kg LPF on the 10th day were 50.00%, 41.18%, and 57.35%, respectively. In addition, 16S rDNA sequencing results showed that 100 mg/kg LPF had a regulatory effect on the intestinal flora of colitis mice. Additionally, Rahabi et al. (117) reported that fish collagen peptide Naticol®Gut could also be used to treat colitis. It directly acts on macrophages, polarizing them into an anti-inflammatory,

TABLE 2 Application of BAPs in the treatment of inflammation of various organs.

Organ	Peptide names	Disease type	Peptide activity	Reference
Skin	LKEKK	Psoriasis	↑ IL-10, IFN-γ ↓ IL-17	(80)
	MHP1-AcN	Psoriasis	↓ IL-6, IL-23, IL-17A	(81)
	AES16-2M	Atopic dermatitis	↓ CD4 T cells ↓ TSLP	(82)
	TPS240	Atopic dermatitis	Inhibition of NF-κB and STAT3 activation	(83)
	AMP-IBP5	Atopic dermatitis	↑ TJ barrier function	(84)
	ARA290	Systemic lupus erythematosus	↓ IL-6, MCP-1, TNF-α ↑ TGF-β Suppressing the level of serum ANAs and anti-dsDNA autoantibodies Inhibiting the inflammatory activation of macrophages Promoting the phagocytic function of macrophages	(85)
Intestinal	rVIPa	Colitis	↓ TNF-α, MPO activity, serum endotoxin, TLR4 ↑ IL-10 ↑ occluding, ZO-1, NF-κB p65, IκBα	(86)
	R7I	Intestinal inflammation	Inhibition of TLR4 and NF-κB expression ↑ SOD and GSH-PX ↓ MDA	(87)
	MOP	Colitis	Inhibiting JAK-STAT pathway's activation Regulating gut microbiota and its metabolites	(88)
	TBP	Ulcerative colitis	↑ SOD and GSH-Px ↓ LPS, IL-6, TNF-α ↑ Gene expression of TJ protein ↑ SCFAs Restoring intestinal flora	(89)
	Cecropin A (1-8)-LL37 (17-30)	Intestinal inflammation	↓ TNF-α, IL-6, IFN-γ ↓ Apoptosis ↓ Markers of jejunal epithelial barrier function	(90)
Lung	PS1-2	Fungal pneumonia	↓ Activity of TLR-2 ↓ TNF-α	(91)
	7-amino acid peptide (7P), (Gly-Gln-Thr-Tyr-Thr-Ser-Gly)	Allergic lung inflammation	↓ Airway hyperresponsiveness ↓ Airway inflammation ↓ Th2 responses	(92)
	IDR-1002	Pneumonia	↓ IL-6, TNF-α	(93)
	Hydrostatin-SN1	Acute lung injury	↓ TNF-α, IL-6, IL-1β	(94)
Joint	AKP	Osteoarthritis	↓ HIF-2α and downstream genes	(95)
	AESIS-1	Rheumatoid arthritis	Downregulation of STAT3 signaling	(96)
	KPs	Adjuvant-induced arthritis	Inhibiting IL-1β-related inflammation and MMPs production	(97)
	GLPP	Rheumatoid arthritis	↓ TNF-α, IL-1β, IL-6, MMPs, BCL-2, OPN, β-Catenin, HIF-1α ↑ Bax Inhibiting NF-κB and MAPK signaling pathways	(98)
	IQW	Ankylosing spondylitis	↓ IL-6, IL-1β, TNF-α ↑ CAT, GSH-PX, SOD	(99)
	Alamandine	Rheumatoid arthritis	↓ IL-6, IL-23 and IFN-γ mRNA expression ↓ TNF-α, IL-6, IL-17 ↑ IL-10	(100)
Eyes	R9-SOCS1-KIR	Uveitis	Inhibiting nuclear factor κB and p-p38 pathways	(101)
	WP-17	Uveitis	Inhibition of NF-κB pathway activation	(102)

(Continued)

TABLE 2 Continued

Organ	Peptide names	Disease type	Peptide activity	Reference
	TSP	Dry eye disease	Regulating Bax/Bcl-2 signal pathway Inhibiting iNOS and COX-2 Moderating ROS/Nrf2/HO-1 axis Apoptosis inhibiting	(103)
Others	P140	Periodontitis	↓ TNF-α, INF-γ ↓ Infiltration of activated lymphocytes	(104)
	Nal-P-113	Periodontitis	↓ IL-1β, TNF-α	(105)
	Bomidin	Periodontitis	Downregulation of MAPK and NF-κB signaling pathways Activation of Keap1/Nrf2 pathway	(106)

↑ and ↓ indicated increase and decrease, respectively.

immunotolerant, and antioxidative phenotype through an MR-dependent mechanism. For enteritis, antibiotics are often used for treatment, but their long-term use can lead to increased antibiotic resistance, posing a significant challenge. Sun et al. (118) reported that AMP R7I with anti-proteolytic properties could reduce inflammatory factors and maintain intestinal barrier function. The histological examination of the intestine showed that the tissue structure in the 20 mg/kg R7I group was basically normalized with only a small amount of isolated epithelial cells, and R7I could restore the normal morphology of the intestine. In addition, this peptide plays a crucial role in the treatment of murine bacterial enteritis and is helpful in finding effective strategies for the treatment of enteritis.

3.3 Lung inflammation

Pneumonia is a prevalent respiratory illness that involves inflammation in the lungs (119). Its occurrence is associated with respiratory viruses, common gram-negative or gram-positive bacteria, and mycobacterium (120, 121). Pneumonia has a complex etiology, and traditional treatment methods mainly involve the use of antibiotics, which can effectively reduce the incidence and mortality of pneumonia. However, issues such as antibiotic resistance, low bioavailability, and strong side effects exist (92, 122). Therefore, there is a necessity to discover novel treatment approaches. BAPs as a novel therapeutic drug may have potential in the treatment of pneumonia. Zhao et al. (92) reported that 7-amino acid peptide (7P), as a synthetic analog peptide, could effectively reduce bronchial contraction, inhibit acute inflammatory cytokines (TNFα, IL-1β and IL-6) and Th2 cytokine responses (IL-5, IL-4 and IL-13), and has certain effects on relieving airway hyperresponsiveness, airway inflammation and Th2 response. The results inferred that 7P could reduce allergic lung inflammation. It made a new option for addressing allergic pulmonary inflammation. Additionally, peptide modification can also be employed to improve the therapeutic effects. Moreira et al. (123) pegylated the synthetic peptide LyeTx I-b derived from natural LyeTx I, and reported that pegylated LyeTx I-b exhibited significant therapeutic effects against multidrug-resistant *Acinetobacter baumannii*-induced pneumonia. LyeTx I-bPEG increased the anti-biofilm activity. At 16 μM and 32 μM, LyeTx I-bPEG reduced the carbapenem-resistant *Acinetobacter baumannii* biofilm by 33 ± 4% and 26 ± 8%, respectively, compared with untreated cells. Furthermore, Jin et al. (124) designed two derived peptides GHbK4R and GHb3K based on the maternal peptide GHb. Vancomycin reduced lung bacteria in mice to 7.8×10^7 CFU/g, whereas GHb3K and GHbK4R decreased lung bacteria to 5.3×10^5 and 5.4×10^5 CFU/g. These results demonstrated that these peptides had significant therapeutic effects in a mouse model of acute pneumonia caused by *Staphylococcus aureus* infection. PS1-2 peptide is active against fluconazole-resistant *Candida albicans*, can inhibit the activity of TLR-2 and the expression of TNF-α, and has anti-fungal and anti-inflammatory functions for intratracheal infection induced by *Candida albicans* (91). However, there is limited research on the use of BAPs for the treatment of human pneumonia. It still needs a good strategy to treat pneumonia.

3.4 Joint inflammation

Arthritis is a common inflammatory disease which affects the joints and surrounding tissues. It can be acute or chronic, leading to joint pain, swelling and difficulty movement in severe cases. Arthritis has a high prevalence and encompasses various types, including osteoarthritis, rheumatoid arthritis, and psoriatic arthritis (125). Osteoarthritis is a progressive disease and a major cause of chronic disability (126). Peptides offer a new therapeutic approach for osteoarthritis. Wu et al. (127) validated that the anti-inflammatory capacity of skipjack tuna elastin peptides in a zebrafish model could inhibit the JAK2/STAT3 signaling pathway, suppress inflammation and protect cartilage. Rheumatoid arthritis is an autoimmune disease that can lead to joint and bone damage (128, 129). For rheumatoid arthritis, Kim et al. (96) reported that a synthetic peptide AESIS-1 could inhibit STAT3-mediated signaling by upregulating SOCS3 expression, resulting in the decrease of Th17 cells. Psoriatic arthritis is a chronic systemic inflammatory disease affecting the skin, joints, and tendons (130). Wixler et al. (131) discovered small splenic peptides (SSPs) in the spleen, which could target dendritic cells and transforming them into tolerant cells, thus differentiating naive CD4 cells into regulatory T cells expressing Foxp3. SSPs had anti-inflammatory effects *in vivo*, and restore peripheral tolerance, effectively inhibiting the development of psoriatic arthritis. In addition, ankylosing spondylitis and gouty arthritis could be treated by using BAPs. Ankylosing spondylitis is

an immune-mediated chronic inflammatory rheumatic disease that most commonly affects the spine (132). Liu et al. (99) reported that BAPs IQW could treat mice with ankylosing spondylitis, delay disease progression, alleviate inflammation in the intervertebral joints, and reduce the concentration of pro-inflammatory factors. Gouty arthritis is caused by inflammation triggered by the deposition of urate crystals in the joints and surrounding tissues (108). Commonly used medications include colchicine, corticosteroids, NSAIDs, and adrenocorticotropic hormone, but these drugs have certain side effects such as nausea and gastrointestinal toxicity. Therefore, there is an urgent need to develop new drugs to treat gouty arthritis (133). Yan et al. (134) described that BAPs mastoparan M (Mast-M) extracted from wasp venom could inhibit the MAPK/NF-κB signaling pathway and reduce oxidative stress, thereby blocking the activation of the NLRP3 inflammasome and effectively treating gouty arthritis. Hence, BAPs have good therapeutic effect on joint inflammation.

3.5 Eyes inflammation

Eye inflammation is a common ocular condition that can occur from the surface of the eye to intraocular tissues (135). As threatened by inflammation, the eye tissues can sustain damage over the short or long term (136). The causes of eye inflammation are varied, including pathogen infections such as bacterial, fungal, and viral infections, as well as non-infectious factors like external environmental stimuli and allergic reactions (137). The treatment of eye inflammation mainly involves the use of anti-inflammatory drugs and antibiotics for medication or surgical methods. However, these approaches have certain drawbacks such as drug side effects and long recovery times. In recent years, more BAPs with therapeutic potential have emerged. Lu et al. (102) designed a peptide called WP-17, which targeted the toll-like receptor 4 (TLR4) to inhibit the activation of the NF-κB pathway. The highest dose of WP-17 (10 μg/eye) strikingly decreased the protein levels of TNF-α and IL-6 in the aqueous humor of rats by 77.26% and 85.67%, respectively. WP-17 has shown promising therapeutic effects in rat uveitis. Similarly, Ho et al. (138) reported that a 29-mer peptide derived from pigment epithelium-derived factor could inhibit the expression of matrix metalloproteinase-9 and pro-inflammatory cytokines on murine dry eye. In addition, Zeng et al. (103) described that tilapia skin peptides (TSP) impeded the generation and development of dry eye disease via inhibition of apoptosis (19.4%), inflammation, and oxidative stress.

3.6 Other inflammation

The oral cavity is an important part of the human body and serves as the starting point of the digestive system. The oral cavity harbors a rich microbial population, constituting the second abundant microbial community in the human body after the gut, with over 700 identified oral microbial species (139, 140). Disruption of the oral microbiota can lead to an increase in local T_H17 cells, which are associated with oral immunity and inflammation (141). Dysbiosis of the oral microbiota can lead to periodontitis, a common oral disease caused by pathogens invading the periodontal tissues such as the gums (142, 143). BAPs can inhibit bacterial growth and reduce inflammation. Akiyama et al. (104) reported the role of peptide P140 in a mouse model of periodontitis, and found that treatment with P140 effectively alleviated inflammation in gingival tissues, reduced lymphocyte infiltration, and lowered the expression of pro-inflammatory mediators. In addition, liver injury can also be treated by bioactive peptides. Zhu et al. (144) described a peptide HEPFYGNEGALR isolated and identified from *Apostichopus japonicus*. This peptide can activate the Nrf2/HO-1 pathway, block the nuclear translocation of NF-κB, alleviate oxidative stress and inflammation, and alleviate acute alcoholic liver injury caused by excessive alcohol intake. Besides, BAPs have a certain ability in the treatment of myocarditis. Cortistatin is a small molecule bioactive peptide (145). Delgado-Maroto et al. (146) reported the therapeutic effect of cortistatin in experimental autoimmune myocarditis, and found that it could inhibit the inflammatory response driven by cardiomyogenic T cells.

3.7 Clinical application of BAPs

Peptides and peptidomimetics are emerging as an important class of clinic therapeutics (147). However, their application is hindered by their poor stability, short half-life, and low retention rate (148). It was reported that cyclic peptide structures had high topological flexibility, and their shape changes without transforming the amino acid composition sequence could not alter their properties (149). Therefore, molecular grafting is a good choice. It has been demonstrated that bradykinin antagonists were conjugated onto cyclic peptide scaffolds for the inflammation treatment (150). And sustained-release peptide analogues can be used for clinical treatment (151). BAPs are widely used to regulate inflammatory pathways and inflammatory factors to treat inflammation in clinics. Brimapitide (XG-102), a peptide bound to the N-terminal sequence of c-Jun, inhibits JNK by competing with endogenous c-Jun. In this way, it suppresses inflammation caused by JNK. This drug is currently under Phase III (149). Thymosin alpha-1 is an immunostimulatory peptide. It can regulate the immune system, enhance T cell function, inhibit the release of pro-inflammatory cytokines, and promote the production of anti-inflammatory cytokines (152). It is clinically used to treat hepatitis B (153). Since one century ago, more than 80 peptide drugs have reached the market for a wide range of diseases, including diabetes, cancer, osteoporosis, multiple sclerosis, HIV infection and chronic pain (154). However, there are still few peptides as clinical drugs for the treatment of inflammation.

4 Anti-inflammatory mechanism of BAPs

4.1 Regulation of the release of inflammatory mediators

Chemical substances released by cells or produced by body fluids during the inflammatory process, which participate in or cause the inflammatory reaction, are referred to as inflammatory mediators. They mainly include prostaglandins, NO, cytokines like interleukins (IL) (e.g., IL-1β, 2, 6, and 8), chemokines, etc. (155). As activated through toll-like receptors (TLR), these innate immune cells induce the release of IL-6 and TNF-α, along with transforming growth factor-β, which facilitates cell proliferation (156). The NF-κB and MAPK are also key pro-inflammatory intermediaries that are produced after TLR activation (157). Cytokines are low molecular weight glycoproteins produced and secreted by different cells, which can regulate the proliferation and differentiation of immune cells (158). They can be divided into two major categories: pro-inflammatory and anti-inflammatory factors. Pro-inflammatory factors such as IL-1β and TNF-α further induce the inflammatory response, while anti-inflammatory factors such as IL-10 can promote the resolution of the inflammatory response (159). Many studies show that BAPs can regulate the release of inflammatory mediators. Tornatore et al. (157) isolated four peptides from eggs white and these peptides exhibited anti-inflammatory activities in colitis mice by inhibiting the production of TNF-α and IL-6 as well as reducing the mRNA-expressions TNF-α, IL-6, IL17, IL-1β, IFN-γ, and MCP-1. Xing et al. (160) reported that bovine bone gelatin peptides could alleviate the additional secretion of inflammatory factors IL-6, NO, and TNF-α induced by lipopolysaccharide (LPS) in RAW264.7 cells to mitigate DSS-induced colitis. Cresti et al. (161) conducted efficacy studies on the synthetic peptide SET-M33 targeting gram-negative bacteria by using an LPS-induced pneumonia model. They found that the peptide effectively reduced the production of pro-inflammatory cytokines KC, MIP-1α, IP-10, MCP-1, and TNF-α.

4.2 Regulation of inflammatory signaling pathways

Inducers like LPS can stimulate and activate key proteins or genes involved in cellular signaling pathways such as NF-κB pathway (162) and MAPK pathway (163). The anti-inflammatory peptides inhibit cell inflammatory responses mainly through the MAPK and NF-κB pathways. NF-κB pathway is the most important way to regulate the transcription of pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α, and also plays a vital role in the expressions of inducible nitric oxide synthase (iNOS) and COX-2 (164). NF-κB is a family of transcription factor proteins, including five subunits: p65 (RelA), p50, p52, Rel, and RelB. After dimer p65/p50 is released into the cytosol, it can be translocated into the nucleus and initiates target gene transcription for pro-inflammatory factors, causing inflammation (164, 165). MAPK can regulate many cellular activities, including proliferation, differentiation, death and immune response. The stimulus and MAP3K phosphorylation can mediate the phosphorylation of the downstream MAP2K and MAPK, which contain three subfamilies: p38, extracellular signal-regulated kinases (ERK1 and ERK2), and c-Jun N-terminal kinase (JNK). In unstimulated cells, JNK mainly exists in the cytoplasm and partly distributes in the nucleus. After being stimulated, JNK accumulates in the nucleus and causes the corresponding gene (IL-1 and TNF-α) expression, resulting in an inflammatory response (166). BAPs inhibit the expression of inflammatory genes by blocking NF-κB and MAPK signaling pathways (Figure 2). The JAK-STAT pathway is also important for inflammatory response, which can regulate hematopoietic cell development and inflammatory cytokines (167). Phosphorylation of JAK and STATs can form the dimer translocated to the nucleus (168). In addition, the peptide transporter PepT1 can transport small BAPs to the bloodstream. Therefore, the role of PepT1 is vital to the bioactivity of BAPs (167). Chei et al. (169) described that acid-hydrolyzed silk peptide (SP) inhibited LPS-induced inflammation by modulating the TLR4 signaling pathway, while clam peptide MMV2 reduced the mRNA levels of inflammation-related genes induced by LPS in adult zebrafish (170). Formyl peptide receptors (FPRs), members of the GPCR family with seven transmembrane domains (171), play important roles in antimicrobial host defence mechanisms. FPRs recognize formylated peptides, non-formylated peptides, synthetic small molecules, and formyl analogs from bacteria and mitochondria to regulate inflammatory responses that lead to chemotaxis, degranulation, and oxidative bursts (172). Jin et al. (173) reported that VLATSGPG (VLA), a DPP-IV inhibitory peptide isolated from the skin of *Salmo Salar*, could inhibit the activation of PERK through the AKT signaling pathway, and increase the expression of IκBα mRNA through the PERK/IκBα pathway, leading to blocking the activation of NF-κB p65 and further cell inflammation. Tsuruki et al. (174) isolated some immunostimulating peptides from soy protein, which had specific binding sites on mouse or human macrophages and could stimulate their phagocytic activity.

4.3 Regulation of reduced oxidative stress response

Oxidative stress is a significant pathological factor that contributes to various inflammatory diseases. Inflammatory responses trigger the excessive generation of reactive oxygen species (ROS) within cells, disrupting the body's free radical metabolism and leading to oxidative stress. Moreover, during oxidative metabolism, excessive ROS can attack cells or tissues, causing structural and functional damage and exacerbating inflammatory reactions (175, 176). BAPs can reduce the generation of ROS. Lee et al. (177) isolated the peptide PPY1 from *Pyropia yezoensis*, and stated that PPY1 significantly decreased the ROS levels in LPS-induced macrophages. Oxidative stress and inflammation are closely related, which can elucidate why NF-κB is the initial mammalian transcription factor to be influenced by oxidation (178). NF-κB plays a crucial role in

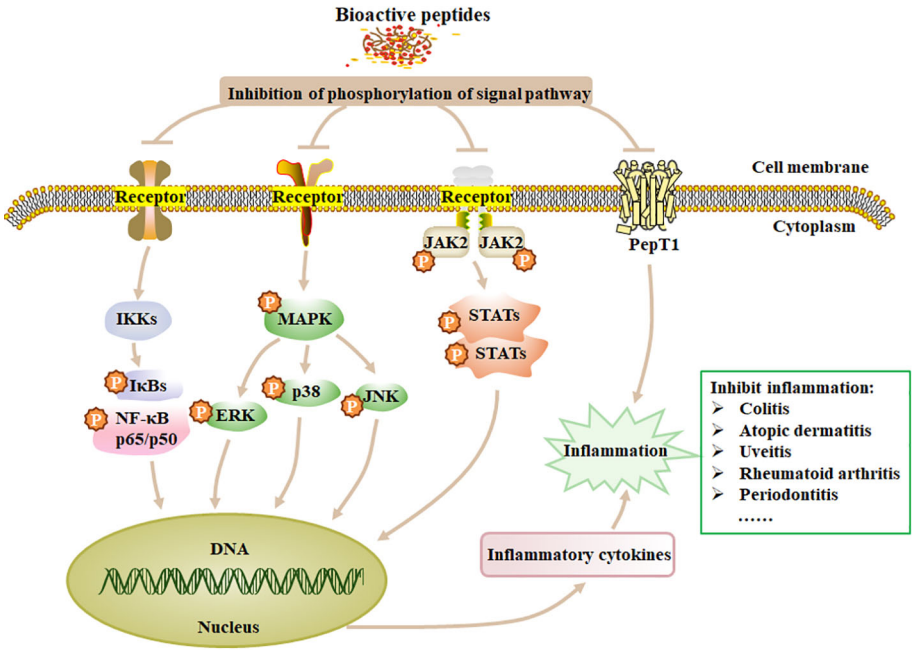

FIGURE 2
The mechanism of anti-inflammation of BAPs. Treatment of inflammation by modulating the four signaling pathways, such as NF-κB, MAPK, JAK and STATs. p, phosphorylation; Ikks, inhibitor of kappa B kinase. Adapted from previous reports (167).

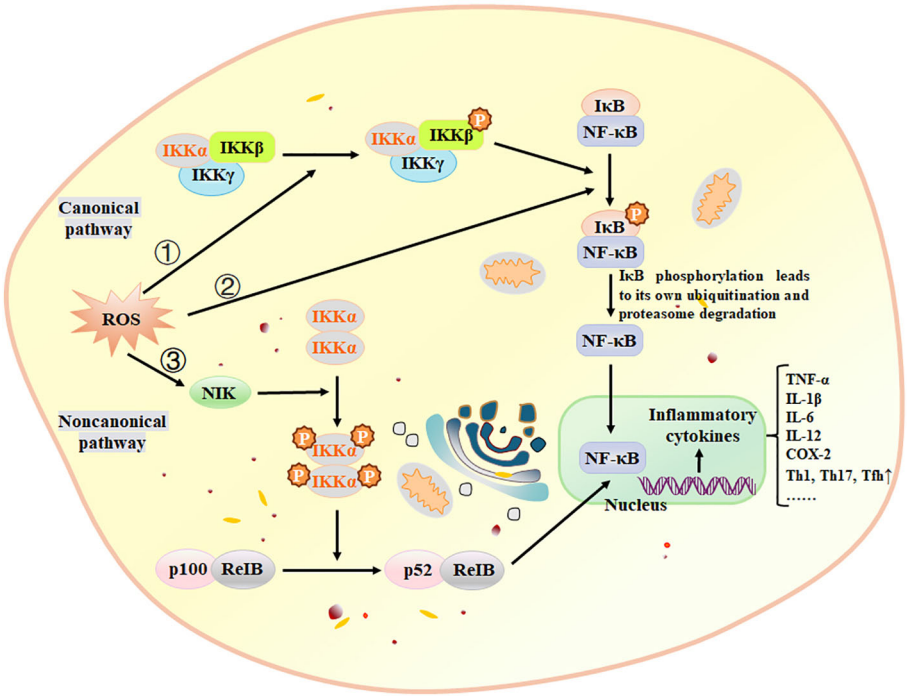

FIGURE 3
ROS activate NF-κB through three pathways. ① Canonical pathway: ROS activates the IKK complex, phosphorylating IκBα. Phosphorylation leads to ubiquitination and proteasomal degradation of IκBα, resulting in nuclear translocation of the NF-κB complex and gene expression through high-affinity binding to κB components, ② ROS directly phosphorylate IκBα, subsequently following the same pathway as the canonical pathway, ③ Noncanonical pathway: NIK is activated by ROS through inhibition of phosphatases and oxidation of cysteine residues. The NF-κB activation pathway relies on IKKα and activates the p52/RelB complex by triggering proteolytic cleavage of the p52/p100 precursor. IKK, IκB kinase; NIK, NF-κB-inducing kinase. Adapted from previous reports (181).

mediating inflammatory responses and is regulated by various mediators, including H_2O_2 and ROS (178). ROS can modulate NF-κB through both the Canonical and Noncanonical pathways (Figure 3). Malondialdehyde (MDA) and glutathione (GSH) are important markers of oxidative stress. MDA is the final product of ROS-induced lipid peroxidation, while GSH is an intracellular antioxidant that protects cells from oxidative stress damage. Peng et al. (54) identified an active peptide, GGAW, which exhibits excellent antioxidant functionality. This peptide effectively reduces the production of ROS, MDA and lactate dehydrogenase (LDH), and increases the activity of SOD and glutathione peroxidase (GSH-PX). Consequently, it enhances cell viability and protects IEC-6 cells from H_2O_2-induced oxidative damage. The Kelch-like ECH-associated protein 1-(Keap1) Nrf2-antioxidant response element is the main antioxidant signaling pathway that prevents oxidative stress and helps maintain the optimum redox steady state *in vivo* (179). Hence, the Nrf2 antioxidant signaling pathway can be stimulated to suppress oxidative stress within the body (167). Fernando et al. (180) reported that AMVDAIAR, a peptide isolated from pepsin hydrolysate of krill enhanced antioxidant enzymes SOD, CAT and GPx, thereby suppressing the oxidative stress in H_2O_2-induced hepatocytes and increasing the expression of Nrf2.

5 Conclusions and prospects

BAPs are widely employed in the treatment of inflammation. This review summarizes the therapeutic effects of BAPs on various inflammatory diseases such as pulmonary, gastrointestinal, dermatological, arthritic, oral and ocular inflammations. It also outlines the anti-inflammatory mechanisms of action of BAPs, which include modulation of inflammatory mediators' release, regulation of inflammatory signaling pathways (NF-κB, MAPK, and JAK-STAT), and reduction of oxidative stress reactions to influence the development of inflammation.

BAPs have promising prospects for the preparation of anti-inflammatory drugs. However, BAPs are commonly implicated with several challenges, encompassing a short half-life, susceptibility to proteases, instability, potential toxicities, and other processing-related issues. Attempts can be made to modify or transform the BAPs, such as by attaching metal ions, targeting groups or nanomaterials to maximize their effectiveness. However, before using BAPs to treat various inflammatory diseases, more experiments are needed to obtain additional data on dosages, pharmacodynamics and pharmacokinetics. Studies should also investigate the differential effects of BAPs on different populations to better understand their efficacy. Furthermore, the anti-inflammatory mechanisms of various types of BAPs require investigation to ensure their safety in clinical applications. Additionally, many peptides face challenges in maintaining stability and functional activity *in vivo* due to inherent limitations of amino acids. BAPs can be encapsulated within nanoparticles to improve their stability. Future efforts should concentrate on finding more methods to overcome these challenges to maximize the efficacy of BAPs. In conclusion, BAPs hold great promise as potential inflammatory therapy. Further research and clinical data are necessary to support their widespread and safe application.

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Conflict of interest

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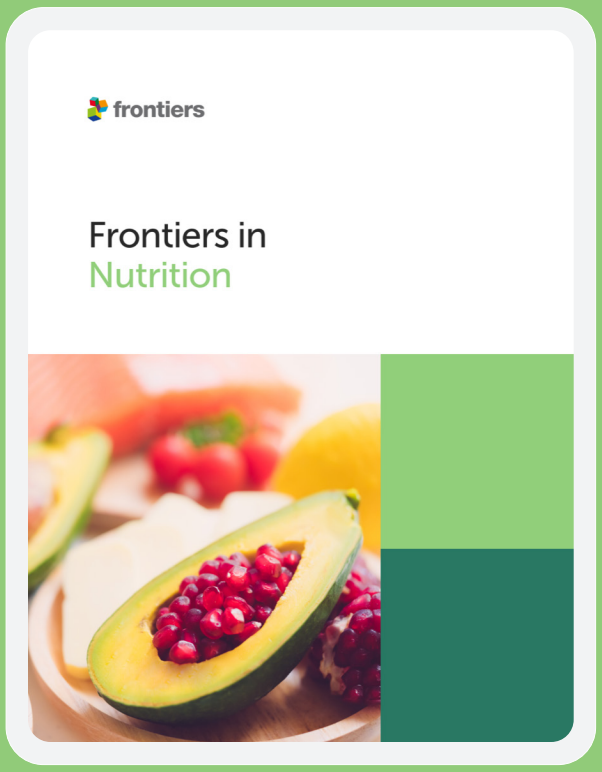

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