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Foods, dietary supplements, and herbal products treating the diseases of the 21st century: Moving from traditional to scientific research

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Foods, dietary supplements, and herbal products treating the diseases of the 21st century: Moving from traditional to scientific research

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Editorial: Foods, dietary supplements, and herbal products treating the diseases of the 21st century: moving from traditional to scientific research

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Editorial on the Research Topic
Foods, dietary supplements, and herbal products treating the diseases of the 21st century: moving from traditional to scientific research

The Research Topic of Frontiers in Nutrition entitled: "*Foods, dietary supplements, and herbal products treating the diseases of the 21st century: moving from traditional to scientific research*" contributed to explore the role of food, dietary supplements, and herbal products in the treatment of the diseases in a scientific mode, paving the road to bridge the gaps in their scientific acceptability.

This Research Topic includes total 10 papers, including four review papers, two systematic reviews, and four original research papers.

The review entitled "*From antiquity to contemporary times: how olive oil by-products and waste water can contribute to our health*" (Albini et al.) addressed the historical recognition of the benefits of olive oil and it is by products in a variety of fields, including cooking, skincare, and medicine. Olive mill waste water (OMWW), a byproduct of olive oil production, causes environmental issues while also providing a rich supply of phytochemicals with health advantages. OMWW extracts have been studied for their anti-angiogenic and chemopreventive properties, with prominent polyphenols including hydroxytyrosol (HT), verbascoside, and oleuperin. A particular extract, A009, displayed anti-angiogenic actions *in vitro* and *in vivo*, outperforming HT alone. A009 also showed possible cardioprotective effects by lowering chemotherapy-induced cardiotoxicity and pro-inflammatory markers in cardiomyocytes.

Several multinational research teams in the paper have tested extracts from OMWW and other olive byproducts for biological activity. According to this paper, the favorable results point to the potential of A009 as nutraceuticals, cosmeceuticals, or dietary supplements, notably in cancer prevention or co-treatment with anticancer conventional treatments. Furthermore, potential of OMWW extracts provide cardioprotective advantages offers up possibilities for use in cardio-oncology.

The review paper entitled "*Modulation of immune response by nanoparticle-based immunotherapy against food allergens*" (Krishna et al.) focuses on food allergies, specifically their treatment using nanoparticles in allergen-specific immunotherapy.

The increased global frequency of food allergies and their associated life-threatening anaphylactic events has resulted in limited treatment options, which primarily provide symptomatic relief. Recent advances in science and clinical practice aim to solve this issue by developing new treatments for allergic diseases. Despite improvements, current allergy immunotherapy has limitations in terms of long-term efficacy and safety, as evidenced by local side effects and the risk of anaphylactic reactions.

Ongoing research into the safety and efficacy of allergen immunotherapy has prompted the development of novel approaches such as intra-lymphatic immunotherapy. Furthermore, the use of nanoparticles in allergen immunotherapy is highlighted as a safer and more effective treatment. This manuscript describes a unique drug delivery approach that involves gradually administering specific allergens in increasing dosages in order to induce desensitization and tolerance. It stresses various administration routes, processes, and the use of nanoparticles in allergen-specific immunotherapy.

The research paper entitled "*Xanthophyll pigments dietary supplements administration and retinal health in the context of increasing life expectancy trend*" (Jurja et al.) addresses the effects of a sub-class of carotenoids, xanthophylls, in retinal health. These supplements are widely recommended for preventing retinal degenerative damage and slowing down the progression of age-related changes. Notably, these dietary supplements are recognized for their total antioxidant activity, as confirmed by the photochemiluminescence method using the Antioxidant Capacity in Lipid soluble-substances procedure. This study involved subjects with comparable ages and retinal age-related degenerative abnormalities, as well as a similar number of healthy individuals with normal retinas. Both groups of subjects were then administered similar dosages of xanthophyll pigments dietary supplements, with variations in the association of xanthophylls with vitamins and oligo-elements. After a three-year supplementation period, the subjects were reevaluated, and this paper emphasis the impact of these supplements on visual health.

The systematic review entitled: "*Impact of omega-3 fatty acids supplementation on the gene expression of peroxisome proliferator activated receptors-γ, α and fibroblast growth factor-21 serum levels in patients with various presentation of metabolic conditions: a GRADE assessed systematic review and dose–response meta-analysis of clinical trials*" (Ahmadi et al.) examined the impact of omega-3 fatty acid supplementation on the gene expression of peroxisome proliferator-activated receptors (PPAR-α and PPAR-γ) and serum fibroblast growth factor-21 (FGF-21) levels in people with various metabolic disorders. The analysis covered 15 trials found by a comprehensive search of various databases until April 2022.

Omega-3 fatty acids supplementation significantly increased PPAR-γ and PPAR-α gene expression compared to the control group. Overall, the results imply that omega-3 fatty acid supplementation may have a favorable effect on the regulation of adipose tissue-related genes in people with diverse metabolic disorders. However, more studies are needed to corroborate these findings and confirm the usefulness of this supplementation strategy in varied groups.

The systematic review entitled "*The metabolic effect of Momordica charantia cannot be determined based on the available clinical evidence: a systematic review and metaanalysis of randomized clinical trials*" (Laczkó-Zöld et al.) is a meta-analysis that assesses the efficacy of *M. charantia* L. (bitter melon) in treating metabolic syndrome, with a particular emphasis on its anti-diabetic properties. The study includes nine randomized controlled human trials with a total of 414 individuals and follow-up periods ranging from 4 to 16 weeks.

The meta-analysis, which followed the PRISMA statement, found no significant effects of bitter melon treatment above placebo in terms of change scores for most parameters. The bitter melon treatment had no significant influence on fasting blood glucose, HbA1c, HDL, LDL, total cholesterol, body weight, BMI, or systolic and diastolic blood pressure readings. The meta-analysis also found no significant changes in ALT, AST, or creatinine levels. The findings highlight the need for additional study, including properly conducted clinical studies with extended durations, to better understand the potential advantages and safety of *M. charantia* in treating metabolic syndrome.

The research paper entitled "*The impact of high-glucose or high-fat diets on the metabolomic profiling of mice*" (Xie et al.) aims to determine the effect of high-glucose and high-fat diets on metabolomic profiles in primary tissues of C57BL/6J mice. Mice were given either a high-glucose or high-fat diet for 8 weeks, and the levels of metabolites in their primary tissues were evaluated. This study highlights the strong impact of dietary composition on the metabolic profiles of primary tissues in mice, implying that metabolomics could be useful for detecting the development of sickness in animal models. When the metabolic profiles of the two diet groups were compared to those of a control group, the study found 32 metabolites in the high-glucose diet (HGD) group and 28 metabolites in the high-fat diet (HFD). The most significantly changed metabolites were amino acids (AAs). However, it is vital to recognize the limitations of this research, and there is still much need for further investigation in this area.

The research paper entitled "*Hypoglycemic effects of Dendrobium officinale leaves*" (Lv et al.) evaluated the hypoglycemic effects and processes of *D. officinale* leaves (EDL), with a focus on a portion of the plant that has received less attention than its stems. Male C57BL/6 mice were fed a conventional or high-fat diet, as well as normal or EDL-containing water, for 16 weeks. Mice fed a high-fat diet and treated with EDL showed considerably lower blood glucose levels and better glucose tolerance, whereas mice fed a low-fat diet showed no such effects. These findings shed light on the hypoglycemic potential of *D. officinale* leaves, helping to better understand the molecular pathways that enhance insulin sensitivity. The findings may help guide future research into isolating specific chemicals from EDL for the potential creation of hypoglycemic medicines, providing a theoretical underpinning for using *D. officinale* leaves in this setting.

The review paper "*Nutrients, herbal bioactive derivatives and commensal microbiota as tools to lower the risk of SARS-CoV-2 infection*" (Romani et al.) focuses on laying a solid scientific foundation and recommending complementary nutritional

methods to aid in the prevention and treatment of SARS-CoV-2 infections. The authors explore the processes of viral entry, highlighting the possible significance of polyunsaturated fatty acids like α-linolenic acid and other micronutrients in suppressing SARS-CoV-2 and its entrance gateways. Herbal-derived pharmacological substances, certain microbial strains, and microbial-derived polypeptides are also addressed for their ability to prevent SARS-CoV-2 infection. Furthermore, the research thoroughly investigates the role of probiotics, micronutrients, and herbal-derived substances in boosting the immune response to SARS-CoV-2. By providing a comprehensive scientific backdrop, the authors hope to create a foundation for considering dietary tools as supplementary strategies in the battle against the current pandemic.

The review entitled "*Mechanism of the antidiabetic action of Nigella sativa and thymoquinone: a review*" (Shaukat et al.) addresses *Nigella sativa* (NS), a plant with a long history of traditional medicine. This review investigates the pharmacological and pharmacokinetic aspects of NS as herbal diabetic medicine, focusing on its effects on oxidative stress and Diabetes mellitus development. NS, notably its thymoquinone (TQ)-rich volatile oil, has received interest for its efficacy and safety in diabetic treatment. However, determining a precise therapeutic dose remains difficult. NS has been shown to reduce insulin resistance, enhance insulin signaling, decrease cyclooxygenase-2, upregulate insulin-like growth factor-1, and avoid endothelial damage in diabetes patients.

The research paper "*Characterizations of microRNAs involved in the molecular mechanisms underlying the therapeutic effects of noni (Morinda citrifolia L.) fruit juice on hyperuricemia in Mice*" (Liu et al.) addresses the therapeutic effects of noni (*Morinda citrifolia* L.) fruit juice on hyperuricemia and the underlying molecular pathways utilizing a potassium oxonate-induced mice model. Mice fed with noni fruit juice presented significantly lower serum uric acid (UA) and xanthine oxidase (XOD) levels. The study shows that noni fruit juice can treat hyperuricemia by decreasing XOD activity and lowering serum UA levels. Furthermore, the noni fruit juice group has considerably lower levels of serum creatinine and blood urea nitrogen than the model group, implying that noni fruit juice improves UA excretion without impairing renal function in mice. The study presented persuasive experimental evidence to warrant future investigation of noni fruit juice as a possible therapy for hyperuricemia.

Research contributions to this topic highlight the wide range of foods, dietary supplements, and herbal products that have shown promise in the treatment of twenty-first century diseases such as SARS-CoV-2, cancer, allergies, and metabolic disorders. These useful discoveries not only broaden our understanding of alternative treatment techniques, but also pave the path for future research and development of new solutions that take advantage of nature's healing capabilities. Embracing natural therapies may provide a holistic and complementary dimension to traditional medical tactics, creating a comprehensive and customized approach to disease management in the modern era.

Author contributions

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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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Nutrients, herbal bioactive derivatives and commensal microbiota as tools to lower the risk of SARS-CoV-2 infection

Arianna Romani[1†], Domenico Sergi[2†], Enrico Zauli[2], Rebecca Voltan[1*], Giada Lodi[1], Mauro Vaccarezza[3], Lorenzo Caruso[1], Maurizio Previati[2*] and Giorgio Zauli[4]

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The SARS-CoV-2 outbreak has infected a vast population across the world, causing more than 664 million cases and 6.7 million deaths by January 2023. Vaccination has been effective in reducing the most critical aftermath of this infection, but some issues are still present regarding re-infection prevention, effectiveness against variants, vaccine hesitancy and worldwide accessibility. Moreover, although several old and new antiviral drugs have been tested, we still lack robust and specific treatment modalities. It appears of utmost importance, facing this continuously growing pandemic, to focus on alternative practices grounded on firm scientific bases. In this article, we aim to outline a rigorous scientific background and propose complementary nutritional tools useful toward containment, and ultimately control, of SARS-CoV-2 infection. In particular, we review the mechanisms of viral entry and discuss the role of polyunsaturated fatty acids derived from α-linolenic acid and other nutrients in preventing the interaction of SARS-CoV-2 with its entry gateways. In a similar way, we analyze in detail the role of herbal-derived pharmacological compounds and specific microbial strains or microbial-derived polypeptides in the prevention of SARS-CoV-2 entry. In addition, we highlight the role of probiotics, nutrients and herbal-derived compounds in stimulating the immunity response.

KEYWORDS

SARS-CoV-2, COVID-19, natural products, phytochemicals, ACE-2, TMPRSS2

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has triggered a devastating global health, social and economic crisis, with more than 664 million cases and 6.7 million deaths (1). Coronaviruses are a group of enveloped, positive-sense, single-stranded RNA viruses that are able to cause a range of diseases in several species including humans (2).

Several different strains of human coronaviruses (HCoV) have been identified to date (3). Among them, SARS-CoV, MERS-CoV, and SARSCoV-2 are highly pathogenic and have resulted in three life-threatening severe respiratory disease outbreaks in the past two decades. Other

HCoV strains [i.e., HCoV-229E (an alpha CoV), HCoV NL63 (an alpha CoV), HCoV-OC43 (a beta CoV), and HCoV HKUI (a beta CoV)] usually cause only common-cold-like mild upper respiratory tract illnesses in humans (3, 4). As these human coronaviruses have a zoonotic origin, it is increasingly likely that there will be more HCoV outbreaks in the future (5). The envelope spike (S) protein of SARS-CoV-2 plays a crucial role in coronavirus pathogenesis, mediating receptor binding, membrane fusion and promoting viral entry into target cells (6). The S protein of coronaviruses is functionally divided into the S1 domain, the receptor binding domain (RBD), and the S2 domain responsible for cell membrane fusion. Virus entry requires S protein priming by cellular proteases which determine the cleavage of S1/S2 domains and allow fusion of the viral envelope with the cellular membrane. SARS-CoV-2 engages angiotensin-converting enzyme 2 (ACE2) as the entry receptor and uses the cellular transmembrane serine protease 2 (TMPRSS2) for S protein priming. ACE2 and TMPRSS2 are expressed in several human cells, including cells of the respiratory and digestive tracts (7, 8).

Original data suggest that the downregulation of TMPRSS2 and/or ACE2 expression on the cell surface could avert viral entry into the host cell and, consequently, infection spreading. It has been shown that knocking out ACE2 expression can block SARS-CoV-2 infection of murine epithelial cells (9), and that ACE inhibition blocks SARS-CoV-2 infection *in vitro* (10, 11).

This review is focused on natural products, including nutrients, bacterial strains, molecules or herbal extracts that target virus entry by interfering with ACE2 binding and/or by preventing TMPRSS2 cleavage. In addition, considering the hyperinflammation rise often associated with SARS-CoV-2 infection (12), we highlight also the anti-inflammatory properties associated with several natural products.

2. Virus structure, biology

The RNA genome of SARS-CoV-2 (29.9 kb) encodes 29 proteins (13). Of these, only four proteins, namely S protein, membrane (M), envelope (E), and nucleocapsid (N), make up the whole virus structure (14). The remaining proteins are non-structural proteins ($n = 16$) and accessory proteins ($n = 9$) that are pivotal in the replication of the virus and the escape of host immunity (15). Similar to SARS-CoV, SARS-CoV-2 utilizes the cell surface receptor ACE2 for cellular entry. Firstly, the SARS-CoV-2 S glycoprotein interacts with surface ACE2 to enter the target cell; in addition, invasion also needs proteolytic activation of the S protein, which is helped by the TMPRSS2 and lysosomal cysteine proteases, cathepsins, available in the target host cell (7, 8). Newly made envelope proteins are inserted into endoplasmic reticulum and Golgi membranes, and the nucleocapsid is formed by the assimilation of nucleocapsid protein with genomic RNA. Then, viral particles are produced into the endoplasmic-reticulum-Golgi intermediate compartment and the virus particles are released by exocytosis. Any of the steps in this viral life cycle are a potential target for anti-SARS-CoV-2 drug discovery.

To rationally target the SARS-CoV-2 life cycle, it is important to better outline at least the more general steps of virus entry. The S protein of SARS-CoV-2 is not only the main mediator of initial virus attachment on the cell surface, but also ignites the complex machinery that allows viral RNA entry into the host cytoplasm by triggering pore formation, both during membrane fusion and endocytosis. Similarly to HIV-1 and Ebola viruses, the preliminary step for S protein priming occurs in the infected cells, during the production of new viral particles (16). During this stage, cellular proteases like furin cleave S proteins into two not-covalently-associated S1 and S2 subunits. The S1 subunit is responsible for the attachment to the obligate SARS-CoV-2 receptor, the ACE2 protein, which in the human body is expressed at high levels in the small intestine, testis, kidney, heart muscle, colon and thyroid gland (17). When the viral and the host cell membrane are proximal, the docking of S1 to ACE2 determines ACE2-dependent conformational changes at the S2 sequence. From this moment, TMPRSS2 becomes pivotal in determining the modalities of virus entry. Sufficient expression or activity of TMPRSS2 at cell surface level allows for S2 cleavage, exposure of fusion peptide sequences, followed by the process by which enveloped viruses merge their membrane with the host cell membrane in a way that the virus can move its genome inside the cell, resulting in the potential production of new virions (18). Of note, membrane fusion is not a spontaneous process, as there are high energy requirements to bring the membranes close together (19, 20). Alternatively to membrane fusion, SARS-CoV-2 can take advantage also from endocytic pathways to reach the cytosol of the host cell. In the absence of S1 priming, the virus binding to one or more copies of ACE2 can trigger the endocytic route. After internalization, and presumably at the stage of late endosome, cathepsin protease activity on S2 determines the exposure of S2 sequences which allow the fusion of viral and endosomal membranes, followed by liberation of viral RNA into the cytoplasm. Interestingly, not only naïve but also opsonized virions can be internalized by the endocytic mechanism. Opsonized SARS-CoV-2 virions are coated with antibodies that mask viral proteins, but the virus can still bind the cell surface and be endocytosed due to the presence of receptors for antibody Fc regions (21).

Of note, as SARS-CoV-2 circulated globally, the viral genome acquired new mutations, some of which have become widespread. Until late 2020, the most notable was the S protein mutation D614G. This variant quickly became dominant, and this rapid spread seems to have been due to increased infectivity, stability, and transmissibility over the ancestral D614 form (22, 23), resulting from a shift to the open configuration of the S protein trimer, which is required for binding to the host ACE2 receptor (23) and host cell entry. Not surprisingly, there are many variants of SARS-CoV-2. Some are believed or have been stated to be of particular importance, due to their potential for increased transmissibility (24), increased virulence, or reduced effectiveness of vaccines against them (25, 26). Studies have demonstrated reductions in neutralizing activity of vaccine-elicited antibodies against a range of SARS-CoV-2 variants, against the Omicron variants in particular, exhibiting partial immune escape. However, evidence suggests that T-cell responses are preserved across vaccine platforms, regardless of the variant of concern (26, 27). As of March 2023, only the Omicron variants are designated as a circulating variant of concern by the World Health Organization (28).

Mechanistic details of these pathways may vary considerably between cell types. Regarding SARS-CoV-2's most important cell target, it is worth noting that the diversity of endocytosis in airway epithelium is currently poorly understood. Dissecting the mechanisms of endocytic viral entry in the respiratory tract may therefore offer a promising therapeutic strategy to treat viral infections.

3. COVID-19 pathogenesis and pathophysiology

The SARS-CoV-2 virus is able to infect a wide range of cells and organs of the body. SARS-CoV-2 is most known for affecting the upper respiratory tract (sinuses, nose, and throat) and the lower respiratory tract (bronchi and lungs). The lungs are mostly affected by SARS-CoV-2 because ACE2 is most abundant on the surface of type II alveolar pneumocytes of the lungs (29, 30). Three common patterns of symptoms have been recognised: one respiratory symptom cluster with cough, sputum, shortness of breath, and fever; a musculoskeletal symptom cluster with muscle and joint pain, headache, and fatigue; a cluster of digestive symptoms with abdominal pain, vomiting, and diarrhea (31).

Genetic predisposition may have a role in COVID-19 pathogenesis. The receptor-binding domain (RBD) of the SARS-CoV-2 S protein binds with high affinity with ACE2 receptor to enter cells. Consequently, ACE2 genetic variants that could affect its gene expression, protein conformation, and protein stability are the one of most uncertain factors involved genetic predisposition to SARS-CoV-2 infection (32). ACE2 is an X-linked gene that harbors a strong variant with tendency to an X-linked dominant inheritance pattern in severely affected patients. It might be a clue to the reason for the higher prevalence and severity of COVID-19 in men than in women (33). Furthermore, the immune-related genetic variants associated with the prior strain of coronavirus, namely SARS-CoV, are suspected to have roles in the genetic predisposition to SARS-CoV-2 infection (34), since SARS-CoV-2 has 80% genetic identity to SARS-CoV (35). Focusing on the genes of the human immune system and relating them to SARS-CoV-2 susceptibility, several lines of evidence strongly support the role of the interferon system (and related cytokines) as the most important determinant of infection control versus infection severity in humans (36–38).

3.1. COVID-19 and calcium metabolism

An important issue in the pathogenesis of SARS-CoV-2 infection is the role of calcium signaling. Of note, at the cellular level, coronavirus infection has been shown to modulate calcium metabolism. The SARS-CoV-2 S protein has two FP domains, FP1 and FP2, and binds to two Ca^{2+} ions for host cell entry (39). SARS-CoV-2 appears to affect cellular function by altering the host Ca^{2+} homeostasis in ways that promote viral infection and reproduction. One mechanism is through disruption of calcium channels and pumps (e.g., voltage-gated calcium channels (VGCCs), receptor-operated calcium channels, store-operated calcium channels, transient receptor-potential ion channels, and Ca^{2+}-ATPase) (40). Furthermore, the E and ORF3a proteins of coronaviruses impact Ca^{2+} homeostasis in the host, by acting as calcium ion channels, enhancing the virion's entry and replication potential (41).The SARS-CoV-2-E protein is a 76-amino-acid (aa) integral membrane protein with one transmembrane domain (TMD) that allows the E protein to form protein-lipid channels in membranes that promote permeability to Ca^{2+} ions. The alteration of Ca^{2+} homeostasis by SARS-CoV-2 proteins promotes SARS-CoV-1/2 fitness and elicits the production of chemokines and cytokines, contributing to pathogenesis. Ion channel activity modulation by the SARS-CoV-1-ORF3a protein also modulates viral release (42). Therefore, when SARS-CoV-2 infects the human body, the resultant dysregulation of Ca^{2+} homeostasis may contribute to morbidity and mortality. COVID-19 patients have been noted to have low serum calcium levels overall (43).

3.2. COVID-19 and oxidative balance

In addition to calcium homeostasis alteration, imbalance between oxidative species and antioxidants also has a proven role in COVID-19 pathogenesis. The presence of oxidative stress in COVID-19 patients was recently assessed in studies that observed a significant reduction in free sulfhydryl groups from patient serum (44), and a mortality-related increase in damaged albumin (45). In addition, several other markers like glial fibrillary acidic protein (GFAP), the receptor for advanced glycation end products (RAGE), high mobility group box-1 protein (HMGB1) and cyclo-oxygenase-2 (COX-2) were found increased in patients with severe COVID-19 (46). Another study outlined, in COVID-19 patients, inflammasome activation correlated with mitochondrial superoxide and lipid peroxidation, suggesting that oxidative stress and inflammation are two sides of the same coin, where inflammation and oxidative stress reinforce each other (47). In particular, high reactive oxygen species (ROS) levels, originating from improper oxidative metabolism and the action of defensive enzymes such as NADPH oxidase, could lead to the formation of oxidized forms of proteins, DNA and lipids that in turn could act as damage-associated molecular patterns (DAMPs) which could trigger further inflammatory reaction (48), ultimately unbalancing antiviral response and inflammation regulation, to unleash a dysregulated cytokine production normally known as cytokine storm (49). So, the pathological oxidative response, followed by reduced nitric oxide production and increased endothelial dysfunction, hyperpermeability and hypercoagulability, leads to a scenario of hyperinflammation and thrombosis, that, together with immunosuppression, constitute the core of COVID-19 disease.

3.3. Host cytokine response

Subjects with severe COVID-19 have symptoms of systemic hyperinflammation and dysregulated immune response. Laboratory findings of increased interleukin-2 (IL-2), interleukin-7 (IL-7), interleukin-6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF), C-X-C Motif Chemokine Ligand 10 (CXCL10), monocyte chemoattractant protein-1 (MCP1), macrophage inflammatory protein-1 α (MIP1α), and tumor necrosis factor α (TNF-α) are indicative of cytokine release syndrome and are suggestive of an underlying immunopathology (36, 50–52). The severity of the inflammation can be linked to the severity of what is known as the cytokine storm. Combatting the cytokine storm has been proposed as an effective treatment since it is one of the leading causes of morbidity and mortality in COVID-19 (36, 53, 54). A cytokine storm is caused by an acute hyperinflammatory response that is responsible for clinical illness in an array of diseases; and in COVID-19, it is related to a worse prognosis and increased fatality. The storm causes acute respiratory distress syndrome and blood clotting events such as thrombosis, strokes, myocardial infarction, encephalitis, acute kidney injury, and vasculitis. The production of IL-1, IL-2, IL-6,

TNF-α, and interferon-gamma (IFN-γ), all crucial components of normal immune responses, become the causes of a cytokine storm.

In addition, key transcriptional factors, such as tumor protein 53 (p53) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and their reciprocal balance, are altered upon SARS-CoV-2 infection (55). Of note, interferon alpha (IFN-α) plays a complex, multi-faceted role in the pathogenesis of COVID-19. Although it promotes the elimination of virus-infected cells, it also upregulates the expression of ACE2, thereby facilitating the SARS-CoV2 virus to enter cells and replicate. A competition of negative feedback loops (via protective effects of IFN-α) and positive feedback loops (via upregulation of ACE2) is assumed to determine the fate of patients suffering from COVID-19 (37, 56, 57). Additionally, subjects with COVID-19 and acute respiratory distress syndrome (ARDS) have classical serum biomarkers of cytokine release syndrome, including elevated C-reactive protein lactate dehydrogenase D-dimer and ferritin levels (36, 58). Systemic inflammation results in vasodilation, allowing inflammatory lymphocytic and monocytic infiltration of the lung and the heart. Of note, pathogenic GM-CSF-secreting T cells were linked to the recruitment of pro-inflammatory IL-6-secreting monocytes and severe lung pathology in COVID-19-infected subjects (36). Wide-spread lymphocytic infiltrates have also been reported at autopsy (59).

3.4. COVID-19 and the central nervous system

Loss of smell, a common symptom, results from infection of the cells of the olfactory epithelium, with subsequent damage to the olfactory neurons. The involvement of both the central and peripheral nervous system in COVID-19 has been reported (60, 61). The virus is not detected in the central nervous system (CNS) of the majority of COVID-19 patients with neurological issues. However, SARS-CoV-2 has been detected at low levels in the brains of those who have died from COVID-19, but these results need to be confirmed (60, 61). While the virus has been detected in cerebrospinal fluid in autopsies, the exact mechanism by which it invades the CNS remains unclear, and it could involve invasion of peripheral nerves due to the low expression levels of ACE2 in the brain (60, 61). The virus may also enter the bloodstream from the lungs and cross the blood–brain barrier to gain access to the CNS, possibly within infected white blood cells (61). Observed individuals infected with SARS-CoV-2 (most with mild cases) experienced an additional 0.2–2% of brain tissue lost in regions of the brain connected to the sense of smell compared with uninfected individuals; infected individuals also scored lower on several cognitive tests. All effects were more pronounced among elderly individuals (61).

3.5. COVID-19 and the gastrointestinal tract

The virus also involves gastrointestinal (GI) organs, since ACE2 is expressed in the glandular cells of gastric, duodenal and rectal epithelia as well in the enterocytes of the small intestine (62). Potential mechanisms on how SARS-CoV-2 can cause damage to the GI tract include a direct virus-induced cytopathic effect through cell entry via ACE2, indirect immune-mediated injury triggered by a systemic inflammatory response to SARS-CoV-2, and disruption of the intestinal microecological "milieu" leading to excessive systemic inflammation which may lead to a cytokine storm. Of particular interest, in our opinion, is the role of direct mucosal damage and the role of the intestinal microbiota. SARS-CoV-2 infection of gut epithelial cells is able to trigger dysbiosis, intestinal inflammation, and GI symptoms (63). The cytopathic viral effect on target intestinal cells leads to the generation of inflammatory signals known as pathogen-associated molecular patterns (PAMPs) and intracellular DAMPs, which stimulate pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I) and other RIG-I-like receptors (RLRs). DAMPs and PAMPs trigger, through the recruitment of specific adaptors, the innate immune response which implicates the production of cytokines and chemokines such as TNF-α, interleukin-1 beta (IL-1β), IFNs, IL-6, CXCL10, MIP1α, MIP1β and MCP1.

3.6. COVID-19 and the cardiovascular system

Additionally, the virus can cause acute myocardial injury and chronic damage to the cardiovascular system. An acute cardiac injury was found in 12% of infected people admitted to the hospital in Wuhan, China, and it is more frequent in severe disease. Rates of cardiovascular symptoms are high, in accordance with the systemic inflammatory response and any immune system disorders during disease progression. However, acute myocardial injuries may also be related to the high expression of ACE2 receptors in the heart (64).

A high incidence of thrombosis and venous thromboembolism occurs in people transferred to intensive care units with SARS-CoV-2 infections and may be related to poor prognosis. Blood vessel dysfunction and clot formation (as suggested by high D-dimer levels caused by blood clots) are likely playing a significant role in mortality, with the incidence of clots leading to pulmonary embolisms, and ischaemic events within the brain (found as complications) leading to death in people infected with SARS-CoV-2. Infection may trigger a chain of vasoconstrictive responses within the body, including pulmonary vasoconstriction, decreasing oxygenation. Moreover, microvascular and capillary damage was found in the brain tissue of people who died from COVID-19 (65–67).

3.7. COVID-19 and blood cells changes

SARS-CoV-2 is also able to cause structural changes to blood cells, in some cases persisting for months after hospital discharge. A low level of blood lymphocytes may result from the virus acting through ACE2-related entry into lymphocytes.

One of the most notable changes seen in patients with COVID-19 is the alteration in their blood cell counts (68). During SARS-CoV-2 infection, there is a decrease in the number of white blood cells, particularly lymphocytes. The decrease in lymphocyte count is associated with the severity of the disease, and patients with severe COVID-19 tend to have lower lymphocyte counts (52). Of note, patient's T cell compartment shows several alterations involving naïve, central memory, effector memory and terminally differentiated cells,

as well as regulatory T cells and PD1 + CD57+ exhausted T cells (52). T cells exhibit indications of exhaustion, such as increased expression of inhibitory receptors like PD-1. This state of exhaustion is marked by functional unresponsiveness, which serves to prevent extensive immune activation and the resultant tissue damage from autoimmune reactions. As a result, it is plausible that activation of these cells in COVID-19 patients not only results in a lack of clonal expansion, as evidenced by decreased proliferation, but also leads to the production of molecules that promote inflammation (52). The levels of immunoglobulin classes and antibodies against common antigens or vaccines in COVID-19 patients' plasma were found to be normal. However, the number of total and naïve B cells decreased, along with decreased percentages and numbers of memory switched and unswitched B cells. Conversely, there was a significant increase in IgM+ and IgM-plasmablasts. B lymphocytes showed normal proliferation index and number of dividing cells per cycle during *in vitro* cell activation. The principal component analysis (PCA) indicated that B-cell number, naïve and memory B cells, but not plasmablasts, clustered with patients who were discharged. On the other hand, plasma IgM level, C-reactive protein, D-dimer, and sequential organ failure assessment (SOFA) score clustered with those who died. In patients with pneumonia, the deterioration of the B-cell compartment could be one of the reasons for immunological failure in controlling SARS-CoV2 (52). During SARS-CoV-2 infection, there is a decrease in the number of red blood cells, leading to anemia (69). Anemia can cause fatigue, shortness of breath, and other symptoms. The decrease in red blood cell count is also associated with the severity of the disease (69). Overall, structural and functional alterations of the blood cell compartment have an important role in the pathogenesis of SARS-CoV-2 infection; recent data highlight the predictive role of these alterations in prognosis and in the long COVID clinical setting (70–72).

4. The role of nutrients in preventing SARS-CoV-2 infection

4.1. Dietary omega-3 fatty acids as a tool to prevent COVID-19

The nutritional status of the host represents a pivotal discriminant influencing the ability of SARS-CoV-2 to enter cells and replicate. In this regard, dietary nutrients are emerging as a potential modulator of SARS-CoV-2 infections. Of these, bioactive fatty acids, like omega-3, may play a role in this context. Omega-3 are polyunsaturated fatty acids derived from α-linolenic acid which represents the precursor of eicosapentaenoic (EPA) and docosahexaenoic acid (DHA). While α-linolenic acid is an essential fatty acid, and therefore can only be obtained from the diet, EPA and DHA can be endogenously synthesised from the mutual precursor or obtained mainly via the consumption of fatty fish or fish oil. Regarding their role as potential nutraceuticals to tackle the COVID-19 pandemic, the supplementation of omega-3 fatty acids has been associated with a lower risk of SARS-CoV-2 infection, at least in women (73). This is supported by the ability of these polyunsaturated fatty acids to protect against viral infections by inhibiting viral entry, localization and replication (74). The impact of omega-3 fatty acids on viral entry into the cells is dictated by their capacity to modulate membrane fluidity and protein complex formation in lipid rafts. In turn, the entry gateway for SARS-CoV-2, ACE2 and TMPRSS2, is most commonly found in lipid rafts (75). Additionally, the size and number of lipid rafts may impact the abundance as well as enzymatic activity of ACE2 and TMPRSS2 (74). Thus, the modulation of lipid rafts by omega-3 fatty acids may affect viral entry into the cells (76). Furthermore, polyunsaturated omega-3 fatty acids interfere with the virus binding to ACE2, with linolenic acid and EPA significantly blocking the entry of SARS-CoV-2 (77).

Aside from representing a nutritional tool potentially inhibiting SARS-CoV-2 entry into the cells, omega-3 fatty acids may also interfere with virus-mediated activation of sterol regulatory element binding proteins (SREBPs) which in turn are pivotal for viral replication. They facilitate viral replication, and modulate cellular lipid metabolism, leading to increased availability of lipid substrates directed towards virion replication membrane formation. Considering the central role of SREBP 1/2 in promoting lipogenesis and its involvement in the virus-mediated rewiring of lipid metabolism, this transcription factor has been proposed as a broad-spectrum anti-viral target (78). Not surprisingly, omega-3 fatty acids have been widely reported to influence lipid metabolism (79), an effect that also relies on their ability to inhibit SREBP1 activation and downregulate SREBP1c (80). Considering this, omega-3 may hinder virus-induced SREBP activation, thereby interfering with viral replication. Additionally, the regulation of cholesterol metabolism by SREBP may represent an additional mechanism explaining the potential role of omega-3 fatty acids in inhibiting SARS-CoV-2 infection. Indeed, cholesterol is also a key component of lipid rafts, and as such, it is crucial in mediating the entry of the virus into the cells (81). Thus, it appears that the ability of omega-3 fatty acids to counter SARS-CoV-2 infection relies on their impact upon lipid metabolism. This possibility is further supported by the fact that lipogenesis modulator drugs, able to hamper fatty acid and cholesterol synthesis, also alter SARS-CoV-2 replication cycle *in vitro* (82). In light of this, the therapeutic potential of lipogenesis modulators against COVID-19 may also apply to nutrients able to affect lipid metabolism, such as omega-3 which may therefore represent a promising nutritional tool to tackle SARS-CoV-2 infection. Despite this, direct evidence gathered through clinical trials, on the ability of omega-3 fatty acids to prevent or at least limit SARS-CoV-2 infection, is still lacking.

Another potential mechanism by which omega-3 fatty acids may interfere with SARS-CoV-2 infection is via the modulation of the activity of the immune system. Particularly, the fatty acid composition of the plasma membranes of phagocytic cells modulates their phagocytic capacity (83), which in turn represents an important step in the immune response against foreign pathogens. In support of this, while omega-3 fatty acids have the potential to enhance the phagocytic activity of neutrophils and monocytes, this effect is negatively correlated with palmitic acid content in the plasma membrane of these cells [84; 85]. Considering that the fatty acid composition of cell membranes closely reflects dietary fatty acid intake, it is plausible that increasing the intake of omega-3 fatty acids may result in an increase of phagocytic activity. Indeed, the supplementation of DHA and EPA at a dose of 1.5 g/day results in an increase in the phagocytic activity of both neutrophils and monocytes (84, 85).

Another mechanism by which omega-3 fatty acids, but also other unsaturated fatty acids, may dampen viral infection is via the disruption of the virus envelope. Aside from the aforementioned unsaturated fatty acids, this effect is also elicited by medium-chain

fatty acids, while short-and long-chain saturated fatty acids do not show anti-viral activity against enveloped viral particles (86).

Additionally, apart from their ability to potentially combat SARS-CoV-2 infection, omega-3 fatty acids may also play a role in dampening the severity of the health complications secondary to the infection (74). Omega-3 fatty acids, given their anti-inflammatory role, represent a valuable aid in countering COVID-19-induced inflammation and therefore prevent the cytokine storm (87). However, these effects are not within the scope of this review, as they are not directly linked with the ability of these fatty acids to hinder SARS-CoV-2 infection and are reviewed elsewhere (74).

4.2. Vitamins and minerals as tools to mitigate the risk of SARS-CoV-2 infection

Vitamin and mineral status, and consequently their intake, are crucial for the host to mount effective defence responses against COVID-19. In this regard, vitamin D has been proposed as a putative preventative or therapeutic nutritional tool in the battle against SARS-CoV-2 infection (88). This paradigm is also supported by the fact that low levels of 25-hydroxyvitamin D3 have been associated with increased susceptibility to acute respiratory tract infections (89). In line with this, emerging evidence points to vitamin D as a potential nutritional tool able to lower the risk of SARS-CoV-2 infection and improve disease outcomes. Indeed, vitamin D supplementation was associated with a 9% decrease in the risk of SARS-CoV-2 infection, an effect that, as described for omega-3 fatty acids, was specific to females (73). On the contrary, low vitamin D status has been associated with a higher susceptibility to SARS-CoV-2 infection (90). From a mechanistic perspective, these effects may be dependent on the ability of vitamin D to support innate antiviral immune responses, including the induction of autophagy and the production of antimicrobial components of the innate immune system, such as cathelicidin (91). In further support of the antiviral effects of vitamin D, its active form, calcitriol, has shown an inhibitory effect against SARS-CoV-2 infection in an *in vitro* model of human nasal epithelial cells (92). Furthermore, vitamin D supplementation, aside from lowering the incidence of the infection, may decrease the severity of the symptomatology as well as the risk of death from COVID-19 (93). Nevertheless, despite the potential benefit of vitamin D supplementation in mitigating the risk of SARS-CoV-2 infection, also supported by the protective effects of this vitamin against acute respiratory infections (94), the data generated up to date do not infer a cause-effect relationship between vitamin D intake and prevention of SARS-CoV-2 infection (88). Additionally, the supplementation of cod oil providing 10 µg of vitamin D daily did not affect the incidence of SARS-CoV-2 infection (95), supporting the possibility that vitamin D supplementation, at least at the dosage provided as part of this study, may not be sufficient to mitigate the risk of SARS-CoV-2 infection. Thus, despite observational studies supporting the role of vitamin D in lowering the risk of SARS-CoV-2 infection (73, 96), the evidence gathered to date do not imply a direct causality between vitamin D status and lower SARS-CoV-2 infection incidence.

Vitamin E may also represent a potential molecule to fight off SARS-CoV-2 infection, as demonstrated by the relationship between α-tocopherol supplementation and the decrease in upper respiratory tract infection (97). The antiviral effects ascribed to vitamin E may be dependent upon its capacity to increase the number of T cells, and their ability to produce IL-2 and enhance the activity of natural killer cells (98). Despite its immunomodulatory potential, possibly involved in lowering the risk of SARS-CoV-2 infection, direct evidence of the role of vitamin E in preventing SARS-CoV-2 infection is still lacking.

Vitamin C is well known for its immune-boosting effects, and as such represents another micronutrient with the potential to lower the risk of SARS-CoV-2 infection. In this regard, vitamin C has been shown to elicit antiviral immune responses underlaid by an increase in IFN-α/β as demonstrated in the early stages of influenza virus infection (99). The antiviral effects exerted by vitamin C also relay on the upregulation of natural killer cells and the induction of cytotoxic T-lymphocyte activity (100, 101). Moreover, supplementation of vitamin C at doses of 1-2 g/day was effective in lowering the risk of upper respiratory tract infections (102). It is not surprising indeed that the highest rate of SARS-CoV-2 infection affected low-middle income countries where there is also a high prevalence of hypovitaminosis C (103), suggesting a putative relationship between vitamin C status and SARS-CoV-2 infection. In further support to the role of vitamin C in the battle against COVID-19, the deficiency of this vitamin has been reported in patients suffering from respiratory infections and patients with pneumonia, relative to healthy controls (104). Interestingly, there is an overlapping between vitamin C deficiency and many risk factors for SARS-CoV-2 infection and severity. Indeed, African-Americans, individuals affected by diabetes, hypertension and chronic obstructive pulmonary disease, not only are at high risk of developing severe, life-threatening symptoms due to SARS-CoV-2 infection, but also experience vitamin C deficiency (105). Moreover, scurvy, the direct consequence of vitamin C deficiency, is associated with defective immune function and increased susceptibility of infections like pneumonia (100, 106). Thus, the rationale for using vitamin C as a nutritional strategy to mitigate SARS-CoV-2 infection risk, is supported by the role of this vitamin as an immune-booster. Additionally, the anti-inflammatory, anti-oxidant and anti-thrombotic effects of this vitamin provide the rationale for its use to decrease the severity of the symptomatology in patients. This notion is supported by fact that individuals with lower serum vitamin C are at higher risk of severe COVID-19 (104), whereas intravenous administration of vitamin C in critically ill COVID-19 patients improved the symptomatology, lowered IL-6 circulating levels (107) potentially countering the cytokine storm, decreased mortality (104) and shortened the stay in the intensive care unit (108). Thus, vitamin C may represent a valuable tool not only to prevent the complications of the infection, but also to dampen the risk of SARS-CoV-2 infection given its antiviral and immunomodulatory properties (109). However, despite its immune boosting effects, which make it promising for primary prevention of viral infections, the direct relationship between vitamin C status and risk of SARS-CoV-2 infection remains to be fully elucidated.

Along with vitamins, some minerals have also been implicated in the prevention of SARS-CoV-2 infection. Of these, zinc may play a crucial role in this context, given its role in antiviral immunity (110). Zinc exploits an immunomodulatory role, as it regulates inflammatory responses, and the proliferation, differentiation and function of leucocytes and lymphocytes; it also promotes the secretion of IFN-α and-γ by leucocytes (111–113). In further support, its deficiency leads to decreased natural killer cell activity and impaired cytokine production by monocytes (114). Furthermore, zinc antiviral effects

also rely on the inhibition of virus-host cell interaction as well as viral replication (110). Zinc, in a concentration-dependent fashion, also dampens ACE2 activity, possibly inhibiting its interaction with the SARS-CoV-2 S protein (115). High intracellular zinc concentration or agents able to enhance intracellular zinc influx inhibit the replication of several RNA viruses (116), which may also include SARS-CoV-2, possibly via inhibition of viral RNA polymerase activity (110). However, the latter effect has only been demonstrated on SARS-CoV *in vitro* (116). The role of zinc in supporting the immune system may also have implications for SARS-CoV-2 infection. In support of this, in a case–control study, symptomatic COVID-19 was significantly lower in individuals receiving zinc supplementation compared to controls (i.e., individuals not receiving zinc supplementation) (117). This suggests that maintaining an adequate zinc status may be instrumental in preventing and mitigating the severity of COVID-19 symptomatology (117). Not surprisingly, indeed, zinc deficiency was associated with acute respiratory distress syndrome and higher mortality rates (118). Thus, the prevention of zinc deficiency represents a promising strategy to support the immune system and possibly prevent the deleterious health consequences linked with COVID-19. However, as reported for the aforementioned nutrients, direct clinical evidence on the ability of zinc to counter SARS-CoV-2 infection is still lacking, which highlights the need for a prudent approach in the supplementation of zinc as a prophylaxis or treatment of COVID-19 (119).

5. Microbiome and COVID-19

5.1. Lung and gastrointestinal microbiome

The positive effect of probiotics on the human health is part of the more general interaction between human beings and the microorganisms populating the surfaces of the external part of the body or the internal cavities. At the intestinal level, the microbiota in a healthy individual is represented by an enormous number of microorganisms, consisting of bacteria, fungi, viruses, archaea and protozoa. Bacteria are represented by five major phyla, namely, *Firmicutes, Bacteroidetes, Actinobacteria, Verrucomicrobia, and Proteobacteria*. In the gut, the taxonomic composition, in terms of different genus and species and of their relative abundances, displays considerable variability among individuals (120).

A resident microbiota is present also in the respiratory tract, although the lower part was formerly believed to be sterile. Here, as reported by Magryś et al. (121), the microbial community is mainly represented by *Bacteroides, Firmicutes* and *Proteobacteria*, but the overall microorganism number is enormously reduced with respect to the intestinal bacterial population, and the species diversity is lower too.

The GI resident microbiota exerts several outstanding functions on human health. Generally, the commensal microorganisms participate in digestive processes, provided that hydrolytic enzymes are able to complete the demolition of otherwise non-digestible foods, and they are a source of several nutrients essential for the host, like vitamins and other nutrients (see below). In addition to the nutritional contribution, resident microorganisms exert both direct and indirect protective roles against exogenous or endogenous opportunistic pathogens integrating the host's natural defenses.

Both intestinal and lung lumina are, in fact, hostile environments for both commensal and pathogen microorganisms. Goblet cells in ciliary and intestinal epithelia secrete a mucus layer, which interferes with the attachment of microorganism populations, and is further enriched by immunoglobulin A (IgA) secretion. In the gut, Paneth cells are characteristic epithelial elements that produce bactericidal substances like lysozyme, secretory phospholipase A2, defensins, defensin-like peptides, and cathelicidins (122). Altogether, mucus and bactericidal substances control and sharpen the survival of microorganisms at the epithelial surface and constitute an actual barrier defending underlying tissues from the aggression of pathologic microorganisms including viruses.

The microorganisms composing the customary commensal microbiome integrate passive host immunity defenses in several ways. First, they limit the adhesion and growth of pathogens to the epithelium surface, because commensals occupy the potential adhesion niches, competing for nutrients, and producing waste metabolites able to interfere with pathogen growth. Moreover, microorganisms continuously stimulate the production of mucus, IgA secretion and integrity of intraepithelial adhesion structures, which represent the first line of immunity defense against pathobionts.

Remarkably, the resident microorganisms entertain continuous crosstalk with the host immunity system which receives continuous stimulation. Members of innate and adaptive immunity, like alveolar or GI macrophages, dendritic cells, and regulatory T cells, are continuously challenged, until they reach a homeostatic state of interactive equilibrium. Importantly, this equilibrium is influenced by the reciprocal interaction between the lung and GI tract, and relative microbiome. This interaction, termed as the gut-lung axis, implies a reciprocal communication that occurs in different ways. Both organs can prime the local immune system whose components can be exchanged trough lymphatic and circulatory vessels. Mainly through the same routes, whole bacteria, bacterial fragments and microbiome-derived metabolites can be exchanged, making it possible to stimulate the immune system in diverse anatomical districts.

Of note, GI microorganisms release a wide number of metabolites including metabolized bile salts, short-chain fatty acids (SCFA), branched-chain amino acids, trimethylamine N-oxide, tryptophan and indole-derivative metabolites and imidazole propionate (123). Among them, SCFAs, (i.e., acetate, lactate, propionate, and butyrate) represent the end products of fermentative microbial metabolism, and at the same time, an important source of energy for colonocytes and a relevant caloric integration for the whole body. More importantly, SCFAs regulate immune cell division and metabolism in peripheral regulatory T cells, macrophages and granulocytes, antigen presentation by dendritic cells, and interleukin and cytokine production, often at distant locations (124–126). SCFAs can regulate genomic transcription, by both binding to specific free fatty acid receptors (FFARs) and inhibiting histone deacetylases (127, 128).

The importance of this stimulation by the microbiome on the immune system is attested by the finding that the use of probiotics has been associated with a reduced risk to develop illnesses with a high inflammatory background such as non-alcoholic fatty liver disease, cancer, obesity, cardiovascular diseases, or diabetes (129). Consistently, mice growing under sterile conditions had macrophages and dendritic cells unable to produce IFN-α, IFN-β, IL-6, TNF, IL-12, and IL-18 when challenged with microbial ligands, highlighting the importance of the resident microorganisms in immune system priming (130).

5.2. COVID-19 and microbiome dysbiosis

During SARS-CoV-2 infection, the microbiota, both at gut and lung level, faces a deep alteration in microorganism composition. In particular, the lung microbiota has been reported to undergo a diminution of the diversity and abundance of several beneficial genera which normally colonize the airways and lungs, such as *Corynebacterium, Streptococcus, Dolosigranulum, Fusobacterium periodonticum*; among them, *Dolosigranulum and Corynebacterium* were found to be significantly more abundant in COVID-19 asymptomatic subjects or those with moderate disease (131). On the other hand, several potential pathogens (such as *Pseudomonaceae, Salmonella, Serratia, Haemophilus influenzae, Moraxella catarrhalis, Prevotella, Veillonella, Staphylococcus, Peptostreptococcus, Clostridium*) appear to be enriched during COVID-19 (131); and in particular, *Prevotella salivae* was found to be a good predictor of respiratory support need in COVID-19 patients (132). Again, the amplitude of the microbial dysbiosis correlates with COVID-19 severity (133, 134).

Although SARS-CoV-2 mainly targets the respiratory system, it also affects several organs, including the digestive system. Here, the ACE2 receptor is expressed not only in the endothelial cells of the GI capillary bed, but also in the brush border of enterocytes and in gastric and colon epithelia which can be a target and replication site of the virus (135). Of note, the symptoms of COVID-19 can include nausea, diarrhea, vomiting and abdominal pain, and detection of SARS-CoV-2 in the feces occurs up to 5 weeks after the resolution of respiratory symptoms (136). Again, an association between the presence of GI symptoms, the severity of lung impairment, and the need for ventilatory support has been proposed. COVID-19 is also associated with alteration of the GI microbiota, mainly showing a diminution of the taxonomical variability of microbial species, with an increase of opportunistic or pathologic species, and a decrease of beneficial species, in particular *Ruminococcaceae and Lachnospiraceae* families (137). Some alterations, like the diminution of *Faecalibacterium prausnitzii,* inversely correlate with the severity of COVID-19 (138).

Noteworthily, a direct cause of microbiome dysbiosis could be represented by the downregulation of ACE2 operated by SARS-CoV-2 at the GI epithelial level. In fact, it has been proposed that the relative abundance of ACE2 in the GI tract is joined to ACE2 capacity to heterodimerize with amino acid transporters, warranting normal amino acid supply to enterocytes. SARS-CoV-2-dependent ACE2 deficiency could lead to an insufficient entry of tryptophan, and should in turn lead to scarce synthesis of antimicrobial peptides, affecting microbiome homeostasis and loss of epithelial integrity (135, 139). On the other hand, ACE2 downregulation can have positive consequences: Zuo et al. (138) observed that several Bacteroides species (*Bacteroides dorei, Bacteroides thetaiotaomicron, Bacteroides massiliensis,* and *Bacteroides ovatus*) are already known to be able to downregulate ACE2 expression in mice, inversely correlated with SARS-CoV-2 content in the feces of COVID-19 patients, suggesting a protective role of Bacteroides against SARS-CoV-2. An ACE2 receptor docking study suggested that ACE2 downregulation may have both positive and negative roles at the intestinal level; however, further research is needed to better characterize this dual effect.

Gut microbiota dysbiosis, typically also characterizing obesity (140), may also underpin the increased risk of COVID-19 severity observed in obese individuals (141, 142). Particularly, this increased susceptibility to develop severe symptoms in response to SARS-CoV2 infection may be dictated by the ability of gastrointestinal microbiota dysbiosis to trigger and sustain chronic inflammation (143). The latter not only typically occurs in obese individuals, but also represents one of the putative pathophysiological mechanisms linking obesity, gut microbiota dysbiosis, and COVID-19 severity (144). Indeed, obesity-related gastrointestinal microbiota dysbiosis, in concert with increased gut permeability (145), contributes to inflammation by promoting systemic endotoxemia which is the direct consequence of the leak of lipopolysaccharides (LPS) through the dysfunctional gut barrier (146). Ultimately, this inflammatory status, fueled by gut microbiota dysbiosis, amplifies the so-called "cytokine storm," thereby predisposing subjects with obesity to more severe COVID-19 symptoms and increased risk of death (147).

Altogether, these data depict a scenario where the microbiome could act as an important environmental factor strongly contributing to the wide variability in the patient response to SARS-CoV-2. On this basis, a preventive and therapeutic approach to SARS-CoV-2 infection appears reasonable and promising, based on probiotic and prebiotic administration. The use of probiotics is aimed to positively stimulate the immunity system, reinforcing the passive barriers to prevent or reduce the possibility of viral and opportunistic pathogen entry both at lung and GI level, and to reduce the disease outcomes, preventing the cytokine storm.

5.3. Probiotics and prebiotics in COVID-19 treatment

The microorganism geni most frequently used as probiotics are *Lactobacillus, Bifidobacterium, Lactococcus, Streptococcus, Enterococcus, and Bacillus,* together with some strains of the genus *Saccharomycetes.* Anyway, the probiotic behavior is not linked to the whole bacterial or fungine genus. In fact, the Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food in 2002 states that probiotics are "live strains of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (148). This definition reserves the health benefit to specifically selected strains, that in addition are required to not have, nor propagate, antibiotic resistance and to be able to maintain the health benefit for the whole process of production, conservation, and distribution of the probiotic.

Probiotics improve the host's health using the same strategies as the microbiome, but in a more effective and often targeted and detectable way. It has been clearly shown that probiotics can modulate the human microbiome and interfere with the growth of opportunistic COVID-19-related pathogens, by competing for docking sites at the epithelial surface. Furthermore, probiotics can interfere with the viral cycle, stimulating innate immunity by activating the inflammasome and the production of interferons and inflammatory cytokines which represent a first line of antiviral defense. Consistently, *Lacticaseibacillus rhamnosus GG* has been used in a neonatal mouse model of influenza as a preventive intranasal treatment. In fact, Kumova et al. showed that intranasal administration of *Lacticaseibacillus rhamnosus GG* can have immunoregulatory functions at the lung level, triggering the type-I IFN pathways via the Toll-like receptor (149).

Andrade et al. showed that, in *in vitro* systems, specific strains like *Lactobacillus plantarum* MPL16 and CRL1506, and *Dolosigranulum*

pigrum 040417, increased the resistance of cultured respiratory epithelial cells to SARS-CoV-2 by inducing the production of type-I and type-III IFNs and transcription of IFN-stimulated genes, thereby potentially improving the innate antiviral response and affecting the early phases of SARS-CoV-2 infection. They noted also that the same strains could reduce cytokine production, contributing to the control of immune cell recruitment to the infection site, and subsequent of inflammatory damage. These strains appear to be promising tools to re-modulate respiratory microbiota and to counteract SARS-CoV-2 infection in the early stages (150).

Another interesting possibility is the capacity of certain probiotics to interfere with viral internalization. Silvestre Ortega-Peña et al. noted that *Staphylococcus epidermidis* is a commensal bacterium abundant in the anterior part of the nose, whose abundance is inversely correlated to serious respiratory infections. The authors not only highlighted that *Staphylococcus epidermidis* could, directly or indirectly, eliminate a wide number of pathogens, but also proposed its use as a probiotic able to prevent the development of COVID-19. Interestingly, they reported that *S. epidermidis* acts not only through the production of type-I and-III IFN pathways, but also by regulating the surface expression of the ACE2 receptor and TMPRSS2 protease (151). The importance of this finding is underlined by reports indicating that other bacteria can both release peptides able to interact with ACE2 receptors, and produce enzyme homologs to ACE2, which potentially can behave as decoy receptors for SARS-CoV-2, attenuating its entry into target cells (152).

In addition to intranasal administration, oral administration of specific probiotic strains has also been shown to be effective in the treatment of COVID-19 disease. The commercial kit Lactibiane Iki, which mixes three different strains *(Bifidobacterium lactis LA 304, Lactobacillus salivarius LA 302, and Lactobacillus acidophilus LA 201)* has been proposed to significantly reduce inflammatory markers in patients infected by COVID-19 and interstitial pneumonia (153). Another commercial product, containing three strains of *Bifidobacterium genus* and specific prebiotics, was found to reduce inflammatory markers, normalize gut microbiota composition, and increase antibody formation in 25 COVID-19 patients (154). Consistently, amelioration of antibody production is reported also by other researchers. In fact, oral administration of *Loigolactobacillus coryniformis K8 CECT 5711* to a group of healthcare workers showed a positive effect on anti-SARS-CoV-2 vaccination, leading to significantly higher antibody production after 81 days of probiotic treatment (154).

Oral administration of nisin, a food-grade peptide obtained from *Lactococcus lactis,* seems to be useful also in preventing the interaction between SARS-CoV-2 and the human ACE2 receptor. Nisin is a pentacyclic antibacterial peptide, present in several natural variants and widely used for cheese manufacturing and preservative. Several nisin variants appear to be able to efficiently interact with the ACE2 receptor and diminish SARS-CoV-2 internalization (155). As stated above, further research is needed to clarify the side effects of potential ACE2 downregulation.

Additionally, beneficial effects on human health can come from the administration of prebiotics (Figure 1). These are not living organisms, but represent types of not-digestible foods, which mainly include oligosaccharides, unsaturated fatty acids, dietary fibers, and polyphenols, and can be fermented by specific gut microorganisms, stimulating their growth and so reprogramming microbiome composition. Prebiotics can be beneficial when administered alone, because they can positively modify the distribution of resident microorganisms. As a note, they can also sustain the growth and survival of probiotic strains and species, with further although not easily predictable additive benefits for host health. On the other hand, large amounts of prebiotic fiber can stimulate their utilization by intestinal microorganisms, generating a large amount of gas, bloating, and discomfort.

6. Bioactive herbal products interfering with SARS-CoV-2 entry

Many phytochemicals with different mechanisms of action have been proposed to possess antiviral activity against SARS-CoV-2 (156). Several natural compounds, like flavonoids, steroids, coumarins, and alkaloids, were reported before the COVID-19 outbreak to possess ACE2 modulatory activity (157), encouraging this research field during the pandemic. Many studies focused on *in silico* molecular docking analysis (158–160), but relatively few molecules have been tested so far on biological systems. For this reason, in this section, we aim to resume the results obtained after assessing herbal products *in vitro* on cell line models.

Glycyrrhizic acid (GA), the main active compound of the root of *Glycyrrhiza uralensis* (licorice), was suggested as a possible candidate for COVID-19 treatment, based on its ability to reduce SARS-CoV-2 invasion by blocking the ACE2-S protein binding, as reported in human embryonic kidney 239 T cells (161). GA is also known for its anti-inflammatory properties, evidenced by the inhibition of *NF-kB* expression and cytokines secretion (162). *In vitro*, Zhao and colleagues confirmed the anti-inflammatory action of GA using an encapsulated formulation of nanoparticles to treat human monocytes (THP-1) and PBMC from healthy donors, stimulated with nucleocapsid (N) protein of SARS-CoV-2. They reported significantly reduced mRNA and protein expression of IL-1α, IL-1β and IL-6 (163).

Stachytarpheta cayennensis, an herbaceous plant from tropical and subtropical areas, was reported to significantly inhibit virus entry in HEK-293 T ACE2 cells (164). The characterization of the extract evidenced the presence of β caryophyllene (BCP), thymol, citral, 1,8-cineole, carvone, and limonene. BCP and limonene were previously proposed by docking studies to bind the S protein and ACE2 (160). BCP is a cannabinoid present in essential oil from common spices (e.g., cinnamon, oregano, black pepper, basil), that has been reported as one of the components with antiviral activity; however, its action tested alone was less effective compared to the total extract from *Stachytarpheta cayennensis* in countering SARS-CoV-2 S pseudovirus infection of HEK-293 T-ACE2 cells. This suggests a combined effect of different molecules in the total extract (164). BCP is also known for its anti-inflammatory proprieties, acting on different pathways including cytokine and chemokine signalling (165). Based on that, Jha and colleagues proposed BCP as a candidate for COVID-19 treatment, even if other studies are needed to verify its anti-inflammatory action (165). In agreement, limonene, the main component of essential oil from *Citrus limon,* was reported to reduce ACE2 protein levels and downregulate ACE2 and TMPRSS2 expression in colorectal adenocarcinoma cell line HT-29 (166). The same action was also shown by geranium essential oils (from *Pelargonium graveolens*), and a significant reduction of ACE2 mRNA

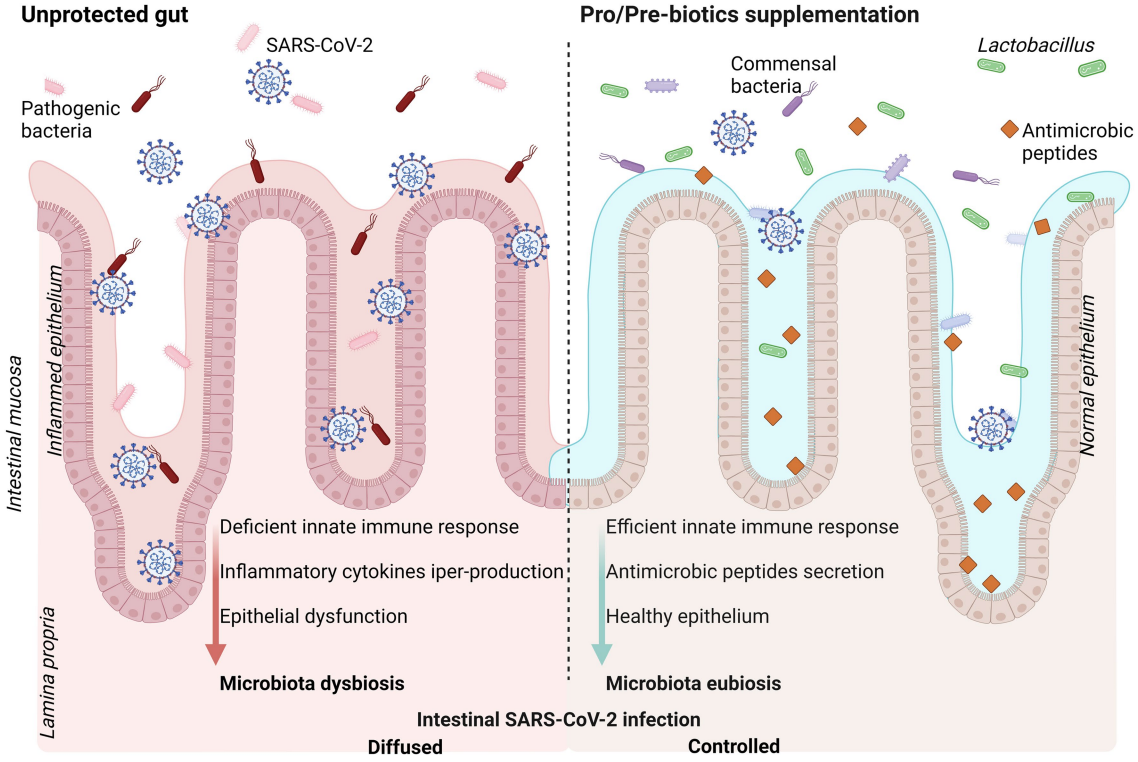

FIGURE 1
Schematic representation of intestinal mucosa exposed to SARS-CoV-2 infection **(left panel)** and positive effects of pro-and pre-biotic dietary supplementation **(right panel)**. SARS-CoV-2 infection promote intestinal dysbiosis enhancing severity of the disease. Supplementation with probiotics and/or prebiotics is suggested as strategy to efficiently counteract the virus infection with better disease outcome. Created with BioRender.com.

and protein levels were also confirmed via major components: citronellol and geraniol tested as single molecules (166). Similarly, the herbal extracts of *Spatholobus suberectus dunn* (SSP) and *Polygonum cuspidatum* root and rhizome showed concentration-dependent entry inhibition in HEK293T cells (167, 168).

Asparagus officinalis extracts, already known for their action in counteracting breast cancer progression (169), were reported to inhibit ACE, with a positive correlation to their content in hydrophobic amino acids and gallic acid (170), suggesting a possible action also on the homolog ACE2. In addition, *Asparagus officinalis* stem extracts evidenced anti-inflammatory action through the inhibition of IL-6 and IL-1β transcription on S1-protein-stimulated macrophages (171). Inhibition of ACE2 and TMPRSS2 transcription and protein expression were also reported in 293 T cells treated for 24 h with 50 μg/mL of Theaflavin extracted from *Camellia sinensis* (172).

A molecular docking study evidenced repression of TMPRSS2 expression by withanone, a withanolide tripertenoid extracted from the root, stems, and leaves of *Withania somnifera*, a medical plant known also as Indian ginseng or winter cherry. *In vitro,* Kumar and colleagues showed that withanone causes a 40–50% reduction in TMPRSS2 expression in breast cancer cells (MCF7) (173). However, it has also been reported that this antiviral effect is associated with cytotoxicity when used at the same dose (40 μM for 48 h). In agreement, cytotoxicity was also reported in hepatocarcinoma (HepG2), breast cancer (MCF7), and normal mammary epithelium (MCF-10) cells when treated with withanone 50 μM for 72 h, while lower concentrations (20 μM) did not evidence cell viability reduction (174). For this reason, studies for possible applications for SARS-CoV-2 treatment should investigate the antiviral efficacy at a concentration of withanone that does not evidence side effects.

Scutellaria barbata (SB) is a widely used herb in Asia, known for its various pharmacological properties including anti-inflammatory and antiviral activities. The anti-inflammatory action of ethanol and ethyl acetate extracts of SB is sustained by phenols, flavonoids, chlorophylls, and carotenoids. These extracts significantly inhibit IL-6 and IL-1β secretion in the macrophage cell line RAW264.7 in a dose-dependent manner (175). Huang et al. reported that aqueous SB extracts, characterized by neo-clerodane diterpenoids and flavonoids followed by polysaccharides, volatile oils and steroids, inhibited the enzymatic activity of TMPRSS2 (176). In particular, 4 mg/mL of extract reduced 54.8% of TMPRSS2 protease activity. Kidney epithelial cell line Vero E6, which express high ACE2 and low TMPRSS2 levels, and human lung carcinoma cells Calu-3, which express high TMPRSS2 levels, were used as cell models to test SB activity. Pre-treatment with SB extract and then infection with SARS-CoV-2 pseudovirus reduced infection in Calu-3 cells but not in VeroE6, suggesting that SB affects TMPRSS2, ultimately reducing virus entry (176).

The same cellular models were used also by Kim et al. (177) to demonstrate that platycodin D (PD), a glycosylated triterpenoid saponin extract from the root of *Platycodon grandiflorum,* inhibited virus entry both in Calu-3 (TMPRSS2-high/positive) and Vero E6 cells (TMPRSS2-low/negative). In this case, the mechanism is still unknown; however, the authors suggested that PD interferes with

virus entry by interacting with cholesterol and preventing virus fusion to the host cells. They demonstrated that cholesterol depletion in host cells decreases 2.5 times the PD effects, supporting the hypothesis that cholesterol facilitates PD action in host cells (177). The same research group, taking advantage of these results, identified the three chemical groups presented on the PD structure that were essential for inhibition of virus entry into the cells, to develop new synthetic saponins. The new molecules efficiently inhibit the fusion to the ACE/TMPRSS2-positive cells (H1299, lung carcinoma cells) with a 2-fold increase in potency compared to the initial natural compound (178). Additionally, phenolic components from *Platycodon grandiflorum* extracts were reported to possess anti-inflammatory activity by reducing IL-6 and TNF-α production in LPS-stimulated macrophage cell lines (RAW 264.7) (179), suggesting multiple bioactive components in the total extract could be useful for COVID-19 treatment.

Similarly to saponin PD, even for astersaponin I (AI), a triterpenoid saponin in *Aster koraiensis*, the observed inhibition of SARS-CoV-2 infection was dependent on effects on cholesterol (180). In this work, the authors demonstrated that treatment with AI induces increasing cholesterol content in the cell and in the endosomal membranes, and that this interferes both with the entry of the virion as well as with syncytium formation. Results of fusion experiments performed in H1299 cells demonstrated that 5 μM of AI inhibited entry and prevented syncytia of more than 90%. The effects were shown for wild-type and D614G variant SARS-CoV-2 and led authors to propose AI as a broad-spectrum agent also against other enveloped viruses.

Following a similar reasoning, epigallocatechin gallate (EGCG), the green tea catechin, has been proposed as a future pan-coronavirus attachment inhibitor due to its effects on cell-surface glycans, as demonstrated by LeBlanc and Colpitts (181). In their work, EGCG treatment of Huh7 and A459 inoculated with seasonal human CoVs, HCoV-229E and HcoV-OC43, inhibited infectivity at low micromolar concentrations ($IC_{50} < 1$ μM), with minimal effects on cell viability. Furthermore, they showed that EGCG was able to inhibit entry of SARS-CoV-1, SARS-CoV-2 and its delta and omicron variants, and WIV1-CoV (a bat coronavirus able to bind human ACE-2) with 15 μM < IC_{50} < 25 μM. Finally, they demonstrated that the antiviral effect of EGCG was caused by the heparan sulfate blocking of virions binding to the cell membrane, with the same mechanism as heparin. Considering that interactions with membrane glycans are shared by many viruses to initiate the infection, and that heparan sulfate proteoglycans are necessary for SARS-CoV-2, the authors conclude that EGCG can be an efficient antiviral, but its low stability and rapid metabolism are important limitations. Nonetheless, other authors have observed a partial reduction of SARS-CoV-2 replication *in vivo* in C57BL/6 mice infected intranasally and treated orally with 10 mg/kg daily of EGCG for 2 weeks (182), indicating a possible future application. Other studies have focused on EGCG and ACE2, observing inhibition of S protein binding to ACE2 receptor by neutralization (183), ELISA (184), and entry or infectivity (184, 185) assays, testing EGCG in the range 0–100 μM on several cellular models. Interestingly, the work of Liu et colleagues reported an efficient reduction of infection when using live SARS-CoV-2 and HcoV-OC43 viruses on Calu-3, HEK-293 T-ACE, and HCT-8 cells, indicating that pre-treatment is fundamental for a significant effect (185). Collectively, these results indicate that EGCG can work both specifically on ACE2, as well as via unspecific interference with heparan sulfates.

Another phenolic compound that was reported to reduce SARS-CoV-2 infection in TMPRSS2-negative Vero E6 cells was the resveratrol tetramer hopeaphenol, a compound extracted from various plants including *Hopea, Vitis,* and *Shorea,* that was suggested to be acting without affecting TMPRSS2. Further studies are required to elucidate its mechanism of action (186).

An herbal mixture called virofree, containing active compounds including quercitin, hesperidin, genistein, daidzein, and resveratrol, was reported to repress protein S binding to ACE2 by *in vitro* biochemical-binding ELISA assay. Tested on Calu-3 cells, virofree dose-dependently decreases the protein expression of ACE2 and TMPRSS2, suggesting an antiviral activity through inhibition of virus entry into the cells (187).

Overall, many phytochemical compounds present in herbs or herbal extracts might be potentially used for relieving SARS-CoV-2 infection, limiting virus entry, or reducing inflammation (Figure 2). However, the identification of bioactive compounds in extract mixtures, the efficient non-toxic antiviral range of concentrations, and the specific mechanisms of action remain as open questions in many cases; thus, further *in vitro* and *in vivo* deepening are needed before moving to clinical trials investigations.

Furthermore, innovative approaches considering the use of nanoparticles to ameliorate the bioavailability of the investigated compound could reconsider some molecules and improve *in vivo* results.

7. Conclusion

The COVID-19 pandemic appears to be a global threat unfortunately lacking robust medical treatments. Several tools have been used to manage COVID-19 symptoms and the clinical aftermath of this illness, including old and new antiviral drugs, and plasma from convalescent patients or purified antibodies. But the most promising medical approach relies upon massive vaccination. Anyway, despite the success of vaccine trials and the presence of different vaccine platforms, several concerns exist, reducing vaccine massive utilization and limiting their efficacy. Social concerns, like logistic and economic issues, together with vaccine hesitancy, can severely limit the dissemination of vaccination. In addition, the quite-fast appearance of new virus variants, together with the wide variability of genetic, health and nutritional status of human beings, can influence the severity of viral illness and consequently interfere with the effectiveness of vaccination in protecting people from severe aftermath of viral infection. In a similar scenario, it seems reasonable that COVID-19 therapeutics can include some supplementary nutritional approaches. In particular, vitamins and nutrients, like vitamins A, E and D, other polyunsaturated lipids and minerals like zinc, can be lowered in at least some part of the population, such as the elderly, or patients suffering from long-lasting subclinical or full-blown inflammatory and oxidative conditions, e.g., arthritis, obesity, diabetes, hypertension, cardiopathies, and cancer. Adequate nutrient supplementation could not only boost the immune system, but also has been shown to prevent viral entry, as reported above, interfering with the process of membrane fusion subsequent to ACE2 docking and TMPRSS2 action.

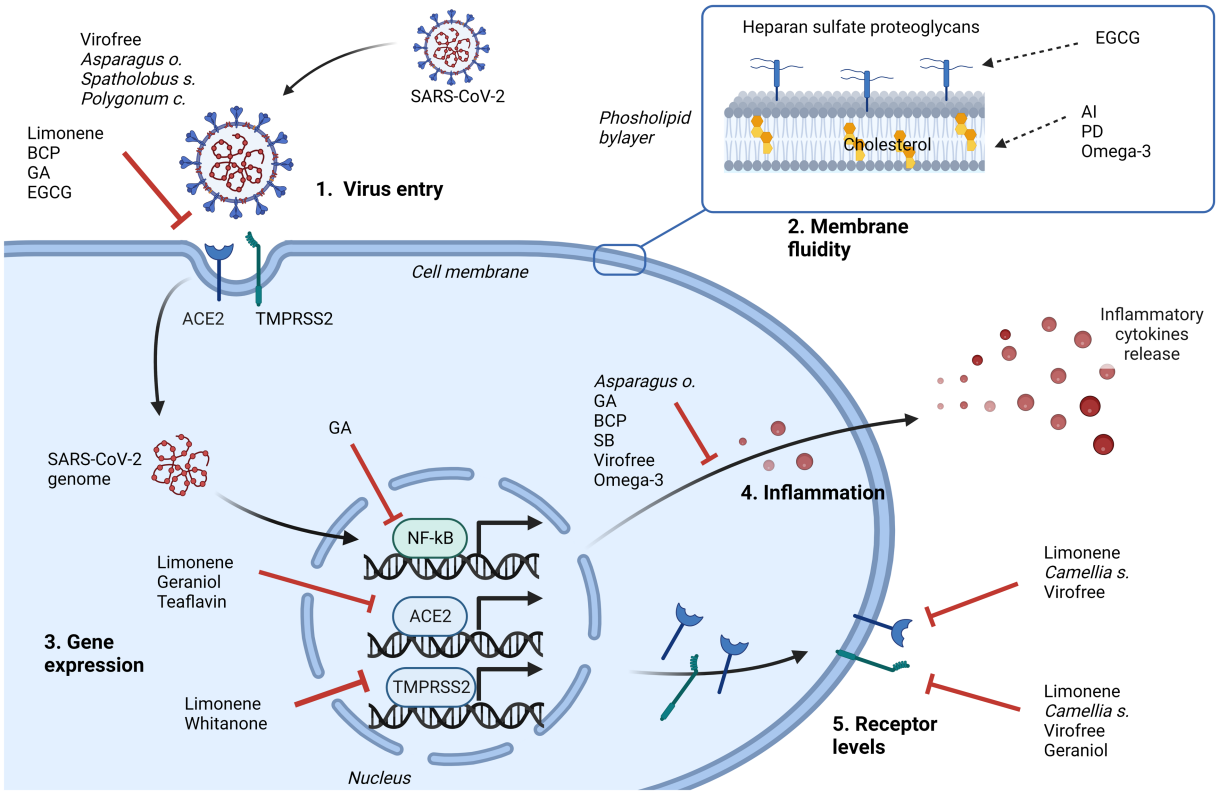

FIGURE 2
Representation of the major cellular events that can be inhibited by natural products in contrasting SARS-CoV-2 infection. Their effects can limit the virus entry, directly interfering with cellular receptor binding (1), or indirectly changing the membrane fluidity (2), and/or can affect some pathways driven by virus. Intracellularly, involved mechanisms can be the downregulation of *NF-kB*, *ACE2* or *TMPRSS2* gene expression (3), the inhibition of cytokines release (4), to control the inflammatory process, and the downregulation of ACE2 and/or TMPRSS2 protein levels (5). AI, astersaponin I; BCP, β caryophyllene; EGCG, epigallocatechin gallate; GA, glycyrrhizic acid; PD, platycodin D; SB, *Scutellaria barbata*. Created with BioRender.com.

Supplementation of prebiotics and probiotics has been largely shown to strongly prompt and reshape the immune system, ultimately also ameliorating antibody production and vaccine effectiveness. Similarly, herbal-derived compounds or extracts from herbal mixtures offer a wide panel of immunoactive substances able to sustain the antiviral response. An interesting feature, shared by probiotics and herbals, is represented by the ability to reduce viral entry, preventing SARS-CoV-2 infection. The entry strategy implicates several interactions with the cell surface, including S protein priming, ACE2 binding and membrane fusion, or, in absence of TMPRSS2 activity, vesicle-mediated endocytosis. Independent of the exact mechanism for viral RNA liberation into the cytoplasm, interaction with the ACE2 protein appears to be the crucial event for viral infection. Of note, extracts from licorice, *Stachytarpheta cayennensis, Spatholobus suberectus dunn* (SSP) and *Polygonum cuspidatum* have been shown *in vitro* to inhibit virus entry, in particular blocking receptor docking or downregulating ACE2 expression. On the other hand, Theaflavin extracted from *Camellia sinensis* and withanone from *Withania somnifera* have been shown to reduce the level of TMPRSS2, while other plants like as *Scutellaria barbata* are effective in reducing TMPRSS2 activity, and consequentially, S protein priming. This latter finding is of particular interest, because TMPRSS2 activity appears to be higher in the respiratory airways and lungs where SARS-CoV-2 exerts its main infectious effects. Also, several strains of probiotics have been shown to reduce viral infection, through both nasal and oral administration. They can act through different but often complementary mechanisms. Probiotics can specifically interfere with viral access to the cell surface, and/or produce peptides able to reduce the interaction between virus and ACE2, like nisin and its derivatives. Decrease of the surface expression of ACE2 receptor and TMPRSS2 protease appears to be an important tool in probiotic antiviral activity. Concurrent triggering of immune reactions represents an important tool in antiviral defense, although IFN activation, which represents a branch of the antiviral action, can paradoxically increase ACE2 expression. This occurrence can in principle increase virus replication, but at the time, evidence for an IFN-mediated increase in COVID-19 severity is lacking; in addition, it has been reported that interferons induce a truncated isoform of ACE2 not supporting virus replication (188). On the other hand, with ACE2 having a role in amino acid transport, at least in the intestine and kidney, its downregulation can potentially imply undesired side effects. This urges further research to clarify all the positive and negative implications of potential ACE2 downregulation.

Lastly, polyunsaturated lipids can alter membrane structure at lipid rafts where ACE2 is localized, thereby influencing viral entry. Of note, several lipids, including polyunsaturated omega-3 fatty acids, linolenic acid, and eicosapentaenoic acid, can directly interfere with virus-binding to ACE2, thereby significantly reducing viral entry. In addition, several bioactive herbal products, including saponins, such as triterpenoid platycodin D and astersaponin I, act on the cholesterol content of lipid rafts, interfering with viral internalization routes. In a

similar way, the green tea catechin epigallocatechin gallate can inhibit viral binding to cell surface glycan and ACE2.

New Omicron variants seem to prefer the endocytosis pathway to TMPRSS2 /ACE2 and membrane fusion (189). Again, further research is needed to explore the contribution of different entry routes for each viral variant. These caveats notwithstanding, the world of natural products represents a huge *reservoir* of biochemical and biological variability, that appears wide enough to offer efficient and hopefully decisive tools to cover, in the general population, the need to counteract viral entry in all its different forms.

Author contributions

GZ, DS, MV, AR, RV, and MP: conceptualization, revision process, and final editing. GZ, DS, MV, AR, RV, MP, EZ, GL, and LC: writing – original draft preparation and writing – review and editing. 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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Hypoglycemic effects of dendrobium officinale leaves

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Introduction: Numerous studies have demonstrated that the stems of *D. officinale* have the effect of lowering blood glucose, but the leaves of *D. officinale* have seldom been investigated. In this study, we mainly studied the hypoglycemic effect and mechanism of *D. officinale* leaves.

Methods: Initially *in vivo*, male C57BL/6 mice were administered either standard feed (10 kcal% fat) or high-fat feed (60 kcal% fat) along with either normal drinking water or drinking water containing 5 g/L water extract of *D. officinale* leaves (EDL) for 16 weeks, and changes in body weight, food intake, blood glucose, etc., were monitored weekly. Next *in vitro*, C2C12 myofiber precursor cells which were induced to differentiate into myofibroblasts and cultured with EDL to detect the expression of insulin signaling pathway related proteins. HEPA cells were also cultured with EDL to detect the expression of hepatic gluconeogenesis or hepatic glycogen synthesis related proteins. Eventually after separating the components from EDL by ethanol and 3 kDa ultrafiltration centrifuge tube, we conducted animal experiments using the ethanol-soluble fraction of EDL (ESFE), ethanol-insoluble fraction of EDL (EIFE), ESFE with a molecular weight of >3 kDa (>3 kDa ESFE), and ESFE with a molecular weight of <3 kDa (<3 kDa ESFE) for intensive study.

Results: The results *in vivo* revealed that the mice fed the high-fat diet exhibited significantly decreased blood glucose levels and significantly increased glucose tolerance after the EDL treatment, whereas the mice fed the low-fat diet did not. The results *in vitro* showed that EDL activated the expression of protein kinase B (AKT), the phosphorylation of AKT, and the expression of downstream GSK3β in the insulin signaling pathway. EDL treatment of HEPA cells confirmed that EDL did not affect hepatic gluconeogenesis or hepatic glycogen synthesis. In the experiment of studying the composition of EDL, we found that the >3 kDa ESFE displayed the effect of lowering blood glucose. In summary, the effect of EDL in lowering blood glucose may bethanole achieved by activating the insulin signaling pathway to increase insulin sensitivity, and the main functional substance was contained within the >3 kDa ESFE.

Discussion: The findings of this study represent a reference point for further exploration of the hypoglycemic effects of *D. officinale* leaves and may assist in both the identification of new molecular mechanisms to improve insulin sensitivity and the isolation of monomeric substances that lower blood glucose. Furthermore, the obtained results may provide a theoretical basis for the development of hypoglycemic drugs with *D. officinale* leaves as the main component.

KEYWORDS

dendrobium officinale leaves, hypoglycemic, type-2 diabetes (T2D), glucose metabolism, traditional Chinese medicine

1 Introduction

Dendrobium officinale refers to a perennial epiphytic herb belonging to the Dendrobium of Orchidaceae genus that is primarily distributed in the southwestern and southeastern provinces of China. Since ancient times, *D. officinale* has been known as the "gold in medicine". At least 190 compounds have been isolated from *D. officinale*, including polysaccharides, phenanthrene, dibenzyl group, sugars and glycosides, essential oils, alkaloids, and other species. There are five main classes of chemicals in *D. officinale*: polysaccharides, dibenzyl compounds and phenanthrene compounds, alkaloids, amino acids, and trace elements and flavonoids (Tang et al., 2017; Ren et al., 2020; Yuan et al., 2020; Chen et al., 2021). The main active ingredient in *D. officinale* is polysaccharides. It is mainly isolated from the stems of Dendrobium officinale with a yield rate of over 30% (Shen et al., 2017).

In recent years, numerous scholars have investigated the pharmacological activity of *D. officinale*, *D. officinale* as a medicinal herbal plant has a long history of being used to attenuate the symptoms of diabetes which is also called "Xiaoke" disease in China. The hypoglycemic efficacy of Dendrobium officinale makes it a common ingredient in Xiaoke decoction for type 2 diabetes treatment (Pang et al., 2015). Dendrobium officinale polysaccharides could decrease the levels of fasting blood glucose, insulin, glycated serum protein, and serum lipid profile and alleviate pancreatic injury as well as the dysregulated metabolism of bile acids and amino acids in type 2 diabetic rats (Chen et al., 2019). In addition, it could regulate the hepatic glucose metabolism via the glucagon-mediated signaling pathways as well as the liver-glycogen structure in HFD/STZ-induced diabetic mice (Liu et al., 2020).

Dendrobium officinale has therapeutic potential in cancer prevention and treatment. Its potential mechanism of action is mainly involved in reducing cancer cell growth and proliferation, triggering apoptosis, and increasing autophagy. Dendrobium officinale could also confer protection against liver injuries and improve liver functions against different forms of liver injuries, such as drug-, chemical-, and acute alcohol-induced injuries and nonalcoholic fatty liver diseases (NAFLD) (Xu et al., 2022). Taken together, these studies indicate that *D. officinale* has a variety of effects, such as antioxidation, hepatoprotective, anticancer, hypoglycemic, anti-fatigue, anti-aging, gastroprotective, and immunomodulatory activities (Tang et al., 2017). Because only the stems of these Dendrobium species are permitted for use according to the Chinese Pharmacopoeia, their leaves are largely discarded.

The global diabetes prevalence in 20–79 year olds in 2021 was estimated to be 10.5% (536.6 million people), rising to 12.2% (783.2 million) in 2045 (Sun et al., 2022). At present, the most commonly used hypoglycemic drugs are metformin, pioglitazone, sulfonylureas, and so on, but their use is associated with many restrictions and can also lead to weight gain, resulting in hypoglycemia and other side effects. As such, new hypoglycemic drugs with fewer side effects and use restrictions are urgently needed. Although *D. officinale* has been confirmed to reduce blood glucose levels, previous studies have primarily focused on the plant stems and the effects of *D. officinale* leaves have rarely been reported. However, *D. officinale* leaves possess chemical constituents similar to those found in stems (Huang et al., 2012; Zhou et al., 2014; Zhang et al., 2017). The biomass of *D. officinale* leaves is considerable, and the ratio of the dry weight of *D. officinale* leaves stems to leaves is approximately 1.6:1 (Zhang et al., 2013; Zeng et al., 2018). Therefore, in this work *D. officinale* leaves were used as the main research object to explore their hypoglycemic effects and obtain insights into the underlying mechanism for further research and development. *Dendrobium officinale* leaves provide a certain theoretical reference for the main components of hypoglycemic drugs.

2 Materials and methods

2.1 Preparation of water extract of *D. officinale* leaves

Dendrobium officinale leaves (500 g) were placed in a heating barrel and tap water (20 L) was added. The resulting mixture was boiled for 30 min then filtered through three layers of medical gauze, repeat twice, and the extracted solution was then poured into a heating barrel for heating and concentration to approximately 1 L. The resulting concentrated solution was allowed to cool then poured into a lyophilization dish, frozen at −80 °C overnight, and lyophilized to obtain the EDL.

The obtained lyophilized water extract was dissolved in ultrapure water (1 L) and absolute ethanol (3 L) was added. The resulting mixture was thoroughly stirred then allowed to stand. The supernatant was removed and centrifuged, and the ESFE and ethanol-insoluble fraction of EDL (EIFE) were lyophilized. The lyophilized ESFE was dissolved in ultrapure water and then transferred to a 3 kDa ultrafiltration centrifuge tube. The sample was centrifuged at 4,000 *g* for 30 min, and then a pipette gun was used to blow up the residual liquid in the upper part of the ultrafiltration tube followed by a second cycle of centrifugation at 4,000 *g* for 30 min. This afforded two fractions with >3 kDa ESFE (upper part of the centrifuge tube) and molecular weight of molecular weight of <3 kDa of ESFE (<3 kDa ESFE) (lower part of the centrifuge tube). Both fractions were lyophilized.

2.2 Animals and treatment

The animal experiments used healthy male C57BL/6J mice, Kunming mice, and db/db mice provided by Changzhou Cavens Laboratory Animal Co. Ltd. The mice were maintained in a controlled environment (12-h light/12-h dark cycle, 50%–60% humidity, and 24°C ± 1 °C ambient temperature) and administered standard laboratory food and water *ad libitum*.

After an acclimation period of 1 week, 40 C57BL/6J mice were divided into four groups: low-fat diet + water (LFD + H2O), LFD + water extract of *D. officinale* leaves (LFD + EDL), high-fat diet + water (HFD + H2O), and HFD + EDL, where the EDL dose was 5 g/L. The feeding lasted for 16 weeks.

Another 40 C57BL/6J mice were also divided into four groups: HFD + H2O, HFD + EIFE, HFD + ESFE, and HFD + EDL. The feeding lasted for 4 months. The ESFE dose was 1.8 g/L, and the EIFE dose was 3.2 g/L. The feeding lasted for 16 weeks.

After an acclimation period of 1 week, 18 db/db mice were divided into three groups: normal diet + water (ND + H2O), ND + EDL, and ND + ESFE. The feeding lasted for 16 weeks.

Thirty Kunming mice were divided into three groups: ND + H2O, ND+<3 kDa ESFE, and ND+>3 kDa ESFE. After an acclimation period of 1 week, the body weights of the mice were measured after overnight fasting, and glucose (1 g/kg) plus either the <3 kDa ESFE (7.875 mg/kg) or the >3 kDa ESFE (28.125 mg/kg) were administered by gavage, then the blood glucose levels were measured after 30, 60, 90, and 120 min. The area under the curve (AUC) was calculated by the trapezoidal method. After measured blood glucose levels, we continued to feed the mice with the two components dissolved in water and normal diet for 2 weeks and measure fasting blood glucose every week.

2.3 Glucose tolerance test

Glucose tolerance tests play an important role in evaluating insulin resistance. In this work, glucose homeostasis was measured by performing intraperitoneal glucose tolerance tests using the C57BL/6J mice after treatment for 15 weeks. The body weights of the mice were measured after overnight fasting, and a drop of blood was collected from the tail vein to measure the basic blood glucose level (t = 0) using a glucose meter (Roche, Accu-Chek Aviva). Next, the mice were administered 1 g/kg d-glucose (2 mg/g) by intraperitoneal injection and the blood glucose levels were measured at 30, 60, 90, and 120 min. The AUC was calculated by the trapezoidal method.

2.4 Cell culture

HEPA and C2C12 cells were used in this study. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM/high glucose, Thermo Fisher Scientific) supplemented with 4 mmol/L L-glutamine (supplier) and 10% fetal bovine serum (Biological Industries Israel Beit Haemek Ltd.). DMEM high-glucose medium containing 2% horse serum was used to differentiate muscle fiber cells for 5–7 days. The cells were maintained at 37 °C in a humidified incubator with 5% CO2.

2.5 Cell treatment

The water extract of *D. officinale* leaves (10 mg/mL) was added to the differentiated muscle fiber cells and HEPA cells after changing the solution to obtain a final EDL concentration in the culture medium of 0, 50, 100, or 200 μg/mL. The cells were then grown in the incubator for 24 h prior to protein extraction.

2.6 Western blotting

Cells or tissue samples were suspended in RIPA buffer (Solarbio, Beijing, China) according to the manufacturer's protocol. Proteins were separated by 10% SDS-PAGE and transferred onto PVDF membranes (EMD Millipore Corporation, Merck Life Sciences KGaA, Darmstadt, Germany). The membranes were then incubated at 4 °C overnight with various primary antibodies (anti-GSK3β, anti-AKT, anti-P-AKT, anti-P-IR (1,150), anti-GAPDH, anti-PEPCK, anti-G6P, and anti-β-tubulin), followed by the appropriate combination of secondary antibodies according to the manufacturer's protocols. Images were obtained using a FluorChem E system (ProteinSimple, Santa Clara, CA, United States).

2.7 Statistical analysis

All data were analyzed using SPSS 17.0 (IBM Corp., Chicago, IL, United States) and GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, United States) for Windows. All values in the text are expressed as the mean ± standard error of the mean. Linear mixed models analysis was used to analyze the body weight data. For other data related to bone metabolism, independent-sample t tests and one-way ANOVA were performed to compare the sham versus model groups and model versus treatment groups using pooled variance, respectively. A probability of $p < 0.05$ was considered significant.

3 Results

3.1 EDL reduces blood glucose and improves insulin sensitivity in mice fed a high-fat diet

To explore the hypoglycemic effect of EDL, 6–7-week-old male C57BL/6J mice were divided into four groups after acclimation for 1 week. The mice were fed either low-fat or high-fat diets with either sterilized drinking water or drinking water containing 5 g/L EDL (*ad libitum*) for 16 weeks (Fang et al., 2015; Cai et al., 2016). The body weight and blood glucose levels were measured throughout the course of the experiment and the data were collated. The results showed that EDL had no significant effect on the body weights of the mice fed either high-fat or low-fat diets (Figure 1A). In addition, EDL had no significant effect on the fasting blood glucose for the low-fat diet groups (Figure 1B). By contrast, for the high-fat diet groups, the fasting blood glucose was significantly lower for the mice administered EDL-containing drinking water (Figure 1C). At the 15th week, we performed glucose tolerance tests. The results showed that EDL administration had little effect on the glucose tolerance for the low-fat diet groups, whereas it significantly reduced the blood glucose 30 min after injection for the high-fat diet groups (Figure 1D). The area under the glucose tolerance curve was also measured, which revealed no significant difference upon EDL administration for the low-fat diet groups but a significant decrease upon EDL administration for the high-fat diet groups (Figure 1E). These results were consistent with those shown in Figure 1D, indicating that EDL increased the glucose

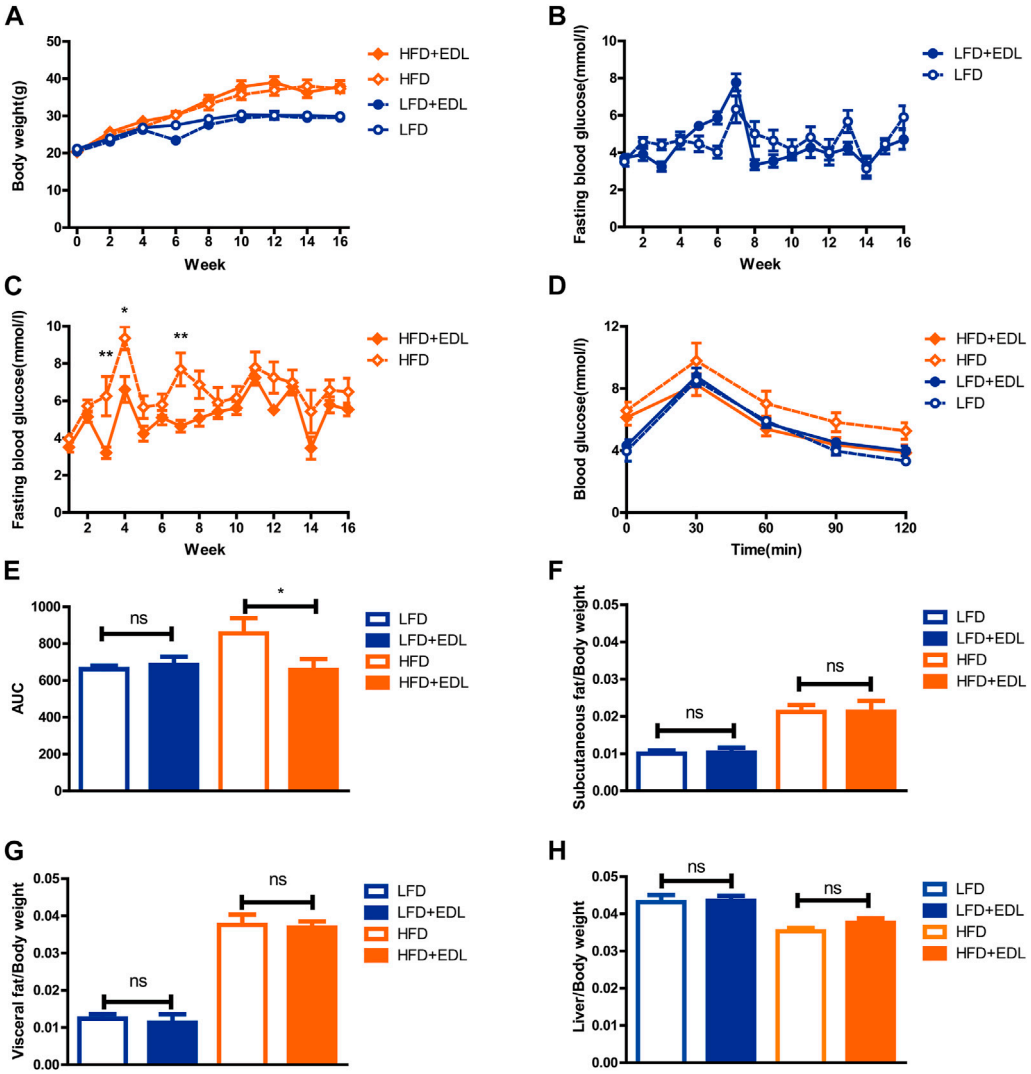

FIGURE 1
Statistical analysis of body weight, fasting blood glucose, subcutaneous fat, visceral fat, and liver tissue weights, and glucose tolerance in male C57BL/6J mice treated with water extract of *D. officinale* leaves. **(A)** Body weight. **(B)** Fasting blood glucose for the low-fat diet groups. **(C)** Fasting blood glucose for the high-fat diet groups. **(D)** Glucose tolerance. **(E)** Area under the glucose tolerance curve (AUC). **(F)** Subcutaneous fat with respect to body weight. **(G)** Visceral fat with respect to body weight. **(H)** Liver tissue with respect to body weight. Ns, not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. LFD:low-fat diet; HFD:high-fat diet; EDL:water extract of **(D)** officinale leaves.

tolerance and improved the intrinsic insulin sensitivity in mice fed a high-fat diet.

After 16 weeks, the mice were euthanized and dissected. The subcutaneous fat, visceral fat, and livers of the mice were removed and weighed. The results revealed that EDL administration did not significantly affect the relative weights of subcutaneous fat, visceral fat, and liver tissue for either the low-fat or high-fat diet groups (Figures 1F–H), in accordance with the total body weight results shown in Figure 1A. It was concluded that the addition of EDL had no significant effect on either the body weight or the relative subcutaneous fat, visceral fat, and liver tissue weights of male C57BL/6J mice for both the low-fat and high-fat diet groups, although it significantly reduced the fasting blood glucose and improved the glucose tolerance and endogenous insulin sensitivity in the mice fed a high-fat diet.

3.2 EDL regulates the expression of proteins related to glucose metabolism in the muscles of mice fed a high-fat diet

Muscle tissue is known to be an important target organ of insulin, and GSK3β is a key downstream protein of the insulin signaling pathway that plays a role in muscle glycogen synthesis (Cross et al., 1995; Welsh et al., 1996; Srivastava and Pandey, 1998). p-IR1150 is the key upstream node of the insulin signaling pathway (White et al., 1985; White et al., 1988). To examine the specific mechanism of action of EDL, the mice fed the various diets for 16 weeks were euthanized, their thigh muscles were dissected, and the muscle proteins were extracted for Western blotting. The results revealed significantly increased expression of the GSK3β and

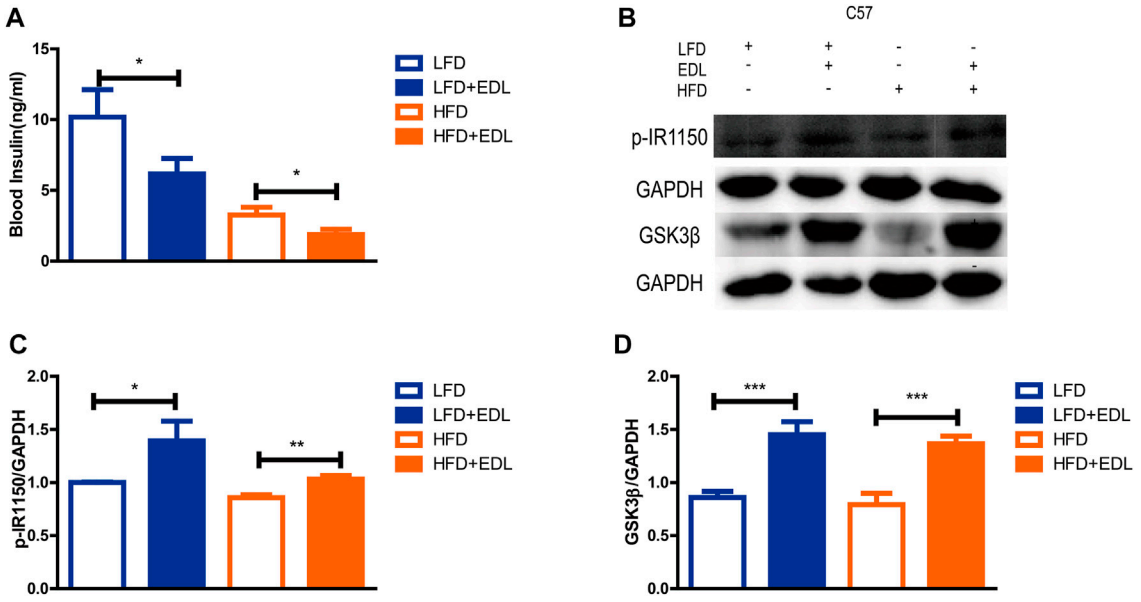

FIGURE 2
Statistical analysis of blood insulin concentration and muscle p-IR1150 and GSK3β protein contents in male C57BL/6J mice treated with water extract of *D. officinale* leaves. **(A)** Blood insulin concentration. **(B)** Western blots showing the expression of p-IR1150 and GSK3β proteins in muscle tissue. **(C)** Expression of p-IR1150 in muscle tissue. **(D)** Expression of GSK3β in muscle tissue. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

p-IR1150 proteins in the muscles of the mice treated with EDL for the both the low-fat and high-fat diet groups (Figures 2B–D), indicating activation of the insulin signaling pathway. It is worthwhile to note that mice treated with EDL exhibited significantly lower blood insulin levels (Figure 2A). These results suggest that lower insulin levels were able to mediate greater effects.

3.3 EDL regulates the expression of proteins related to glucose metabolism in C2C12 cells

The results described in the previous subsection suggested that EDL reduces fasting blood glucose levels and improves glucose tolerance in mice fed a high-fat diet by activating the insulin signaling pathway. The next step was to conduct *in vitro* experiments with myofibroblasts differentiated from C2C12 cells. We thus measured the expression and phosphorylation of AKT in myofibroblast cells after treatment with various concentrations of EDL (Cross et al., 1995; Hajduch et al., 2001). The activation level of akt is determined by the ratio of p-AKT to AKT expression. The results revealed that the activation level increased with increasing EDL concentration in a concentration-dependent manner (Figure 3C). The similar increase in GSK3β expression suggests that the insulin signaling pathway may be activated in myofibroblasts (Figures 3A,E). In addition, HEPA cells were treated with EDL. The results showed no significant changes in G6P (Au et al., 2000) or PEPCK (Rodgers et al., 2005) expression with increasing EDL concentration (Figures 3B,D,F). These results suggest that the decrease in fasting blood glucose and improved glucose tolerance in mice administered EDL were not attributable to hepatic glycogen synthesis.

3.4 ESFE may be the main contributor to reducing blood glucose

Earlier in this section, we demonstrated that EDL reduced the fasting blood glucose and improved glucose tolerance in mice fed a high-fat diet. In an effort to determine the main components of EDL responsible for these effects, we used ethanol to separate the isolated EDL into an ESFE and an EIFE. Male C57BL/6J mice (n = 40) were then divided into four groups and treated with water, EIFE, ESFE, or EDL alongside a high-fat diet while monitoring their fasting blood glucose and glucose tolerance. The results revealed that treatment with ESFE or EDL led to a reduction in the fasting blood glucose compared with the normal drinking water treatment group, whereas treatment with EIFE did not (Figure 4A). Similarly, the glucose tolerance experiments demonstrated that the blood glucose concentration decreased 30 min after injection for the mice in the EDL and ESFE groups compared with those in the control group, indicating that EDL and ESFE improved the glucose tolerance of mice fed a high-fat diet (Figure 4B). Figure 4C shows the areas under the glucose tolerance curves, which were lower for the mice in the EDL and ESFE groups, further indicating that EDL and ESFE promoted improved glucose tolerance in mice fed a high-fat diet. After 16 weeks, the subcutaneous fat, visceral fat, and liver tissue were weighed. The results showed no significant changes in the relative weight of liver tissue for the EDL and ESFE groups compared with the control (Figure 4F), whereas the relative weights of subcutaneous fat and visceral fat were significantly reduced (Figures 4D,E). In order to study whether ESFE still works under hyperglycemia, we examined the effect of ESFE on

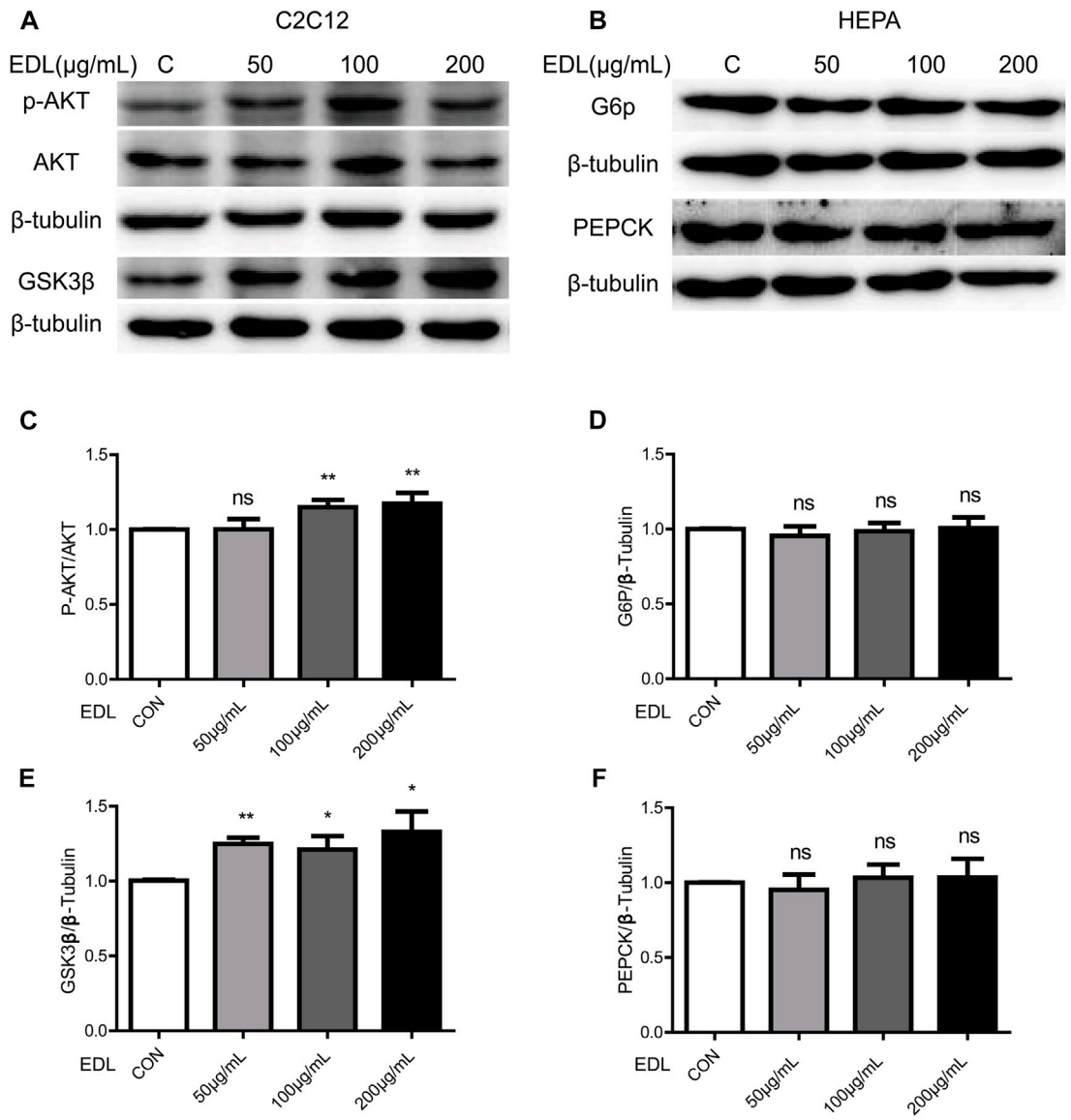

FIGURE 3
Statistical analysis of the expression of proteins related to the insulin signaling pathway and glucose metabolism in wild-type C2C12 cells and HEPA cells treated with water extract of *D. officinale* leaves. **(A)** Expression of AKT and GSK3β proteins in wild-type C2C12 cells after treatment with water extract of *D. officinale* leaves. **(B)** Expression of G6P and PEPCK proteins in wild-type HEPA cells treated with water extract of *D. officinale* leaves. **(C)** Expression of p-AKT protein in wild-type C2C12 cells after treatment with water extract of *D. officinale* leaves. **(D)** Expression of G6P protein in wild-type HEPA cells treated with water extract of *D. officinale* leaves. **(E)** Expression of GSK3β protein in wild-type C2C12 cells after treatment with water extract of *D. officinale* leaves. **(F)** Expression of PEPCK protein in wild-type HEPA cells treated with water extract of *D. officinale* leaves. Ns, not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

db/db mice, and the results revealed that ESFE inhibited the blood glucose increase with increasing body weight (Figures 4G,H). These results further confirmed that ESFE reduced blood glucose.

3.5 The main component responsible for reducing blood glucose is > 3 kDa ESFE

The results described in the previous subsection indicated that the ESFE fraction of EDL contained the main component responsible for reducing blood glucose. We considered the possibility of isolating the active species on the basis of molecular weight and thus passed the ESFE fraction through a 3 kDa ultrafiltration tube to separate the constituents into a <3 kDa ESFE and a >3 kDa ESFE. These two fractions were separately administered to Kunming mice along with glucose by gavage. The results revealed that the >3 kDa ESFE significantly decreased the blood glucose measured 30 min after administration, which was confirmed by a significant decrease in the area under the blood glucose curve (Figures 5A,B). Next, we continued to feed the mice with the two components dissolved in water and normal diet for

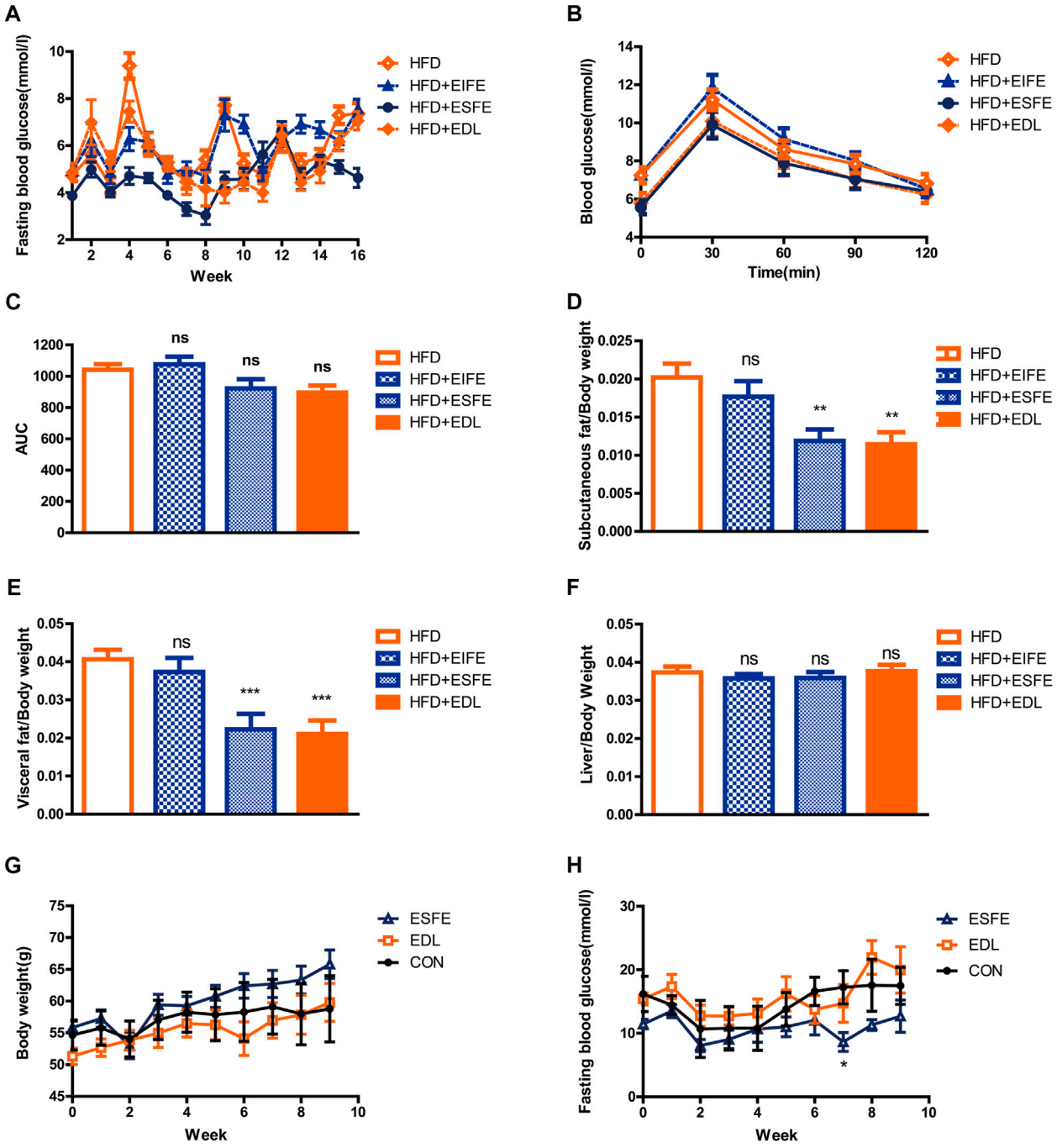

FIGURE 4
Statistical analysis of fasting blood glucose, subcutaneous fat, visceral fat, and liver tissue contents, and glucose tolerance in male C57BL/6J and db/db mice treated with EDL and its various fractions. **(A)** Fasting blood glucose in C57BL/6J mice. **(B)** Glucose tolerance in C57BL/6J mice. **(C)** Area under the glucose tolerance curve (AUC). **(D)** Subcutaneous fat with respect to body weight in C57BL/6J mice. **(E)** Visceral fat with respect to body weight in C57BL/6J mice. **(F)** Liver tissue with respect to body weight in C57BL/6J mice. **(G)** Body weight for db/db mice. **(H)** Fasting blood glucose in db/db mice. Ns, not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. ESFE: ethanol-soluble fraction of EDL; EIFE: ethanol-insoluble fraction of EDL.

2 weeks. The results confirmed that the >3 kDa ESFE significantly inhibited the increase in blood glucose in Kunming mice (Figure 5C). On the basis of these results, we conclude that the active component responsible for the hypoglycemic effect of the water extract of *D. officinale* leaves was contained mainly in the >3 kDa ESFE.

4 Discussion

At present, common hypoglycemic drugs include metformin, pioglitazone, sulfonylureas, and so on. Metformin is the primary drug recommended for the treatment of type 2 diabetes. It decreases insulin resistance and hepatic gluconeogenesis, reducing the glucose

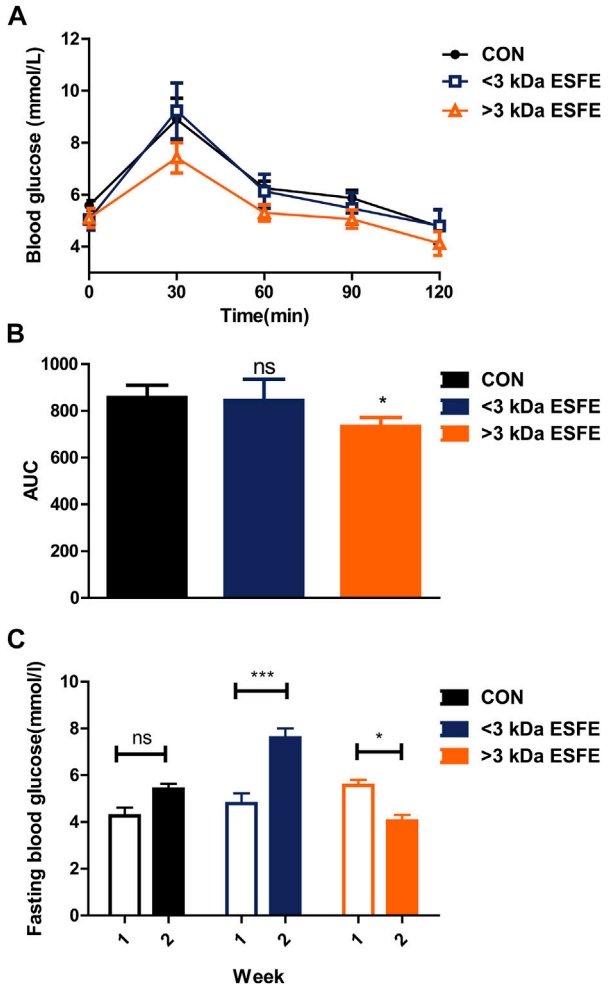

FIGURE 5
Statistical analysis of glucose tolerance and fasting blood glucose for male Kunming mice treated with the <3 kDa and >3 kDa ethanol-soluble fractions of EDL. **(A)** Oral glucose tolerance. **(B)** Area under the glucose tolerance curve (AUC). **(C)** Fasting blood glucose over 1–3 weeks ns, not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. >3 kDa ESFE: EFSE with a molecular weight of >3 kDa; <3 kDa ESFE: ESFE with a molecular weight of <3 kDa.

concentration without increasing the risk of hypoglycemia. Because metformin is excreted via the urine, a good glomerular filtration rate is needed (Inzucchi et al., 2014). Moreover, metformin should be used cautiously in patients with congestive heart failure or liver dysfunction owing to the risk of lactic acidosis (American Diabetes, 2019). Pioglitazone is an insulin sensitizer that acts at the transcription level and is characterized by good results, low cost, and no risk of hypoglycemia when used as a monotherapy. It can even be used with poor glomerular filtration rates and is safe for patients with cardiovascular disease (Schneider et al., 2008). However, pioglitazone is associated with weight gain and fluid retention and is therefore contraindicated in the case of congestive heart failure. In addition, the use of this drug in older people at risk of falling is not recommended because it has been shown to increase the risk of non-osteoporosis fractures (Viscoli et al., 2017), and it should also be avoided in patients with or at a high risk of bladder cancer (Lewis et al., 2015). Sulfonylureas stimulate insulin secretion by promoting the depolarization of β-cell membranes. They are highly effective and economical but should be used with extreme caution because of the high risk of hypoglycemia and weight gain. Although these compounds are commonly applied as hypoglycemic drugs, their use has numerous limitations and side effects, such that it is crucial to develop new hypoglycemic drugs with fewer side effects and use restrictions.

In this regard, *D. officinale* has been demonstrated to exhibit promising anti-hyperglycemia activity, such as not significantly reducing blood glucose and insulin levels in normal mice while increasing serum insulin levels and reducing serum glucagon levels in streptozotocin-induced diabetic rats. Furthermore, it has been reported to reduce serum glucose levels and increase hepatic glycogen levels in epinephrine-induced hyperglycemic mice (Wu et al., 2004). Among the varieties of *D. officinale*, *D. officinale* has the best hypoglycemic effect (Pan et al., 2014).

In traditional Chinese medicine, *D. officinale* preparations are primarily prepared using the stems of the plant, and most previous research into treating hypoglycemia with *D. officinale* has also focused on the stems. By contrast, the hypoglycemic effects of *D. officinale* leaves have received comparatively little attention. In this study, we thus selected *D. officinale* leaves as the research object to determine whether they also decrease blood glucose levels and explore the underlying mechanism. We first conducted *in vivo* experiments in a mouse model to study the potential hypoglycemic effects of EDL treatment. During 16 weeks of feeding, we observed no significant hypoglycemic effect in mice fed a low-fat diet, whereas in mice fed a high-fat diet there was a decrease in blood glucose. This is similar to the results reported in previous studies for the hypoglycemic effects of *D. officinale* stems. Glucose tolerance tests of mice fed a high-fat diet revealed that EDL significantly improved the glucose tolerance and enhanced endogenous insulin sensitivity. Analysis of the protein expression levels in muscle tissue suggested that EDL mediates this effect by activating the insulin signaling pathway, although EDL also appeared to reduce the plasma insulin levels. These findings indicate that EDL improves insulin sensitivity while reducing the plasma insulin concentration, thus making plasma insulin more effective at lower levels. *In vitro* analysis of protein expression in myofibroblasts also confirmed that EDL activated the insulin signaling pathway. Meanwhile, the results of treating HEPA cells with various concentrations of EDL indicated no effect on hepatic gluconeogenesis and hepatic glycogen synthesis. Next, we separated the components of EDL into two fractions based on their ethanol solubility, namely, the ESFE and the EIFE. Because we had confirmed that EDL displayed no significant hypoglycemic effect in normal mice, we treated mice fed a high-fat diet with EDL, ESFE, or EIFE with water as the control group. After 16 weeks of feeding, we found that treatment with ESFE or EDL reduced blood glucose levels, increased glucose tolerance, and improved insulin sensitivity compared with the control group, whereas treatment with ESFE did not. These findings indicate that the ESFE fraction contained the main active component(s) responsible for the observed effects of EDL.

By confirming ESFE fraction of EDL contained the main component responsible for reducing blood glucose, we separated ESFE fraction with the molecular weight into a <3 kDa ESFE and a >3 kDa ESFE through a 3 k Da ultrafiltration tube. Based on the two very few fractions, we chose Kunming mice for short-term experiment

to verify which fraction can decrease blood glucose. After Kunming mice for a week's rest at the laboratory, we first performed an oral glucose tolerance test to Kunming mice through mixing glucose with a <3 kDa ESFE and a >3 kDa ESFE by Intragastric administration. The results revealed that the >3 kDa ESFE significantly decreased the blood glucose measured 30 min after administration, which was confirmed the >3 kDa ESFE could improve Glucose tolerance. Next, we continued to feed the two components to the mice for 2 weeks. The results confirmed that the >3 kDa ESFE significantly inhibited the increase in blood glucose in Kunming mice. On the basis of the two experiment results, we conclude that the active component responsible for the hypoglycemic effect of EDL was contained mainly in the >3 kDa ESFE.

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 animal study was reviewed and approved by Experimental Animal Ethics Committee, Yunnan Agricultural University.

Author contributions

CF conceived and designed the experiments. ML, QL, and XH performed the experiments; ML QL, XD, YuL, and YaL analyzed the data; CF contribute dreagents/materials/analysis tools. ML, QL, and CF wrote the manuscript. 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1163028/full#supplementary-material

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Characterizations of microRNAs involved in the molecular mechanisms underlying the therapeutic effects of noni (*Morinda citrifolia* L.) fruit juice on hyperuricemia in mice

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Background: Hyperuricemia is generally defined as the high level of serum uric acid and is well known as an important risk factor for the development of various medical disorders. However, the medicinal treatment of hyperuricemia is frequently associated with multiple side-effects.

Methods: The therapeutic effect of noni (*Morinda citrifolia* L.) fruit juice on hyperuricemia and the underlying molecular mechanisms were investigated in mouse model of hyperuricemia induced by potassium oxonate using biochemical and high-throughput RNA sequencing analyses.

Results: The levels of serum uric acid (UA) and xanthine oxidase (XOD) in mice treated with noni fruit juice were significantly decreased, suggesting that the noni fruit juice could alleviate hyperuricemia by inhibiting the XOD activity and reducing the level of serum UA. The contents of both serum creatinine and blood urine nitrogen of the noni fruit juice group were significantly lower than those of the model group, suggesting that noni fruit juice promoted the excretion of UA without causing deleterious effect on the renal functions in mice. The differentially expressed microRNAs involved in the pathogenesis of hyperuricemia in mice were identified by RNA sequencing with their target genes further annotated based on both Gene Ontology and Kyoto Encyclopedia of Genes and Genomes databases to explore the metabolic pathways and molecular mechanisms underlying the therapeutic effect on hyperuricemia by noni fruit juice.

Conclusion: Our study provided strong experimental evidence to support the further investigations of the potential application of noni fruit juice in the treatment of hyperuricemia.

KEYWORDS

hyperuricemia, mice, *Morinda citrifolia* fruit juice, microRNA, xanthine oxidase, uric acid, creatinine, blood urine nitrogen

1. Introduction

Hyperuricemia in either males or females is generally defined based on the solubility of uric acid (UA), i.e., the serum UA concentration over 7.0 mg/dL or 416 mmol/L (1), which is caused by either excessive UA production (2) and/or decreased excretion in kidney and intestine, ultimately leading to excessive increase of serum UA concentration (3, 4). Furthermore, hyperuricemia is well known as an important risk factor for the development of various medical disorders, e.g., hypertension, diabetes, cardiovascular complication, metabolic syndrome, atherosclerosis, stroke, gout, and kidney diseases (5–7), even though it is recommended that the asymptomatic hyperuricemia may not be treated (8). Unfortunately, the commonly applied clinical treatments of hyperuricemia, i.e., the urate-lowering therapies, including the use of xanthine oxidase (XOD) inhibitors and other uricosuric drugs, often cause severe side effects (9). According to the American College of Rheumatology Guidelines for Management of Gout Part I in 2012, the first-line treatment strategy for lowering the content of UA is to use XOD inhibitor, i.e., allopurinol (10), while the application of allopurinol is frequently associated with multiple side-effects, including increased toxicity with low glomerular filtration rate resulting in accelerated risk of allopurinol hypersensitivity syndrome, hepatotoxicity, and Stevens-Johnson syndrome (11). Therefore, it is clinically urgent to develop effective and safe drugs in the therapeutic treatment of hyperuricemia. Recently, probiotics have been revealed with potential treatment and prevention of hyperuricemia. For example, the serum UA levels are significantly decreased in mice by the treatments of *Lactobacillus fermentum* F40-4 (12) and *Lactiplantibacillus pentosus* P2020 (13). In addition, oral delivery of nanoparticles with uricase has shown protective effects on mice with hyperuricemia (14).

Notably, plants have a long history of being widely used in traditional medicines for the treatment of various diseases (15). Medicinal plants have also played an important role in drug discovery, development, and production (16). In particular, the plants of *Morinda citrifolia* L., popularly known as noni, are one of the commonly used traditional medicinal plants discovered by Polynesian ancestors and have been used in Polynesia and almost worldwide for over 2000 years (17). Traditionally, noni has been used in the treatment of various diseases and medical disorders, including cancer, infection, cold, flu, diabetes, hypertension, arthritis, gastric ulcer, sprain, depression, senility, muscle ache, and pain (18). Furthermore, the modern pharmacological studies have revealed the anti-inflammatory, antioxidant, hepatoprotective, and immunomodulatory effects of noni (19). In our previous studies, the noni fruit juice showed significantly therapeutic effect on acute gouty arthritis, which is closely related to hyperuricemia, with a group of differentially expressed microRNAs (miRNAs) involved in the pathogenesis of acute gouty arthritis in mice identified using the high-throughput RNA sequencing (RNA-Seq) (20). Furthermore, studies have shown that the inhibitory effect of noni fruit juice on the enzymatic activities of XOD could explain the underlying mechanisms ameliorating gout and gout-like diseases (21). To date, the molecular mechanisms regulating the therapeutic effects of noni fruit juice on the treatment of hyperuricemia are unclear. Therefore, it is necessary to further investigate the biochemical and molecular associations between noni fruit juice and hyperuricemia in order to provide the experimental evidence to support the potential application of noni fruit juice in the clinical treatment of hyperuricemia.

The molecular mechanisms underlying hyperuricemia have been extensively investigated with various genetic factors and metabolic pathways involved in the occurrence and treatment of hyperuricemia identified. For example, a group of miRNAs involved in hyperuricemia have been identified (22). The miRNAs are small non-coding RNAs, with an average of 22 nucleotides in length and widely found in various organisms (23), functioning to regulate the stability of target messenger RNAs (mRNAs) by selectively binding to specific sites. With the rapid advancements in the understanding of the regulatory functions of miRNAs involved in various biological activities, it is highly expected that the targeted studies focusing on the exploration of the relationships between miRNAs and various types of human diseases would significantly enhance the identification and further development of novel therapeutic targets in the treatments of these medical disorders. For example, the increasing evidence has suggested that the development of hyperuricemia and gout is regulated at the post transcriptional level with strong involvement of miRNAs (24). Furthermore, studies have revealed abnormal expressions of miRNAs involved in hyperuricemia and the association between serum UA concentration and altered expressions of miRNAs (25). In particular, miRNAs could regulate the expression of urate transporter genes, e.g., miR-34a regulates the mRNA of the *solute carrier family 22 member 12* (*SLC22A12*) gene to ultimately repress the uric acid transporter 1 (URAT1) expression in the animal model of hyperuricemia (26). These studies strongly indicate that miRNAs could be potentially used as the therapeutic biomarkers for the clinical prevention and treatment of hyperuricemia.

The objective of this study was to investigate the effect of noni fruit juice on the alleviation of hyperuricemia and the underlying molecular mechanisms in mouse model of hyperuricemia induced by the uricase inhibitor potassium oxonate (PO) using biochemical and high-throughput sequencing analyses. The levels of a group of four hyperuricemia related biochemical factors, i.e., serum UA, XOD, creatinine (Cr), and blood urine nitrogen (BUN), were detected in the mouse model of hyperuricemia treated with either noni fruit juice or allopurinol. The RNA-Seq was used to identify the differentially expressed miRNAs involved in the pharmacological regulation of hyperuricemia by noni fruit juice. The target genes of these differentially expressed miRNAs were further annotated using the both Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases to explore the metabolic pathways and molecular mechanisms underlying the therapeutic effect on hyperuricemia by noni fruit juice. This study strongly demonstrated the regulatory functions of miRNAs in the treatment of hyperuricemia in mice by noni fruit juice, providing strong experimental evidence to support the further investigations of the biochemical and molecular associations between noni fruit juice and hyperuricemia as well as the potential application of noni fruit juice in the clinical treatment of hyperuricemia.

2. Materials and methods

2.1. Production of noni fruit juice

Fresh and mature noni fruits were purchased from the Fiji Pacific Noni Biotechnology Co., Ltd. (Hainan, China). These fruit materials were locally collected (Nadi, Fiji) and kept frozen at −20°C until further use. The frozen fruits were processed as previously reported

(20). Briefly, the frozen fruits were first thawed at room temperature and then rinsed using sterile water. After being air dried, the fruits were cut into small pieces each of ~5 mm in thickness and crushed by a fruit crusher to make the fruit pulp, which was heated to ~25°C, added with pectinase (0.225 g/L) under constant stirring, and then inoculated with *Lactobacillus plantarum* (1%) to incubate for 4 h in an incubator (Shanghai Yiheng Scientific Instrument Co., Ltd., Shanghai, China) with constant temperature at 40°C. Then, the 20-mesh filter cloth was used to filter the fruit pulp, which was fermented for 20–30 d at 38°C. Finally, the supernatant was collected with a siphon, filtered, sterilized for 20 min at 80–82°C, and aseptically collected as the fermented noni fruit juice used in this study.

2.2. Animal treatments and induction of hyperuricemia in mice

A total of 60 male Kunming mice with an average body weight of 20 ± 2 g and the animal certificate number of SCXK (Liao)-2015–0001 were purchased from Liaoning Changsheng Biotechnology Co., Ltd., Benxi, China. The selection of male animals was based on the general knowledge that the male mice were prone to developing hyperuricemia than female mice. During the week of adaptive feeding, these 60 mice were randomly and evenly separated into six treatment groups as follows: the normal control group contained the mice treated with regular food and water, the model group treated with uricase inhibitor PO, the positive control group treated with both PO (250 mg kg^{-1}; MedChemExpress, Shanghai, China) and allopurinol (5 mg kg^{-1}; Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China), and three noni fruit juice groups treated with both PO and noni fruit juice of 3.3 mL kg^{-1} d^{-1} (low-dose), 6.6 mL kg^{-1} d^{-1} (medium-dose), and 13.2 mL kg^{-1} d^{-1} (high-dose), respectively. The selection of these three dose levels was based on the results of our pre-experiments showing significant variations in different groups of mice. The mice were treated in compliance with the principles of laboratory animal care and the guide for the care and use of laboratory animals approved by the Ethics Committee of Jilin University (approval # 2018SY0602). All mice were caged and fed *ad libitum*, under natural sunlight and constant temperature and humidity, and were regularly cleaned and disinfected. The mice in the noni fruit juice group with the optimal effects on hyperuricemia related biochemical factors were selected for further RNA-Seq analysis.

To make the mouse model of hyperuricemia, the treatment with PO was used to induce hyperuricemia in mice based on the previous study (27). Except for the normal control group, each mouse in other five treatment groups was orally administered with PO at 8 am for 7 d to induce hyperuricemia. In 1 h, the mice in the noni fruit juice and the allopurinol groups were orally given noni fruit juice and allopurinol, respectively, for 7 d. The successful establishment of mouse model of hyperuricemia was determined by the significantly elevated level of serum UA ($p < 0.0001$) compared with the normal control group. On day 7, 1 h after the treatments, the mice were euthanized by inhalation of carbon dioxide gas.

2.3. Biochemical and statistical analyses

The whole blood sample was collected from each mouse 1 h after the 7th administration of required treatments, coagulated at room temperature for about 1 h, and then centrifuged for 5 min at 3000 rpm/min to obtain the serum. The contents of serum UA (1), XOD (9), Cr (28), and BUN (29) in the serum of each mouse were measured by the specific detection kits purchased from Nanjing Jiancheng Biotechnology Co., Ltd. (Nanjing, China) according to the manufacturer's instructions.

All data were expressed as the mean ± standard error of the mean. All statistical analyses were performed using the one-way analysis of variance (ANOVA) to determine the level of significance based on $p < 0.05$.

2.4. High-throughput RNA sequencing and microRNA analyses

In order to further explore the molecular mechanisms regulating the effect of noni fruit juice and allopurinol on reducing the level of serum UA in mice with hyperuricemia, each representative serum sample was randomly selected for RNA sequencing. A total of 200 µL serum sample were added with 1 mL QIAzol Lysis Reagent (QIAGEN, Germany) in the sample tube, vortexed, and incubated at room temperature for 5 min. Then, the mixed sample was added with a total of 200 µL chloroform/isoamyl alcohol (24,1; v:v) solution, vortexed for 15 s, incubated at room temperature for 3 min, and then centrifuged for 8 min at 12,000 × *g* and 4°C. The supernatant was absorbed and added with anhydrous ethanol twice the volume of the supernatant, mixed well, and purified through the column. The sample was washed once with 700 µL buffer RWT and twice with 500 µL buffer RPE, and centrifuged for 2 min at room temperature and 12,000 × *g*, with the purification column moved to a new collection tube and added with 20 µL RNA-free water, incubated at room temperature for 1 min, and centrifuged for 2 min at room temperature and 12,000 × *g* to elute RNA, which was detected with Agilent 2100 Bioanalyzer (Agilent Technologies Co., Ltd., Beijing, China). Nanodrop was used to detect the salt ion pollution in the RNA samples.

The small RNA library was constructed as previously reported (20). The RNA-Seq was performed by combinatorial Probe-Anchor Synthesis (cPAs) to obtain the miRNAs by next generation sequencing platform BGISEQ-500 (BGI, Shenzhen, China) (30, 31). The raw data were filtered to remove tags of low quality, with 5′ primer contaminant, without 3′ primer, without insertion, with poly A, and shorter than 18 nt, to obtain the clean tags, which were mapped to the reference genome and the miRbase database (22nd edition) using Bowtie2 (32) and mapped to Rfam using the function "cmsearch" (33). Novel miRNAs were predicted using miRDeep2 (34). The expression levels of miRNAs were calculated by the transcripts per million kilobase transcript (TPM) method (35), which eliminated the effects of sequencing variations. The TPM method was performed using the following formula: $TPM = (C*10^6)/N$, where C represented the count of miRNAs in a sample and N the total number of reads mapped to the reference genome. Based on the detection method of differentially expressed genes (DEGs) as previously reported (36), a strict in-house algorithm was developed to screen the DEGs of miRNAs between two samples with the multiple hypothesis test corrections set up (37) to determine the domain value of p by controlling the false discovery rate (FDR) (38).

Based on the default parameters, both MiRanda (39) and TargetScan (40) were used to predict the target genes of the differentially expressed miRNAs. The functional annotation and

enrichment analyses of the target genes were further performed based on Gene Ontology (GO)[1] and Kyoto Encyclopedia of Genes and Genomes (KEGG)[2] databases, respectively. GO database is an international standardized gene function classification system, providing a set of dynamically updated standard vocabulary to comprehensively describe the attributes of genes and gene products in organisms, categorized into three groups of genes, i.e., molecular function, cellular component, and biological process, respectively. GO annotation was performed to identify the functional GO terms that were significantly enriched in the target genes corresponding to the differentially expressed miRNAs compared with the genomic background, ultimately detecting the biological functions that were significantly related to the target genes corresponding to the differentially expressed miRNAs. First, all target genes corresponding to differentially expressed miRNAs were directed to the GO database to calculate the number of genes of each GO term, and then the hypergeometric test was performed to identify the GO entries that were significantly enriched in the target genes corresponding to the differentially expressed miRNAs compared with the whole genome background. Based on GO::Termfinder[3], a strict in-house algorithm was developed to perform the GO annotation analysis with the calculated p-value corrected by Bonferroni (37). The corrected value of $p \leq 0.05$ was used as the threshold and the GO term meeting this condition was defined as the GO term significantly enriched in the target gene corresponding to the differentially expressed miRNAs. The enrichment analysis of metabolic pathways based on KEGG database was performed to investigate the biological function of targe genes of the differentially expressed miRNAs (41) and to identify the metabolic pathways that were significantly enriched by the target genes of differentially expressed miRNAs compared with the whole genome background based on Q-value ≤ 0.05.

3. Results

3.1. Effects of noni fruit juice on the levels of serum uric acid, xanthine oxidase, creatinine, and blood urine nitrogen in mice

Compared with the normal control group, the serum UA level of mice in the model group was significantly increased with the treatment of PO ($p < 0.0001$), indicating that the mouse model of hyperuricemia was successfully established (Figure 1A). Compared with the model group, the levels of serum UA in the allopurinol group (i.e., the positive control; $p < 0.0001$) and the noni fruit juice groups were significantly decreased ($p < 0.05$, $p < 0.001$, and $p < 0.0001$ for low-, medium-, and high-dose of noni fruit juice groups, respectively). These results revealed the therapeutic effect of noni fruit juice on hyperuricemia in mice in a dose-dependent manner. Compared with the normal control group, the content of XOD in mice of the model group was significantly increased with the treatment of PO ($p < 0.0001$; Figure 1B). Compared with the model group, the contents of XOD in both allopurinol ($p < 0.0001$) and noni fruit juice groups ($p < 0.01$, $p < 0.001$, and $p < 0.0001$ for low-, medium-, and high-dose noni fruit juice groups, respectively) were significantly reduced. These results showed that noni fruit juice could effectively reduce the enzymatic activity of XOD with curative effect on inhibiting the UA synthesis. To evaluate the changes in renal function of mice treated with noni fruit juice, the contents of serum Cr and BUN were measured in mice of hyperuricemia. The results showed that noni fruit juice could effectively alleviate the renal injury in mice of hyperuricemia. Compared with the normal control group, the content of serum Cr in the model group was significantly increased ($p < 0.0001$), whereas the contents of serum Cr in mice of both the allopurinol ($p < 0.0001$) and the noni fruit juice groups ($p < 0.01$ and $p < 0.001$ for medium- and high-dose noni fruit juice groups, respectively) were significantly lower than that of the model group (Figure 1C), though no significant difference was revealed in the Cr content between the low-dose noni fruit juice group and the model group of mice. Similar patterns were revealed in the changes of the contents of BUN (Figure 1D), i.e., compared with the normal control group, the contents of BUN in mice of the model group were significantly increased ($p < 0.0001$), whereas the contents of BUN in mice of both the allopurinol ($p < 0.0001$) and noni fruit juice groups ($p < 0.05$, $p < 0.001$, and $p < 0.0001$ for low-, medium-, and high-dose noni fruit juice groups, respectively) were significantly lower than that of the model group. These results suggested that the noni fruit juice did not cause deleterious impact on the renal function in mice with hyperuricemia. Due to its optimal effects on these biochemical indices, the mice in the high-dose noni fruit juice group were used for further RNA-Seq analysis.

3.2. RNA sequencing

3.2.1. Statistics of microRNAs

In order to further explore the molecular mechanisms regulating the effect of noni fruit juice on the contents of hyperuricemia related biochemical indices in mice of hyperuricemia, the miRNAs of different groups of mice were sequenced using the BGISEQ-500 sequencing technology. The raw sequencing data were deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI)[4] database under the BioProjects PRJNA910471 and PRJNA719968. The raw data were filtered to obtain the clean tags, which were annotated using miRBase and Rfam databases to identify the known miRNAs, with the remaining unknown miRNAs used to predict novel miRNAs (Table 1 and Supplementary Table S1).

3.2.2. Differentially expressed microRNAs

Differential expression analysis of miRNAs was performed using Expdiff with the PossionDis method based on FDR ≤ 0.001 and the absolute value of Log2Ratio(Fold Change) ≥ 1 as the default thresholds to determine the significance of expression variation and identify the differentially expressed microRNAs between samples (Figure 2 and Supplementary Table S2). The results showed that the largest numbers

1 http://geneontology.org/; accessed on 15 March 2022.

2 https://www.genome.jp/kegg/; accessed on 15 March 2022.

3 http://www.yeastgenome.org/help/analyze/go-term-finder/; accessed on 15 March 2022.

4 http://www.ncbi.nlm.nih.gov/sra/; accessed on 09 December 2022.

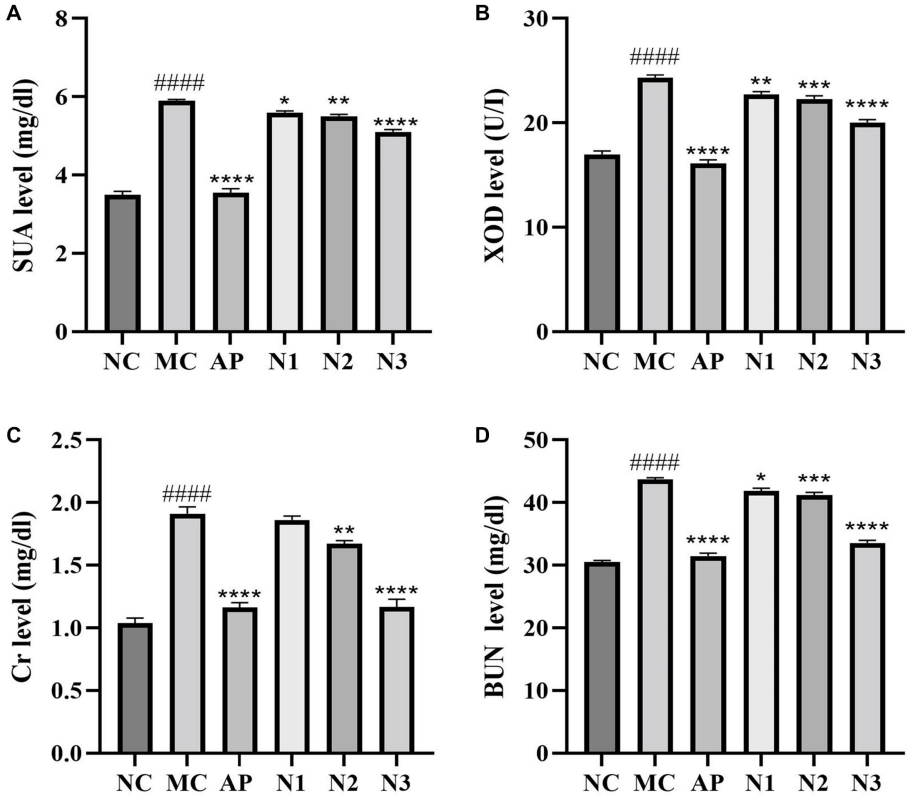

FIGURE 1
Effects of noni fruit juice on the contents of **(A)** serum uric acid (SUA), **(B)** xanthine oxidase (XOD), **(C)** creatinine (Cr), and **(D)** blood urine nitrogen (BUN) in six groups of mice, i.e., the normal control (NC), the model (MC), the positive control treated with allopurinol (AP), and low-dose (N1), medium-dose (N2), and high-dose (N3) noni fruit juice groups. Significant differences are determined based on $p<0.0001$ (####) compared with the normal control group and $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), and $p<0.0001$ (****), compared with the model group, respectively.

TABLE 1 Summary of microRNA sequencing data obtained from four groups of mice based on the BGISEQ-500 sequencing technology.

Sample	Raw tag	Clean tag (%)	Mapped tag (%)	Known miRNA	Novel miRNA
Normal control group	29,268,292	23,957,783 (81.86)	21,749,074 (90.78)	271	2,146
Model group	33,333,333	24,026,953 (72.08)	21,562,608 (89.74)	248	1,887
Noni fruit juice group	28,679,191	12,187,451 (42.5)	11,301,855 (92.73)	201	4,289
Allopurinol group	28,571,428	23,892,444 (83.62)	20,602,381 (86.23)	163	2,134

The percentage of clean tag is calculated as (clean tag counts/raw tag counts) × 100%. The percentage of mapped tag is calculated as (mapped tag counts/clean tag counts) × 100%.

of differentially expressed miRNAs were detected in the noni fruit juice group compared with the other three groups of mice, i.e., a total of 3,280 and 3,184 up-regulated miRNAs were revealed in the pairwise comparisons of the normal control group vs. noni fruit juice group and the model group vs. noni fruit juice group, respectively, while a total of 3,333 down-regulated miRNAs were detected in the pairwise comparison of the noni fruit juice group vs. allopurinol group. These results evidently revealed the significant effect of noni fruit juice on mice with hyperuricemia. Further studies are needed to explore the explicit functions of these differentially expressed miRNAs in the development and treatment of hyperuricemia in mice.

3.2.3. Prediction of the target genes of the differentially expressed microRNAs

To further determine the genes involved in the molecular mechanisms regulating the effect of noni fruit juice on the contents of hyperuricemia related biochemical indices in mice, the target genes of differentially expressed miRNAs were predicted using both TargetScan and miRanda (Figure 3 and Supplementary Table S3). A total of 13,912, 13,942, 13,921, 13,950, 13,917, and 13,944 target genes were detected in the six pairwise comparisons of four groups of mice, i.e., the normal control vs. model groups, the normal control vs. noni fruit juice groups, the normal control vs. allopurinol groups, the model group vs. the noni fruit juice groups, the model vs. allopurinol groups, and the allopurinol vs. the noni fruit juice groups (Supplementary Tables S4–S9), respectively.

3.2.4. GO functional annotation of target genes of differentially expressed microRNAs

In order to further explore the effect of noni fruit juice on hyperuricemia in mice at the genomic level, the target genes of the differentially expressed miRNAs were further annotated based on

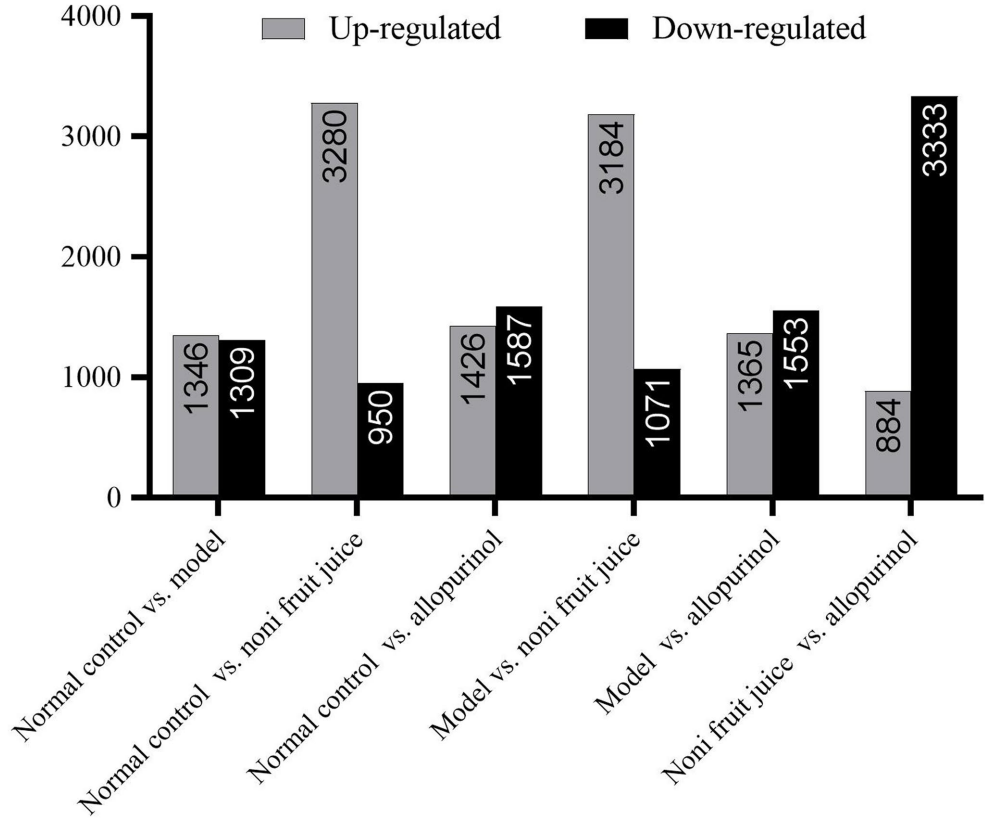

FIGURE 2
Summary of the differentially expressed microRNAs (up-regulated and down-regulated) identified in six pairwise comparisons of the four groups of mice, i.e., the normal control, the model, the noni fruit juice, and the allopurinol groups.

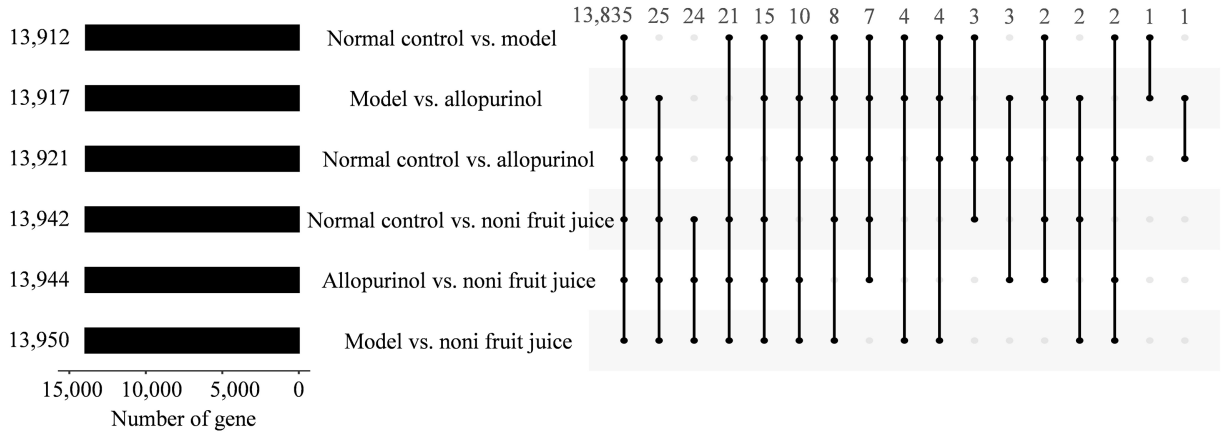

FIGURE 3
Target genes of differentially expressed microRNAs predicted by both TargetScan and miRanda in the six pairwise comparisons of the four groups of mice, i.e., the normal control, the model, the allopurinol, and the noni fruit juice groups. Numbers on the left indicate the total number of genes identified in the six pairwise comparisons; numbers on the top indicate the number of genes shared (i.e., intersection values) among the pairwise comparisons indicated by dots and lines.

the GO database (Figure 4). The results showed that the most target genes of the differentially expressed miRNA were annotated in the category of biological process, followed by the categories of cellular component and molecular function, of the GO database. A total of 26 GO functional groups were revealed in the category of biological processes, e.g., the GO terms in cellular process, single-organism process, and metabolic process. In the category of cellular component, a total of 19 GO terms were annotated, with most target genes annotated in the GO terms of cell and cell part, followed by organelle. The category of molecular mechanism contained a total

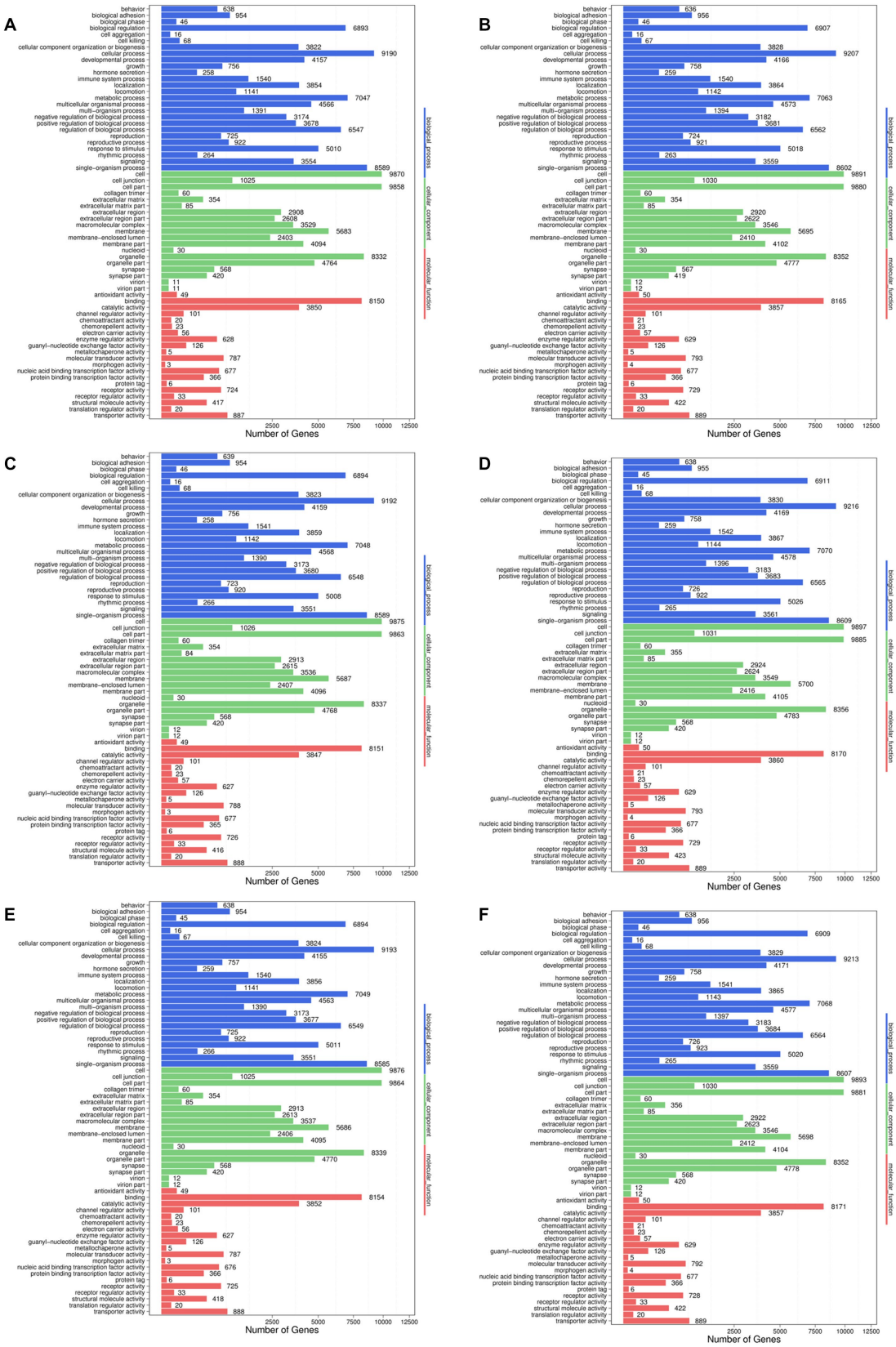

FIGURE 4
Functional annotations of the target genes of differentially expressed microRNAs based on the Gene Ontology (GO) database identified in the pairwise comparisons of the four groups of mice, i.e., the normal control group vs. model group **(A)**, the normal control group vs. noni fruit juice group **(B)**, the normal control group vs. allopurinol group **(C)**, the model group vs. noni fruit juice group **(D)**, the model group vs. allopurinol group **(E)**, and the allopurinol group vs. noni fruit juice group **(F)**. The GO terms are grouped in to three categories, i.e., biological process, cellular components, and molecular function.

TABLE 2 Target genes and the expression patterns of the differentially expressed microRNAs involved in the metabolism of uric acid based on Gene Ontology (GO) annotation in the six pairwise comparisons among the four groups of mice, i.e., the normal control, the model, the allopurinol, and the noni fruit juice groups.

GO term and gene annotated	Control vs. model	Control vs. noni fruit juice	Control vs. allopurinol	Model vs. noni fruit juice	Model vs. allopurinol	Allopurinol vs. noni fruit juice
Urate biosynthetic process (GO:0034418)						
NM_021463.4 (Prps1)	(2↑,2↓)/(30↑,26↓)	(2↑,5↓)/(80↑,14↓)	(5↑,1↓)/(31↑,31↓)	(0↑,3↓)/(76↑,19↓)	(5↑,1↓)/(29↑,36↓)	(1↑,6↓)/(77↑,14↓)
NM_013632.4 (Pnp)	(0↑,1↓)/(31↑,26↓)	(1↑,2↓)/(77↑,15↓)	(0↑,2↓)/(32↑,33↓)	(1↑,1↓)/(75↑,20↓)	(0↑,1↓)/(33↑,38↓)	(2↑,0↓)/(81↑,16↓)
NM_001164370.1 (Mipoll)	(2↑,1↓)/(20↑,24↓)	(1↑,4↓)/(61↑,15↓)	(1↑,1↓)/(26↑,32↓)	(1↑,3↓)/(63↑,14↓)	(1↑,2↓)/(27↑,30↓)	(1↑,4↓)/(63↑,16↓)
Urate metabolic process (GO:0046415)						
XM_006503685.3 (predicted: facilitated glucose transporter, Slc2a9, transcript variant X3)	(3↑,5↓)/(129↑,127↓)	(3↑,10↓)/(343↑,66↓)	(3↑,6↓)/(129↑,152↓)	(3↑,7↓)/(326↑,73↓)	(2↑,2↓)/(128↑,146↓)	(3↑,6↓)/(352↑,69↓)
XM_006503881.3 (predicted: Gckr, transcript variant X1)	(0↑,0↓)/(13↑,5↓)	(0↑,0↓)/(27↑,4↓)	(0↑,0↓)/(9↑,9↓)	(0↑,0↓)/(25↑,7↓)	(0↑,0↓)/(10↑,15↓)	(0↑,0↓)/(24↑,3↓)
NM_009198.3 (sodium phosphate, Slc7a1, transcript variant 1)	(0↑,1↓)/(7↑,12↓)	(0↑,1↓)/(26↑,7↓)	(1↑,1↓)/(15↑,13↓)	(0↑,0↓)/(29↑,7↓)	(1↑,0↓)/(14↑,8↓)	(0↑,2↓)/(25↑,7↓)
NM_013632.4 (Pnp)	(0↑,1↓)/(31↑,26↓)	(1↑,2↓)/(77↑,15↓)	(0↑,2↓)/(32↑,33↓)	(1↑,1↓)/(75↑,20↓)	(0↑,1↓)/(33↑,38↓)	(2↑,0↓)/(81↑,16↓)
NM_009203.3 (organic anion/ cation transporter, Slc22a12)	(0↑,0↓)/(2↑,2↓)	(0↑,0↓)/(4↑,2↓)	(0↑,0↓)/(0↑,4↓)	(0↑,0↓)/(3↑,3↓)	(0↑,0↓)/(0↑,2↓)	(0↑,0↓)/(4↑,2↓)
NM_021463.4 (Prps1)	(2↑,2↓)/(30↑,26↓)	(2↑,5↓)/(80↑,14↓)	(5↑,1↓)/(31↑,31↓)	(0↑,3↓)/(75↑,19↓)	(5↑,1↓)/(29↑,36↓)	(1↑,6↓)/(77↑,14↓)
NM_134069.3 (sodium phosphate, Slc7a3, transcript variant 1)	(2↑,0↓)/(17↑,21↓)	(0↑,2↓)/(59↑,6↓)	(2↑,0↓)/(19↑,25↓)	(0↑,4↓)/(62↑,7↓)	(1↑,1↓)/(15↑,20↓)	(0↑,4↓)/(64↑,5↓)
NM_025807.3 (monocarboxylic acid transporters, Slc16a9)	(3↑,2↓)/(48↑,59↓)	(0↑,4↓)/(136↑,53↓)	(4↑,2↓)/(63↑,83↓)	(0↑,5↓)/(137↑,42↓)	(3↑,1↓)/(66↑,64↓)	(0↑,5↓)/(143↑,38↓)
NM_001164370.1 (Mipoll)	(2↑,1↓)/(20↑,24↓)	(1↑,4↓)/(61↑,15↓)	(1↑,1↓)/(26↑,32↓)	(1↑,3↓)/(63↑,14↓)	(1↑,2↓)/(27↑,30↓)	(1↑,4↓)/(63↑,16↓)
XM_006506148.3 (predicted: TED:ATP-binding cassette, WHITE, Abcg2, transcript variate X1)	(1↑,0↓)/(8↑,21↓)	(0↑,1↓)/(42↑,12↓)	(0↑,1↓)/(18↑,26↓)	(0↑,2↓)/(43↑,5↓)	(0↑,1↓)/(19↑,17↓)	(0↑,1↓)/(46↑,9↓)
NM_008061.4 (glucose-6-phosphatase, G6pc)	(2↑,4↓)/(77↑,80↓)	(2↑,6↓)/(186↑,51↓)	(2↑,6↓)/(64↑,101↓)	(2↑,4↓)/(183↑,52↓)	(2↑,3↓)/(62↑,92↓)	(3↑,2↓)/(210↑,40↓)
Urate transport (GO:0015747)						
NM_134069.3 (sodium phosphate, transcript variant 1)	(2↑,0↓)/(17↑,21↓)	(0↑,2↓)/(59↑,6↓)	(2↑,0↓)/(19↑,25↓)	(0↑,4↓)/(62↑,7↓)	(1↑,1↓)/(15↑,20↓)	(0↑,4↓)/(64↑,5↓)
NM_009203.3 (organic anion/ cation, transporter, Slc22a12)	(0↑,0↓)/(2↑,2↓)	(0↑,0↓)/(4↑,2↓)	(0↑,0↓)/(0↑,4↓)	(0↑,0↓)/(3↑,3↓)	(0↑,0↓)/(0↑,2↓)	(0↑,0↓)/(4↑,2↓)
XM_006506148.3 (predicted: TED:ATP-binding cassette, WHITE, Abcg2, transcript variant X1)	(1↑,0↓)/(8↑,21↓)	(0↑,1↓)/(42↑,12↓)	(0↑,1↓)/(18↑,26↓)	(0↑,2↓)/(43↑,5↓)	(0↑,1↓)/(19↑,17↓)	(0↑,1↓)/(46↑,9↓)

Data are presented as number of (known)/(novel) microRNAs. Symbols "↑" and "↓" indicate up-regulation and down-regulation, respectively.

of 20 GO functional groups, with the most target genes annotated in the GO term of binding, followed by catalytic activity. Subsequently, these most annotated GO functional groups were further explored to investigate the molecular mechanisms underlying the effect of noni fruit juice on lowering the content of serum UA in mice with hyperuricemia.

It is commonly known that UA is the final product of the purine metabolism in human, while hyperuricemia is mainly caused by either excessive UA produced by purine degradation pathway or insufficient excretion of UA by interfering with the UA uptake transporters and secretory transporters. Therefore, as one of the main pathways involved in the pathology of hyperuricemia, the urate biosynthetic process, urate metabolic process, and urate transport were further characterized based on GO annotation of target genes of differentially expressed miRNAs identified in six pairwise comparisons of the four groups of mice (Table 2 and Supplementary Table S10). The results

showed that in the biological process category of GO database, many up-regulated and down-regulated miRNAs were involved in the positive and negative regulations of the metabolic pathways of urate biosynthetic process, urate metabolic process, and urate transport. These results strongly indicated that the urate biosynthetic process, urate metabolic process, and urate transport pathways were involved in the regulatory function of noni fruit juice in mice with hyperuricemia, while these target genes of the differentially expressed miRNAs annotated in these GO terms were probably involved in the urate biosynthetic process, urate metabolic process, and urate transport. The known and novel miRNAs involved in the urate biosynthetic process, urate metabolic process, and urate transport were summarized in the pairwise comparisons of the four groups of mice (Table 3 and Supplementary Table S10). Further studies are needed to investigate the explicit regulatory functions of these miRNAs in the occurrence of hyperuricemia and the molecular mechanisms regulating the alleviation effect of noni fruit juice on hyperuricemia in mice.

3.2.5. KEGG metabolic pathway enrichment analysis of the target genes of differentially expressed microRNAs

Generally, different genes coordinate with each other to perform their biological functions. In order to comprehensively explore the explicit functions of the target genes of the differentially expressed miRNAs in mice with hyperuricemia, the enrichment analyses of the target genes of the differentially expressed miRNAs in the six pairwise comparisons of the four groups of mice were performed based on the KEGG database to determine the main intracellular signal transduction and metabolic pathways involved in the occurrence of hyperuricemia. The results showed that the target genes of the differentially expressed miRNAs were enriched in six categories of metabolic pathways in the KEGG database, including Cellular Processes, Environmental Information Processing, Genetic Information Processing, Human Diseases, Metabolism, and Organismal Systems (Figure 5). The target genes were highly enriched in the pathways of the Environmental Information Processing, e.g., the Signal transduction, while the "Cancers: Overview" and "Infectious diseases: Viral" were the top two highly enriched pathways in Human Diseases. In the category of Metabolism, the target genes were highly enriched in Global and overview maps. The target genes were highly enriched in both Immune system and Endocrine system of the Organismal Systems. The target genes were relatively less enriched in both categories of Cellular Process and Genetic Information Processing. Among the top 20 most enriched pathways, the target genes of the differentially expressed miRNAs were enriched the most significantly in the metabolic pathways (Figure 6). Subsequently, the regulatory mechanisms of noni fruit juice involved in mice with hyperuricemia were further investigated based on these most enriched metabolic pathways. Future studies are needed to clarify the functions of the target genes enriched in the metabolic pathway involved in the occurrence of hyperuricemia and the role of noni fruit juice in attenuating hyperuricemia in mice.

Because the UA was the final product of the purine metabolism (with XOD playing the rate-limiting role in the terminal step of purine metabolism that converted xanthine to UA) and the results of our biochemical analysis revealed the decreased contents of both serum UA and XOD in mice with hyperuricemia treated with either noni fruit juice or allopurinol, the xanthine dehydrogenase/oxygenase (KEGG orthology K00106) in the KEGG pathway of purine metabolism (KEGG pathway map00230) was further investigated to characterize the genes and differentially expressed miRNAs related to XOD in order to explore the molecular mechanisms regulating the decreased levels of XOD in mice with hyperuricemia (Table 4 and Supplementary Table S11). The results showed that a large number of known and novel miRNAs were involved in the molecular regulation of XOD in the purine metabolism (Table 5 and Supplementary Table S11). Further studies are needed to verify the findings revealed in this study, i.e., the miRNAs regulated the production of XOD and ultimately the decreased level of serum UA in mice with hyperuricemia and the noni fruit juice regulated XOD to play its therapeutic role in the treatment of mice with hyperuricemia.

4. Discussion

In this study, both biochemical and next generation high-throughput RNA-Seq analyses were performed to explore the effect of noni fruit juice on hyperuricemia in mice. The results of biochemical analysis showed that the noni fruit juice could significantly reduce the contents of serum UA and XOD in mouse model of hyperuricemia induced by PO. The RNA-Seq analysis showed that the decrease of the serum UA and XOD levels was probably caused by the inhibited expression of XOD by noni fruit juice (below). The molecular and pharmacological mechanisms underlying the effect of noni fruit juice on hyperuricemia in mice were further investigated based on GO annotation and KEGG enrichment analyses of the target genes of differentially expressed miRNAs.

4.1. Variations in the levels of hyperuricemia related biochemical factors caused by the treatment of noni fruit juice

In recent years, studies on hyperuricemia have attracted increasing attention worldwide due to its elevated incidence rate in the populations of young people. However, the effective medicines used in the clinical treatment of hyperuricemia to decrease the blood UA are sparse (42). Therefore, it is urgent to develop novel drugs to reduce the level of blood UA. To date, several animal models of hyperuricemia have been established to develop new drugs for the treatment of hypouricemia. Among these models, PO has been widely used to induce high UA in rodents due to its capability of preventing uricase from degrading UA to generate allantoin (43) as well as its advantages of fast operation and low cost (44). Therefore, the PO was used to induce hyperuricemia in mice in this study. Furthermore, the concentration of serum UA is an important parameter of human health, while hyperuricemia is generally diagnosed based on the abnormally high level of blood UA (45). Therefore, the change of serum UA homeostasis is closely related to hyperuricemia. Our results showed that in 7 days, the serum UA level in mice of the model group treated with PO was significantly higher than that in the normal control group. These results were consistent with those previously reported (27), indicating that the mouse model of hyperuricemia induced by PO

TABLE 3 Expression patterns of target genes and the known differentially expressed microRNAs involved in uric acid production based on Gene Ontology (GO) annotation in the six pairwise comparisons among the four groups of mice, i.e., the normal control, the model, the allopurinol, and the noni fruit juice groups.

GO term and gene annotated	Control vs. model	Control vs. noni fruit juice	Control vs. allopurinol	Model vs. noni fruit juice	Model vs. allopurinol	Allopurinol vs. noni fruit juice
Urate biosynthetic process (GO:0034418)						
NM_021463.4 (Prps1) ↑	let-7e-5p; miR-181b-5p	let-7e-5p; miR-181b-5p	let-7e-5p; miR-33-5p; let-7a-5p; let-7c-5p; let-7b-5p	—	miR-33-5p; let-7e-5p; let-7c-5p; let-7a-5p; let-7b-5p	miR-181b-5p
NM_021463.4 (Prps1) ↓	miR-124-3p; miR-33-5p	miR-124-3p; miR-33-5p; let-7d-5p; let-7c-5p; let-7b-5p	miR-124-3p	let-7d-5p; let-7c-5p; let-7b-5p	miR-181b-5p	miR-33-5p; let-7c-5p; let-7d-5p; let-7a-5p; let-7b-5p; let-7e-5p
NM_013632.4 (PnP) ↑	—	miR-214-3p	—	miR-214-3p	—	miR-140-3p; miR-214-3p
NM_013632.4 (Pnp) ↓	miR-7051-5p	miR-7051-5p; miR-140-3p	miR-140-3p; miR-7051-5p	miR-140-3p	miR-140-3p	—
NM_001164370.1 (Mipoll) ↑	miR-429-3p; miR-205-5p	miR-199a-5p	miR-485-5p	miR-199a-5p	miR-485-5p	miR-199a-5p
NM_001164370.1 (Mipoll) ↓	miR-485-5p	miR-532-5p; miR-429-3p; miR-485-5p; miR-205-5p	miR-429-3p	miR-429-3p; miR-532-5p; miR-205-5p	miR-429-3p; miR-532-5p	miR-532-5p; miR-429-3p; miR-485-5p; miR-205-5p
Urate metabolic process (GO:0046415)						
XM_006503685.3 (predicted: facilitated glucose transporter, Slc2a9, transcript variant X3) ↑	miR-7052-3p; miR-145a-3p; miR-484	miR-199a-5p; miR-214-3p; miR-709	miR-211-5p; miR-328-3p; miR-145a-3p	miR-709; miR-199a-5p; miR-214-3p	miR-211-5p; miR-149-5p	miR-709; miR-214-3p; miR-199a-5p
XM_006503685.3 (predicted: facilitated glucose transporter, Slc2a9, transcript variant X3) ↓	miR-500-3p; miR-709; miR-7051-5p; miR-6914-3p; miR-149-5p	miR-7052-3p; miR-145a-3p; miR-500-3p; miR-7051-5p; miR-6914-3p; miR-204-5p; miR-149-5p; miR-501-3p; miR-484; miR-328-3p	miR-501-3p; miR-7052-3p; miR-500-3p; miR-709; miR-7051-5p; miR-6914-3p	miR-7052-3p; miR-145a-3p; miR-484; miR-501-3p; miR-204-5p; miR-328-3p; miR-149-5p	miR-7052-3p; miR-501-3p	miR-145a-3p; miR-211-5p; miR-149-5p; miR-204-5p; miR-328-3p; miR-484
NM_009198.3 (predicted: Gckr, transcript variant X1) ↑	—	—	miR-382-5p	—	miR-382-5p	—
NM_009198.3 (predicted: Gckr, transcript variant X1) ↓	miR-7a-5p	miR-7a-5p	miR-7a-5p	—	—	miR-382-5p; miR-7a-5p
NM_013632.4 (Pnp) ↑	—	miR-214-3p	—	miR-214-3p	—	miR-140-3p; miR-214-3p
NM_013632.4 (Pnp) ↓	miR-7051-5p	miR-7051-5p; miR-140-3p	miR-140-3p; miR-7051-5p	miR-140-3p	miR-140-3p	—
NM_021463.4 (Prpsl) ↑	let-7e-5p; miR-181b-5p	let-7e-5p; miR-181b-5p	let-7e-5p; miR-33-5p; let-7a-5p; let-7c-5p; let-7b-5p	—	miR-33-5p; let-7e-5p; let-7c-5p; let-7a-5p; let-7b-5p	miR-181b-5p
NM_021463.4 (Prpsl) ↓	miR-124-3p; miR-33-5p	miR-124-3p; miR-33-5p; let-7d-5p; let-7c-5p; let-7b-5p	miR-124-3p	let-7d-5p; let-7c-5p; let-7b-5p	miR-181b-5p	miR-33-5p; let-7c-5p; let-7d-5p; let-7a-5p; let-7b-5p; let-7e-5p

(Continued)

TABLE 3 (Continued)

GO term and gene annotated	Control vs. model	Control vs. noni fruit juice	Control vs. allopurinol	Model vs. noni fruit juice	Model vs. allopurinol	Allopurinol vs. noni fruit juice
NM_134069.3 (sodium phosphate, Slc17a3, transcript variant 1) ↑	miR-6996-5p; miR-1934-5p	—	miR-1934-5p; miR-211-5p	—	miR-211-5p	—
NM_134069.3 (sodium phosphate, Slc17a3, transcript variant 1) ↓	—	miR-204-5p; miR-122-5p	—	miR-6996-5p; miR-1934-5p; miR-204-5p; miR-122-5p	miR-6996-5p	miR-211-5p; miR-1934-5p; miR-204-5p; miR-122-5p
NM_025807.3 (monocarboxylic acid transporters, Slc16a9) ↑	miR-7117-3p; miR-6948-3p; miR-151-5p	—	miR-330-5p; miR-6948-3p; miR-151-5p; miR-151-3p	—	miR-330-5p; miR-6948-3p; miR-151-5p	—
NM_025807.3 (monocarboxylic acid transporters, Slc16a9) ↓	miR-7051-5p; miR-124-3p	miR-124-3p; miR-7051-5p; miR-151-3p; miR-326-3p	miR-124-3p; miR-7051-5p	miR-7117-3p; miR-6948-3p; miR-151-3p; miR-326-3p; miR-151-5p	miR-7117-3p	miR-330-5p; miR-6948-3p; miR-151-3p; miR-326-3p; miR-151-5p
NM_001164370.1 (Mipoll) ↑	miR-429-3p; miR-205-5p	miR-199a-5p	miR-485-5p	miR-199a-5p	miR-485-5p	miR-199a-5p
NM_001164370.1 (Mipoll) ↓	miR-485-5p	miR-532-5p; miR-429-3p; miR-485-5p; miR-205-5p	miR-429-3p	miR-429-3p; miR-532-5p; miR-205-5p	miR-429-3p; miR-532-5p	miR-532-5p; miR-429-3p; miR-485-5p; miR-205-5p
XM_006506148.3 (predicted: TED:ATP-binding cassette, WHITE, Abcg2, transcript variant X1) ↑	miR-346-5p	—	—	—	—	—
XM_006506148.3 (predicted: TED:ATP-binding cassette, WHITE, Abcg2, transcript variant X1) ↓	—	miR-193b-3p	miR-193b-3p	miR-193b-3p; miR-346-5p	miR-346-5p	miR-193b-3p
NM_008061.4 (glucose-6-phosphatase, G6pc) ↑	miR-671-5p; miR-17-5p	miR-214-3p; miR-709	miR-485-5p; miR-3074-2-3p	miR-709; miR-214-3p	miR-485-5p; miR-3074-2-3p	miR-709; miR-214-3p; miR-93-5p
NM_008061.4 (glucose-6-phosphatase, G6pc) ↓	miR-8116; miR-485-5p; miR-709; miR-574-5p	miR-3074-2-3p; miR-17-5p; miR-8116; miR-93-5p; miR-485-5p; miR-574-5p	miR-17-5p; miR-8116; miR-574-5p; miR-709; miR-470-5p; miR-93-5p	miR-17-5p; miR-3074-2-3p; miR-671-5p; miR-93-5p	miR-17-5p; miR-671-5p; miR-93-5p	miR-3074-2-3p; miR-485-5p
Urate transport (GO:0015747)						
NM_134069.3 (sodium phosphate, Slc17a3, transcript variant 1) ↑	miR-6996-5p; miR-1934-5p	—	miR-1934-5p; miR-211-5p	—	miR-211-5p	—
NM_134069.3 (sodium phosphate, Slc17a3, transcript variant 1) ↓	—	miR-204-5p; miR-122-5p	—	miR-6996-5p; miR-1934-5p; miR-204-5p; miR-122-5p	miR-6996-5p	miR-211-5p; miR-1934-5p; miR-204-5p; miR-122-5p
XM_006506148.3 (predicted: TED:ATP-binding cassette, WHITE, Abcg2, transcript variant X1) ↑	miR-346-5p	—	—	—	—	—

(Continued)

TABLE 3 (Continued)

GO term and gene annotated	Control vs. model	Control vs. noni fruit juice	Control vs. allopurinol	Model vs. noni fruit juice	Model vs. allopurinol	Allopurinol vs. noni fruit juice
XM_006506148.3 (predicted: TED:ATP-binding cassette, WHITE, Abcg2, transcript variant X1) ↓	—	miR-193b-3p	miR-193b-3p	miR-193b-3p; miR-346-5p	miR-346-5p	miR-193b-3p

Symbols "↑" and "↓" indicate up-regulation and down-regulation, respectively. Symbol "—" indicates miRNAs not detected.

was successfully established. Furthermore, the results of biochemical analysis showed that the content of serum UA in mice of hyperuricemia was significantly decreased by the administration of either noni fruit juice or allopurinol, suggesting the alleviation effect of noni fruit juice on hyperuricemia. It was noted that although the UA level was not restored by the treatment of noni fruit juice to the level of the normal control group of mice, the UA levels in both the noni fruit juice and the allopurinol (the positive control) groups were significantly different from that of the model group, suggesting that the noni fruit juice probably altered the production of UA with different mechanisms from that of the allopurinol. Future studies are needed to clarify the molecular mechanisms underlying the alleviation effects of both noni fruit juice and allopurinol on hyperuricemia.

As one of the key enzymes in the production of UA in human, XOD plays the rate-limiting role in the terminal step of purine metabolism that converts hypoxanthine to xanthine, which is used to generate UA (46); as the XOD activity is increased *in vivo*, the purine metabolism is increased, ultimately resulting in the increased UA synthesis. In our study, the biochemical analysis showed that the content of serum XOD in mice of the model group was significantly increased, compared with that of the normal control groups. As one of the effective inhibitors of XOD, allopurinol has been commonly used in the treatment of gout and hyperuricemia for decades (47). As expected, the serum XOD level in mice with hyperuricemia was significantly reduced by the treatment of allopurinol, even below the level of that in the normal control group. Similarly, compared with the model group, the serum XOD level in mice with hyperuricemia was significantly reduced by the treatment of noni fruit juice, suggesting that the noni fruit juice probably functioned to reduce the content of UA by inhibiting the enzymatic activities of XOD, as reported in the previous studies *in vitro* showing that noni fruit juice could inhibit the XOD activity (21). These results were consistent with those previously reported (27), revealing the strong inhibitory effect of both allopurinol and noni fruit juice on XOD.

The UA is mainly excreted by kidney, while insufficient excretion of UA leads to kidney damage (48). Both Cr and BUN are important indicators of renal function (49). In particular, the renal damage is accompanied by the increased levels of serum Cr and BUN, indicating decreased clearance of Cr and urea, respectively (28). Our results were consistent with those reported previously, showing that in comparison to the model group, the contents of serum Cr and BUN in mice with hyperuricemia were significantly reduced by the treatment of either noni fruit juice or allopurinol ($p < 0.0001$), suggesting that the noni fruit juice could promote the excretion of UA without causing deleterious effect on the renal functions of the mice.

4.2. MicroRNAs and metabolic pathways involved in the therapeutic treatment of hyperuricemia in mice by noni fruit juice

Hyperuricemia is diagnosed by excessive UA production and/or decreased excretion of UA from mainly kidney and slightly intestine, ultimately leading to excessive increase of serum UA concentration (3). The UA homeostasis in human is a complex and highly hereditary process, including the biosynthesis of metabolic urate, the reabsorption of renal urate, and the excretion of renal and extrarenal urate (50). As the final metabolic product of endogenous and exogenous purines in human, UA is excreted in human, instead of allantoin in some other animals (51, 52). The UA in plasma is filtered out in glomerulus of the kidney and then transported bidirectionally along renal tubules. This process includes renal tubule reabsorption, re-secretion, and reabsorption after secretion. The transport of UA in kidney is mainly accomplished by UA transporters, which act synergistically to maintain the steady level of UA. The abnormality of these transporters could cause UA to be excreted in urine and accumulated in the body, ultimately causing the hyperuricemia.

In order to further explore the pharmacological and molecular mechanisms underlying the effect of noni fruit juice on the therapeutic treatment of hyperuricemia in mice, the differentially expressed miRNAs and their target genes were identified in mice using the next generation high-throughput RNA-Seq technology. The target genes were further annotated by the GO database and enriched by the KEGG database to reveal the metabolic pathways involved in the pharmacological and molecular mechanisms regulating the therapeutic treatment of hyperuricemia in mice by noni fruit juice.

To date, a few dozens of miRNAs involved in hyperuricemia have been identified (22, 53–56) with many of them revealed in our study, showing various expression patterns largely consistent with those previously reported. For example, studies have shown that the expression of miR-143-3p is significantly decreased in mice with hyperuricemia to cause the increased activity of the glucose and fructose transporter GLUT9 (57), while our results showed that the miR-143-3p was significantly increased by the treatment of noni fruit juice. Furthermore, the down-regulation of miR-92a is involved in the KLF2-VGEFA axis and the angiogenesis in hyperuricemia (58), while our results revealed significantly increased expression of miR-92a by the treatment of noni fruit juice. The expression of miR-9 is increased by epigallocatechin gallate (EGCG) to regulate the NF-κB and JAK–STAT pathways in NRK-49F cells (59). Similarly, our results showed that the expression of miR-9 was significantly increased by the treatment of noni fruit juice. It was worthy of noting that the expression patterns of some of these miRNAs were different between

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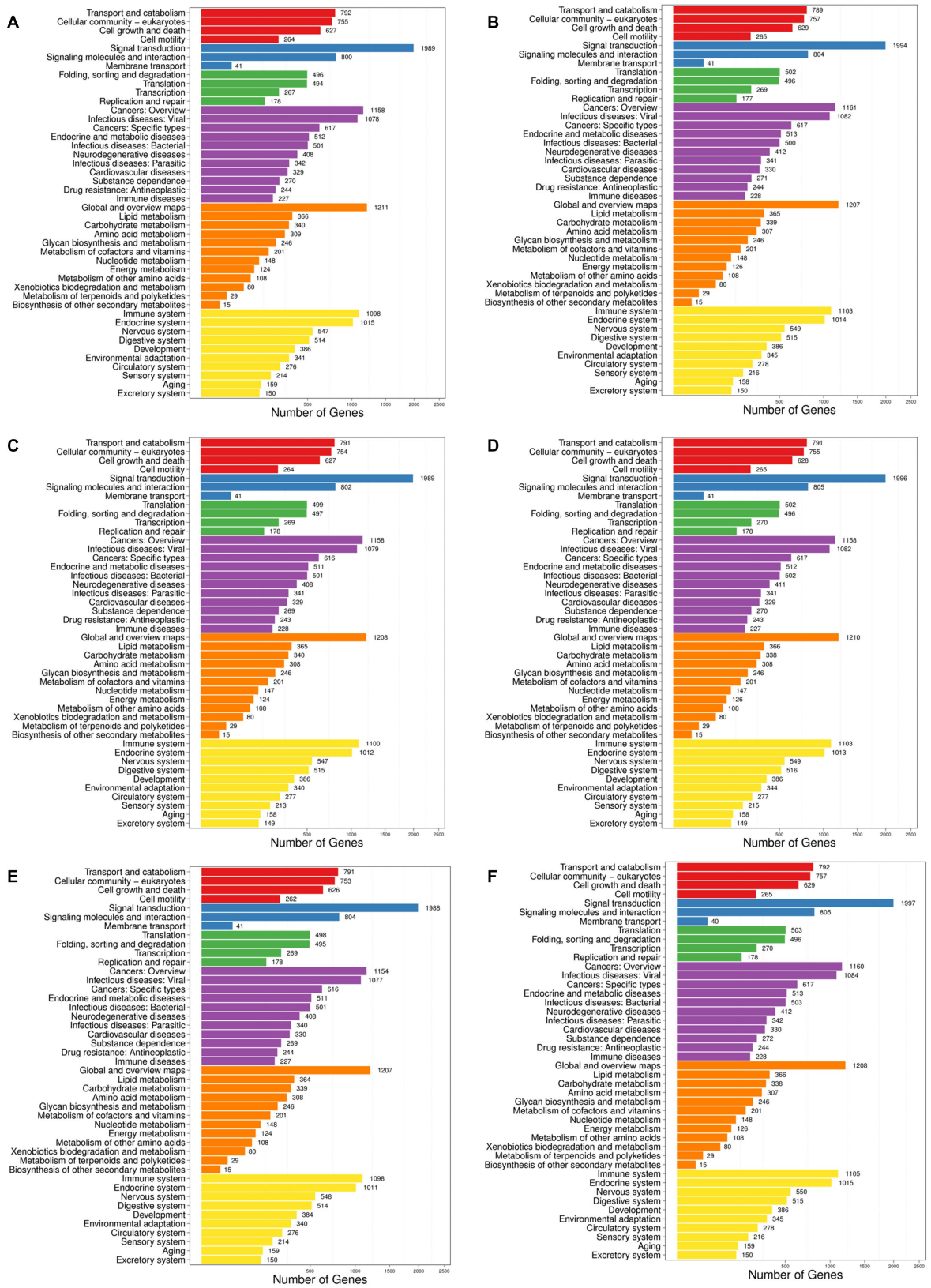

FIGURE 5
Metabolic pathway enrichment analysis of the target genes of differentially expressed microRNAs based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database identified in the pairwise comparisons of four groups of mice, including the normal control vs. model groups **(A)**, the normal control vs. noni fruit juice groups **(B)**, the normal control vs. allopurinol groups **(C)**, the model vs. noni fruit juice groups **(D)**, the model vs. allopurinol groups **(E)**, and the allopurinol vs. noni fruit juice groups **(F)**. The six categories of metabolic pathways in KEGG database are shown in six different color blocks, i.e., red, blue, green, purple, orange, and yellow, representing cellular processes, environmental information processing, genetic information processing, human diseases, metabolism, and organismal systems, respectively. Number of genes represents the target genes of the differentially expressed miRNAs.

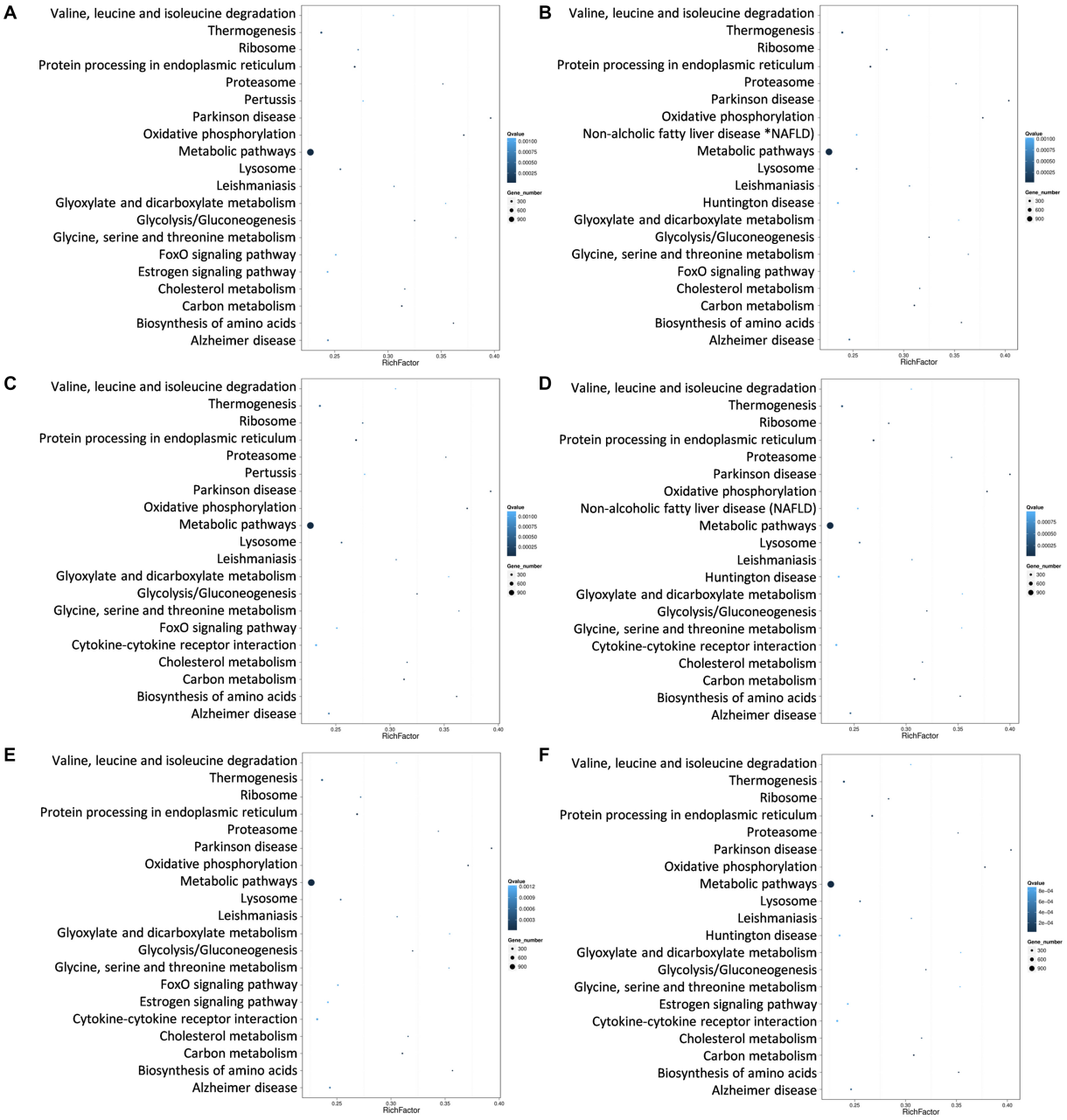

FIGURE 6
Scatter plots of the top 20 enriched pathway terms based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database of the target genes of the differentially expressed microRNAs identified in the pairwise comparisons among the four groups of mince, including the normal control vs. model groups **(A)**, the normal control vs. noni fruit juice groups **(B)**, the normal control vs. allopurinol groups **(C)**, the model vs. noni fruit juice groups **(D)**, the model vs. allopurinol groups **(E)**, and the allopurinol vs. noni fruit juice groups **(F)**. The Rich Factor is the ratio of the target gene number annotated in the pathway term to all gene number annotated in the pathway term; the bigger the Rich Factor, the greater the degree of enrichment. The *Q*-value is the corrected *p*-value, ranging from 0 to 1; the lower the *Q*-value, the greater the level of enrichment.

the treatments of noni fruit juice and allopurinol, suggesting the varied molecular mechanisms regulating the alleviation effects of noni fruit juice and allopurinol. For example, the expression of miR-181a, which is closely related to the production of UA and renal damage in the chronic kidney disease by the down-regulation of the TLR/NF-κB pathway (60), was significantly up-regulated by the treatment of allopurinol but down-regulated by the treatment of noni fruit juice in our study, whereas the expressions of several miRNAs (e.g., miR-451a, miR-155-5p, and miR-149-5p) were significantly down-regulated by PO, noni fruit juice, and allopurinol, suggesting the varied functions of PO, noni fruit juice, and allopurinol in the development and treatment of hyperuricemia.

The GO annotation results showed that a group of target genes of differentially expressed miRNAs were annotated in urate biosynthetic process, urate metabolic process, and urate transport related to the etiology of hyperuricemia with the known and novel miRNAs identified in the pairwise comparisons of the four groups of mice. Further studies are needed to identify the explicit functions

TABLE 4 Expression patterns of differentially expressed microRNAs in the six pairwise comparisons among the four groups of mice, i.e., the normal control, the model, the allopurinol, and the noni fruit juice groups, with the target genes related to xanthine dehydrogenase/oxygenase involved in the metabolic pathways of hyperuricemia based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis.

XOD related gene	Control vs. model	Control vs. noni fruit juice	Control vs. allopurinol	Model vs. noni fruit juice	Model vs. allopurinol	Allopurinol vs. noni fruit juice
NM_011723.3 (Xdh)	(6↑,4↓)/(77↑,79↓)	(1↑,10↓)/(223↑,42↓)	(1↑,5↓)/(75↑,94↓)	(1↑,8↓)/(209↑,48↓)	(1↑,5↓)/(80↑,98↓)	(0↑,5↓)/(226↑,35↓)
NM_026670.4 (zinc finger, Zmym1)	(0↑,0↓)/(1↑,3↓)	(0↑,0↓)/(2↑,2↓)	(0↑,0↓)/(0↑,5↓)	(0↑,0↓)/(2↑,1↓)	(0↑,0↓)/(0↑,2↓)	(0↑,0↓)/(3↑,0↓)

Data are presented as number of (known)/(novel) microRNAs. Symbols "↑" and "↓" indicate up-regulation and down-regulation, respectively.

TABLE 5 Known microRNAs of the pairwise comparisons among the four groups of mice, i.e., the normal control, the model, the allopurinol, and the noni fruit juice groups, with the target genes related to xanthine dehydrogenase/oxygenase involved in the metabolic pathways of hyperuricemia based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis.

XOD related gene	Control vs. model	Control vs. noni fruit juice	Control vs. allopurinol	Model vs. noni fruit juice	Model vs. allopurinol	Allopurinol vs. noni fruit juice
NM_011723.3 (Xdh) ↑	miR-93-3p; miR-346-5p; miR-15a-5p; miR-486b-3p; miR-484; miR-15b-5p	miR-497a-5p	miR-3074-2-3p	miR-497a-5p	miR-3074-2-3p	—
NM_011723.3 (Xdh) ↓	miR-195a-5p; miR-6953-3p; miR-7051-5p; miR-574-5p	miR-486a-3p; miR-3074-2-3p; miR6953-3p; miR-7051-5p; miR-15a-5p; miR-15b-5p; miR-484; miR-195a-5p; miR-574-5p; miR-486b-3p	miR-486a-3p; miR-574-5p; miR-195a-5p; miR-6953-3p; miR-7051-5p	miR-486a-3p; miR-3074-2-3p; miR-346-5p; miR-93-3p; miR-15a-5p; miR-15b-5p; miR-484; miR-486b-3p	miR-486a-3p; miR-346-5p; miR-93-3p; miR-15a-5p; miR-15b-5p	miR-3074-2-3p; miR-15a-5p; miR-484; miR-15b-5p; miR-486b-3p

Symbols "↑" and "↓" indicate up-regulation and down-regulation, respectively. Symbol "—" indicates miRNAs not detected.

of these novel miRNAs in the treatment of hyperuricemia in mice, while some of the known miRNAs are discussed here due to their functions related to hyperuricemia as previously reported. For example, the miR-214-3p was highly expressed in mice of the noni fruit juice group. Studies have shown that the level of serum miR-214 in patients with hyperuricemia is lower than that in healthy controls, showing negative correlation with the level of UA (56). These results suggested that the noni fruit juice was probably involved in the urate biosynthetic process and urate metabolic process by up-regulating the expression of miR-214-3p, ultimately decreasing the level of serum UA in mice with hyperuricemia. Furthermore, the miR-17-5p was significantly up-regulated in the model group of mice. These results were consistent with those previously reported, showing that hsa-mir-17-5p was significantly up-regulated in plasma of patients with hyperuricemia and gout compared with normal subjects ($p < 0.001$) (53). Moreover, the miR-17-5p was significantly down-regulated in both the noni fruit juice and the allopurinol groups, suggesting that the noni fruit juice and allopurinol could reduce the expression of miR-17-5p. Therefore, these results indicated that both noni fruit juice and allopurinol reduced the level of serum UA in mice with hyperuricemia, probably by inhibiting the expression of miR-17-5p, which was likely involved in the metabolic pathways of urate biosynthetic process, urate metabolic process, and urate transport. Further studies are necessary to explicitly identify the regulatory functions of miR-17-5p and underlying molecular and pharmacological mechanisms in the metabolic pathways of urate biosynthetic process, urate metabolic process, and urate transport in mice with hyperuricemia. In addition, previous microarray studies revealed the down-regulation of miR-149-5p by the treatment of allopurinol in mice, while the miR-149-5p expression was significantly up-regulated in UA-stimulated hepatocytes (61). Furthermore, as one of the target genes of miR-149-5p, the *fibroblast growth factor 21* (*FGF21*) expression was inhibited by the overexpression of miR-149-5p, while the UA-induced lipid deposition was decreased in the hepatocytes, indicating that the expression of miR-149-5p was significantly up-regulated by UA in hepatocytes to increase the lipid accumulation in hepatocytes via the interaction between miR-149-5p and FGF21 (61). These results were consistent with the findings revealed in our study, showing that the expression of miR-149-5p was differentially altered in mice of both noni fruit juice and model groups and involved in the urate metabolic process. Future studies are need to explore the explicit functions of miR-149-5p involved in the pathogenesis of hyperuricemia.

The KEGG enrichment analysis of the target genes of the differentially expressed miRNAs in the pairwise comparisons among the four groups of mice identified the significantly enriched metabolic pathways involved in the development and treatment of hyperuricemia. Future studies are needed to further explore the functions of these metabolic pathways and miRNAs enriched in the therapeutic effects of noni fruit juice on hyperuricemia. For example, as one of the key enzymes involved in the biosynthesis of UA in the purine metabolism, the XOD, also known as xanthine oxidoreductase or XOR, is coded by the xanthine dehydrogenase gene *XDH* (62). Therefore, the XOD related genes involved in the purine metabolism were further evaluated based on KEGG database to identify the known and novel differentially expressed miRNAs in the pairwise comparisons among the four groups of mice. However, the relationships between these miRNAs and hyperuricemia are rarely reported in the literature. Further studies are needed to identify the explicit functions of these miRNAs involved in hyperuricemia. For example, our results showed that the miR-93-3p was significantly up-regulated in the model group and significantly down-regulated in both the noni fruit juice and allopurinol groups. Studies have shown that miR-93-3p is one of the 10 diagnostic biomarkers in patients with acute kidney injury in the intensive care units (63), suggesting that the increased expression of miR-93-3p in the model group in our study could be related to the renal injury in mice of the model group and could be potentially used as the diagnostic biomarker of renal injury in mice. Future studies are needed to explore the regulatory functions of these miRNAs involved in the therapeutic treatment of hyperuricemia in mice by noni fruit juice and allopurinol. For example, the results of the TargetScan revealed that as the target gene of miR-214-3p, *SLC22A12* encodes the glucose and fruticose transporter URAT1 (22, 59), which functions as the exchanger of UA and anion, ultimately altering the serum UA level through UA reabsorption in human kidney (64, 65). Our results showed the expression of miR-214-3p was significantly increased by the treatment of noni fruit juice. Therefore, the URAT1 has become an important and potential target for the treatment of hyperuricemia (66), and future studies are needed to identify the regulatory functions of miR-214-3p in the synthesis of URAT1 and its effect on the absorption of UA and the treatment of hyperuricemia in mice.

Studies have shown that some miRNAs are involved in both hyperuricemia and gout (22, 67). For example, the expression of miR-146a was significantly increased in patients with gout and involved in both NLRP3 and MyD88/NF-κB pathways (68). Similar results were revealed in our study, showing that the expression of miR-146a-5p was significantly increased in the model group and significantly decreased in both noni fruit juice and allopurinol groups, suggesting the therapeutic effect of noni fruit juice on hyperuricemia and its involvement in the NLRP3 and MyD88/NF-κB pathways. Furthermore, studies have shown that the expression of miR-223-3p is significantly down-regulated in the mouse model of pouch synovium with the expression of NLRP3 inhibited by the overexpression of miR-223-3p, ultimately alleviating the inflammatory effect of gout (69). Our results showed that the expression of miR-223-3p was significantly decreased in both noni fruit juice and allopurinol groups and significantly increased in the model group, suggesting the potential participation of miR-223-3p in the NLRP3 pathway. Moreover, studies have shown that the expression of miR-155 is increased in the gouty arthritis model with the expression level of SHIP-l inhibited and production of proinflammatory cytokines enhanced (70). Our results showed that the expression of miR-155-5p was significantly increased in the model group, suggesting the important roles that miR-155-5p played in the occurrence of hyperuricemia in mice. In addition, a group of miRNAs have been identified as biomarkers of hyperuricemia (53) with some of them revealed in our study with significant regulations in their expressions and correlations with the contents of serum Cr, including miR-17-5p, miR-18a-5p, miR-223-3p, miR-146a-5p, and miR-155-5p, in the noni fruit juice, model, and allopurinol groups in our study. Future studies are necessary to identify the explicit functions of these miRNA involved in the various metabolic pathways related to hyperuricemia.

To date, numerous studies have shown that the natural products, i.e., traditional Chinese medicines, have been revealed with alleviation effect on hyperuricemia, in particular, altering the content of hyperuricemia related biochemical factors (e.g., serum UA, Cr, and BUN) and inhibiting the XOD activities (26, 29, 71–79). For example, an empirical formula Xie-Zhuo-Chu-Bi-Fang is used to treat mice with hyperuricemia, significantly decreasing the content of serum UA, down-regulating the expression of URAT1, and up-regulating miR-34a (26). Furthermore, as a hydrogenated derivative of berberine, dihydroberberine is revealed with effective inhibition of XOD, significantly decreasing the contents of serum Cr and BUN and down-regulating the renal mRNA and protein expression of XOD as well as several other hyperuricemia related biochemical factors in mice with hyperuricemia (29). Together with the findings revealed in our study, these natural products provide a wide spectrum of potentially promising therapeutic treatments of hyperuricemia. Studies have shown the various types of polysaccharides are involved in the treatment and prevention of hyperuricemia, suggesting that the noni fruit juice probably contained the same or similar chemical compositions as the polysaccharides as previously reported (80). Further studies are necessary to identify these functional substances in noni fruit juice (81).

5. Conclusion

In this study, we investigated the therapeutic effect of noni fruit juice on mice with hyperuricemia using both biochemical and RNA-Seq analyses and characterized the variations in the contents of hyperuricemia related biochemical factors and differentially expressed miRNAs involved in the molecular and pharmacological mechanisms regulating the therapeutic treatment of hyperuricemia in mice. The results showed that the treatment of noni fruit juice caused significant decrease in the serum UA levels and the contents of XOD, Cr, and BUN in mice with hyperuricemia. The results of RNA-Seq analysis revealed a group of differentially expressed miRNAs involved in the pathogenesis of hyperuricemia in mice. The functional annotation and enrichment analysis of the target genes of differentially expressed miRNAs were performed based on GO and KEGG databases. Our study provided strong experimental evidence to support the therapeutic and pharmacological effects of noni fruit juice on the

treatment of hyperuricemia in mice and to support the agricultural and nutritional development of noni plants due to their potential clinical significance in the treatment of hyperuricemia.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://ncbi.nlm.nih.gov/bioproject/PRJNA910471.

Ethics statement

The animal study was reviewed and approved by the Ethics Committee of Jilin University with the approval # 2018SY0602.

Author contributions

YL, XJL, FS, HL, and ZDL: conceptualization, formal analysis, and writing—original draft preparation. YL, XJL, HL, and ZDL: methodology. MW: software. CC: validation. XHL: investigation. YS: resources. ZYL: data curation. FS, HL, and ZDL: writing—review and editing and supervision. YY: visualization. HL and ZDL: project administration. XJL, HL, and ZDL: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

ZYL was employed by Qingdao Haoda Marine 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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1121734/full#supplementary-material

SUPPLEMENTARY TABLE 1
Lists of predicted known and novel microRNAs in the four groups of mice.

SUPPLEMENTARY TABLE 2
List of differentially expressed microRNAs based on the six pairwise comparisons among the four groups of mice.

SUPPLEMENTARY TABLE 3
Target gene intersection of differentially expressed microRNA predicted by TargetScan and miRanda in the pairwise comparisons of the four groups of mice.

SUPPLEMENTARY TABLE 4
Target genes identified by both TargetScan and miRanda of the differentially expressed microRNAs of the normal control and model groups of mice.

SUPPLEMENTARY TABLE 5
Target genes identified by both TargetScan and miRanda of the differentially expressed microRNAs of the normal control and noni fruit juice groups of mice.

SUPPLEMENTARY TABLE 6
Target genes identified by both TargetScan and miRanda of the differentially expressed microRNAs of the normal control and allopurinol groups of mice.

SUPPLEMENTARY TABLE 7
Target genes identified by both TargetScan and miRanda of the differentially expressed microRNAs of the model and the noni fruit juice groups of mice.

SUPPLEMENTARY TABLE 8
Target genes identified by both TargetScan and miRanda of the differentially expressed microRNAs of the model and allopurinol groups of mice.

SUPPLEMENTARY TABLE 9
Target genes identified by both TargetScan and miRanda of the differentially expressed microRNAs of the allopurinol and noni fruit juice groups of mice.

SUPPLEMENTARY TABLE 10
List and expression patterns of microRNAs with the target genes involved in urate biosynthetic process, urate metabolic process, and urate transport, as well as the positive and negative regulations of urate biosynthetic process, urate metabolic process, and urate transport assembly based on the Gene Ontology (GO) annotation in the six pairwise comparisons of the four groups of mice.

SUPPLEMENTARY TABLE 11
List and expression patterns of both known and novel microRNAs targeting xanthine oxidase (XOD) in purine metabolism pathways related to hyperuricemia based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis in the six pairwise comparisons of the four groups of mice.

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The impact of high-glucose or high-fat diets on the metabolomic profiling of mice

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Objective: Diets high in glucose or fat contribute to an increased prevalence of the diseases. Therefore, the objective of the current research was to observe and evaluate the impact of dietary components on different metabolomic profiles in primary tissues of mice.

Methods: For 8weeks, diet with high-glucose or-fat was given to C57BL/6J mice. The levels of metabolites in the primary tissues of mice were studied using gas chromatography-mass spectrometry (GC-MS) and analyzed using multivariate statistics.

Results: By comparing the metabolic profiles between the two diet groups and control group in mice main tissues, our study revealed 32 metabolites in the high-glucose diet (HGD) group and 28 metabolites in the high-fat diet (HFD) group. The most significantly altered metabolites were amino acids (AAs; L-alanine, L-valine, glycine, L-aspartic acid, L-isoleucine, L-leucine, L-threonine, L-glutamic acid, phenylalanine, tyrosine, serine, proline, and lysine), fatty acids (FAs; propanoic acid, 9,12-octadecadienoic acid, pentadecanoic acid, hexanoic acid, and myristic acid), and organic compounds (succinic acid, malic acid, citric acid, L-(+)-lactic acid, myo-inositol, and urea). These metabolites are implicated in many metabolic pathways related to energy, AAs, and lipids metabolism.

Conclusion: We systematically analyzed the metabolic changes underlying high-glucose or high-fat diet. The two divergent diets induced patent changes in AA and lipid metabolism in the main tissues, and helped identify metabolic pathways in a mouse model.

KEYWORDS

diet, glucolipid, tissue, gas chromatography-mass spectrometry, metabolome

Introduction

High-energy food intake is a characteristic of the Western-style diet (1). Dietary intake patterns are linked with glucolipid metabolic disorders which are a series of diseases associated with the metabolic disturbance of glucose and lipids (2). Glucolipid metabolic disorders can lead to many health problems such as obesity, diabetes, hypertension, coronary heart disease, and steatohepatitis (2). The incidence of obesity and diabetes has also risen in younger individuals with morbidity significantly increasing in children and young people (3, 4). To mitigate these

diseases, dietary education is usually recommended. Studies on glucolipid metabolism investigating metabolic disorders in target tissues support findings of how dietary changes influence health.

Metabolomic has played a critical role in assessing and diagnosing health and disease of individuals; it allows for a thorough examination of low-molecular-weight molecules in biological samples (5). Multiple metabolomic techniques such as gas chromatography–mass spectrometry (GC-MS) and liquid-chromatography mass spectrometry (LC-MS), have been employed to uncover the specificity and complexity of metabolic alterations within animal and human tissues (6). The goal of metabolomics is to develop diagnostic and mechanistic biochemical biomarkers that can monitor changes in the metabolic homeostasis of individuals (7). This requires the identification of specific metabolites that play a role in health and disease. In the field of nutritional metabolomics, small molecule chemical profiling is used to promote dietary information integration to forecast health and complicated pathobiology, complex biosystems research, and underline the essential role of nutrition and food in health and disease integrated biosystem models (8).

Diets have long been suspected by researchers to have a role in the development of obesity and other metabolic diseases, however, in the last few decades they have been the focus of a comprehensive investigation. To date, most studies that have revealed the intimate interplay between diet style and metabolism have been performed on a limited number of samples or tissues in animal models (9, 10). There are few studies to evaluate the metabonomic changes of the main tissues in animal models comprehensively. Additionally, there is currently a lack of data comparing the effects of the glucose or fat diet on the metabolism of main tissues. Therefore, in order to evaluate the role of diet in metabolomic variations of the main tissues in animal models comprehensively. The experimental design of this work is the first to compare the metabolomic responses of the main tissues in mice fed with different dietary glucose and fat levels by using the GC/MS-based metabolomics approach. Consequently, the results of this study enhance our understanding of the metabolic alterations that occur in mice under an obesogenic environment and exploring optimal nutritional requirements and feeding regimes.

Materials and methods

Experimental animal and dietary intrusion

Eventually, the acquired 8-week-old male C57BL/6J mice were kept in a colony room providing a 12/12 h light/dark cycle at 22–23°C, respectively. Before being utilized for experiments, all mice were given 7 days to acclimate. Following that, the mice were categorized randomly into three groups: control group, high-glucose diet (HGD) group, high-fat diet (HFD) group, and each group with 7 mice ($n = 7$ per group). The composition of diet provided to each group as Ain-93 M (control); 75.9% carbohydrate, 14.7% protein and 9.4% fat (high-glucose diet); 25% carbohydrate, 15% protein, and 60% fat (high-fat diet) at Jiangsu Synergy Pharmaceutical & Biological Engineering Co., Nanjing, CHA (Supplementary file 1). For 8 weeks, all diets were given *ad libitum*. Mice were weighed weekly and their food and drink intake were assessed twice a week. According to national rules and with the agreement of Jining Medical University's ethics committee (Protocol #JNMC2020DWRM0076), animal studies were conducted.

Tissue sampling

Following a 12-h overnight fast, mice were euthanized with 1% sodium pentobarbital (50 mg/kg). Subsequently, the tail artery was punctured via needle to evaluate total cholesterol (TC), triglycerides (TG), and glucose levels, from the pricked blood sample. A portable glucometer was used to monitor blood glucose levels (ACCU109 CHEK, Roche, IN). Wako Inc. (Richmond, VA, United States) kits were used to quantify TG and TC. To isolate serum, the blood was immediately drawn and centrifuged at 5,000 rpm for 6 min. Furthermore, the mice were slaughtered by dislocation of the cervical. On an ice surface, the mice were instantly dissected. 0.9% physiological saline was used to cleanse the whole heart, liver, brain, and kidney samples before freezing them in liquid nitrogen and preserving them in storage at −80°C for future use.

Sample pretreatment for GC-MS

Serum Samples Preparation: The 350 µL methanol (having 100 µg/mL Internal Standard Heptadecanoic acid, 98% purity; lot: SLBX4162) was mixed with 100 µL serum. The prepared mixture was then centrifuged at 4°C at 14,000 rpm for 10 min. Later, the obtained supernatant was transferred to a 2-mL tube and dried under nitrogen gas at 37°C. The extracts were then mixed with 80 µL of O-methyl hydroxylamine hydrochloride (purity: 98.0%; lot: LG10T16, 15 mg/mL in pyridine; J&K Scientific Ltd. Beijing, CHA) and incubated at 70 °C for 90 min. After that, each sample was subjected to 100 µL N, O-bis-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA +1% TMCS, v/v; lot: BCBZ4865, Sigma-Aldrich, MO, United States), incubated for 60 min at 70°C. Before GC–MS analysis, the prepared samples were vortexed, centrifuged at 4°C for 2 min at 14,000 rpm, and filtered via a 0.22-µm filter membrane.

Tissue samples: For this experiment, methanol (Thermo Fisher Scientific, Waltham, MA, United States) was used to homogenize the 50 mg of tissue (kidney, liver, heart, and brain), transferred to a 2-mL tube with 50 µL 1 mg/mL IS. Mixtures were further centrifuged for 10 min at 14,000 rpm and 4°C to isolate the solid particles. The remaining procedure was similar to that of serum samples.

GC-MS analysis

A 7890B gas chromatograph (GC) machine was utilized in conjunction with a 7000C mass spectrometer (MS) and an HP-5MS fused silica capillary column (Agilent Technologies, CA, United States) for GC-MS analysis. Each 1 µL aliquot of the derivatized solution was processed in splitting operation at a flow rate of 1 mL/min of helium gas through the column (50: 1). The GC temperature protocol started at 60°C for 4 min, then raised by 8°C/min to 300°C for 5 min. The temperatures of the injection, transfer line, and ion source were 280°C, 250°C, and 230°C, respectively. Electrospray ionization was used to record 20 scans per second across 50–800 m/z.

Multivariate analysis

Weight, blood glucose, TG, and TC levels were compared using a two-way ANOVA. For all measurements, the mean ± standard error of the mean was used. Significantly, the obtained was visualized and interpreted via GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, United States) with applied a p-value < 0.05.

Data processing and metabolite identification

Analysis of unknown preprocessing of GC data was performed using MassHunter (Agilent Technologies, CA, USA). The raw data were converted to the m/z data format. We created a library containing all QC samples, and the NIST (U.S. National Institute of Standards and Technology) 14 GC-MS library was used to identify the unknown metabolites from QC library. Further, the data were analyzed by alignment, retention time correction, baseline filtration, and deconvolution. Then, the metabolites with similarity >80% were considered as structurally identified. Afterwards, a new spectrum library named "New Library" was obtained, and the metabolites of samples were identified using this New Library. Finally, an integrated data matrix composed of the peak index (RT-m/z pair), sample name, and corresponding peak area was generated. Subsequently, the peak area from the data matrix was normalized using Microsoft Excel.

The statistical analyses were performed using **SIMCA-P v14.0** (Umetrics, Umea, Sweden). Additionally, orthogonal projection to latent structures discriminant analysis (**OPLS-DA**) was used to distinguish amongst the control, HGD, and HFD group with applied significant cutoff value as a p-value and variable importance in projection (VIP) values set as <0.05 and >1.0, respectively. Moreover, using permutation testing, the HGD and HFD groups were given one last look-over (200 permutations). We conducted our statistical analyses using **SPSS** Inc.'s (Chicago, IL) Statistical Package for the Social Sciences (19.0). To conduct functional analyses, **MetaboAnalyst v5.0** and the Kyoto Encyclopedia of Genes and Genomes (**KEGG**[1]) were utilized. A raw p-value < 0.05 was classified as significant, and an impact value >0 was considered to be significant. In earlier research, several standard metabolomic analysis methodologies were used (11).

Results

Metabolic changes induced by HGD and HFD

At the start of the trial, the average body weight was 18.94 ± 0.34 g (Table 1). Compared to the beginning of the experiment, each group's animals gained weight by the study's end, the control group gained 3.54 ± 0.82 g, the HGD group gained 6.83 ± 0.79 g, the HFD group gained 9.43 ± 0.41 g. Mice in the HGD and HFD diet groups gained considerably more weight than mice in the control group after 8 weeks on the diets (p < 0.05). Furthermore, the weight of the HFD group was increased more obviously compared with the HGD group (p < 0.05). The fasting blood glucose concentration was increased significantly in the HGD and HFD groups (p < 0.01) than the control group, as well as the TG and TC (p < 0.05). And the glucose, TG and TC increased in the HFD group as compared to that of the HGD group (p < 0.01).

Serum and tissue sample GC-MS total ion chromatograms

Consequently, representative GC–MS TICs of quality control (QC) serum and tissue samples (liver, heart, kidney and brain) from control groups and HGD mixture, and control groups and HFD mixture exhibited robust signals and high RT repeatability. The details of TICs among different tissues could be observed in Supplementary file 2.

Metabolomics analyses of tissue sample and serum

The parameters obtained from the OPLS-DA showed efficient model that evidently separated the control and two diet groups (serum: R2X = 0.671, R2Y = 0.968, Q2 = 0.573; heart: R2X = 0.867, R2Y = 1, Q2 = 0.826; liver: R2X = 0.907, R2Y = 1, Q2 = 0.877; brain: R2X = 0.822, R2Y = 0.99, Q2 = 0.548; kidney: R2X = 0.692, R2Y = 0.95, Q2 = 0.78) (Figures 1A–E). Parameter values near to 1.0 imply a model that is stable and predictable. In Figures 1F–J, the blue Q2 points regression line crosses the vertical axis (on the left) below zero, indicating that the OPLS-DA models are not overfitting.

Identification of metabolites

OPLS-DA with VIP and p-value of the t-test are the standard criteria for potential metabolites. VIP >1.0 and p < 0.05 in comparison to the control group indicated differences in the metabolites between the two diet groups. In addition, FC (Fold Change: HGD group/control group or HFD group/control group) >1 indicated that the metabolite has an upward trend, while FC < 1 indicated a downward trend. The **HGD** group alone changed 32 metabolites including AA derivatives, FAs, nucleosides and bases, TCA cycle, and other metabolites. There were 9 increased metabolites and 2 decreased metabolites in serum. In heart tissue, HGD led to 12 up-regulated altered metabolites. In liver tissue, 12 differential metabolites including 11 up-regulated and 1 down-regulated metabolites. In brain tissue, 10 up-regulated altered metabolites were identified. In addition, 7 up-regulated differential metabolites in kidney tissue. The **HFD** changed 28 metabolites including AA derivatives, FAs, and other metabolites. Ten metabolites (9 up-regulated and 1 down-regulated) in serum, 10 up-regulated in heart, 7 up-regulated metabolites in liver, 4 up-regulated metabolites in brain, 6 up-regulated metabolites in kidney were observed. Table 2 contains detailed metabolite findings.

Analyzing the data for the discovered metabolites, we additionally used heatmaps (MetaboAnalyst v5.0) to identify two separate clusters with low overlap for most metabolites in two diet groups (HGD groups: Figures 2A,C,E,G,I; HFD groups: Figures 2B,D,F,H,J).

1 http://www.kegg.jp

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TABLE 1 The dietary effect on body weight and serum biochemical parameters.

Group		Initial weight (g)	Final weight (g)	Glucose (mg/dl)	TG (mg/dl)	TC (mg/dl)
CON	Mean ± SD	18.93 ± 0.35	22.47 ± 1.17	99.07 ± 2.71	32.0 ± 2.09	53.26 ± 4.24
HGD	Mean ± SD	18.80 ± 0.36	25.63 ± 1.15	164.43 ± 4.39	61.20 ± 4.39	70.13 ± 6.81
	Versus CON	1.000	0.022*	0.000*	0.000*	0.015*
	Versus HFD	1.000	0.032*	0.000*	0.003*	0.006*
HFD	Mean ± SD	19.10 ± 0.30	28.53 ± 0.71	189.10 ± 3.60	79.40 ± 3.31	90.93 ± 3.56
	Versus CON	1.000	0.001*	0.000*	0.000*	0.000*
	Versus HGD	1.000	0.032*	0.000*	0.003*	0.006*

TC, total cholesterol; TG, total triglyceride. Values are means ± SD, $n = 7$, *p-value significant.

Analysis of metabolic pathways

Furthermore, metabolites were examined using MetaboAnalyst v5.0[2] and KEGG database (see footnote 1) to investigate the metabolic pathways among diets and the control group. The following are some important pathways (raw $p < 0.05$, impact >0; Table 3): glyoxylate and dicarboxylate metabolism; D-glutamine and D-glutamate metabolism; arginine biosynthesis; Alanine, aspartate, and glutamate metabolism; phenylalanine, tyrosine, and tryptophan biosynthesis; glutathione metabolism; phenylalanine metabolism; and galactose metabolism are all included in the HGD group (Figures 3A,C,E,G,I). Moreover, biosynthesis of phenylalanine, tyrosine, and tryptophan; main bile acid biosynthesis; arginine biosynthesis; D-glutamine and D-glutamate metabolism; arginine and proline metabolism; alanine, aspartate, and glutamate metabolism; and linoleic acid metabolism were all studied in the HFD group (Figures 3B,D,F,H), but no data of kidney meet the screening criteria in HFD group. A summary of metabolites and metabolic pathways is shown in Figure 4.

Discussion

Pathophysiology and metabolic disturbances are produced by glucolipid metabolic diseases. For excess models and nutritional deficiencies, high-throughput metabolomics data is becoming accessible, which is important for understanding the complicated nutritional interactions of complete organisms (8). As a result, we conducted primary tissue metabolite profiling in mice and observed alterations in a few metabolites. Long-term use of HGD and HFD resulted in a weight increase in the current research. The progressive accumulation of body fat was the major cause of weight increase. Furthermore, the HGD and HFD groups showed severe metabolic impairments, including increased glucose, TG, and TC blood levels, indicating that HGD and HFD induced obesity and disrupted glucose homeostasis, which is consistent with earlier results (12).

We compared the differences in serum and main tissue metabolite levels between the two divergent dietary interventions. In both HGD and HFD, the majority of the metabolites whose concentrations altered were associated with AA, lipids, and energy metabolism (Table 2; Figure 3). Additional support for the impact of nutrition on the metabolome was found through the discovery of specific metabolites, such as the amino acids L-valine, L-isoleucine, L-leucine, phenylalanine, and L-threonine, as well as phenylalanine and serine. Metabolic pathways significantly associated with HGD and HFD were consistent with altered dietary patterns, including amino acid metabolism and lipid or fatty acid-related metabolic pathways (Table 3; Figure 4). In these pathways, there are several same pathways in both diet groups, included alanine, aspartate, and glutamate metabolism, arginine biosynthesis, phenylalanine, tyrosine, and tryptophan biosynthesis, and D-Glutamine and D-glutamate metabolism. And there are three pathways in each diet group, glutathione metabolism, phenylalanine metabolism, galactose metabolism in HGD group, and primary bile acid biosynthesis, arginine and proline metabolism, linoleic acid metabolism in HFD group. Therefore, in light of the distinct metabolic pathways observed in each group, we can select the most suitable biomarker for modulating glycolipid metabolism.

Obesity has been highly related to detrimental glucose profiles, and leads to the development of type 2 diabetes (T2DM) (13). In this research, both HGD and HFD were linked to insulin dysfunction, with the high-energy diets promoting increased insulin resistance and raised blood glucose levels. Consequently, the L-valine, L-leucine, L-isoleucine, tyrosine, phenylalanine, and serine levels underwent significant alternation in the two diet groups of our study. Increase in branch-chain amino acids (BCAAs) and phenylalanine may attenuate insulin sensitivity and enhance insulin resistance (14). Researchers have studied amino acids and related metabolites in normoglycemic individuals and suggest that aromatic and BCAAs could be predictors of diabetes (15). The branched-chain amino acids, L-valine and L-leucine, also have important roles in inflammation and energy metabolism (16, 17). Accumulating evidence supports the theory that a high-energy diet, metabolic imbalance, and inflammation interact in states of over-nutrition (12, 18). Further research confirmed that consumption of HGD and HFD seems to be linked to unfavorable alterations in metabolic profiles that are associated with inflammation (19, 20).

HGD seemed have a greater impact on murine metabolism than HFD. Our study noted that HGD increased the diversity of metabolites in mice. Glucogenic (valine, glycine, serine, alanine, proline, and asparagine) and ketogenic (isoleucine, tyrosine, leucine, and phenylalanine) amino acids have substantially

2 http://www.metaboanalyst.ca

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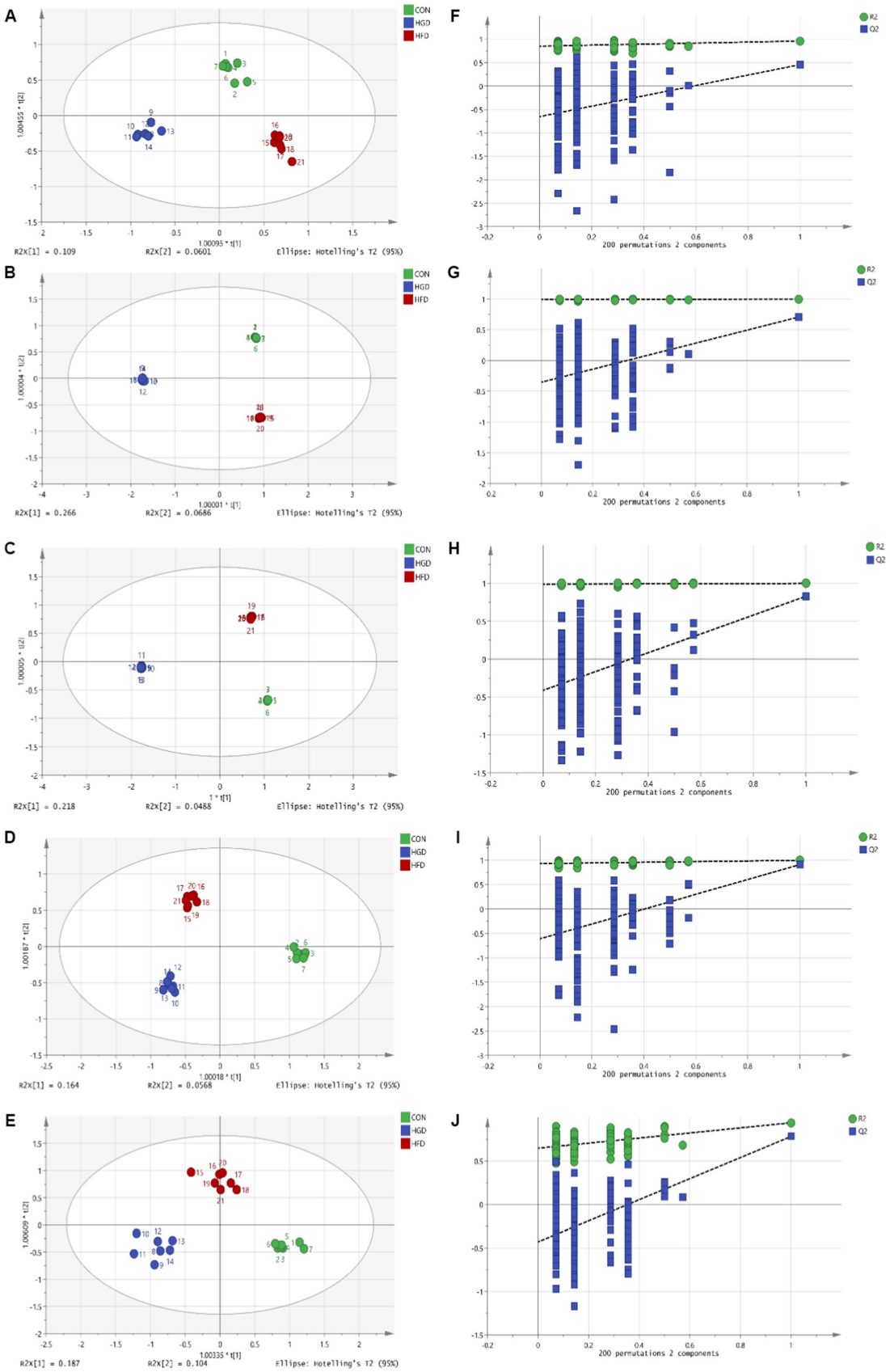

FIGURE 1
OPLS-DA scores (**A**: serum, **B**: heart, **C**: liver, **D**: brain, **E**: kidney) and 200 permutation tests (**F**: serum, **G**: heart, **H**: liver, **I**: brain, **J**: kidney) for two diet groups and a control group in the OPLS-DA models.

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TABLE 2 Metabolomics of target tissues in mice as a result of treatment with HGD and HFD.

	HGD				HFD			
Tissue	Metabolites	HMDB	VIP	Trend	Metabolites	HMDB	VIP	Trend
Serum	L-Alanine	HMDB0000161	2.23	↑	Lysine	HMDB0000182	2.52	↑
	Glutamate	HMDB0000148	3.04	↑	Glycine	HMDB0000123	1.59	↑
	Glycine	HMDB0000123	1.20	↑	Proline	HMDB0000162	1.58	↑
	L-Leucine	HMDB0000687	1.88	↑	L-Alanine	HMDB0000161	2.05	↑
	Tyrosine	HMDB0000158	2.08	↑	Tyrosine	HMDB0000158	1.81	↑
	L-Aspartic acid	HMDB0000191	1.45	↑	Glycerol	HMDB0000131	1.40	↑
	Propanoic acid	HMDB0000237	1.83	↓	Aminomalonic acid	HMDB0001147	1.12	↓
	Succinic acid	HMDB0000254	1.66	↑	Cholesterol	HMDB0000067	1.44	↑
	Myo-inositol	HMDB0000211	1.89	↑	Palmitate	HMDB0000220	1.48	↑
	d-Glucose	HMDB0000122	1.89	↑	Myo-inositol	HMDB0000211	1.59	↑
	Uric acid	HMDB0000289	1.36	↓				
Heart	L-Alanine	HMDB0000161	1.64	↑	d-Glucose	HMDB0000122	1.98	↑
	Hexanoic acid	HMDB0000535	1.48	↑	Inositol	HMDB0000211	2.12	↑
	L-Valine	HMDB0000883	1.65	↑	Adenosine	HMDB0000050	2.60	↑
	L-Isoleucine	HMDB0000172	1.55	↑	L-Alanine	HMDB0000161	1.46	↑
	Uracil	HMDB0000300	1.62	↑	Aspartic acid	HMDB0000191	1.10	↑
	Serine	HMDB0062263	1.48	↑	L-Threonine	HMDB0000167	1.78	↑
	Aspartic acid	HMDB0000191	1.52	↑	Hexane	HMDB0029600	1.06	↑
	L-Glutamic acid	HMDB0000148	1.62	↑	propanoic acid	HMDB0000237	1.21	↑
	Phenylalanine	HMDB0000159	1.42	↑	L-Glutamic acid	HMDB0000148	1.54	↑
	Tyrosine	HMDB0000158	1.08	↑				
	Myo-Inositol	HMDB0000211	1.37	↑				
	Adenosine	HMDB0000050	1.45	↑				
Liver	Alanine	HMDB0000161	1.13	↑	Valine	HMDB0000883	2.21	↑
	Acetamide	HMDB0031645	1.80	↓	4-Aminobutanoic acid	HMDB0000112	2.09	↑
	Succinic acid	HMDB0000254	1.55	↑	Myristic acid	HMDB0000806	2.32	↑
	L-Valine	HMDB0000883	1.33	↑	d-Proline	HMDB0003411	1.66	↑
	Urea	HMDB0000294	1.37	↑	Glycine	HMDB0000123	2.95	↑
	Glycine	HMDB0000123	1.37	↑	9,12-Octadecadienoic acid	HMDB0000673	2.87	↑
	Aspartic acid	HMDB0000191	1.21	↑	9-Octadecenamide	HMDB0002117	1.94	↑
	Pentadecanoic acid	HMDB0000826	1.15	↑				
	Galactinol	HMDB0005826	1.31	↑				
	D-Mannose	HMDB0000169	1.38	↑				
	D-Myo-Inositol	HMDB0000211	1.25	↑				
	Adenosine	HMDB0000050	1.16	↑				
Brain	Phosphoric acid	HMDB0002142	1.01	↑	L-Aspartic acid	HMDB0000191	2.44	↑
	Norleucine	HMDB0001645	1.83	↑	Uridine	HMDB0000296	1.45	↑
	L-Aspartic acid	HMDB0000191	1.57	↑	L-(+)-Lactic acid	HMDB0000190	1.37	↑
	Aminomalonic acid	HMDB0001147	1.55	↑	L-Alanine	HMDB0000161	1.39	↑
	Malic acid	HMDB0000744	1.21	↑				
	L-5-Oxoproline	HMDB0000267	1.09	↑				
	L-Phenylalanine	HMDB0000159	1.39	↑				
	Citric acid	HMDB0000094	1.12	↑				
	L-[+]-Lactic acid	HMDB0000190	1.21	↑				
	Phosphorylethanolamine	HMDB0000224	1.59	↑				
Kidney	Glycine	HMDB0000123	1.92	↑	L-Alanine	HMDB0000161	1.24	↑
	L-Leucine	HMDB0000687	1.13	↑	L-Leucine	HMDB0000687	1.53	↑
	L-Aspartic acid	HMDB0000191	1.40	↑	L-Valine	HMDB0000883	1.28	↑
	Tyrosine	HMDB0000158	1.56	↑	Pentanedioic acid	HMDB0000661	1.39	↑
	Glycerol	HMDB0000131	1.71	↑	Phosphoric acid	HMDB0002142	2.00	↑
	Xylitol	HMDB0002917	2.03	↑	Myo-Inositol	HMDB0000211	1.24	↑
	Phosphoric acid	HMDB0002142	1.26	↑				

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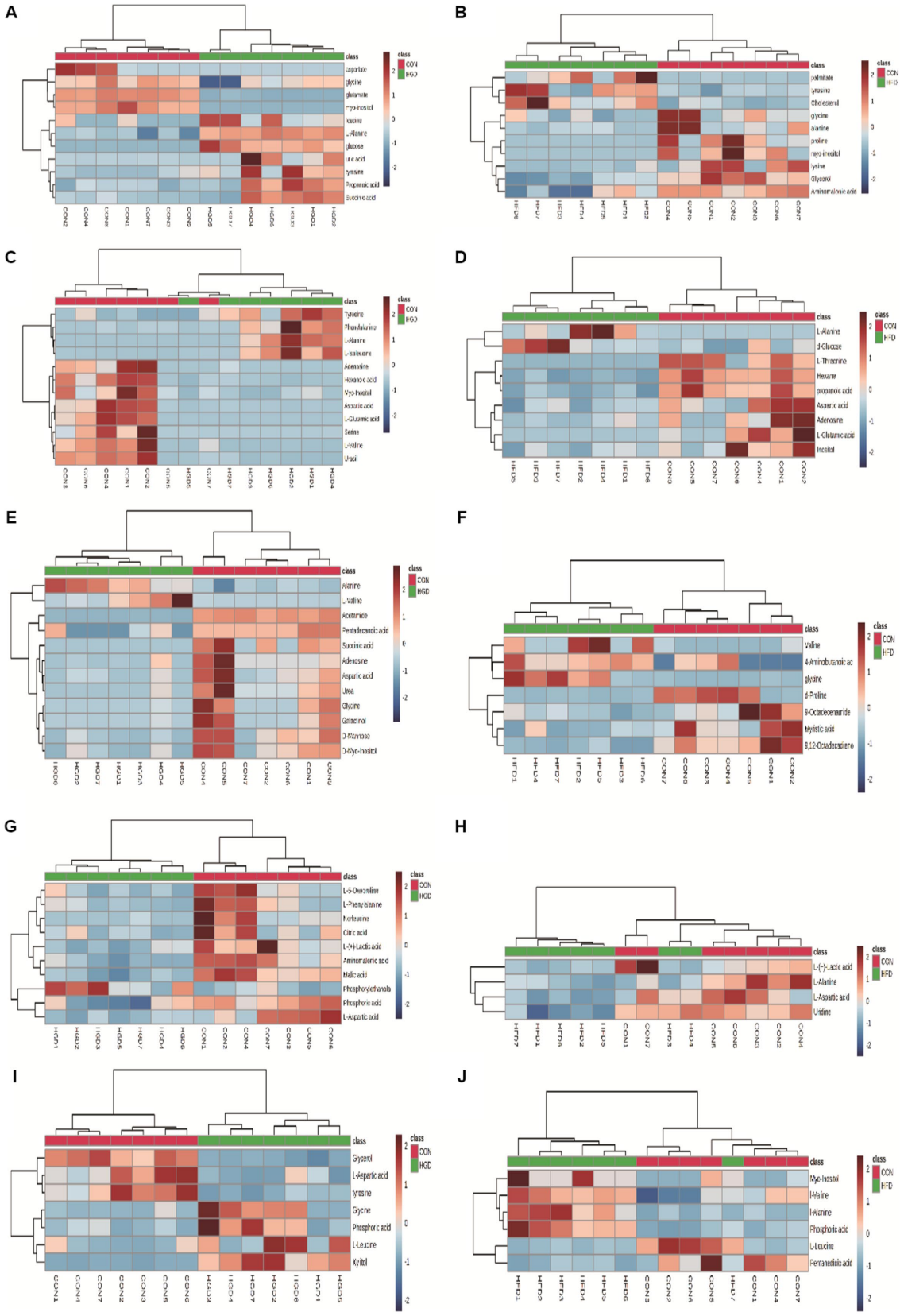

FIGURE 2
Heatmaps of metabolite expression differences between HGD and HFD groups: serum (**A**: HGD, **B**: HFD), heart (**C**: HGD, **D**: HFD), liver (**E**: HGD, **F**: HFD), brain (**G**: HGD, **H**: HFD), kidney (**I**: HGD, **J**: HFD). The shades of red and blue signify upregulation and downregulation; the darker the color, the greater the shift. Samples and metabolites are represented by columns and rows, respectively.

TABLE 3 Pathway analysis by MetaboAnalyst 5.0.

	HGD			HFD		
Tissue	Pathway	Raw p	Impact	Pathway	Raw p	Impact
Serum	Alanine, aspartate and glutamate metabolism	2.89E-05	0.42	Phenylalanine, tyrosine and tryptophan biosynthesis	2.63E-02	0.50
	Arginine biosynthesis	4.21E-03	0.12	Primary bile acid biosynthesis	3.51E-02	0.06
	Glutathione metabolism	1.65E-02	0.11			
	Glyoxylate and dicarboxylate metabolism	2.14E-02	0.11			
	Phenylalanine, tyrosine and tryptophan biosynthesis	2.89E-02	0.50			
	D-Glutamine and D-glutamate metabolism	4.31E-02	0.50			
Heart	Phenylalanine, tyrosine and tryptophan biosynthesis	3.46E-04	1.00	Alanine, aspartate and glutamate metabolism	4.49E-04	0.42
	Alanine, aspartate and glutamate metabolism	1.13E-03	0.42	Arginine biosynthesis	2.78E-03	0.12
	Phenylalanine metabolism	3.68E-03	0.36	D-Glutamine and D-glutamate metabolism	3.54E-02	0.50
	Arginine biosynthesis	5.02E-03	0.12			
Liver	Alanine, aspartate and glutamate metabolism	1.13E-03	0.22	Arginine and proline metabolism	1.20E-02	0.02
	Galactose metabolism	1.83E-02	0.04	Linoleic acid metabolism	2.31E-02	1.00
Brain	Alanine, aspartate and glutamate metabolism	1.37E-02	0.22	Alanine, aspartate and glutamate metabolism	1.96E-03	0.22
	Phenylalanine, tyrosine and tryptophan biosynthesis	2.63E-02	0.50			
Kidney	Phenylalanine, tyrosine and tryptophan biosynthesis	1.85E-02	0.50			

different related metabolites. Feeding mice a high-energy diet induces tissue-specific changes that ultimately result in the onset of metabolic disorders. For example, the heart is a "metabolic omnivore" and can utilize various fuel sources including fats, sugars, ketone bodies, lactate, and amino acids (21). Ketogenic amino acids can be utilized by the liver to form ketone bodies, which are then transported to other organs (22). Unlike the muscle and liver, brain tissue obtain energy from both ketones and glucose. Moreover, glycogenolysis and gluconeogenesis occur in the liver as well as in renal proximal tubules to maintain a steady energy supply to the brain (23).

Aromatic amino acids have a close relationship with body metabolism and can have a significant impact on liver function. Studies have shown that changes in plasma aromatic amino acids can be indicative of atypical liver function and enhanced protein catabolism (24). These changes are often associated with various health conditions, such as liver diseases, metabolic disorders, and cancer, and can be used in clinical diagnosis and treatment. In our investigation, oxidative stress-related metabolites including L-glutamic acid and serine were dramatically altered. A high-glucose diet has been shown to enhance oxidative stress in the livers of mice, potentially leading to diabetes and metabolic syndrome (25). Furthermore, in other disease models, elevated tyrosine, proline, glycine, and alanine are thought as indicators of mitochondrial malfunction (26).

Obesity is a non-communicable condition characterized by the buildup of excessive fat. Significantly, obesity and changes in the gut flora and metabolites are readily caused by a high-fat diet (27, 28). Glucose was the primary source of energy for HGD-fed mice, but lipids were utilized more often in the HFD group. HFD also disrupts normal cellular metabolic programming and perturbs the activities of the regulators of nutrient homeostasis, including mTOR, AMPK,

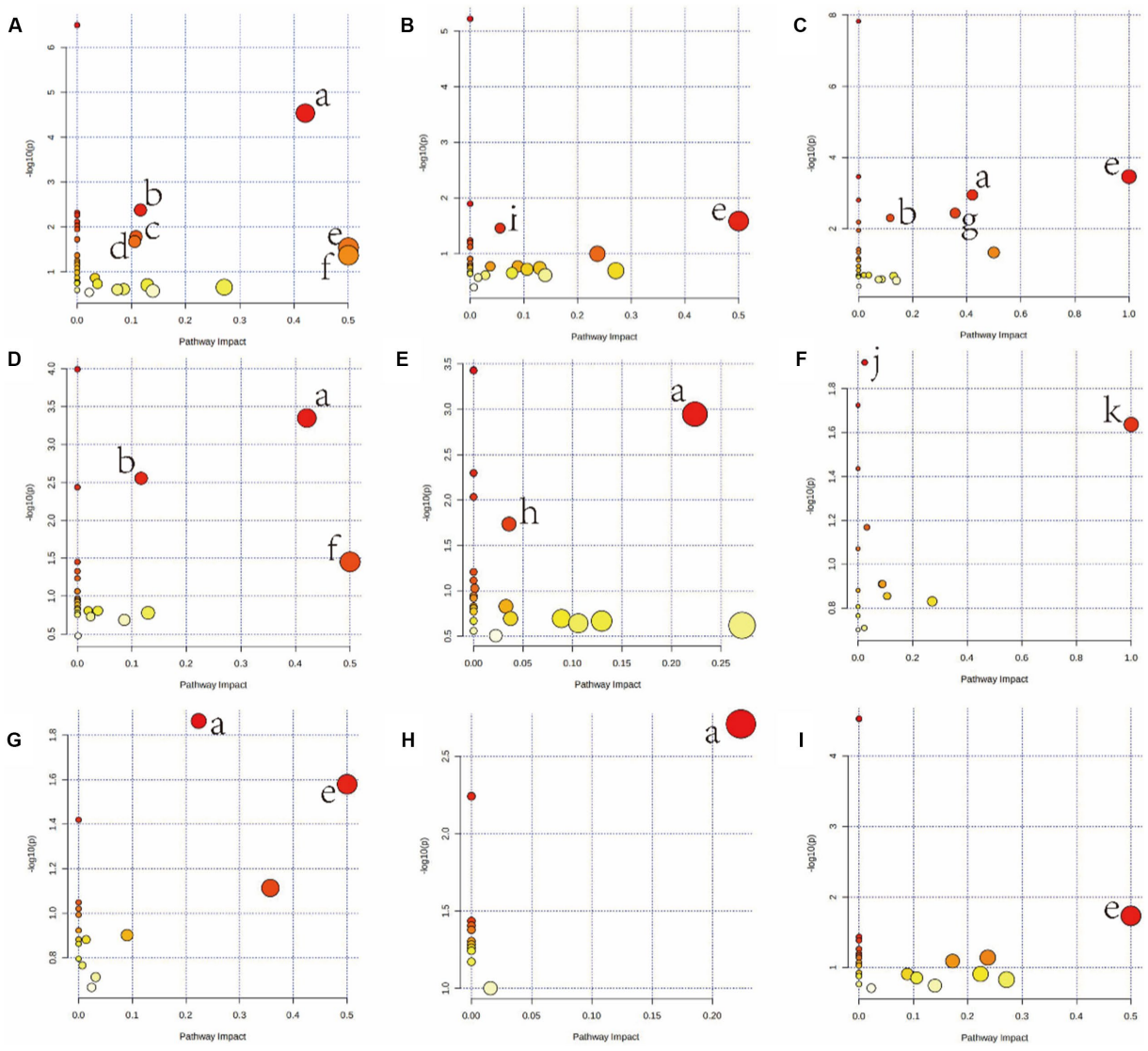

FIGURE 3
Summary of pathway analysis using MetaboAnalyst v5.0. Serum (**A**: HGD, **B**: HFD); heart (**C**: HGD, **D**: HFD); liver (**E**: HGD, **F**: HFD); brain (**G**: HGD, **H**: HFD); kidney (**I**: HGD). **(a)** Alanine, aspartate, and glutamate metabolism. **(b)** Arginine biosynthesis. **(c)** Glutathione metabolism. **(d)** Glyoxylate and dicarboxylate metabolism. **(e)** Phenylalanine, tyrosine, and tryptophan biosynthesis. **(f)** D-Glutamine and D-glutamate metabolism. **(g)** Phenylalanine metabolism. **(h)** Galactose metabolism. **(i)** Primary bile acid biosynthesis. **(j)** Arginine and proline metabolism. **(k)** Linoleic acid metabolism.

and CREB, which contribute to metabolic diseases (29–31). Our study has demonstrated that the lipid metabolism of blood, cardiac muscle, and liver is susceptible to the effects of HFD, resulting in alterations of lipid levels in these tissues. When mice were given high-fat diet, lipid metabolism was found to be greatly elevated, and genes linked to the production and breakdown of fat in the liver were shown to be significantly increased (32). In our study, for mice in HFD, most of the metabolites in tissues were related to lipid metabolism, such as glycerol, cholesterol, palmitate, myristic acid, propanoic acid, 9,12-Octadecadienoic acid, and myo-inositol, as well as few AAs. The saturated fatty acid palmitate can induce a mixed inflammatory response by upregulating inflammatory and endoplasmic reticulum (ER) stress genes, and increasing the expression of appetite-stimulating NPY (neuropeptide Y) neurons leading to difficulty in weight management (33). 9,12-octadecadienoic acid is able to reduce the risk of cardiovascular disease and exert a certain protection on the cardiovascular system (34). Myristic acid also produces a significant increase in systemic inflammatory response syndrome and shows potential as a biomarker in the diagnosis of septic patients (35). Moreover, myristic acid improves hyperglycemia and reduces body weight, and could be used in the treatment of diabetes (36).

In addition, long-term consumption of HFD affects brain health, decreases hippocampus volume, and impairs cognitive function (37, 38). In our study, HFD supplied alanine, glycine, and 4-aminobutanoic acid, which exert negative effects on the brain. This may explain the

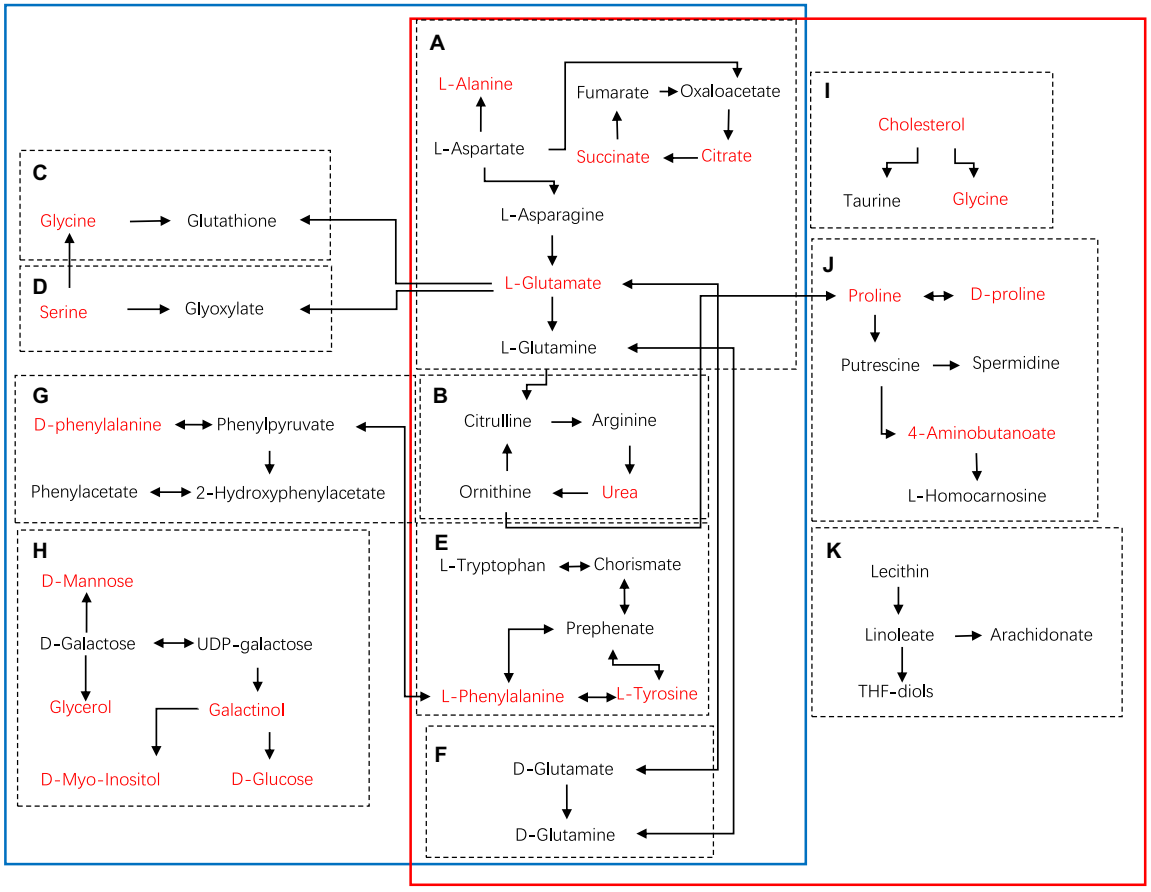

FIGURE 4
Schematic diagram of metabolic pathways using KEGG in main tissues (serum, heart, liver, brain, kidney) of HGD and HFD compared to the controls (blue solid line: HGD group; red solid line: HFD group). **(A)** Alanine, aspartate, and glutamate metabolism. **(B)** Arginine biosynthesis. **(C)** Glutathione metabolism. **(D)** Glyoxylate and dicarboxylate metabolism. **(E)** Phenylalanine, tyrosine, and tryptophan biosynthesis. **(F)** D-Glutamine and D-glutamate metabolism. **(G)** Phenylalanine metabolism. **(H)** Galactose metabolism. **(I)** Primary bile acid biosynthesis. **(J)** Arginine and proline metabolism. **(K)** Linoleic acid metabolism. Metabolites marked in red represent the significant biomarkers found in main tissues.

association between high fat intakes and increased incidence of depression and anxiety in individuals with obesity.

Finally, our findings show that whereas both HGD and HFD impacted tissue metabolomes, they exhibited different metabolic signatures. The dietary intervention had a significant impact on tissue metabolomes in mice, indicating that metabolomics might be a useful tool for detecting illness development in animal models. However, our research is not without limitations, and there is still much more to be explored in this field. In the next phase of our study, we plan to include additional groups, such as those following a high-fructose diet and a high-fiber diet, to further investigate the effects of different dietary components on the body's metabolism. Furthermore, we will concentrate more on the influence of varying nutrient consumption quantities on an individual's metabolic health.

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 Jining Medical University's ethics committee (Protocol #JNMC2020DWRM0076).

Author contributions

DX, YGu, and PJ designed the study. SZ, CG, YL, RY, YGa, and HL performed the experiments. DX, YZ, and XX analyzed the data. DX, YZ, ZR, and PJ wrote the manuscript. 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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1171806/full#supplementary-material

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Xanthophyll pigments dietary supplements administration and retinal health in the context of increasing life expectancy trend

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Introduction: Medicine faces nowadays the trend of increasing life expectancy of human population, with the resulting increase of degenerative age related diseases prevalence, combined with the risks of less tempered sun radiations environment exposure. Under these circumstances, our work pointed out on evaluating the effect of some xanthophyll pigments dietary supplements, actually widely recommended, for prevention of retinal degenerative damages and for slowing down the progression of such age related changes if they have already occurred. These dietary supplements are already well known for their total antioxidant activity, proven by photochemiluminescence method using Total Antioxidant Capacity in Lipid soluble-substances procedure.

Materials and methods: The study recruited a number of 120 subjects equally divided on genders. The lot included a first group of 60 patients with comparable ages (all of them over 50 years and divided in 2 segments of age: 50-60 and over 60) and suffering from comparable retinal age-related degenerative abnormalities (mild/ medium severity age-related macular degeneration according to Wisconsin Age-Related Maculopathy Grading System) , and a second group, considered control, including a similar number of healthy, normal retina subjects belonging to same age and gender categories. There were evaluated at baseline the eye medical status and the retinal risk by specific methods: complete eye check-up, Amsler grid, specific standardized questionnaires focused on visual function and its impact on the quality of current life. Both groups, patients and control, received similar dosages of xanthophyll pigments dietary supplements including lutein and zeaxanthin during 18 months after baseline; at the end of this supplementation period a new evaluation was conducted. In the second part of the research all subjects involved received a new dietary supplement in which the same xanthophylls were enriched with C and E vitamins and oligo-elements Zinc and Copper. At the end of three years duration supplementation, the subjects were reevaluated and the paper presents the conclusions on the matter, pointing on the impact of xanthophyll supplements on visual health.

Results: Correlation tests were applied to the complete set of data. Correlation tests have values between -1 and +1. The value -1 represents the negative correlation (reverse proportionality) meanwhile the value +1 represents the positive correlation (direct proportionality). The charts show the curves that are fitting experimental data. The dependence is linear in nature, and the value R2, as

it approaches more the value 1, represents a better match with the experimental data (the data are in a percentage of approximately 99% on these straight lines of type y = ax + b). In the charts, there were noted the average values of the scores for healthy control patients with "Control", and the average values of the scores for the patients with existing age related degenerative retinal pathology at baseline with "Patients".

Discussion: The retinal function and the impact of visual condition on health were both evaluated at baseline, 18 months and 36 months after baseline, by visual acuity, ophthalmoscopy fundus examination, Amsler test and by asking the subjects to answer the visual function questionnaires: EQ-5D, NEI-VFQ-25, as measures of health status quality and of the influence on welfare. The study revealed that under supplementation both control healthy subjects and patients with known degenerative retinal pathology included in the 50-60 years of age group evolved almost the same way, leading to the conclusion that administered xanthophyll pigments-based supplements, simple or enriched, managed to slow down the progression of abnormal degenerative vision loss to a rate comparable to physiological aging-related vision loss. It was also observed that intake of xanthophyll pigments dietary supplements preserved the general health condition and maintained relatively constant vision on the entire 36th months follow-up research duration in patients presented with existing age related degenerative retinal pathology at baseline. For healthy subjects, evaluation showed an improvement in results after dietary supplementation, with maintenance of constant vision and a significantly increase of general condition, in a positive sense. For subjects over the age of 60 dietary supplements intake was even more effective compared to younger group in providing better control of degenerative processes.

KEYWORDS

xanthophyll pigments, dietary supplements, visual health, retina, degeneration

1. Introduction

The role of food in preserving ocular health was mainly ignored for many years except for the known classical recommendation of consuming carrots. The focus of researchers on detecting valuable correlations of nutrition, dietary supplements intake and ocular health developed quite recently, with encouraging results despite the small volume of definitive data. Antioxidants, carotenoids or various nutrients might have a good impact on pathology related to aging processes of the ocular tissues, ranging from macular degeneration to dry eye syndrome (1).

Though it would be obviously premature to develop distinctive guidelines on dietary supplements targeted at those worried about the quality and preservation of visual function, what is effective in prophylaxis of cardiovascular disease and cancer might also work in combating visual deterioration, as the identical mechanism of oxidative stress, common in the pathogenesis of cardiac pathology and various age-related conditions, seems to alter ocular health. The mechanisms of occurence of many ocular ageing-related phenomena are as yet incompletely elucidated, however there is nowadays increasing acceptance of the impact of certain factors in the development of these phenomena: oxidative aggression, inflammation, toxicity from blue light cumulated exposure, retinal pigment epithelium (RPE) cells malfunction, poor blood irrigation in the foveal choroid (1).

The idea that oxygen, the vital need for all living organisms, is equally associated with a toxic potential gets more and more widely clarified and agreed. There is increasing data supporting the destructive influence of reactive oxygen intermediates (ROI) in pathologies related to ageing of ocular tissues, the notion of oxidative stress including all damage caused by unstable and reactive oxygen metabolites (2).

Oxidative stress occurs when imbalances occur in the body between the production of reactive oxygen (including free radicals) and the detoxification capacity of reactive intermediates. Basically, when a cell is exposed to more reactive oxygen compounds, it can degrade.

Thus, oxidative stress contributes to ageing processes and the pathophysiology of degenerative diseases.

Depending on their chemistry, their origin location, their tropism onto certain targets in the body, their allegiance to the free radical or non-radical subgroups, ROI can be categorised. There are many types of free radicals. In humans, the most significant are free oxygen radicals (reactive oxygen species). Examples include singlet oxygen, hydrogen peroxide, superoxides and hydroxyl anions. There are two common forms of free radicals: reactive oxygen species (ROS) and reactive nitrogen species (RNS). Examples of ROS include: superoxide anion, hydrogen peroxide, highly reactive hydroxyl radical and peroxyl radical. RNS are often considered a subclass of ROS and include: nitric oxide, nitrous oxide, peroxynitrite, nitroxyl anion and peroxynitrous acid.

Free radicals are atoms or molecules that contain odd electrons, which tend to reach chemical stability. The process can involve a number of reactions. When a free radical 'steals' an electron from a molecule, that molecule becomes a free radical because it is missing an electron - and so on, generating a veritable cascade of cytotoxic reactions. Free radicals can damage the body's DNA, which contains genes as well as proteins, lipids, cell membranes, causing disease. Antioxidants help to maintain physiological levels of free radicals to maintain their physiological function and prevent pathological effects caused by the action of oxidative stress, as this is precisely the state of imbalance between ROS and the properties of antioxidants. In these circumstances, ROS outperform antioxidants due to increased levels, deficient antioxidant defence or a combination of the two, attacking biological structures (3).

The retina is particularly exposed to altering by ROI aggression (4, 5). This particular sensitivity is due to several factors. A first such factor is the very high oxygen consumption in the retina. In addition to this, there is the massive presence of polyunsaturated fatty acids (PUFA) in the photoreceptor structure of the retina. The chemical structure of PUFA includes hydrogen atoms, which provide an electron, the perfect target for ROI and thus for oxidative destruction. In addition, by definition, retinal function involves photosensitization phenomena under the action of visible radiation, which is certainly ROI-producing. The phagocytosis function of the RPE also is the one that generates hydrogen peroxide (non-radical species).

Lutein and its stereoisomer, zeaxanthin, belong to the carotenoids xanthophyll group.

Compared to hydrocarbon carotenoids, for example β-carotene and lycopene, lutein and zeaxanthin have two hydroxyl groups, on both sides of the molecule. Both groups have an essential contribution regarding their biological role and in identifying appropriate chemical methods for the determination of these xanthophylls.

Xanthophylls such as Lutein (3R, 3′R, 6′R)-β,ε-carotene-3,3′-diol), MW: 568.88 g/mol and Zeaxanthin all-trans-(3R, 3′R)-β-carotene-3,3′-diol), MW: 568.9 g/mol, with identical molecular formulas (C40H56O2), isomers, but not stereoisomers, distinguished strictly by location of the double bond in one final ring, emphasize a specificity given by their presence as carotenoids in certain eye tissues, being strongly represented in the macula, a little portion of the retina in charge with central vision and visual accuracy. Lutein and zeaxanthin exist in the lens, another eye tissue essential for vision (6). Currently, macular degeneration is the leading factor for vision loss in populations from developed regions, defined as progressing, degenerative, non-reversible damages of the central retinal zone (macula), which is responsible for detailed vision. It has a yellowish coloration given by a yellow pigment. Macular degeneration develops progressively affecting over 5% of people over 65 years of age, so that it tends to become a public health problem of the 21st century.

The dry form of macular degeneration has no effective treatment yet but is responsive to nutraceuticals including vitamins and minerals or lutein and zeaxanthin, as major carotenoids concentrated in the macula of human retina (6). Lutein and zeaxanthin are known as plants generated pigments, found in yellow to reddish colour fruits and vegetables. Both are chemically pretty alike, very slightly differentiated by the atomic layout of the molecules. Each are strong antioxidants and provide plenty of positive impacts on health condition, specially known for eyes protection (6, 7).

Lutein is present in many biological systems, such as bacteria, algae, yeasts, plants, usually existing in flowers, grains, fruits and vegetables (8, 9). Meanwhile lutein is present in foods of animal and fish origin, and as a pharmacy nutritional supplement, so that we can refer to a multiple choice lutein market (10). Carotenoids are very healthy due to their high antioxidant activity (11). Lutein and zeaxanthin prevent and limit the ocular damage from UV radiation and contribute decisively for brain development (12–14). Other carotenoids have the capacity to prevent the appearance of low-density lipoprotein (LDL) and thus contribute towards heart protection (15–17).

1.1. Oxidative stress has major impact in the age-related macular degeneration

Pathogenesis (18, 19). Some studies emphasize the antioxidative capacity of astaxanthin, zeaxanthin, lutein, along with ascorbic acid and tocopherol acetate, by various procedures including spectrophotometric, fluorimetric and chemiluminescence methods, confirming that xanthophylls have an increased antioxidant potential. Due to this property, they can be assigned the first option position in combating retinal oxidative damage, an important step in preventing or slowing down the progression of AMD (20).

Zeaxanthin, a non-provitamin A carotenoid similar to lutein, was demonstrated to have significant positive impact on human health due to its capacity to capture and neutralize free radicals, providing antioxidant effects and reducing inflammation. This carotenoid presents beneficial impact on eye, skin, liver and cardiovascular health (21).

Literature emphasizes that lutein and zeaxanthin have a special affinity for RPE (retinal pigment epithelium) cells (22). Moreover, it was found that ARPE-2 cells (a human RPE cell line), same as RPE cells had a double affinity for lutein and zeaxanthin compared to the affinity for beta-carotene, when treated with 3 different pigments (23).

Macula is a specialized region in the retina of humans, centered by the foveola, which provides the clearest vision. Due to their major presence inside macular retina, lutein and zeaxanthin provide a protector shield against blue light (24). They behave as powerful antioxidants and neutralize ROS resulting from photoexcitation (25, 26). Degeneration processes can damage macula, mostly in subjects over 65 years of age. This risk highlights the importance and necessity to increase the dietary intake of lutein as a strategy to reduce the incidence of macular degeneration and cataracts (25, 27–29).

The carotenoid profile of human cells is known to include six such pigments; to those already mentioned above are added α-carotene, β-carotene, β-cryptoxanthin and lycopene (30, 31). Human plasma includes several such pigments, depending on diet, while few pigments can concentrate in certain tissues, as is the case with lutein and zeaxanthin in macula lutea (32–36).

The possibilities to identify, quantify and monitor the antioxidative activity of various molecules *in vivo* are reduced due to the unavailability of suitable biomarkers. For the moment investigators have no method to evaluate oxidative stress reaction and total antioxidant activity in the animal kingdom. All that can be detected and measured so far are lipoprotein fragments from animals or humans that have consumed carotenoids through food

or supplementation. This method has been used by researchers quite recently and involves introducing carotenoids into the LDL molecule or target membrane. It has been found that increasing carotenoid intake through increased fruit and vegetable intake or supplementation decreased the degree of oxidation of LDL particles (37–39).

However, it should be noted that higher consumption of fruit and vegetables increases plasma levels not only of carotenoids, but also of vitamin C, polyphenols and flavonoids, which are also agents with antioxidative activity and to which any decrease in LDL oxidation can be attributed (40). Other research has demonstrated the presence of antioxidant activity also in the case of lycopene and other carotenoids (41), with the caveat that some authors found that the association of lutein or lycopene to beta-carotene, with already demonstrated antioxidant efficacy, paradoxically resulted in an amplification of LDL oxidation (42).

Consequently, carotenoids known as tetraterpenoids behaved as a shield to protect photosynthetic structures against oxidative stress induced by ROS (43). Their functions in nature are multiple, involving growth, signaling oxidative stress, determining sex-linked color patterns or constituting a precursor of vitamin A for numerous vegetal varieties (44–46). It is precisely because of these defensive capacities that carotenoids have led to the idea of a correlation between their concentrations in the body and the prophylaxis or therapy of many types of pathology (47–50).

Other studies revealed that lutein has higher antioxidative activity than different carotenoids, both *in vivo* and *in vitro*, being able to neutralize superoxide and hydroxyl radicals, and to block lipid peroxidation (51).

Lutein also significantly decreased the destructive impact of oxidative stress by reducing membrane permeability for oxygen (52). Other research has demonstrated the superior antioxidant efficacy of lutein compared to β-carotene in combating auto oxidation of lipids in cell cultures (53). Lee and collaborators have shown the anti-inflammatory and immunosuppressive effect of dietary lutein (54, 55). Studies have also shown the antioxidant defensive action of lutein on liver cells in humans (56, 57).

Starting from scientific literature regarding a few studies on total antioxidant capacity through photo chemiluminescence method, applied of two type antiaging vegetal dietary supplements such as Lutein, Zeaxanthin (58–66) the goal of this study was to highlight variations of treatment response potentially connected to different antioxidative potential of concerned antiaging vegetal dietary supplements, consisting in soft capsules based on vegetal pigments (Lutein 10 mg + Zeaxanthin 2 mg), or same active principles combined with vasoprotective and antioxidant capacity components (vitamin C, vitamin E, Zinc and Copper) (67–71).

2. Materials and methods

In this study the impact of xanthophyll pigments dietary supplements administration has been studied by evaluating the retina, both morphological and functional.

Two groups of subjects were studied.

The first group included 60 subjects with mild/medium severity macular retina abnormalities - early age-related macular degeneration (AMD) according to Wisconsin Age-Related Maculopathy Grading System (AREDS 11- step severity scale), including 26 subjects (13 women +13 men) of 50–60 years of age and 34 subjects (19 women +15 men) older than 60 years.

The second group (control group) consisted of 60 subjects with healthy eyes: 30 subjects (15 women +15 men) of 50–60 years of age and 30 subjects (15 women +15 men) older than 60 years.

Both groups of subjects received xanthophyll pigments dietary supplements containing 10 mg of Lutein +2 mg of Zeaxanthin, for 18 months after baseline. Afterwards, the retinal function and the impact of visual condition on health were evaluated by asking the subjects to answer the visual function questionnaires.

In the second part of the study, the same groups of subjects received a different xanthophyll pigments dietary supplement, containing the same active principles as the first one, enriched with vitamin C, vitamin E, Zinc and Copper, for another 18 months duration.

At the end of this second supplementation period, a new evaluation was conducted.

The retina, as first peripheral nervous structure which provides visual sensation, was evaluated by visual acuity, visual field, Amsler Test, fundus camera images and macular pigment optical densitometry (MPOD apparatus). In order to quantify the impact of administered xanthophyll pigments dietary supplements on preservation of retinal abilities, subjects were asked to answer The EuroQol EQ-5D-5L Questionnaire. They were also asked to score on The EuroQol Visual Analogue Scale EQ-VAS Questionnaire. The 25 Items Visual Function Questionnaire VFQ- 25 was also applied to all involved subjects. All these questionnaires offer preference-based measure of health status which is frequently used in clinical trials, observational studies and other health surveys. They are standardized measures of health status, here including also the visual function, developed in order to provide a simple, generic measure of health for clinical and economic appraisal (72).

Resulting scores were afterwards collected, summarized and statistical analysis was carried out.

3. Results

Correlation tests were conducted on the complete set of data. Correlation tests have values between −1 and + 1. The value −1 represents the negative correlation (reverse proportionality) meanwhile the value +1 represents the positive correlation (direct proportionality). The charts show the curves that are fitting the experimental data. The type of dependence is linear in nature, and the value R^2, as it approaches more the value 1, represents a better match with the experimental data (practically shows that the evolution of the data is of a linear type, and the data are in a percentage of approximately 99% on these straight lines of type $y = ax + b$).

In the following charts, there were noted the average values of the scores for healthy subjects with "control," and the average values of the scores for the patients with existing age related degenerative retinal pathology at the time of presentation to the ophthalmologist, with "patients."

The first chart represents the evolution of 1st score for the 50–60 years age category, starting from the day of the first eye check-up until the end of the 36th months xanthophyll pigments-based dietary supplementation. The value R^2 shows a match of the experimental data with the fitting curve in proportion of 99% for the control and 98% for the patients, and the value of 'b' from the equation

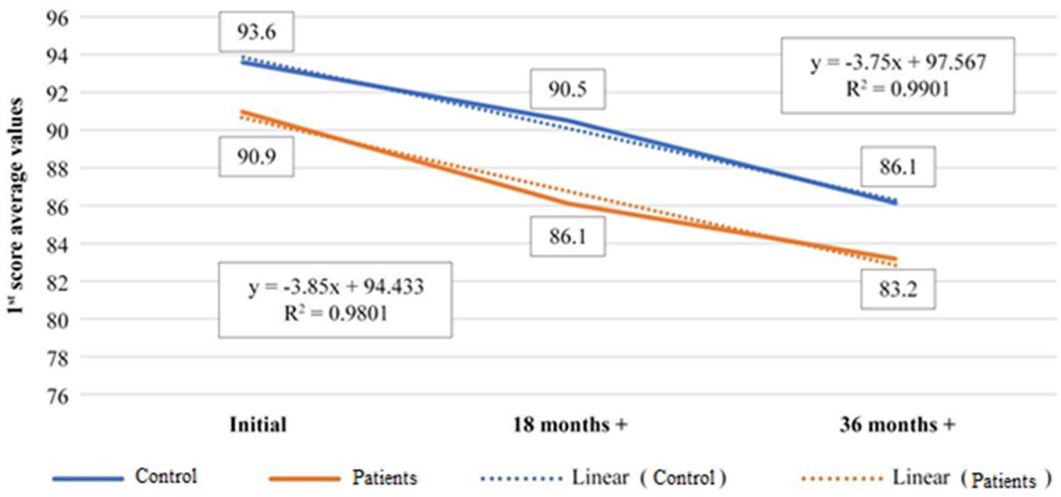

FIGURE 1
Representation of evolution of 1st score average value, during 36th months treatment time for 50–60 years old age category, control versus patients.

TABLE 1 Average values of 1st score for 50–60 years old age category, control versus patients.

50–60 years old		Initial	18 months+	36 months+
	Control	93.6	90.5	86.1
	Patients	90.9	86.1	83.2

TABLE 2 Positive correlation between control and studied patients (50–60 years old, age category).

		Patients
Control	1	
Patients	0.971039	1

TABLE 3 Negative correlation between control 1st and 2nd score (50–60 years old, age category).

	1st Score	2nd Score
1st Score	1	
2nd Score	−0.88192	1

y = ax + b, represents the slope of the fitting curve. This means, the evolution of the data is almost similar, the slope difference of the linear fitting curve being very small, b = 97.567 for control and b = 94.433 for the studied patients (Figure 1; Table 1).

The correlation between the patients and controls in the age group 50–60 years is strongly positive (0.97; Table 2), which means that the two curves are correlated, meaning that both healthy subjects and those with known pathology evolve in the same way as a result of the administered dietary supplementation.

The second chart refers to the relationship between the 1st score and the 2nd score for the same age group 50–60 years old. The data correspond to a linear evolution of the 1st score in proportion of 99%, while for the 2nd score the match with the linear evolution is 85%. The correlation test run for the two parameters for the controls, category 50–60 years old, shows a high negative correlation of the two scores for them which means that 1st score and 2nd score are in reverse proportionality relation.

The correlation coefficient is (−0.88; Table 3) while the maximum negative value can be (−1; Figure 2; Table 4).

It was observed that the general health condition associated with vision remains relatively constant for patients presented to the doctor with present pathology, while for healthy subjects (controls), 2nd score shows an improvement in results after supplementation, with a significantly increase of general condition, in a positive sense.

Figure 3 refers to the entire set of data, controls and patients, both categories of age, 50–60 and 60+ years old. The average of 1st score over the 36th months dietary supplementation time, are fitting the linear evolution in percentage of 99% in both controls and patients. The slope of the fitted curve is slightly higher for the patients (b = 88.22) than for controls (b = 92.56). That means that the dietary supplementation as a whole is less efficient in patients with known pathology than in healthy subjects, but the difference is not significant, as the average t-test shows. The correlation coefficient is 0.99 (Table 5), meaning the data are in a strong positive correlation, both, controls and patients evolve similarly under dietary supplementation (Figure 3; Table 6).

Figure 4 refers to the evolution of the 1st score for the age category 60+ years old. The comparison made between controls and patients average values of the 1st score over the entire dietary supplementation period, is fitting the linear evolution in proportion of 99%, for both categories. The slope of the fitting curves for the patients is higher (b = 89.967) than in controls (b = 92.867), but the data are positively correlated to 99% (Table 7), meaning that both sets of data analyzed are in direct relation and the results are closely dependent (Figure 4; Table 8).

Figure 5 shows linear dependence of 1st score with a match of 99%, but the slope of the 60+.

age category curve is lower (b = 91.867) than 50–60 age category (b = 97.567). This means that dietary supplementation is more effective in the case of 60+ age category, or that degenerative processes slow down with age (presumably due to age slowing down local metabolism), the dietary supplementation having better result in subjects over 60 years old. The correlation of the results of 1st score for the two age categories is also highly positive (0.997; Table 9), meaning that there is a direct proportionality between them and the

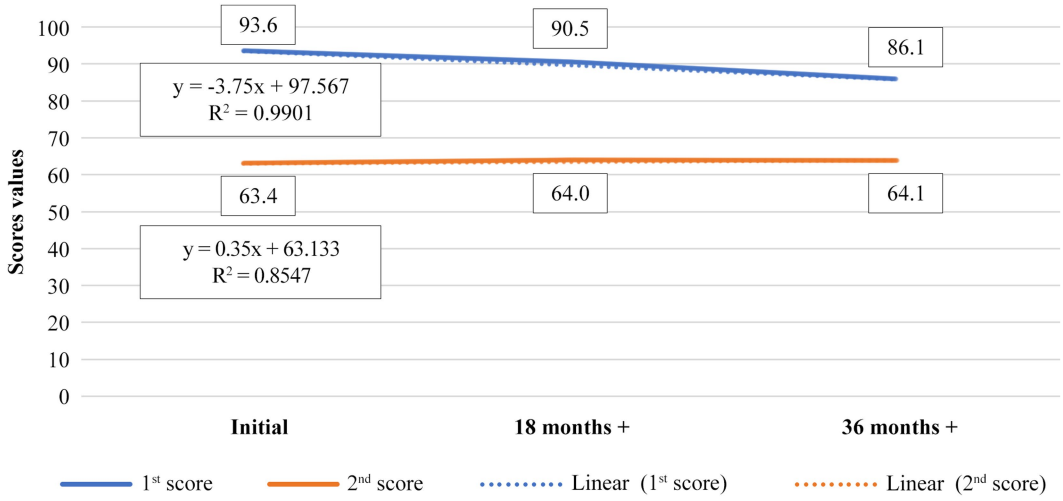

FIGURE 2
Representation of evolution of 1st score average value versus 2nd score average value, during 36th months treatment time for control, 50–60 years old age category.

TABLE 4 Average values of 1st and 2nd score for 50–60 years old age category, control.

Control 50–60 years old		1st Score	2nd Score
	Initial	93.6	63.4
	18 months+	90.5	64
	36 months+	86.1	64.1

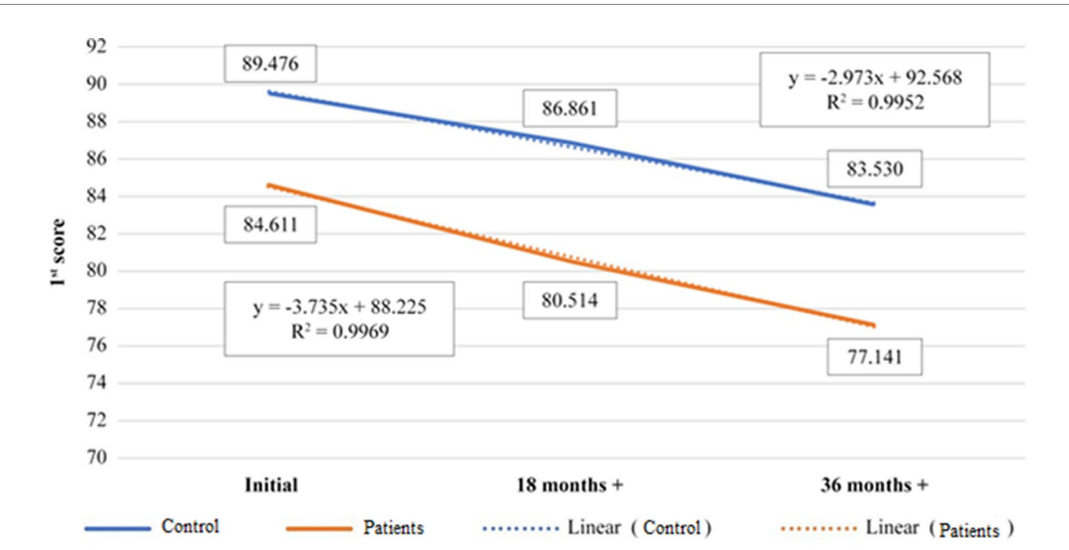

FIGURE 3
Representation of evolution of 1st score average value, control *vs* patients, during 36th months treatment time, 50–60+ years old age category.

TABLE 5 Positive correlation between control and patients 1st score (50–60+ years old).

50–60+ years old	Control	Patients
Control	1	
Patients	0.992159	1

TABLE 6 Average values of 1st score for 50–60+ years old age category, control vs. patients.

50–60+ years old		Control	Patients
	Initial	89.476	84.611
	18 months+	86.861	80.514
	36 months+	83.53	77.141

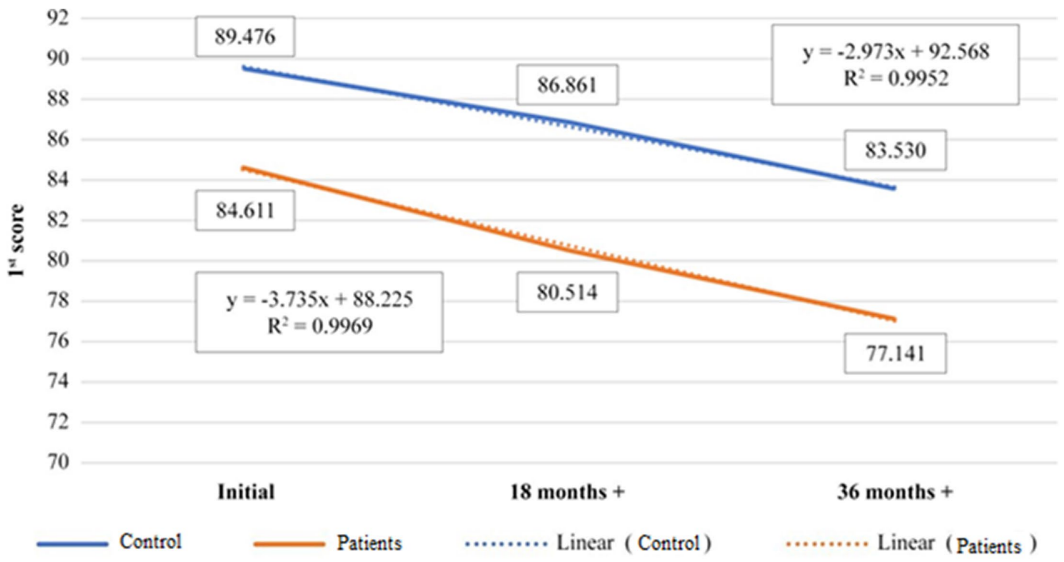

FIGURE 4
Representation of evolution of 1st score average value, control versus patients, during 36th months treatment time, 60+ years old age category.

TABLE 7 Positive correlation between control and patients 1st score (60+ years old).

	Control	Patients
Control	1	
Patients	0.999996	1

TABLE 8 Average values of 1st score for 60+ years old age category, control *vs* patients.

Category 60+ years old		Control	Patients
	Initial	89.5	85.8
	18 months+	87.3	81.9
	36 months+	84.85	77.6

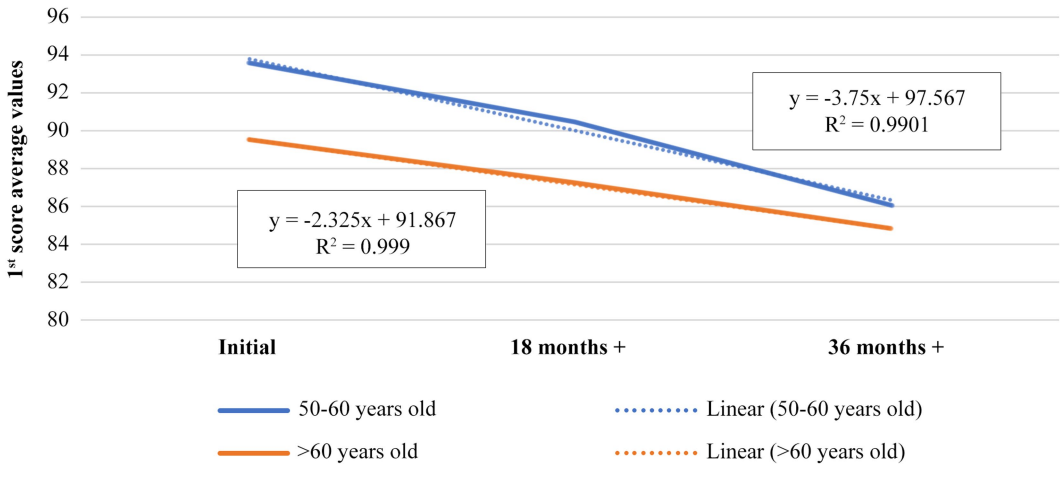

FIGURE 5
Representation of evolution of 1st score average value for patients, during 36th months treatment time, 50–60 versus 60+ years old age category.

TABLE 9 Positive correlation between the values of the patients 1st score, 50–60 versus 60+ years old category.

1st score	50–60 years old	60+ years old
Patients 50–60 years old	1	
Patients > 60 years old	0.99764	1

TABLE 10 Average values of 1st score for patients, 50–60 vs. 60+ years old age category.

1st Score	Patients	Initial	18 months+	36 months+
Patients	50–60	93.6	90.5	86.1
Patients	>60	89.5	87.3	84.85

evolution is positive for both, but the results are better for 60+ age category (Figure 5; Table 10).

Score 2 for the patients (subjects with existing degenerative retinal pathology) evolves upwards (in a negative sense) for both age categories, with a faster evolution in the age period 50–60 years. After 60 years the rate of deterioration of health in relationship with sight, is somewhat slower. Score 3 has an almost identical evolution in both age categories, the deterioration of near vision being maintained in approximately equal rates in patients.

Score 1 starts from significant differences between the patients and controls and evolves in the sense of health deterioration for both categories (with present pathology and those without initial pathology), with a slightly lower rate for controls than those with initial pathology.

The differences between the groups remain significant even after 36 months dietary supplementation, the degradation is higher in those who initially presented retinal degenerative pathology, and the data are in a positive correlation.

Score 2 shows an increasing evolution in the sense of decreasing health and vision with age, more pronounced at patients, while the controls 2nd score remains relatively constant throughout the supplementation, proving that xanthophyll pigments dietary supplementation manages to maintain the initial retinal state of healthy subjects throughout the study.

Score 3 shows a relatively constant evolution for the controls, which proves efficacy of xanthophyll dietary supplementation, and an increase in the patients, the growth rate being relatively small, although the difference between the initial and final state is significant.

4. Discussion

The retinal function and the impact of visual condition on health were both evaluated at baseline, 18 months and 36 months after baseline, by visual acuity, ophthalmoscopy fundus examination, Amsler test and by asking the subjects to answer the visual function questionnaires: EQ-5D, NEI-VFQ-25, as measures of health status quality, and of the influence on welfare.

The applied xanthophyll pigments-based dietary supplementation proves to be effective in case of people with existing degenerative retinal pathology, managing to keep a linear evolution of their visual health condition, the speed of progressive deterioration being slowed down. Under supplementation the vision decrease was done gradually with reduced speed, near to the age-related physiological rate.

It was no possible to compare the visual status evolution between subjects which received xanthophyll pigments dietary supplements and subjects who did not, because the authors of the research considered it unethical to deprive any study participant of a potentially beneficial and side effects-free supplement. However, the evolution of the 1st score, with strongly positive correlation and almost similar slope of the fitting curves for both patients and controls in the age group of 50–60 years showed that both healthy subjects and patients with known degenerative retinal pathology evolved almost the same way as a result of the administered xanthophyll pigments-based, simple or enriched, dietary supplements. This revelation of our data analysis brings a strong argument in favor of considering xanthophyll pigments dietary supplements a credible and necessary resource in the management of age degenerative retinal damages.

It was also observed, according to the 2nd score, that intake of xanthophyll pigments dietary supplements (both simple and enriched) preserved the general health condition and maintained relatively constant vision on the entire 36th months research duration for the patients presented to the doctor with existing age related degenerative retinal pathology at baseline. For healthy subjects, 2nd score showed an improvement in results after dietary supplementation, with a significantly increase of general condition, in a positive sense.

According to the average t-test, despite the fact that xanthophyll pigments dietary supplements as a whole are less efficient in patients with known retinal pathology than in healthy subjects, the difference is not significant, observation which leads us once more to the idea of a real positive effect.

Subjects who initially did not show retinal degenerative changes maintained their health and constant vision during xanthophyll pigment dietary supplements intake duration.

The studied dietary supplementation had better results for people over the age of 60, where the degenerative processes were better controlled under xanthophyll pigments intake, compared to younger groups. An explanation of this finding might be that retinal degenerative processes slow down with age (presumably due to age slowing down local metabolism). All the more this should encourage the administration of dietary supplements.

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 studies involving human participants were reviewed and approved by Institutional Review Board Ethics Committee of Ovidius University Constanta. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SJ, TN-P, MMH, and B-SN-P were involved in literature research and wrote the manuscript. MV supported the statistical analysis and reviewed the results. SJ, TN-P, and B-SN-P conceived, planned, and followed the execution of the experiments. SJ and VC provided patient samples. All authors contributed to the article and approved the submitted version.

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.2023.1226686/full#supplementary-material

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Modulation of immune response by nanoparticle-based immunotherapy against food allergens

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The increasing prevalence of food allergies worldwide and the subsequent life-threatening anaphylactic reactions often have sparse treatment options, providing only symptomatic relief. Great strides have been made in research and in clinics in recent years to offer novel therapies for the treatment of allergic disorders. However, current allergen immunotherapy has its own shortcomings in terms of long-term efficacy and safety, due to the local side effects and the possibility of anaphylaxis. Allergen-specific immunotherapy is an established therapy in treating allergic asthma, allergic rhinitis, and allergic conjunctivitis. It acts through the downregulation of T cell, and IgE-mediated reactions, as well as desensitization, a process of food tolerance without any allergic events. This would result in a protective reaction that lasts for approximately 3 years, even after the withdrawal of therapy. Furthermore, allergen-specific immunotherapy also exploits several routes such as oral, sublingual, and epicutaneous immunotherapy. As the safety and efficacy of allergen immunotherapy are still under research, the exploration of newer routes such as intra-lymphatic immunotherapy would address unfulfilled needs. In addition, the existence of nanoparticles can be exploited immensely in allergen immunotherapy, which would lead to safer and efficacious therapy. This manuscript highlights a novel drug delivery method for allergen-specific immunotherapy that involves the administration of specific allergens to the patients in gradual increasing doses, to induce desensitization and tolerance, as well as emphasizing different routes of administration, mechanism, and the application of nanoparticles in allergen-specific immunotherapy.

KEYWORDS

allergen immunotherapy, food-induced anaphylaxis, oral immunotherapy, sublingual immunotherapy, nanoparticles

1 Introduction

Allergy is an immune system response, an IgE-mediated hypersensitivity reaction that is triggered upon exposure to antigens that are known as allergens. Allergens can be found in the form of faunal products (dander and house dust mites), food, drugs, or flora. Among these, food allergens are perilous, which makes food allergies one of the most prevailing conditions that are life-threatening. Food allergy is a common condition that affects up to 10% of the general population (1). Food allergy, a type 1 hypersensitivity reaction, arises when the immune system attacks the allergens, due to the presence of IL-4 in T cells. Following that, the T cells differentiate into Th2 cells. Then, these Th2 cells produce IL-4, IL-5, and IL-13, which leads to the production of IgE (2) (3). Then, during the elicitation phase, the allergen attaches to IgE and couples with FcRI on the surface of the effector cells (mast cells, eosinophils, and basophils), initiating a rapid release of pro-inflammatory mediators such as histamine and leukotrienes, which induce the allergy symptoms (4). In a recent epidemiological study involving 333,200 children in the US, the occurrence of food allergy was found to be 6.7%. The current scenario shows that there is an increasing prevalence of food allergies, both in children and adults, with life-threatening allergic reactions. However, the refrainment of food allergens and the first-line treatment of anaphylaxis with adrenaline symbolize the current standard of care in food allergy (5). In this scenario, allergen immunotherapy (AIT) plays a pivotal role in the treatment of food allergen-induced anaphylaxis, as food avoidance is ineffective, difficult, and could cause a deterioration to patients' quality of life, with regard to persistent IgE-mediated food allergies. Even though AIT is a curative, permanent curation, it is difficult to achieve. The main aim of FA-AIT (food allergen-allergen immunotherapy) is sustained unresponsiveness that would help patients to consume normal food without any allergy exposure (6).

The disadvantages of older approaches of treatment are not much effective due to lower bioavailability. The use of nanoparticles has produced sensitive, cost-effective, time-consuming, and selective methods that can replace conventional methods used in recent years. However, each nanomaterial has demonstrated a distinct potential for certain allergens or classes (7). It helps to design novel allergen immune therapy strategies. Thereby, it has an significant impact on the safety, efficacy on the food allergen induced allergic reaction (8). In addition, allergen-delivery systems based on nanoparticles are now possible because to advancements in nanotechnology, which can be used as potential adjuvants in allergen-specific immunotherapy. Since of their improved bioavailability and focused distribution of therapy molecules, the use of nanoparticles offers the possibility of a remedy for allergic reactions that is more effective than other methods now in practice. Consequently, nanotechnology-based allergen delivery strategies have the goal of developing an innovative and potentially fruitful strategy for allergy immunotherapy (9).

Recent years have seen a sharp increase in the number of individuals who are affected by food allergies, despite the fact that immunotherapy still has numerous significant drawbacks that require to be corrected. These negatives include longer intervention periods (months or years), prevalent hospital, significant expenditures, a greater likelihood of adverse events throughout therapy, and a reduction of sustainability in desensitization. Additionally, there is a higher chance of adverse events during therapy. This paper discusses the recent discoveries in innovative allergen-specific treatments for food allergy, as well as a synopsis of food allergy grouping, the mechanism involved in immune response, as well as the advancements in research and unique delivery breakthroughs.

2 Food allergy

Food allergies are generally categorized as IgE-mediated food allergies, mixed food allergies (mediated by IgE-dependent and IgE-independent mechanisms) and non-IgE-mediated food allergies, based on the type of mediators involved (2). Some of the major food allergens with their allergenic constituents are listed in the given table (Table 1).

2.1 IgE mediated food allergy

According to an increasing body of research, IgE antibodies and mast cells may serve not just as effectors of acute hypersensitivity, but also as amplifiers during first antigen exposure (10). When an individual gets exposed to a food allergen, this exposure induces an initial immune response. On subsequent exposure to the same allergen, it triggers IgE mediated response with the involvement of mast cells and basophils that results in an immediate expression

TABLE 1 List of a few major food allergens with their allergenic constituents.

Food	Threshold level (mg)
Egg white Egg yolk	2.9 (Pasteurized whole egg) 0.13 (Whole raw), 0.2 (raw white), 10 (cooked white)
Cow's milk	15 mg
Wheat	15 mg
Peanut	0.25 (Ground), 1.25 (crushed)
Crustacean shellfish	Not reported

Abbreviations: APCs, Antigen-presenting cells; Th2, T helper 2; AIT, Allergen immunotherapy; FA-AIT, Food allergen -allergen immunotherapy; GIT, Gastrointestinal tract; OAS, Oral allergy syndrome; EoE, Eosinophilic esophagitis; FPIES, Food ; rotein induced enterocolitis syndrome; FPIP, Food protein induced proctocolitis; FPE-Food protein enteropathy; GALT, Gut associated lymphoid tissue; Innate lymphoid cells 2 , ILC2; DCs, Dentritic cells; OIT-Oral immunotherapy; SLIT, Sublingual immunotherapy; EPIT, Epicutaneous immunotherapy; OFC-Oral food challenge; MALT, Mucosal associated lymphoid tissue; MCT, Microcrystalline tyrosine; MPL, Monophosphoryl lipid; HAP, Hydroxyapatite scaffold; PLGA, Poly lactic co glycolide; NP, Nanoparticles; VLP, Virus like particles; PEG, Polyethylene glycol; PCL, Poly(ϵ-caprolactone); PVP, Polyvinylpyrrolidone

of symptoms. Crosslinking takes place as a result of the attachment of allergen-derived epitopes to IgE molecules. This crosslinking is what causes the release of prepared inflammatory mediators like histamine. This results in the appearance of allergic responses, which is then followed by the creation and release of leukotrienes, platelet-activating factors, and other cytokines that continue to perpetuate the allergic inflammation. The gastrointestinal tract (GIT), the skin, and the respiratory system are the primary organs that are impacted by this kind of allergy, and it frequently causes systemic symptoms. This is the most frequent kind of condition that can be fatally allergic to food (11). IgE-mediated food allergies develop when the essential immunological components that support tolerance and avoid innocuous food antigens from being mistakenly identified as pathogens lose their integrity. Clinical symptoms typically appear minutes to hours after consumption, which typically have a quick onset. Additionally, it raises the possibility of adverse or deadly reactions. While atopic reactions to peanuts, tree nuts, and shellfish typically last into adulthood, IgE-mediated allergies to cow's milk, egg, wheat, and soy are more likely to be outgrown.

The food antigen crosses the mucosal barrier and get processed by the dentritic cells. This process results in the activation of dentritic cells, which in turn stimulates naive T cells to develop a T helper cell 2 (Th2) phenotype. This stimulates inflammatory signals that lead to the production of food antigen-specific IgE by food Ag (antigen)-specific B cells, hence fostering a state of sensitization and allergy. IgE that is specific to a food antigen binds to basophils' and mast cells' FceRI (high-affinity IgE receptor) receptors. When exposed to an antigen, mast cells and basophils' IgE and IgE receptors cross-link, releasing prepared mediators (histamine, tryptase, platelet activating factor, prostaglandins, and leukotrienes) into the bloodstream and hastening the onset of symptoms.

Gastrointestinal manifestations can include oral tingling, pruritus, swelling, nausea, abdominal pain and vomiting. Respiratory effects include wheezing and airway inflammation. Skin manifestations include flushing, urticaria, angioedema and pruritus. Systemic responses may also occur, such as hypothermia.

Variants of IgE-mediated food allergy include oral allergy syndrome (OAS), in which individuals with allergic rhinitis produce IgE molecules that are crossreactive with fruit, or vegetable-protein epitopes. Another variant of IgE-mediated food allergy occurs in individuals who produce IgE antibodies that are specific for the red meat carbohydrate galactose-α-1,3-galactose, rather than specific for a protein epitope (5, 12).

2.2 Mixed food allergy

Atopic symptoms include things like delayed food allergy-associated atopic dermatitis (6–48 hours after exposure), that is put by the presence of T helper 2 (Th2) cells, and eosinophilic gastrointestinal illnesses like eosinophilic esophagitis (EoE). Milk allergies and the eosinophilic infiltration of tissues brought on by IgE-independent mechanisms are common triggers for these reactions (13). Currently, research is being conducted to identify how the IgE-mediated and non-IgE-mediated pathways augment food-induced anaphylaxis (14). Some of the examples of mixed food allergies include atopic dermatitis, asthma, eosinophilic gastroenteritis, eosinophilic gastritis, and eosinophilic esophagitis. Mixed food allergies are frequently implicated in causing gastrointestinal symptoms in children and adults, but they most likely will remain underdiagnosed, as the evidence-based protocols needed to diagnose and treat these diseases are still lacking (15).

2.3 Non-IgE-mediated food allergy

Non-IgE-mediated food allergy is defined as an immune reaction that occurs against the food, but not via the synthesis of IgE, and it does not activate the mast cells or basophils (16). This allergy is mediated by allergen-specific T cells that primarily affect the GIT and cause food protein-induced enterocolitis syndrome (FPIES), food protein-induced proctocolitis (FPIP), and food protein enteropathy (FPE). This type of allergy is also known as a sub-acute or chronic inflammatory process (17).

The food protein-induced allergy proctocolitis of infancy, food protein-induced enterocolitis syndrome (FPIES), pulmonary hemosiderosis (Heiner syndrome), and celiac disease are all examples of non-IgE-mediated reactions. Non-IgE-mediated food allergies are immunologic reactions to food that take place in the absence of conspicuous IgE antibodies that are specific to the food, in the skin or serum, and as a result, they possess a variety of distinct pathogenetic pathways. Non-IgE-mediated reactions begin slowly, and they are mostly T cell-driven, with the involvement of other cells, as well as macrophages, eosinophils, or neutrophils.

Lethargy, pallor, and frequent excessive vomiting are typical acute symptoms of FPIES, which often appear 1 to 4 hours (mostly 2 hours) after consumption. Rice and milk are frequently mentioned as FPIE triggers, followed by soy, oats, eggs, and poultry. Presently, a biomarker or *in vitro* test are unavailable for the diagnosis of FPIES or the food allergy that it causes, as there is no concrete evidence that suggests which cell is ultimately responsible for antigen identification and reaction initiation (17).

3 Mechanism of the immune response

The immune system is categorized into two parts: innate and adaptive immunity. Innate immunity is a nonspecific immune mechanism that occurs rapidly upon exposure to antigens in the body. The innate immune response is mediated by the skin as a barrier, chemicals in the body, and the immune cells. Whereas, adaptive immunity is an antigen-specific immune mechanism that is more complex than an innate response. The immunological response is chiefly dependent upon the T lymphocytes, also known as T helper cells—Th1 and Th2. The TH1 cells trigger the release of variant chemical mediators as a first-line defense mechanism against invasion from harmful pathogens in normal individuals. Whereas, in an atopic individual, Th2 cells with their mediators uplift the immunological system to recognize the food allergens as an invader and produce a response against these

allergens (18). ILC2 is type 2 innate lymphoid cells. It is a type of innate lymphoid cell, which is derived from lymphoid progenitor and belongs to the lymphoid groups. They are subsets of lymphoid cells. These cells lack recombinant activating gene and lacks B and T cell receptor. ILC2 secretes large amounts of IL-5, IL-9 and IL-13. ILC2 plays an important role in allergic reactions. These cytokines can activate the eosinophils, mast cells and plays a role in food allergen immune response. ILC2 resides in the mucosal tissues. They mainly lack lineage markers. They secrete large number of cytokines, activates immune cells and cause pathologic as well as physiologic changes.

In case of food allergy IgE mediated is the most common. In this scenario ILC2 plays an important role. After allergic sensitization, IL-25 and Il-2 obtained from CD4+ cells Th2 cells stimulates ILC2 and produce Il-13 which increases IgE mediated food allergy (19, 20). It mediates the immunosuppressive effect. It acts by activation of eosinophils and IL-5 through an independent pathway. ILC2 plays a role in homeostasis and helps in transition from innate to adaptive immunity (21, 22).

An individual undergoes allergen sensitization, resulting in an allergic response. The allergens are detected by antigen-presenting cells (APCs) such as macrophages and dendritic cells, which are abundantly present on the mucosal surface of the body. These allergens that interact with the APCs are subsequently absorbed and presented on the major histocompatibility complex class II (MHCII) to T cells. This helper T cell differentiates into Th2 in the presence of IL-4, which releases inflammatory markers such as IL-4, IL-5, IL-10, and IL-13. This leads to a massive production of IgE by B cell-derived plasma cells. Then, these allergens bind to the high-affinity IgE receptor (FcεRI) that is present on mast cells and basophils. This is followed by the production of IL-25, IL-33, and TSLP by the epithelial cells, which further affect the Th2 response (23). Consequently, they would release IgE with the expansion and differentiation of Th2 cells (24).

Once released, IgE binds to the high-affinity receptor on the surfaces of mast cells and basophils. Upon subsequent exposure to the same allergen, the allergic reaction is stimulated as described above, which leads to degranulation, followed by the release of inflammatory mediators such as histamine, prostaglandins, leukotrienes, and other cytokines. Therefore, this would cause hypersensitivity reactions that affect different organs including the GIT, skin, and the respiratory system (25). The mechanism of immune response is shown in Figure 1.

The immune mechanism of food allergy involves the tolerance, sensitization and desensitization.

3.1 Tolerance

The healthy immune system becomes tolerant to food, which can be characterized as unresponsiveness towards potential food allergens. Immune tolerance is an active condition that begins with the uptake of food antigens in the small intestine, which is filled with gut-associated lymphoid tissue (GALT) (26). In a set of actions that would sustain immune tolerance, various cell types function together to transfer antigens from the gut lumen to the lamina propria. The lymphoid tissue present antigens, which trigger a T-cell response in the lymphoid tissue, and revert the immune effector cells to the gut. Paracytosis and transcytosis are the two mechanisms by which food antigens can be captured from the gut lumen. Dendritic cells (DCs) and/or macrophages, which are myeloid cells, are able to capture potential dietary antigens from the gut lumen. Specialized cells such as the M

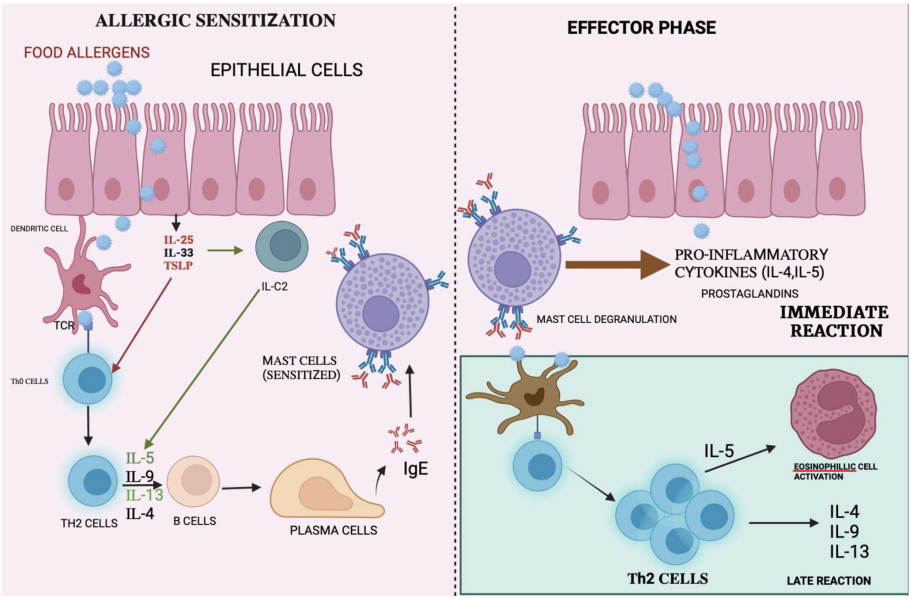

FIGURE 1
Mechanism of immune reactions: Allergen are presented to the T-cells by APC causing differentiation of T-cells to T-helper cells. The interaction with interleukins causes the plasma cells to release IgE which binds on mast cells causing mast cell degranulation thereby releasing pro-inflammatory cytokine, which causes immune reactions.

(microfold) cells, situated near the GALT, would be able to endocytose the antigen (27). CD103+ DCs, for example, is an antigen that can pass the epithelial barrier through transcytosis or translocation with the help of mucin-secreting goblet cells. It captures the antigen from the lumen, either through a tight junction, or by creating transcellular pores in M cells. A chemokine receptor CX3C extends dendrites between the epithelial cells through the secretion of IL-10. The CD103+ DCs would also produce transforming growth factor-β (TGFβ) and retinoic acid during migration from the lamina propia to the lymph nodes. CD103+ DCsmay also induce the differentiation of naive CD4+ T cells into FOXP3−, IL-10-secreting T cells, and possibly type 1 regulatory T cells (Tr1 cells). The movement of these cells to the lymph node can improve tolerance towards food allergens. Furthermore, the retinoic acid produced during the migration can also induce Treg cell expression of integrin α4β7. Human clinical trials demonstrating hypomethylation of the FOXP3 locus of T reg cells in those individuals who establish and maintain functional tolerance in response to oral immunotherapy, have provided the necessary information regarding the role of Treg cells in oral tolerance (OIT) (28).

3.2 Sensitization

When APC in the gut mucosal epithelial cells come in contact with the allergen, they recruit dendritic cells and convert them into pro allergic phenotype (DC2). DC2 takes up the allergen molecules and move into the lymph nodes, present it to the naive T cells and develops Th2 and TFH subsets. These subsets promote maturation of B cell which leads to allergen-specific IgE production through class-switch recombination. The produced IgE molecules binds to the surface of basophils and mast cells through high affinity receptors thereby sensitizing the individual. When the sensitized individual is re exposed to the allergen, it causes the crosslinking of bound IgE molecules, degranulation of mast cells and release of vasodilatory and chemoattractive mediators (29).

3.3 Desensitization

The exact mechanism of desensitization in food allergy is still unclear. Desensitization of mast cells and basophils mainly occur due to anaphylaxis during immunotherapy. It mainly occur after first administration of immunotherapy agents. It has been hypothesised that in food allergy the desensitization occurs through same mechanism as the exact mechanism is lacking.

There is evidence that desensitization to MC is both allergen-specific and reversible. Increasing dosages of allergen can cause IgE-FcRI complex internalization, which makes mast cells insensitive to allergen stimulation. This is one of the molecular mechanisms. Internalization of the TgE-FcRI complex, reduced calcium-paired calcium flow in mast cells, and dysregulation of the STAT6 pathway are some of the proposed mechanisms of desensitization. There is evidence from a variety of *in vitro* experiments to support the hypothesis that increasing dosages of allergen cause IgE-FcRI complex internalization in mast cells, which in turn renders MCs resistant to allergen challenge. Desensitization is successful whether there is a complete or partial reduction of IgE in the patient (30).

4 Management of food allergy: Current insights

Food allergy is a global health concern that has caused a tremendous reduction in the quality of life of those affected. The current paradigm is a stringent avoidance of food allergens and self-management through the usage of epinephrine auto injectors during an emergency. However, it is crucial to acknowledge that accidental exposure to food allergens can result in life-threatening anaphylaxis (25). Additionally, antihistamines, corticosteroids, and oxygen treatment have been undertaken for the mitigation of allergic symptoms (31). Presently, several treatment approaches are under investigation, which comprise allergen-specific immunotherapy and allergen-nonspecific approaches.

Allergen-specific approaches include the desensitization of atopic individuals with food allergens via oral, sublingual, and epicutaneous immunotherapy; the adoption of recombinant proteins, and a paradigm of an extensively heated diet containing milk and egg (5). Allergen's nonspecific approach is mediated by monoclonal IgE antibodies and the use of Chinese herbal formulations, which are still under clinical trials (32).

4.1 Allergen-specific immunotherapy

Allergen immunotherapy, which is also known as allergen shots, focuses on the administration of gradually incremented doses of allergen that render two possible payoffs: desensitization and tolerance. Desensitization is defined as transitory hypo-responsiveness, owing to an unceasing allergen exposure that would ultimately end up with an increased threshold of reactivity to the food allergen. Tolerance, on the other hand, is the ability to consume allergic food even after the withdrawal of therapy (33).

4.1.1 Mechanism of AIT

AIT is mediated through immunomodulation, which affects the T lymphocytes. The findings have suggested that there is an increase in CD8+ cells, along with an increased production of IL-4 and IFN-γ, which may be a result of an increased TH1 response or the downregulation of Th2 cells. Moreover, the generated Treg cells would inhibit the cytokine response (34). There is also a marked reduction in basophils and eosinophils that ultimately lead to a declined IgE-mediated release rate of histamine. During the initial course of AIT, an elevated serum concentration of IgE is observed, which gradually declines during therapy. An immense increase in the level of IgG-blocking antibodies can be interpreted as successful AIT. The production of IgG2a, IgG2b, and IgG3, and the synthesis of IgG1 are associated with the Th1 and Th2 responses, respectively (35). The activation of IgE is suppressed by the action of IgG through interaction with IgE before it can crosslink with the

IgE receptors on mast cells and basophils (36), as shown in Figure 2 below.

4.1.2 Routes of administration

Allergen immunotherapy is an effective strategy to treat food allergies, despite questions regarding the clinical safety and the unorthodox method of delivery in AIT. Current research, however, is focusing on alternative routes and delivery methods that are safer and more convenient for supplying the allergenic extracts. AIT comprises three major forms of treatment, upon which research is focused:

1. Oral immunotherapy (OIT);
2. Sublingual immunotherapy (SLIT);
3. Epicutaneous immunotherapy (EPIT).

The alternative routes include subcutaneous, transcutaneous, intranasal, and intra-lymphatic immunotherapy.

4.1.2.1 Oral immunotherapy

Oral immunotherapy (OIT) has been subjected to clinical trials over the last few years, and it is believed to be a potent therapy for food allergies. OIT is associated with the consumption of steadily incrementing doses of allergen extract that are usually combined with a vehicle. Several OIT protocols exist, which primarily depend upon the dose and the type of food (milk, peanut, egg, etc.). These food allergens can be obtained from any ordinary grocery stores. This, however, would raise a significant query regarding the safety and quality of the therapy. Therefore, regulated standards and guidelines from the Food and Drug Administration have been set up to ensure the safety and quality of the therapy (37).

The maximum OIT protocol is a three-phase program involving:

i. *Initial escalation phase*—This phase is characterized by a dose escalation on the first day of therapy, with 6 to 8 doses of allergenic extract, starting from the very smallest dose, typically 0.1 mg, which is gradually incremented to a maximum of 25 mg. This phase is designed to establish a sub-threshold level and maintenance.

ii. *Build-up phase*—The dose would vary significantly in escalation, which is typically monitored by a physician over weeks to months.

iii. *Dose maintenance*—The dose and the period of treatment would differ between different studies, with a huge increment in the dose, up to 4000 mg, and a range from months to years (38).

4.1.2.1.1 Immunological changes

The principal mechanism is not completely understood, and it is still under investigation. Based on several studies conducted, a significant increase in the food-specific IgG4 antibody was observed, with a decline in basophil and mast cell activation and response. After a few months, there was a deviation observed from the Th2 to the Th1 profile. A heightened action of CD4+ and CD25+ cells were observed, together with the suppression of the immune system by regulatory T cells (39, 40).

4.1.2.1.2 Efficacy and safety profile

Shreds of evidence from recent clinical trials have indicated that OIT would lead to desensitization and clinical tolerance among food-allergic patients. However, there is a paucity of sustained unresponsiveness (SU), which is defined as no increment in reactivity during the oral food challenge (OFC). Moreover, the scarcity of long-term follow-up data of the patients discharged is another concern to consider. It is also evident that most of the

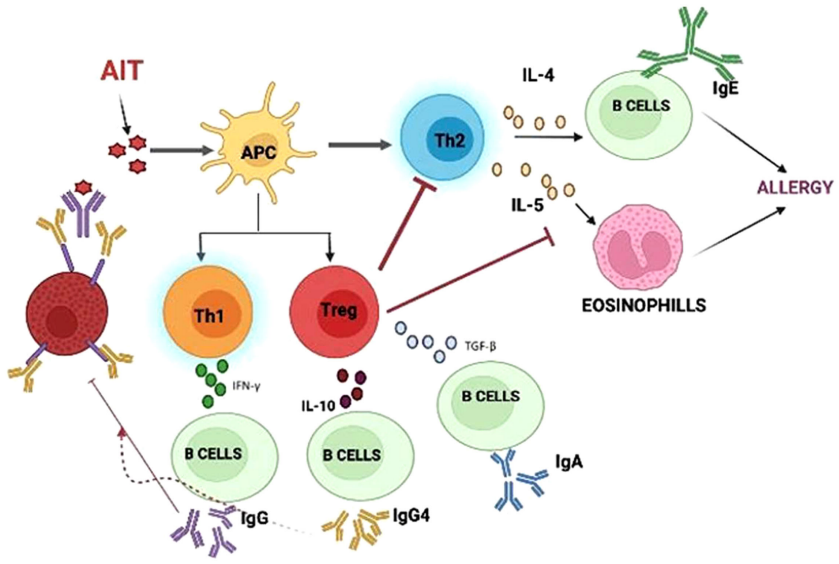

FIGURE 2
Mechanism of Allergen immunotherapy: AIT molecules interact with APC causing regulatory cells to inhibit further steps of immune response.

discharged patients have terminated the intake of allergic food, due to gastrointestinal adverse effects (37).

Adverse events are frequent during OIT, which are similar to all food types. Oral pruritus is the most common local reaction, which is mild and requires either no treatment or antihistamine therapy. Severe gastrointestinal symptoms such as abdominal pain, vomiting, nausea, and occasional reflux would lead to a discontinuation of OIT in most patients. Some cases with severe symptoms, however, may be associated with exercise, infection, hormonal changes during menstruation, or allergen co-exposure. Approximately 36% of therapy withdrawal is said to be due to adverse events, in which intolerable GI symptoms have contributed significantly. Moreover, eosinophilic esophagitis has been reported as well, through meta-analysis (41).

4.1.2.2 Sublingual immunotherapy

Sublingual immunotherapy (SLIT) involves the sublingual administration of the allergenic extract in gradually incrementing doses to achieve desensitization and tolerance (42). SLIT exhibits a much safer profile compared to OIT, but it has been found to be less efficacious than OIT. In this strategy, the allergenic extract can be used in the form of drops, tablets, or lyocs (43, 44). The allergenic extract is administered sublingually and must be held beneath the tongue for a few minutes. It can either be spit out or swallowed afterwards. The dose usually falls within a range of 1–10 ug of allergenic extract, which is smaller than in OIT. SLIT exploits the antigen presenting cells (APCs—Langerhans cells), which results in desensitization and tolerance. SLIT, however, is not presently advised in the treatment of food allergies, as it is still under investigation due to the exhibition of desensitization in clinical trials (45). This therapy also follows the dose-escalation and build-up phases.

4.1.2.2.1 Mechanism of SLIT

Oral mucosa is the site application of SLIT. It is rich in APCs and acts as the principal key in therapy, inducing tolerance. Several mechanisms of tolerance have been suggested which include:

i. Lack of mucosal-associated lymphoid tissue (MALT);
ii. Limited number of inflammatory cells (basophils, eosinophils, and mast cells);
iii. Existence of lamina propria-limiting antigen absorption;
iv. IgA secretion restricting antigen penetration;
v. Interferon-ɣ-producing Th1 lymphocytes and regulatory T cells invoking immunosuppression that is mediated by cytokine release (IL-10) (46).
vi. Presence of Pru p 3 and Ara h 9 proteins (47).

The epithelial tissue, lamina propria, and submucosal layer of the oral mucosa are abundant with APCs, particularly the dendritic cells, which play a pivotal role in the tolerogenic mechanism. Antigens at the site are snatched by the dendritic cells within half an hour, and drift to the regional lymph nodes, with the degradation of the antigen into fragments that are presented to the T cells.

In a retrospective comparative study between OIT and SLIT among food-allergic patients, it was evident that patients who received SLIT had rendered a low-threshold dose and faced difficulties in OFC. This indicated that SLIT was less efficacious than OIT, but exhibited a safer profile. SLIT also was found to be more effective among children than adults. The majority of adverse events were confined to local reactions, such as oro-mucosal pruritus being cleared up without any treatment. Furthermore, there were no systemic side effects reported (48).

4.1.2.3 Epicutaneous immunotherapy

Epicutaneous immunotherapy (EPIT) utilizes the novel delivery of antigen that explores the administration of an allergen-containing epicutaneous patch to stimulate the Langerhans cells abundant in the epidermis layer of skin that ultimately would downregulate the effector cell responses. EPIT usually offers a better safety profile with a diminished risk of adverse events associated with the non-vascularized nature of the epidermis. EPIT has been subjected to research, as it presents a promising platform for immunotherapy, with proven efficacy and a self-administrable form (49).

4.1.2.3.1 Immunization, safety and efficacy of EPIT

Epicutaneous therapy primarily aims at the epidermal layer of skin that is described by some salient features: the barrier function by keratinocytes, and the scrutiny of immunity by keratinocytes, Langerhans cells, and non-vascularized nature. Langerhans cells (LCs) are involved in portraying the cellular immune response, whereas the dendritic cells (DCs) in the dermis regulate the B-cell response. The Langerhans cells as APCs will effectively present the antigen and increase the CD8+ cells, while the dendritic cells are involved in the induction of IgA. IL-10 and IL-4 have been found to be secreted with the activation of LCs eliciting Th2-type responses. On the other hand, the DCs(dendritic cells) induce pro-inflammatory cytokines and ultimately, the TH1 response. According to recent studies, it was observed that different types of epithelial cell damage stimulate different molecular pathways that induce the secretion of specific cytokines that portray the immune response, both innate and adaptive. Less intensive epithelial cell damage (abrasion) could also trigger the release of cytokines such as IL-25 and IL-33, which in turn would induce the Th2 response (31, 50).

EPIT (epicutaneous immunotherapy) is found to be more efficacious than other therapies, with the advantage of abundant LCs in the skin. EPIT is a needle-free treatment that is self-administrable. Through this therapy, there is an increased serum IgG level, along with a declined IgE level. Furthermore, clinical studies conducted have reported local adverse effects such as local erythema and eczema, as well as pruritus and urticaria, with no serious systemic adverse events. The non-vascularized nature of epidermal cells contributes to the lower systemic side effects. In short, the EPIT is found to be efficacious, and with a safe profile (51).

4.1.3 Alternative routes

4.1.3.1 Subcutaneous immunotherapy

Subcutaneous immunotherapy (SCIT) involves antigen administration by subcutaneous injection, with steadily incremented doses of the allergen, under the surveillance of a physician (52). Currently, SCIT is an effective treatment for patients with asthma and allergic rhinitis. However, SCIT is not prescribed for atopic dermatitis and food allergy (53). Several attempts were made in the past to utilize SCIT in food allergies, focusing on peanut allergy, which was efficacious, but ultimately resulted in severe systemic reactions that deemed this approach unsuitable. Numerous studies are still programmed to utilize this approach with a minimal risk of anaphylaxis, focusing on alternate routes of therapy or other modifications to the allergenic extract (54).

An IgE antibody treatment with omalizumab or adjuvants has made improvements to the efficacy and safety of SCIT allergenic extracts by reducing IgE levels significantly, and diminishing systemic side effects. Aluminum salts, mainly aluminum hydroxide, have been extensively explored as adjuvants in SCIT therapy. The mechanism of immunization in SCIT is similar to other immunotherapies that comprise the activation of DCs (dendritic cells), antigen presentation, and the stimulation of Treg cells mediating a downregulating Th2 response. SCIT, in combination with alum, has exhibited a TH1 response. Alas, the safety of normal SCIT is questioned, due to the presence of local side effects and severe systemic ADRs (55, 56).

5 Adjuvants in AIT

The ultimate goal of current research in allergen immunotherapy is to improve the efficacy and safety of AIT. This is achieved by developing the vaccines, along with their adjuvants, to help with reducing the dose of allergen administered. An adjuvant is a pharmaceutical aid that acts by modulating the immune response against the delivered antigen. An adjuvant requires some ideal characteristics, as follows:

- Should trigger a TH1 response;
- Should be non-mutagenic, non-carcinogenic, and non-teratogenic;
- Should be free from pyrogens;
- Stable

The adjuvants act in different ways from the depot system, rendering a slow-release does that targets the APCs and that confers an immunomodulating action. Adjuvants can act as delivery systems and immunomodulating agents (36).

Some of the adjuvants used in AIT are described below.

Alum is a first-line adjuvant that is widely used in AIT, and it is known for its fascinating property of immunomodulation and stability, along with depot formation. The antigens are adsorbed onto the alum surface, mostly by electrostatic interactions that are slowly released into the tissue and lymphatic organs. Then, the APCs take up the released antigens to process them, before presenting them on the APC surface, which would trigger the immune response (57). Three main mechanisms of alum as an adjuvant in AIT are established through studies that have been conducted.

i. Immunomodulating action through depot formation;

ii. Allergen-specific antibody production by NLRP3 inflammasome activation;

iii. Self-DNA or uric acid release triggers the activation of immature dendritic cells.

Alum, however, has an issue of non-biodegradability; therefore, research regarding its safety and toxicity must be highlighted (58).

Calcium phosphate is not widely utilized as an adjuvant in AIT (59). It is a biodegradable and biocompatible adjuvant that acts through immunomodulation via depot formation. Studies have shown that it is able to induce high IgG levels and diminish IgE levels (58).

Microcrystalline tyrosine (MCT) acts as an adjuvant in AIT, and exhibits an excellent absorption capacity, due to its biodegradable and biocompatible nature. MCT also forms a depot system that can trigger an immune response(58, 60).

Monophosphoryl lipid A (MPL) stimulates the immune response by activating the APCs, thereby inducing a TH1 response. It also activates monocytes and macrophages, fastening the process of antigen presentation. It is found to be less toxic, but due to its poor availability, it is often combined with other adjuvants to enhance its efficacy(58, 61).

6 Nanoparticles: A promising platform in AIT

Nanoparticles (NPs) are defined as nano-scaled particles with a dimension of 1–100 nm which are greatly explored in drug delivery systems. Depending on the components used in synthesis and their structure, nanoparticles can be categorized as organic and inorganic nanoparticles (53, 62). The detailed classification of nanoparticles and its preparation is given in the Figures 3 , 4 below.

Even though the conventional AIT has been found to be effective in clinical trials, serious systemic side effects are presented that have limited their application. Nanoparticles have the potential to be a promising platform in AIT, by encapsulating the allergens. NPs offer advantages as both carrier systems and adjuvants. Encapsulation benefits the allergen with triple functions, namely protection from enzymatic or acidic degradation, and delivery and co-delivery, resulting in targeted drug delivery with a reduced dose of antigen. This in turn would enhance the local drug concentration that has escaped from the immune system. The side effects of AIT can be limited, or to a certain extent, even prevented, by utilizing the peculiar feature of nanoparticle shielding of allergens which would restrict their identification through IgE that is present on the immune cells. NPs can be modified to meet the specific requirements for various routes of administration; for example, protection from gastric acids in oral delivery. Besides, drug tolerance could be improved with the targeted drug delivery of nanoparticles (63–66).

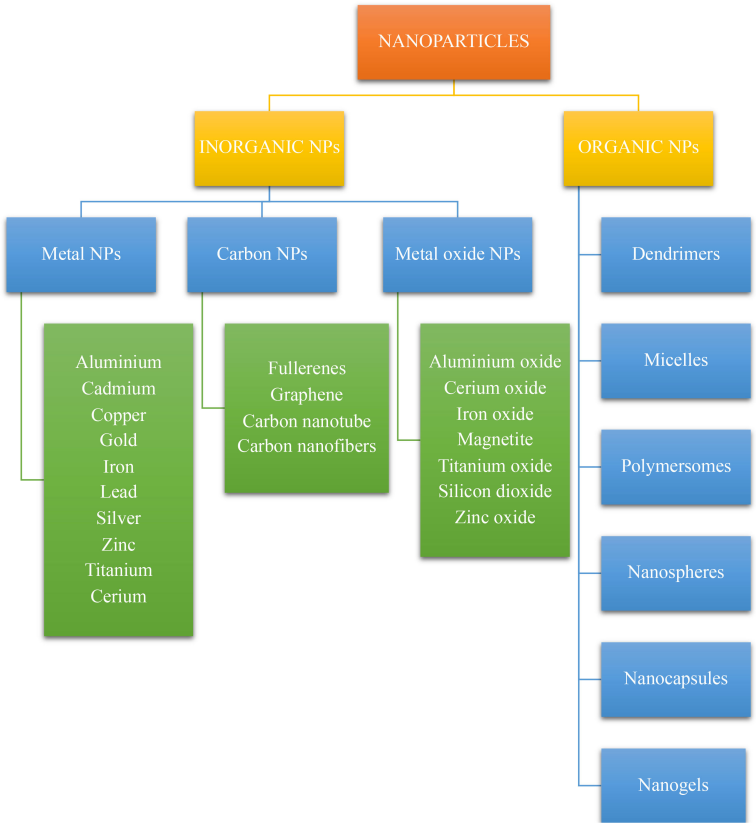

FIGURE 3
Classification of nanoparticles.

The physicochemical characteristics of NPs and the attachment of specific ligands for specific targeting have greatly influenced their adjuvant effects. The encapsulation efficiency and the release pattern of a drug from a polymeric nanoparticle depends on the solid-state solubility, which is defined as the miscibility of the drug with the carrier vehicle solution (67). The particle size could affect its penetration into tissues, as well as its entry into blood vessels and the lymphatic system. The potential for antigen protection at the site of administration, and the maintenance of the stability and exertion of depot effect are attributed to the chemical nature, shape, and solubility of the NPs. The NPs also exert a direct immunosuppressive effect on the immune cells, with a prolonged circulation time. The NPs are currently being investigated for their immunomodulating effect, which could open doors to new allergen formulations in AIT (68). An attempt was made by several scientists to classify nanoparticles for the preparation of the allergen complex. Nanoparticles used in AIT can be broadly categorized as being biodegradable and non-biodegradable NPs, as represented in Figure 5 (56, 68).

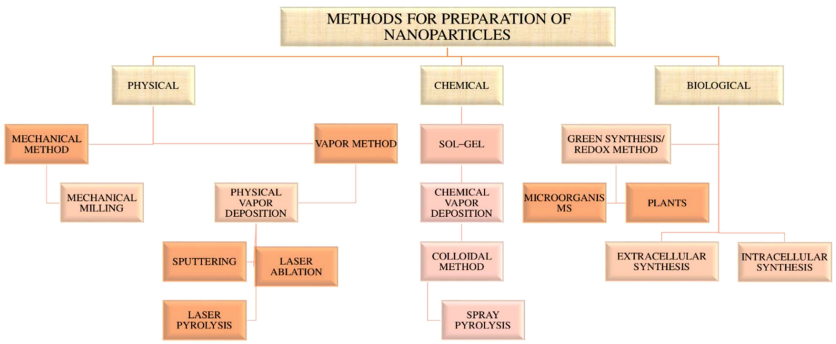

FIGURE 4
Flow chart of preparation of nanoparticles.

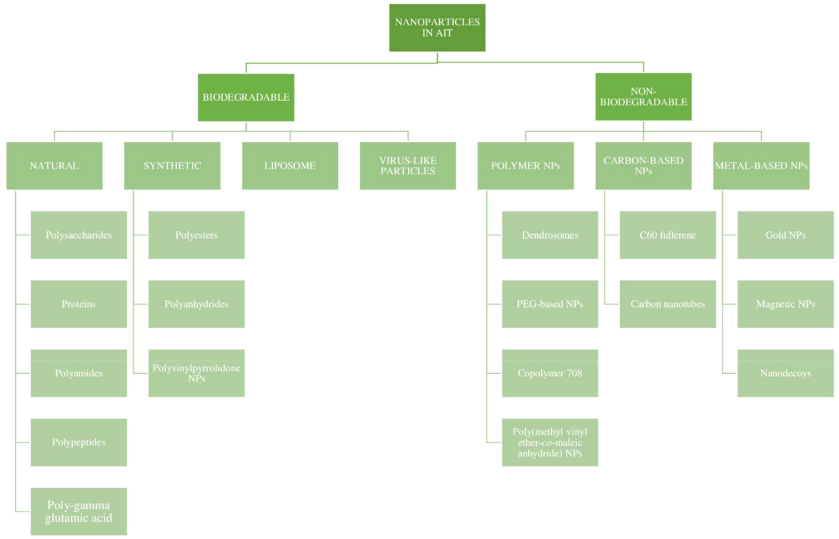

FIGURE 5
Types of nanoparticles in AIT.

6.1 Biodegradable nanoparticles

Over the past few years, various polymers have been used in formulating nanoparticles. Biodegradable nanoparticles are one of the polymers exploited intensely in the field of drug delivery systems. These nanoparticles offer numerous advantages, such as sustained/controlled release of the drug, biocompatibility, and small size; and they act as a carrier for bioactive molecules, such as proteins and peptides. Biodegradable nanoparticles comprise natural polymers (polysaccharides, proteins, polypeptides, polygammaglutamic acid, and polyamides), synthetic polymers (polyesters, polyanhydrides, and polyvinylpyrrolidones), liposomes, and virus-like particles (36, 69), as shown in Figure 6.

6.1.1 Natural polymers

Natural polymers mainly consist of polysaccharides and polypeptides. Meanwhile, chitosan, alginate, dextran, and cellulose derivatives are some of the polysaccharide polymers. Natural polymers also comprise proteins such as collagen, gelatin, globulin, and albumin (70).

6.1.1.1 Synthetic polyamides

Polyhydroxyethylaspartamide (PHEA) is one of the synthetic polypeptides that is widely utilized in drug delivery, due to its biodegradable nature and aqueous solubility. Protamine, another polyamide, has been extensively used in AIT trials. A study with protamine-based nanoparticles that are complexed with Ara h2 for peanut allergy have exhibited increased IgG antibody and diminished IgE antibody levels, and a lower reactivity upon SC administration in mice (71). Poly(gamma-glutamic acid) (ɣ, PGA) is a polypeptide, with dual functions as a carrier and an adjuvant, that has demonstrated efficacy in an investigation of ɣ-PGA with OVA, upon IV, SC, or IP administration in mice (72).

6.1.1.2 Polysaccharides

Chitosan, a cationic polymer (linear polysaccharide) is extensively used in formulating nanoparticles that can maintain the immunogenicity of an allergen, along with having its own advantages of biocompatibility, biodegradability, and non-toxicity. Past studies had indicated its success in AIT in murine models with OVA and peanut, in which it had induced a Th1 response. Gelatin, a protein derived from animals, has shown a decrease in allergic symptoms when formulated as NPs. Other than that, sodium alginate allergens have exhibited a significant increase in IgG1 and IgG4 levels, along with diminished IgE levels, during immunotherapy in clinical trials.

6.1.1.3 Polypeptides

Peptides derived from allergens are the most promising approaches for AIT. Based on the allergen's basic structure, this novel treatment employs soluble synthetic allergen fragments of various lengths (73). Peptide-based vaccinations can be categorized into those that employ T-cell peptides, and those that use IgE-mediated peptides, depending on the length of the fragments and their capacity to induce tolerance (74, 75). Peptides have the ability to control the genes and activate the pathways involved in the tolerogenic response, particularly in the specific case of olive pollen. Through this, the combination of five short Ole e 1 that are derived from dodecapeptides would be able to stop the proliferative response to olive pollen associated with the release of IL-10 and IL-35, thus regulating the cytokines in allergic individuals. Additionally, these peptide combinations are incapable of activating basophils, a requirement for the creation of a novel peptide vaccine (76).

The mechanism of polypeptides was investigated in clinical trials using a mixture of peptides from Fel D 1 and from bee venom allergen. According to these studies, changes in both cellular and

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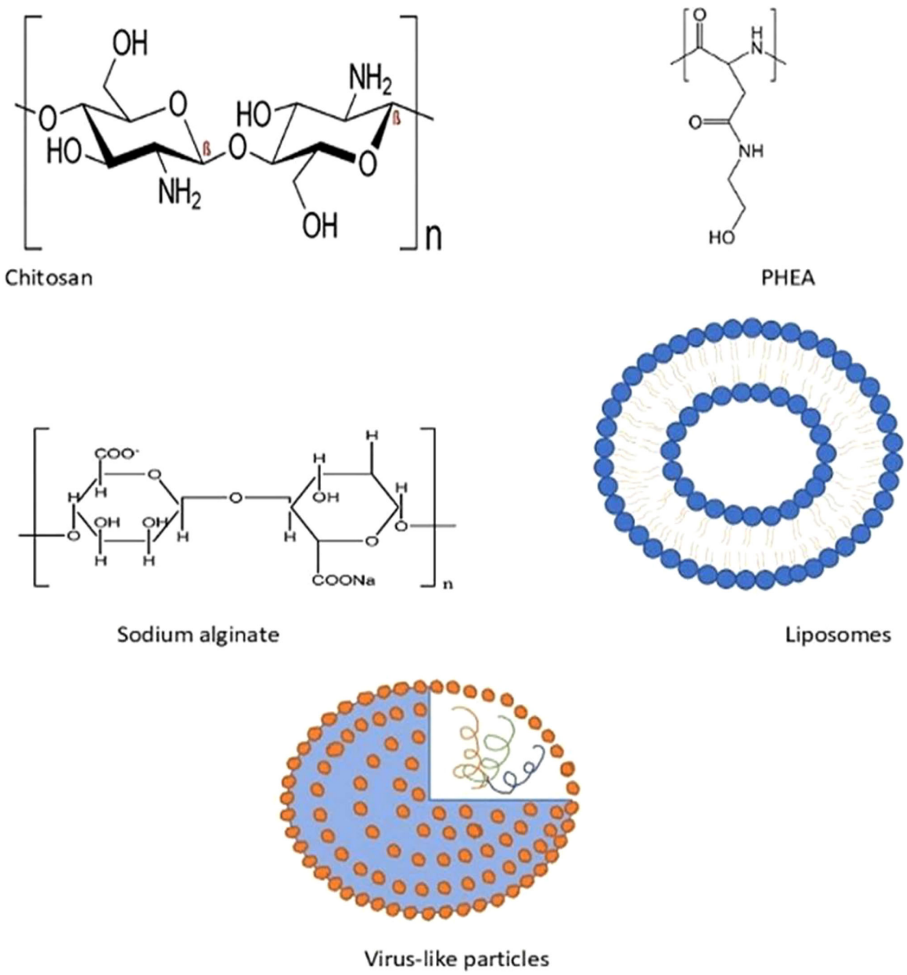

FIGURE 6
Some of the biodegradable nanostructures in AIT.

humoral immunity were observed. Moreover, these peptides have their own limitations, as B-cell epitopes on short peptides are weak in humoral immunotherapy (77, 78).

Another study that centralized on the usage of peptides in skin has shown that there is a significant increase in the number of Th1 cells ($CD4^+$/IFN-γ^+) and $CD25^+$ cells, suggesting that a combination of immune deviation (Th2 to Th1) and regulation (the recruitment of regulatory T cells) may be important in controlling the responses to allergen post-therapy (79). There are several other studies indicating that peptides in AIT have produced satisfactory results, in which peptide-induced tolerance was observed after intradermal administration, and not observed during inhalation (80).

6.1.1.4 Poly-gamma glutamic acid

Certain Bacillus strains produce high molecular weight polypeptides known as poly(gamma-glutamic acid) (or -PGA), made up of linked glutamic acid units and carboxylate side chains. The amphiphilic characteristics of the hydrophobically modified -PGA copolymers makes it possible to create nanoparticles through a straightforward process (81, 82). Further research has revealed that these particles could activate human monocyte-derived dendritic cells, thus significantly increasing the production of chemokines and inflammatory cytokines, as well as costimulatory molecules and immunomodulatory mediators that are essential for effective T-cell priming. Additionally, *in vitro* research using grass pollen allergen Phleum pratense-loaded -PGA nanoparticles and monocyte-derived dendritic cells has revealed an increase in allergen-specific IL-10 production and the proliferation of autologous CD4+ memory T cells. As for allergen-specific immunotherapy, these systems appear to be innovative and effective adjuvants and antigen carriers (82).

A study by Touseefn et al. has revealed that (POSS)-grafted polyurethane (PU) is a good nanomaterial for muscle tissue renewal. Thus, it can be inferred that this nanomaterial is a good material for immunotherapy (83). Another study by the same author outlines that a HAP (hydroxyapatite scaffold) with N. sativa grafts can be combined for use as implants. Interestingly, N. sativa-grafted HAP has demonstrated a remarkable potential as effective antiosteoporotic scaffolds, inheriting antioxidant and anti-inflammatory properties; as a result, they can be used as forthcoming material for allergic reactions (84).

6.1.2 Synthetic polymers

Polyesters: Poly(lactic-co-glycolide) (PLGA) and polylactic acids have been extensively researched in drug delivery systems, due to their biodegradability and biocompatibility nature (85). These polymers have been subjected to extensive studies in AIT to identify their potential to specifically target antigen-presenting cells (APCs). The acidic degradation products might negatively affect the stability of the loaded antigen, which can be addressed by the use of copolymers such as PEG. Several studies have shown that the application of PLGA-based NPs has superior effects on free antigens. The oral administration of PLGA-based NPs in mice has displayed a Th1 immune response with an elevated IgG level, and diminished IgE levels, while other studies have shown a conversion from Th2 to Th1 immune response (86). Poly(ϵ-caprolactone) (PCL) is another polymer that is biocompatible, biodegradable, and semicrystalline. The *in vivo* degradation of PCL is much slower than PLGA; therefore, it can be used for the prolonged delivery of drugs/antigens. Treatment with OVA-PCL in mice had evoked an enhanced IgG level and lowered IgE levels, which resulted in declined allergic symptoms.

6.1.2.1 Polyanhydrides

The stability of encapsulated antigen is maintained with polyanhydride, due to its non-toxic and reduced acidic degradation products, which make them more suitable for antigen delivery than polyesters. The bio-adhesive properties of these polymers would contribute to the Th1 response, due to its enhanced interaction between the antigen and APCs in the mucosa. An enhanced Th1 response and Treg cytokines were observed with spray-dried peanut protein-loaded NP immunization in mice. Oral immunization with polyanhydride NPs containing cashew nut proteins (single dose) led to the induction of Th1 and Treg immune responses, exhibiting their immunomodulatory properties, as seen in a study conducted by Pereira et al. (87).

6.1.2.2 Polyvinylpyrrolidones

There are several studies that imply an interest in employing polyvinylpyrrolidone (PVP) to generate promising allergen delivery nanocarriers, despite having less workers than the polymers discussed previously (36). For instance, Aspergillus fumigatus was enclosed in poly(vinylpyrrolidone) nanoparticles by Madan et al., who were able to exhibit intact integrity, immunoreactivity, and persistent allergen release in just 9 weeks (88).

In addition to these polymers, a study by Touseef et al. has shown that polyvinyl alcohol can be used to encapsulate L. gasseri, which possesses extensive usage in food technology. Based on this study, it can be implied that the nanomaterial polyvinyl alcohol with modification can be used for the management of food allergies, by improving its stability (89).

6.1.3 Liposomes

Liposomes are small spherical vesicular structures comprising phospholipid bilayer that encapsulate hydrophilic drugs, thus insulating them from the aqueous environment. Liposomes have been continuously explored for drug delivery and their adjuvant effects, as they can enhance the bioavailability and solubility of the targeted drug, as well as optimizing specific targeting at the site of action without unwanted side effects. It has been proven that liposomes are able to elicit immunomodulatory functions. Several studies with allergen-loaded liposomes ended up with a conclusion that they were effective in inducing a Th1 immune response and an increase in Treg cytokines. These liposomes were found to be safe and effective in AIT, with pollen grains, dust mites, and cat allergen, which resulted in declined serum IgE levels. However, human clinical trials of AIT with allergen-loaded liposomes concluded that liposomes were not suitable for AIT, due to its systemic safety issues. The modified liposomes triggered CD8+ Treg, which subdued the allergic symptoms in the murine food allergy model. Contrary to that, liposomes loaded with refined protein Per a 9 were found to be effective in mitigating allergic symptoms. Aliu et al. (90) investigated the efficacy of liposomal delivery of allergen through SLIT, and concluded that prophylactic SLIT with OVA liposomes were more effective in excluding allergic symptoms than the free OVA AIT (91). Nevertheless, systemic safety in humans must be addressed, to enhance the effectiveness of liposomes in AIT (69).

6.1.4 Virus-like particles

Virus-like particles (VLPs) are self-assembling and non-infectious nanoparticles that have been utilized in drug delivery systems or as adjuvants. In a randomized clinical trial, treatment with VLPs among house dust mite-allergic patients helped with alleviating allergic symptoms by mediating a Th1 immune response. The SC administration of Fel d 1 in mice was found to be efficacious, without triggering any systemic side effects (92). Adeno-associated VLP, when administered in mice, elicited heightened IgG levels and diminished allergen-specific IgE without inducing any anaphylactic reactions. Thus, VLPs can be considered as an option to enhance the efficacy of AIT.

6.2 Non-biodegradable nanoparticles

Researches have been conducted on non-biodegradable NPs, which are made up of different materials, such as gold, silica, and polymers, for immunization in antigen delivery, by presenting them to the immune system for an extended period. Polymer NPs were found to be effective against the OVA peptide, eliciting a shift to the Th1 response. The conjugation of OVA with a copolymer of N-vinylpyrrolidone and maleic anhydride in AIT resulted in a remarkable decline of IgE levels, with an enhanced IgG level. Polyethylene glycol (PEG) is a water-soluble, non-biodegradable polymer that has been explored extensively in drug delivery. In human trials, the encapsulation of allergens with PEG NPs led to a targeted allergen delivery, and triggered the Th1 immune response, which further proved their potential as adjuvants in AIT. However, the safety profile of these NPs is still under research, despite its biocompatible nature. Further research must be conducted to fulfill the needs of these NPs, particularly its degradation in the body, as the non-clearance of these carriers may end up with unwanted side effects (69).

6.2.1 Polymer nanoparticles

Dendrimers, which are branched polymeric nanoparticles, were found to have a role in decreasing IgE, along with a decreased Th1 response (93). In another pre-clinical study by Garaczi et al., the topical administration of nanoparticles, by mixing with poly(ethylene imine) with OVA pDNA, reduced nasal symptoms of rhinitis and induced balanced Th1/Th2 responses. When the copolymers of ethylene oxide and propylene oxide (poloxamine) nanospheres were examined for their therapeutic effects on asthmatic mice, a drop in inflammatory cytokines in the BAL, and a reduction in airway hyperreactivity were observed. PEG, an aliphatic polyether that is used in medicinal applications, is the most well-known synthetic water-soluble polymer. Due to its distinct characteristics, PEG is also known as the "gold standard" for biomedical applications (94). PEG is a non-biodegradable polymer; hence, cleavage sites must be included for the regulated delivery of proteins by PEG-based NP in AIT to take place. In order to achieve this, Pohlit et al. created acid-labile PEG macromonomers that would break down at pH 5 (the physiological pH inside the endolysosome). During *in vitro* tests in humans, the encapsulation of allergens into these NPs led to targeted administration, the activation of T-cell proliferation, and allergen shielding from identification by IgE antibodies (95). A study by Maria et al. shows as a sublingual immunotherapy glycosylated nanostructures can induce a long lasting tolerance in lipid transfer proteins (LTP) induced allergey.LTP allergy is mainly generated from fruits such as peach and apple (96).

6.2.2 Carbon-based nanoparticles

Carbon tubes are the most commonly investigated nanoparticles for drug targeting (97). A study on the immunomodulatory activity of these carbon tubes revealed that they can either promote or suppress the immune response, and they can be used as an appropriate adjuvant. It is also important to highlight that the negative or toxic effects of these nanotubes were also reported (98). Another study by Ryan et al. also demonstrated that C 60 fullerene had an inhibitory effect on IgE-mediated release from mast cells and basophils. Furthermore, it had the ability to block signaling molecules and cytoplasmic ROS, which prevented the release of histamine and anaphylaxis (99). In multiple pre-clinical studies, the administration of tetraglycolate fullerenes or a water-soluble form of C60 fullerene to OVA-sensitized mice caused a shift in cytokine production from Th2 to Th1, as well as a reduction in airway inflammation, eosinophilia, and bronchoconstriction, which were brought on by the production of an anti-inflammatory P-450 eicosanoid metabolite or an increase in Foxop 3 and fillagrin m RNA (100, 101).

6.3.3 Metal-based nanoparticles

Gold nanoparticles have been one of the most commonly used drug delivery systems for anti-inflammatory and antioxidant effects (102).Through magnetic NPs coated in dextran and coupled to either bovine b-lactoglobulin or ovomucoid, Marengo et al. proposed a potential delivery platform for AIT, as it was observed, through the utilization of confocal laser scanning microscopy to confirm internalization, that human monocytes absorbed conjugated NPs more readily than nonconjugated NPs (103, 104).

The reported studies of nanoparticles for allergen mediated immunotherapy is given in Table 2 below.

7 Future prospects

Allergen mediated immunotherapy is novel therapeutic form established for the management of the common allergen sources. Allergen immunotherapy, which act by repeat administration of allergen extracts and can offer a permanent solution for allergic reactions. Recently administration through oral especially sub-lingual route has emerged safe and effective.

The discovery of molecular allergology has made it feasible to create recombinant allergens, which has led to greater accuracy in allergy diagnosis as well as in the selection of patients for allergen immunotherapy. In regards to efficacy or safety, recombinant allergen immunotherapy does not now provide any benefits over entire allergen extracts that are currently accessible; however, in the future, recombinant hypoallergenic variants might provide such advantages. Personalized vaccinations containing significant allergens or hypoallergenic modifications may one day be employed in immunotherapy. These injections would be tailored to patients' individual IgE sensitivity profiles.

Ongoing research into the prevention of other food allergies, such as those to prawns, cashews, and milk, is being conducted on infants who are considered to be at risk for developing those allergies. This is because early introduction of peanut as a primary prophylactic method has been shown to be extraordinarily effective while also being completely risk-free. As a result, it makes perfect sense to contemplate the primary preventative measures for allergic reactions.

The application of nanotechnology in immunotherapy for the treatment of food allergies is a relatively new platform. It contributes to the development of a new treatment approach for improving the clinical safety and effectiveness of immunotherapy. The use of nanoparticles in AIT is being ramped up as a result of the results obtained thus far. The impact that NP has on the immune system is relevant to allergen immunotherapy, which is a form of treatment for allergic reactions. For this reason, more research is required to show the positive effects of various delivery methods and adjuvant formulations in human studies.

8 Conclusion

Food allergies are becoming more common, with increasing severity, which have sparked a great interest to discover commercial treatment strategies. Nanoparticles, DNA vaccines, adjuvants, and combination therapies are some of the methods being investigated to reduce the negative effects of AIT, as well as to increase the effectiveness and development of permanent oral tolerance. Native and modified allergens can be produced by using recombinant technology with less allergenicity. Future research into hypoallergenic IT agents may be particularly significant, due to

TABLE 2 Reported studies of nanoparticles for allergen mediated immunotherapy.

Allergen source	Nanoparticle component	Species	Findings of studies	Reference
Ara h 2 extracted from raw peanuts	Protamine-based nanoparticles (proticles) with CpG-oligodeoxynucleotides	BALB/c mice	Because they decrease the Th2-dominated immune response that is triggered by an allergen, biodegradable nanoparticles based on protamine and containing CpG-ODN are a new carrier system that can be used for allergy immunotherapy.	(71)
Raw or roasted peanuts	Poly(anhydride) nanoparticles	C57BL/6 mice	A pro-TH1 immune response was induced as a result of oral immunization with poly (anhydride) NPs, particularly those formulations that were spray-dried.	(105)
Peanut allergen gene (pCMVArah2)	Chitosan–DNA nanoparticles	AKR/J mice	Oral allergen immunization conating chitosan-DNA nanoparticle is very effective in anaplylatic reaction and can be used for prophylactic management.	(106)
Roasted peanut extract	Polymer conjugate of mannosamine to the copolymer of methyl vinyl ether and maleic anhydride	CD1 mice	Evidence from previous studies suggests that pretreatment with BLG-Pep+CpG/NP modifies the DC phenotype, suppresses the formation of Th2 cells, and enhances the activity of regulatory T cells and helper T cells, all of which may assist to inhibit the growth of allergen-specific CMA.	(107)
Peanut protein allergen Ara h 2	Poly(lactide-co-glycolide acid) (PLGA) nanoparticle	Liver sinusoidal endothelial cells (LSECs)	The targeted distribution of carefully selected T-cell epitopes to naturally tolerogenic liver APC was shown to have the potential to build an effective therapy platform for peanut allergy anaphylaxis	(86)
Wheat allergen in milk	Gold nanoparticles		Gold nanoparticle-based lateral flow (LFIA) strips rapidly detected gliadin in negative milk with a visible limit of detection of 25 ng/mL and a computed LOD value of 6.56 ng/mL. Positive samples tested with LFIA strips yielded responses that were significantly consistent with sandwich enzyme-linked immunosorbent results. Consequently, the LFIA strip rapidly and accurately detects the wheat allergen in milk.	(108)
Cows milk allegen	PLGA nanoparticles	mice	This study reported that oral administration of PLGA nanoparticle encapsulate beta lactoglobulin peptide can prevent rise in serum BLG specific IgE	(109)

rising concerns regarding the clinical safety of food allergy therapy methods.

The pervasiveness of food-induced anaphylaxis has led to research in AIT, which is an impressive and powerful tool in mitigating allergic symptoms. Diversified routes of administration and delivery systems would be able to enhance the effectiveness of AIT. Although the efficacy of AIT was established through numerous studies, its safety profile should be examined continuously to further prove its advantages over other therapies. Intralymphatic and intranasal routes of allergen delivery can be utilized to improve effectiveness. The targeted drug delivery of nanoparticles into the lymphatic system is much more efficacious than conventional delivery system, as it can bypass the systemic side effects. Nanoparticles have displayed their effectiveness in allergen immunotherapy, but the toxicity assessment must be highlighted, to ensure the safety profile. Abundant AIT trials have been conducted, but attentive studies must be conducted to fulfill the required expectations of safety and systemic side effects. AIT is the core component in the management of allergic diseases; therefore, its advantages should be established. AIT with versatile routes of administration such as SLIT, and delivery systems such as nanoparticles, could open many doors for more promising platforms in the prevention of food-induced anaphylaxis.

In the context of trials associated with AIT, establishing the baseline outcome measurement that can be used to predict the likelihood of a successful AIT, and to track the immunological response to intervention during the course of the AIT, both in the short, and long term, remain as a challenge. These goals can be accomplished by standardizing the outcome metrics used across each clinical study.

Even though the usage of nanotechnology has grown in popularity in recent years for immunization procedures, allergen immunotherapy is still in its infancy stage. Despite the positive outcomes of *in vivo* nanoparticle investigations, a deeper comprehension of nanoparticle–allergen complexes and their molecular interactions with the immune system is necessary. Investigating the effect of the nanoparticle–allergen complex at each mechanistic step underlying the immune response, such as internalization, maturation, antigen processing, presentation, and the activation of T cell differentiation, can lead to the development of safe and effective adjuvants for AIT. Therefore, more research on the immunomodulatory effects of polymeric NPs is essential to expand our knowledge, and consequently, our capacity to design more precise and efficient allergen-specific immunotherapies.

Author contributions

Conceptualization, VV and SK. Data collection, VV, SK and AT. Formal analysis, AT, SF and SK. Writing—original draft preparation, SK, AT, RS, and VV. Writing—review and editing, JK, RS, SAF, SM and MN. Supervision, VV. 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.

Publisher's note

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Mechanism of the antidiabetic action of *Nigella sativa* and Thymoquinone: a review

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Introduction: Long used in traditional medicine, *Nigella sativa* (NS; Ranunculaceae) has shown significant efficacy as an adjuvant therapy for diabetes mellitus (DM) management by improving glucose tolerance, decreasing hepatic gluconeogenesis, normalizing blood sugar and lipid imbalance, and stimulating insulin secretion from pancreatic cells. In this review, the pharmacological and pharmacokinetic properties of NS as a herbal diabetes medication are examined in depth, demonstrating how it counteracts oxidative stress and the onset and progression of DM.

Methods: This literature review drew on databases such as Google Scholar and PubMed and various gray literature sources using search terms like the etiology of diabetes, conventional versus herbal therapy, subclinical pharmacology, pharmacokinetics, physiology, behavior, and clinical outcomes.

Results: The efficiency and safety of NS in diabetes, notably its thymoquinone (TQ) rich volatile oil, have drawn great attention from researchers in recent years; the specific therapeutic dose has eluded determination so far. TQ has anti-diabetic, anti-inflammatory, antioxidant, and immunomodulatory properties but has not proved druggable. DM's intimate link with oxidative stress, makes NS therapy relevant since it is a potent antioxidant that energizes the cell's endogenous arsenal of antioxidant enzymes. NS attenuates insulin resistance, enhances insulin signaling, suppresses cyclooxygenase-2, upregulates insulin-like growth factor-1, and prevents endothelial dysfunction in DM.

Conclusion: The interaction of NS with mainstream drugs, gut microbiota, and probiotics opens new possibilities for innovative therapies. Despite its strong potential to treat DM, NS and TQ must be examined in more inclusive clinical studies targeting underrepresented patient populations.

KEYWORDS

diabetes mellitus, *Nigella sativa*, antioxidant, thymoquinone, anti-glycemic, gut microbiota

The rising prevalence of diabetes mellitus and the need for new treatment approaches

DM is an ancient scourge, with evidence of its description first appearing in pre-biblical medical writings (1). It's complicated pathophysiology consists of metabolic derangements marked by chronically elevated blood glucose, resulting from abnormalities in insulin action, secretion, or both (2–4). The name itself was coined in the 2nd century CE derived from the Greek word "diabaínein" which means "a passing through," alluding to the disease's generally profuse urination. The term "mellitus" was added much later in the 16th century because of the sugar found in the urine of people with diabetes (5, 6). In addition to its effect on sugar regulation, DM negatively impacts many important physiological processes, including fat and protein metabolism (2). Chronic hyperglycemia characteristic of DM correlates with long-term damage, dysfunction, and failure of multiple organs, including the eyes, kidneys, nerves, heart, and blood vessels.

The categorization of DM has long baffled medical professionals. Numerous classifications have been proposed, including the now-defunct insulin-dependent (ID) and non-insulin-dependent (NID) types of DM, which have been replaced with a nomenclature more consistent with the disease's accepted etiology (7). DM is currently characterized clinically into four primary categories and two subtypes (8). Type 2 diabetes mellitus (T2DM) is the most prevalent worldwide, with estimates ranging from 85 to 95%, followed by type 1 (9, 10). The gestational and other diabetes types are rarer and entail a broader range of causes, such as pancreatitis, genetic defects, and endocrinopathies (2). Type 1 DM (T1DM) is distinguishable from T2DM because the former is an autoimmune disorder where pancreatic beta-cells are destroyed, while the latter is characterized by progressively dysfunctional glucose regulation attributable to a combination of insulin resistance and pancreatic beta-cell destruction (11, 12). T2DM and prediabetes are often associated with a broader disorder known as "metabolic syndrome" (13). Differentiating between these various diabetes types is challenging since more than one type can manifest in a single patient (13), prompting calls for a revision of how the disease is classified (2).

Diabetes mellitus (DM) morbidity and mortality have become serious worldwide health concerns in both developed and developing nations (14, 15) straining the world economy (16). Recent controversial claims argue that the disease burden has shifted to developing countries, exacerbating the problem (17, 18) because developing countries already house 79 percent of the world's diabetes population (19), and the majority comprises of young people belonging to the lowest socioeconomic strata (20). Public health organizations tasked with epidemiological monitoring of DM have struggled to explain prediabetes's soaring frequency and incidence and the seeming failure to diagnose the condition globally (21). Diabetes is a prime example of the so-called "over-nutrition disease" that is often connected with a surfeit of nutrients and dietary richness (22, 23). "Extra-nutritional" variables, such as the usage of bisphenol A (BPA) in food processing and packaging, also contribute to the spread of diabetes and accompanying comorbidities (24). Societies that have recently transitioned to modern lifestyles have been particularly hard hit by T2DM (14). Asians appear more vulnerable to T2DM, including those in Pakistan, where its incidence has exceeded projections (25). The entire subcontinent of South Asia has been labeled the "diabetes capital of the world" (26). Current estimates indicate the number of DM sufferers globally at 451 million; this figure is projected to rise to 693 million by 2045 (27). Additionally, a whopping 374 million are estimated to be prediabetic, a physiological state that usually leads to full-blown T2DM, a number that is projected to climb by an additional 200 million in the next decades (28). Due to their inadequate healthcare infrastructure, developing nations such as Pakistan will be the hardest hit by these dismal projections (25); thus, traditional medicine will assume even greater importance for the general population in the coming years (14, 29).

The deteriorating situation has spurred several recommendations, including from the WHO (30–32), to investigate the use of plant-based therapies for DM in conjunction with conventional treatments to sustainably and affordably combat the DM epidemic (14, 30, 33). The inability of Western medicine to produce a treatment for DM is another major factor driving the demand for novel alternative medication. Allopathy relies on managing DM with oral hypoglycemic and hypolipidemic drugs with suboptimal therapeutic outcomes and potentially severe side effects (15, 34). The four most common oral hypoglycemics, sulfonylureas/insulinotropics, biguanides, α-glucosidase inhibitors, and thiazolidinediones, have demonstrated efficacy but also safety concerns (35). Natural plant-derived compounds are being increasingly investigated as alternatives to synthetic antioxidants to safely treat numerous oxidative stress-related diseases and conditions (36). Due to their greater molecular variety compared to synthetics (37), medicinal plants have given many therapeutic compounds for treating human ailments, including antidiabetics (38–40).

Methodology

A comprehensive literature search was conducted with publicly available web-based search engines and databases, PubMed, Scopus, ScienceDirect, Web of Science, Google Scholar, and other sources (R&D reports, graduate theses, and dissertations). The search was based on keyword combinations such as DM and etiology, oxidative stress and DM, herbal medicine and DM, challenges of conventional diabetes treatment, herbal medicine and DM, *N. sativa* and black seed oil, *N. sativa* and DM, *N. sativa* and phytoconstituents, thymoquinone and mechanism of action, antioxidant activity, gut microbiota, and DM. Research and review articles published in English from 1985 to 2023 were included. We also checked the references cited in the retrieved articles and reviews to avoid missing pertinent studies. The research articles were managed using EndNote software, version X9 (Thomson Reuters). Conference proceedings or abstracts, non-original research such as letters, protocols, editorials, commentaries, duplicated literature, clinical trials lacking robust controls, and papers dealing with homeopathic agents were excluded. A total of 481 relevant articles were found, which were exhaustively examined. An attempt was made to include literature from the disparate disciplines of human physiology, plant taxonomy, microbiology, and pharmacochemistry related to NS and its therapeutic potential in DM. The review incorporates the findings of 16 clinical trials.

The role of herbal medicine formulations in DM treatment

The rebirth of interest in traditional herbal therapy is due, in part, to the progressively dwindling returns of the reductionist

paradigm of drug development prevalent in the industry (25, 41, 42). A notable example is the ineffectiveness of conventional medications in treating chronic diabetes and their inability to address insulin sensitivity and secretion at the same time (43). Even metformin (metf), the US Food and Drug Administration's (FDA) recommended front-line medicine for DM (44), is devoid of contraindications (45, 46), yet it fails to exert its therapeutic effect due to patient noncompliance (47). Herbal products, as part of the broader notion of integrative medicine, can be used to supplement standard allopathy or to completely replace it, a concept known as complementary and alternative medicine (CAM) (48). CAM is more commonly used in patients with chronic DM, with most patients preferring to supplement rather than replace their orthodox drug regimens (49). The holistic approach inherent in herbal medication gradually strengthens the body's healing abilities and can better be described as preventive rather than curative.

Herbal therapy is based on the utilization of multi-ingredient formulations to achieve a combinatorial impact, with surprising effectiveness when compared to modern pharmaceutics, which is overwhelmingly centered on using single target molecules for treating complicated chronic condition like diabetes (50–52). According to WHO, 80 percent of the world's population still relies on traditional medicine for healthcare (53), making it a legitimate element of the global healthcare network (54). Interestingly, many of the drugs used in modern medicine have botanical origins. The primary antidiabetic drug Metf was originally obtained from the French lilac *Galega officinalis* (44, 55). Certain plant materials that are rich in antioxidants, have been found effective in treating diabetes (56–58). A considerable body of evidence underscores the importance of herbal medicines in the treatment of diabetes (59), particularly those classified as spices (60–63). However, due to a lack of compelling evidence, many in the medical community remain skeptical of the utility of herbal medicine for DM treatment (64). This has prompted efforts to better understand the safety and efficacy of CAM products, practices, and interactions, often using radical trans-disciplinary approaches such as reverse pharmacology (49, 65).

It is worth noting that simply consuming antioxidant rich plants foods cannot be considered herbal therapy because the antioxidant molecules are enmeshed in the food matrix, where dosage and bioavailability can be problematic. Individual plant-derived antioxidants such as vitamins and polyphenols for diabetes treatment, on the other hand, have only partially succeeded due to stability issues and differences in the physiologies of lab animals compared to humans. Despite these challenges, it has been demonstrated that combining different antioxidants can have a synergistic effect, and such formulations are becoming increasingly popular (66). Because of the complexities of diabetes, where oxidative stress is so deeply intertwined with multiple metabolic pathways, therapies including herbal ones that have the twin capacity of antioxidant renewal and ROS route blocking would have the best chance of success (67, 68). Many front-line contemporary drugs currently used for diabetes treatment, such as thiazolidinediones, metf, and glucagon-like peptide-1 (GLP-1) agonists, owe their effectiveness to antioxidant activity and glucose-lowering capability (66).

Taxonomy, biogeography, and ethnomedicinal importance of *Nigella sativa*

NS is an erect, annual flowering herb 20–90 cm tall, is one of the 20 species belonging to the genus *Nigella* L. (Family Ranunculaceae, Order Ranunculales, Class Magnoliopsida, Division Tracheophyta, Kingdom Plantae), accorded the taxonomic serial number of 506,592 by Integrated Taxonomic Information System (ITIS) (69), all the species withing this genus having utility as either food or medicine (70). The black tint of its discoid seeds occurs once they are exposed to air and is the source of many of its colloquial names "Black cumin" (71), "Black caraway" (72) "Alkamoun Alaswad" (73), or Black seed (74). Pertinent is the distinction between black cumin and black caraway from true cumin and true caraway, the latter two being the seeds of *Cuminum cyminium* L., and *Carum carvi* L, respectively, belonging to family Umbellifera (72). Despite the taxonomic impasse of dividing the Nigellae tribe into genera or sections, the consensus splits it into three genera, of which only *Nigella* is found in South Asia. *Komaroffia* and *Garidella*, on the other hand, are common throughout southern Europe and central Asia (74). NS stands out from the other *Nigella* spp. because its seed's volatile oils are particularly rich in TQ (75), making it a promising candidate for both traditional, modern evidence-based phytomedicine with preventive and therapeutic potential (76–79). It remains one of the most widely researched medicinal herbs (80) and the one most frequently cited throughout medical history as the ultimate "cure-all" (81), having been described by the Greeks, the Bible (82), and in traditional Arab and Islamic medicine (TAIM) where it is exalted as a prophetic medicine (83). Besides having culinary value (84), its seeds and oil are believed to have holistic medicinal properties and are used in many ancient medicinal schemas such as Ayurvedic, Siddha, Unani, Chinese, and Islamic (76, 85–86). NS and its oil have been labeled GRAS (generally regarded as safe) by the USFDA for use as a spice (87) but given only a qualified GRAS approval for use as a dietary supplement (88). It is one of the most frequently purchased herbal supplements in many of the world's leading markets (89). The Aegean and Irano-Turanian regions have been considered the evolutionary fountainhead of NS (90); however, a broader nativity claim is also made that includes North Africa and southwest Asia. Egypt produces the best commercial quality though it is also found growing wild in the Middle East, sub-continent, and Mediterranean countries (90, 91). India is its largest global producer (92). NS has an extensive array of pharmacological activities against various ailments and is considered a sacred herb in various religions, and a native, health-promoting plant in traditional medicine (78). In recent decades, researchers have found that NS has anti-inflammatory, and hepato-, neuro-, and gastro-protective effects (76, 78). It has also been substantiated that NS and its chemical constituents exert a nephroprotective effect by normalizing kidney function and reversing tubular damage along with suppression of biochemical alterations (93, 94).

Chemical composition of NS

NS seeds (NSS) have been the focus of study since the latter part of the 19th century (73) as they are the principal source of the herb's bioactive components, and their volatile oil consists mainly of

alkaloids, terpenes, and phenolics (90, 95). However, volatile oils comprise only 0.4–2.5% of the total seed content (96), the fixed oil being the principal part at 36–38% (87). The most active constituent is TQ (97, 98), discovered in 1963 (75). TQ makes up 18 to 57% of the volatile oil (96, 99), with the exact composition depending on species, seed chemotype, and oil extraction method (90, 100). The TQ content in NS oil (NSO) can be as low as 0.05 mg/ml to as high as 7.2 mg/ml (101). Some traditional processing practices employ solvents to effectively remove the TQ from NSS in an attempt to make them safer for people with specific health concerns (102). Other volatiles of interest are thymohydroquinone, pinene, p-cymene, and dithymoquinone. In addition to TQ, other potent radical scavengers include dithymoquinone, trans-anethole, thymol, and carvacrol, except dithymoquinone (90). In addition to these, carvacrol (5–12%), 4-terpinol (2–6%), and thymol are also present (83). NSS also contains limonene, citronellol, and two types of alkaloids, isoquinoline (nigellimin and nigellimin N-oxide) and pyrazole (nigelliden and nigellicin) (78, 87). TQ is present in several plant families besides the Ranunculaceae, but NS stands out as its richest source (103, 104), a claim worth investigating since members of the convergently co-evolved family Lamiaceae have shown amounts of TQ in their floral parts far exceeding those of NS (104, 105).

The essential oil of NSS is also high in polyunsaturated fatty acids, linoleic acid (50–55%), eicosanoic acid (4%), and monounsaturated fatty acids such as oleic (20%). NSS contains substantial phenolic compounds such as salicylic acid, quercetin, tocopherols, and phytosterols such as β-sitosterol (90).

The nexus of DM and oxidative stress revisited

Oxidative stress (OS) is a central concept in biology and medicine. Since its introduction in 1985, it has evolved from a simple imbalance in oxidative species in cells and tissues (106) to a much more complex interaction between reactive oxygen species (ROS) and receptors, signaling pathways, and antioxidant defenses. It incorporates a loss of homeostasis in a cell's many redox-driven physiological processes, defined as "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and molecular damage" (107). Depending on the degree of oxidative stress, it can be either harmful (distress) or helpful (eustress) (108). The pivotal role of redox control in cellular functions describing redox homeostasis as "aurea mediocritas" the golden mean of healthy life (109) has led many researchers to view T2DM as a redox disease (110).

OS is brought about by two broad classes of molecules, reactive oxygen species (ROS), such as H_2O_2, ·OH, and O2-and reactive nitrogen species (RNS), such as NO and NO_2. These are naturally generated as byproducts of metabolic activities (111), but their accumulation can damage biological macromolecules like proteins, lipids, and nucleic acids (112). Under normal circumstances, with a robust antioxidant system, these molecules do not constitute a threat; only when the antioxidant response is compromised or oxidant levels rise too rapidly do they cause oxidative damage leading to diseases such as T2DM (113, 115). Free radicals are species containing one or more unpaired electrons, and this incomplete electron shell accounts for their high reactivity (116, 117). The most common free radical is superoxide anion (O_2^{-}.) (118), generated by the action of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (119). The most unstable and destructive is the hydroxyl radical (·OH) formed by the reaction of H_2O_2 with metal ions (Fenton reaction) which damages lipids through peroxidation, triggering a chain of adverse events (113).

Peroxynitrite is a potent pro-oxidant (114) implicated as the causative agent of the cardiovascular endothelial dysfunction seen in T2DM (120). ROS can be generated via many pathways within cells; however, metabolic activities occurring in the mitochondria and endoplasmic reticulum and enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase are the usual causes associated with OS-mediated onset of T2DM (66) (Figure 1). However, it has been argued that terms like total antioxidant capacity (TAC) and reactive oxygen species (ROS) are too general and prone to

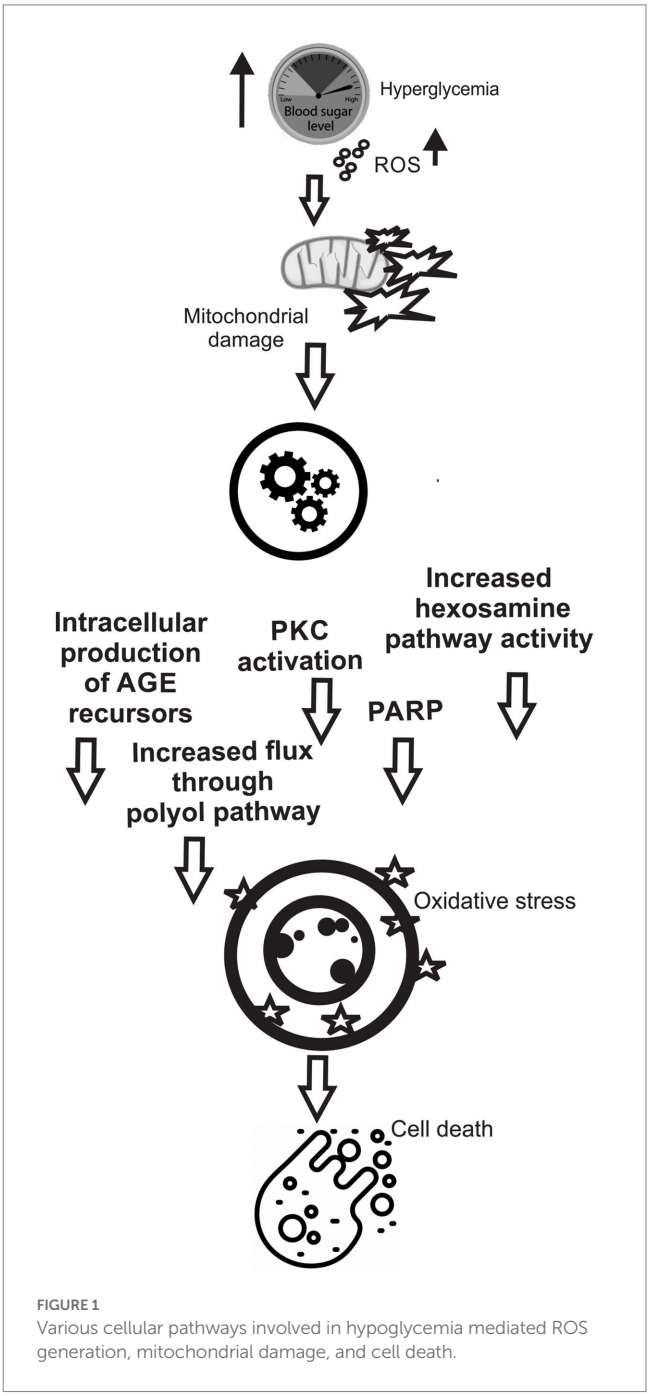

FIGURE 1
Various cellular pathways involved in hypoglycemia mediated ROS generation, mitochondrial damage, and cell death.

misinterpretation when applied to the complex system of redox and oxidative components inherent in OS. Careful identification of the individual oxidant and antioxidant moieties and their behaviors allows for a better understanding of the mechanics of OS (121, 122).

The nexus of OS with diabetes has remained a matter of scientific debate since the 1980s (123), their association being explicitly propounded in the "common soil" hypothesis (124). OS plays a primary role in the pathogenesis of DM (125, 126) as manifested through many enzymatic, non-enzymatic, and mitochondrial mechanisms (127). Free radicals and peroxides are produced in large quantities in T2DM via glucose oxidation and non-enzymatic protein glycation, which can overwhelm antioxidant defense mechanisms leading to cellular damage (119), a phenomenon termed "hyperglycemic or metabolic memory" (128). Auto-oxidation of glucose generates hydroxyl radicals, while membrane-associated xanthine oxidase and nitric oxide synthase generate free radicals, ROS, and RNS (129). Accumulating these oxidants can result in the formation of lipid peroxidation products such as the highly hazardous malondialdehyde (MDA) and acrolein generated by free radical-driven peroxidation of polyunsaturated fatty acids like arachidonic and linoleic acids (113). Heightened MDA levels are typical of diseases with an OS component (130). Although ROS and free radicals are involved in the proinflammatory cytokine-mediated beta-cell injury, not all free radicals generated are implicated in their destruction, as in the NO case (131). High ROS levels also reduce the bioavailability of nitric oxide (NO) generated by endothelial cells, whose multifunctional signaling role is crucial to vascular integrity (132). The proper functioning of cellular antioxidant systems is the mainstay in limiting oxidative damage (129), especially in the context of DM (133).

DM harms the tissues by increasing the non-enzymatic formation of advanced glycation end products (AGEs) through the Maillard reaction and glucose auto-oxidation, leading to loss of protein function. The binding of AGEs to the receptor for AGE (RAGE) induces NADPH oxidase-1 to produce more ROS (134, 135) in addition to activating atherogenesis-promoting signal transduction mechanisms (136) like mitogen-activated protein kinase (MAPK) that further magnifies the inhibition of NO formation by AGEs (137). AGE-induced oxidative stress and the ensuing diabetic progression ultimately depend on the cellular balance between RAGE and AGER1; AGER1 binds and degrades AGEs while RAGE promotes oxidative stress and has a freer rein in chronic diabetes since AGER1 removal of AGE is suppressed (138).

Recent research suggests that mitochondria unavoidably generate significant ROS through oxidative phosphorylation (139). The ROS and RNS generated in mitochondria pass out into the cellular milieu, harming cytoplasmic organelles and damaging macromolecules (140), making DNA more vulnerable to mutagenesis (141). Critical cellular homeostases pathways such as autophagy and mitophagy that clear the cell of damaged macromolecules and organelles such as mitochondria and endoplasmic reticula appear less active in people with diabetes, although the mechanism is unknown (66). The diametrically opposed concept of mitohormesis proposes a dual dose-dependent action of ROS species, wherein, when produced by mitochondria in typically "small" amounts, they have a salubrious instead of a noxious effect. Some researchers proposed that DM may be a consequence of slowed-down mitochondrial machinery, which can restore health if restored to normal levels of superoxide production (142, 143). Intriguingly, a mitohormetic effect in animals has been demonstrated for the front-line antidiabetic metf (144). However, ambiguities remain since the studies did not consider the damaging effects of reductive stress that precedes oxidative stress in hyperglycemia on mitochondria and malfunctioning feedback mechanisms (142). Nonetheless, the importance of reducing the physiological factors that cause oxidative stress in conjunction with the use of natural antioxidant products, known as the "optimal redox" (OptRedox) approach, is being studied as a public health policy to stem the rising incidence of T2DM in global populations, particularly those of the very young (145). Instead of the common "detect and treat" medical practice of redox medicine, a more targeted use of inhibitors and agonists impacting oxidative stress linked biochemical pathways is hypothesized as a more successful therapeutic approach, while supporting empirical proof is currently a pipedream (108).

NS as an antidiabetic agent

ROS levels rise throughout the progression of diabetes, and they are known to be implicated in the destruction of beta cells. TQ's lack of effect on the transcription factor nuclear factor kappa-B (NF-κB), whose activation by OS is a prelude to diabetes, has not lessened its value as a potential therapeutic (146). It is still a powerful inhibitor of the inflammatory pathways that underpin autoimmune illnesses like T1DM, particularly those involving MAPKs (147, 148) and several *in vivo* and *in vitro* studies substantiate the antidiabetic efficacy of NS (38, 149–153). In comparison to fixed oil, the essential oil of NS is thought to have more effective antioxidant activity (154), and if stored appropriately, the antioxidant capacity of the NS oil rises over time despite having a lower TQ content than when it was first extracted (101). Both the NS volatile oil and its principal bioactive ingredient, TQ, are known to improve hyperglycemia and hyperlipidemia (83). In animal studies, NSO's anti-hyperglycemic effect was equivalent to, if not superior to, metf, the principal hypoglycemic medicine now in use (155). Because this was a one-time trial, larger-scale research using more NS parameters may provide a clearer picture. NS has been linked to pancreatic islet regeneration, and the antidiabetic mechanism maybe due to NS's ability to increase insulin secretion by boosting β-cell proliferation (156–158). In addition, NS extracts can decrease the body's inflammatory and OS markers (159) as well as boost skeletal muscle glucose uptake and adenosine monophosphate-activated protein kinase (AMPK) activity (160). NS and TQ repress gluconeogenesis in the liver (161) by explicitly targeting the enzymes glucose-6-phosphatase and fructose-1, 6-biphosphatase (162) and also retard glucose absorption in the alimentary tract while enhancing glucose tolerance in rats (163). Lowering increased glucose uptake in diabetics by inhibiting the intestinal glucose transporter, sodium-glucose linked transporter 1 (SLGT1) through bioactive compounds represents a promising target for novel drug development (164). Controlling the postprandial glycemic spike in T2DM patients by blocking the digestive enzymes α-glucosidase and α-amylase with NS as opposed to clinical drugs that tax the gut physiology is also a feasible strategy (165). The feeding of NS extract decreased lipid peroxidation and increased antioxidant enzymes such as Superoxide dismutase (SOD), Catalase (CAT), and Glutathione peroxidase (GPx) in the organs of rats with chemically-induced diabetes (166,

167). The antidiabetic benefit of ground NSS via an antioxidant-based mechanism has been proven in large-scale clinical trials involving T2DM patients (168, 169). However, the same could not be said of NSO in such trials (170, 171) even though NSO supplementation causes a marked reduction in oxidative stress in healthy individuals (172). A host of confounding factors related to intervention methodology and the quality of the tested herbal product have been cited as the cause of this inconsistency (173). In a first-of-its-kind clinical trial comparing NS as monotherapy to metf in the treatment of diabetic patients, the former failed to achieve therapeutic outcomes (174). It could that the NS quantity was subtherapeutic (1,350 mg/day) since studies suggest that NS doses of less than 2 g are clinically inconsequential (168). On the basis of a meta-analysis of relevant clinical trials, the general consensus is that NS supplementation is an effective treatment for T2DM (175). Chronic T2DM marked by hyperglycemia-mediated insulin resistance remains a therapeutic challenge and the ameliorative impact of TQ has just lately come to light (176). TQ can also improve levels of insulin receptors improving insulin action, low levels of which are the cause of insulin resistance and type 2 DM (177). TQ can dramatically reverse the diabetes associated drop in Glut-2 levels (177), a transporter protein responsible for glucose transfer between the liver and blood and its reabsorption by the kidneys (178). By suppressing oxidative stress, reducing low-density lipoprotein (LDL), raising high-density lipoprotein (HDL-C), and lowering total blood cholesterol, TQ also mitigates cardiovascular complications such as atherosclerosis that accompany the course of diabetes (162, 179, 180).

It has been discovered that all forms of NS, including oil, water extracts, dried and crushed seed portions, show substantial hypoglycemic potential, particularly those based on aqueous extraction (181). Since this form of extraction gives the lowest TQ content (182), it implies the presence of active compounds in NS seeds other than TQ, of which there are over a hundred, many of which are unknown (183), but which may be equally beneficial in diabetes management (184). Multiple clinical trials (Table 1) and related studies (Table 2) evaluating different oral quantities advised for NSS, oils, and TQ have demonstrated that NS ingestion does not result in acute or chronic toxicity (219, 220) and is deemed safe among the hundreds of candidate medicinal plants and the oral antidiabetics currently available (76). However, few significant clinical trials on the safety and efficacy of NS have been conducted, highlighting the need for additional study (221). Some trial results, such as the one evaluating the suitability of NS supplementation for diabetic patients undergoing hemodialysis, have not yet been published (222). Even if unfavorable reactions in human subjects are uncommon, it is prudent to proceed with caution and prudence. There is some evidence linking NSS and essential oil to negative health effects in laboratory animals (223, 224). Consumption of NSS has been associated with inhibition of the drug-detoxifying enzymes cytochrome P450 2D6 (CYP2D6) and cytochrome P450 3A4 (CYP3A4), which raises the specter of unforeseen drug–drug interactions and prescription drug toxicity (225). Reaction to these enzymes is a key aspect of the protocols followed by worldwide drug regulatory authorities, including the FDA, when reviewing innovative drug candidates (226). TQ inhibits CYP2C19 and CYP3A4 *in vitro*; the latter is involved in the biotransformation of various oral antidiabetic drugs, a concern which must be investigated through *in vivo* studies (227).

Hepatoprotective and lipid-lowering effects of NS

Due to the features of several new medications, the necessity for hepatoprotection is becoming an increasing concern (228). Acute hepatotoxicity can lead to liver cancer (229), and almost half of drug-induced hepatoxicity cases were attributed to acetaminophen/paracetamol overdosing (230). The liver is the primary site of drug detoxification and is particularly vulnerable to OS (231). NS, because of its protective activity against an array of natural and synthetic toxins (232), including xenobiotics (233), and because of its relative safety and potent antioxidant and anti-inflammatory effects, is ideal for reducing the side effects of neoadjuvant therapy of the cancerous liver before ablative surgeries (228). NSS extracts stabilized lipopolysaccharide-induced hepatotoxicity by normalizing levels of aspartate aminotransferase (AST), Alanine aminotransferase (ALT), and Alkaline phosphatase (ALP) (234). Similarly, NSO effectively raised antioxidant enzyme levels and improved liver function in malathion-induced liver dysfunction (235) and hypervitaminosis (236). TQ also normalized hepatic OS and lowered cholesterol levels, which were elevated due to a cholesterol-rich diet (237). Research has demonstrated that TQ can normalize the amounts of the liver enzymes such as oxidized glutathione (GSSG), SOD, and MDA and enhance reduced glutathione (GSH), essentially protecting against oxidant damage to the liver (238). NSS also augmented the hepatoprotective effect by enhancing CAT, and GPx activity (215, 239). Liver damage due to therapeutic drug overdose is a growing health concern (240). Presently, N-acetylcysteine is the sole clinical intervention for acetaminophen overdose, but its drawbacks such as poor bioavailability, high costs, and side effects warrant exploration for a new, natural curative (221, 241–243). Acetaminophen (APAP)-related hepatotoxicity was effectively countered in experimental rats receiving a combination of α-Lipoic acid (ALA) and TQ as assessed by a decrease in ALT and ALP function and down-regulation of cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGFR1, flt-1) expression (216). Another study suggested that TQ's mechanism for attenuating APAP-induced acute liver injury involved inhibition of the entire MAPK family with simultaneous activation of the AMPK pathway (221). High doses of NS supplements over prolonged periods can reduce ALP levels substantially, but because of the imprecision of NS's dose, duration and effect on liver parameters, its approval as a treatment for liver ailments has remained elusive (244).

Antioxidant activity of NS and TQ

Since the 1990s, the notion of antioxidants has been in the public eye, and their role in disease prevention a subject of interest. They have been defined as "any substance able to eliminate ROS and derivatives (RNS, or reactive sulfur species, RSS), directly or indirectly, acting as an antioxidant defense regulator, or reactive species production inhibitor" (245). Using antioxidants to complement diabetes therapy has gained much prominence, and many compounds of plant origin and vitamins have been scrutinized as possible candidates, each with its challenges and shortcomings (246–249). NS essential oil has been markedly better at radical scavenging than many commercially available synthetic antioxidants

TABLE 1 Clinical trials for NS and TQ supplementation on DM outcome.

Sr. No.	Dose and form of NS	Duration of treatment	Trial/Test design	Outcome	References
1	NS capsules: 500 mg/capsule used as 2 g/day	1 year	Randomized clinical trial 114 T2DM patients (18–60 Y) I: Control (charcoal-placebo) II: NS group	• Significant decrease in FBG, HbA1c and TBAR • Significantly elevated TAC, SOD and GSX levels • Significantly low insulin resistance and upregulated β-cell activity • Long term NS supplementation is better than oral hypoglycemics in controlling glycemia and oxidative stress in T2DM patients	(169)
2	NS tea: 5 g/day	6 months	41 T2DM patients +25 healthy controls (identical in age) I: Control: NS tea II: NS tea + oral antidiabetic drug	• Significant decrease in FBG, PPBG, and HbA 1c • Significant decrease in AST serum bilirubin, blood urea ALT, and serum creatinine • NS tea is recommended as supplemental antidiabetic therapy.	(185)
3	NS/capsule: 1 g, 2 g, and 3 g	3 months	Randomized controlled trial of 94 T2DM female patients (mean age range: 44.91–49.63 Y) I: NS capsule 1 g/day (n = 16) II: NS capsule 2 g/day (n = 18) III: NS capsule 3 g/day (n = 17)	• NS (1 g/day) minor improvement in all the measured parameters from the baseline. • NS (2-3 g/day) significant reductions in FBG, 2hPG, and HbA1 • No significant change in BW. • Reduction in insulin resistance ($p < 0.01$) • Increase in β-cell function ($p < 0.02$) after 12 weeks of treatment. No adverse effects on renal and hepatic functions with either dose	(168)
4	NSS: 250 Mg*trigonella foenum-graecum*: 250 mg	3 months	100 T2DM patients (30 to >40 Y) Male and female I: Control (Glibenclamide) II: Intervention (NS + *Trigonella foenum-graecum* seeds + Glibenclamide)	• Significant increase in serum HDL levels • No significant change in serum creatinine and triglyceride levels	(186)
5	NSO:1,350 mg/day	3 months	66 newly diagnosed T2DM patients (≤6 months) (18-60Y) I: Metf II: NS oil capsule,	• NS was inferior to Metf in glycemic control (in lowering FBG, 2 h pp., and A1C or increasing %B) • NS was comparable to Metf in significantly lowering weight, WC, and BMI. • NS was comparable to Metf in its effects on fasting insulin, %S, IR, ALT, TC, LDL, HDL, TG, and TAC. • Metf showed a significant increase in AST and creatinine compared to NSO.	(174)
6	TQ: 50 mg/kg	90 days	60 T2DM I: 1 metf +1 TQ II: 1 metf +2 TQ III: 1 metf	• Glycated hemoglobin (HbA1c) levels decrease after 3 months of TQ intake. • A more significant reduction in FBG and postprandial blood glucose was also observed in TQ receiving groups compared to metf alone	(17)
7	NSO: 3 g/day	12 weeks	72 T2DM patients (30–60 Y) I: Treatment (NSO) II: Placebo (sunflower oil)	• Insignificant BW and BMI reduction • Dietary intake in both groups changed compared to baseline. • Significant changes in FBS, HbA1c, TG, and LDL-c • Insulin level and insulin resistance decreased and • Insignificant increase in HDL-c	(170)
8	NS soft gel capsules: 500 mg Twice/day	8 weeks	A randomized controlled 43 (23 women, 20 men) T2DM participants (30–60 Y) I: NSO II: sunflower oil	• NSO significantly decreased FBS, HbA1c, total cholesterol, TG, LDL-c, BMI, waist circumference, SBP, and DBP. • No significant change in HOMA-IR and HDL-c	(187)
9	NSO: 2.5 ml twice/day	6 weeks	60 patients (50 males and 10 females) with obesity, diabetes, dyslipidemia I: Met + atorvastat II: Met + atorvastat + NSO	• Significant improvement in total cholesterol, LDL-c, and FBG	(188)
10	NSO equivalent to 0.7 g of seed/day	40 days	41 T2DM patients 1st 40 days NS treatment, following 40 days placebo treatment	• Significant decrease in FBG and increase in insulin and AST levels • No changes in platelet count, total leukocyte count, and ALT blood urea comparable to baseline levels.	(189)
	NSS	40 days	Male + female T2DM patients (30-60Y) 1st 40 days NS treatment, following 40 days placebo	• Improved levels of BGL, INS, and lipids • Decreased fasting blood glucose, TC, LDLc, TG, HDL	(190)
11	NS: 1 g, 2 g and 3 g	30 days	45 diabetic patients I: NS 1 g (glucose <180 mg/dL) II: NS 2 g (glucose 180–220 mg/dL) III: NS 3 g (glucose >220 mg/dL)	• Significant improvement in blood glucose (2 g NS shows better performance) • Negligible improvement of lipid profile in all groups.	(191)

(Continued)

TABLE 1 (Continued)

Sr. No.	Dose and form of NS	Duration of treatment	Trial/Test design	Outcome	References
12	NSO: 5 ml/daily	3 months	70 T2DM patients (30 males, 40 females) I: NSO II: Mineral oil (placebo)	• Significant decrease in the blood levels of fasting and 2 h postprandial glucose and HbA1c and BMI • No side effects	(192)
Prediabetic/metabolic syndrome					
13	NSO capsule: 450 mg twice /day	6 months	Open-label, randomized prospective comparative 117 prediabetic patients (18–65 Y) I: LM group, a calorie-restricted diet with moderate exercise II: Metf group, Metf tablet 500 mg/day for initial 2 weeks, then same was given twice/day III: NS soft gelatin capsules containing 450 mg NSO twice daily Group II and III did not follow a lifestyle management program (LM)	• NS was statistically like Metf in improving anthropometric glycemic parameters and SIRT1 gene expression. • NS improved lipid panel and suppressed inflammation • Significantly reduced TNF-α and Castelli risk index-I • NS may represent a promising intervention for obese prediabetic subjects	(193)
14	NS powder: 1.5 g/day In combination, NS: 900 mg/day	8 weeks	Double-blind, randomized, 250 healthy male (MetS participants) (44 ± 13.3 Y) I: Powdered NSS II: NS powder + Turmeric powder III: Placebo of Ispaghul husk	Week 4: • Showed improvement in BMI, WC, and BF%. • The combination improved all parameters except HDL-c with lower FBG and LDL-c compared to placebo. Week 8: • Reduced lipids and FBG, • Combination group with a 60% dose of the individual herbs showed an improvement in all parameters from baseline. • Reduced BF%, FBG, cholesterol, TG, LDL-cholesterol, and CRP, but raised HDL-cholesterol.	(194)
15	NSO 500 mg/day	8 weeks	80 metS patients (52 male, 38 females) 20–70 Y (majority 40-60Y) I: Met + Astorvastatin + Aspirin II: NSO + Aspirin	• NS significantly lowered FBG, PPBG and HbA1c after 8 weeks. The NS group showed significant improvement in FBG, PPBG, HbA1c, and LDL cholesterol. • NS is safe and an effective remedy for patients with metabolic syndrome	(195)
Safety/toxicity studies					
16	TQ: 10, 20, 100, 200, 400 mg capsules	1 to 20 weeks	18 adult patients with solid tumors or hematological malignancies (at least 18 Y) with an Eastern cooperative oncology group performance status (ECOG) of ≤2; received TQ orally at a starting dose of 3, 7, or 10 mg/kg/day. Dose escalation was done using a modified Fibonacci design.	• No side effects or systemic toxicities were reported, and the maximum tolerated dose (MDT) was not identified. • No anti-cancer effects were observed. • Tolerable oral TQ dose ranging from 75 mg/day to 2,600 mg/day	(196)
17	TQ: 200 mg capsules	90 days	70 healthy adult volunteers (phase I randomized, double-blinded, placebo-controlled trial clinical trial) Each participant received a single daily dose of 200 mg/day, 10–20 min before bedtime.	• No significant alterations in the hematological parameters • No significant changes in the biochemical parameters of liver function • (ALT, AST, ALP), renal function (serum creatinine and urea) • 5% TQ v/v in NS oil at a dose of 200 mg/adult/day is safe for human consumption and ought to be clinically evaluated for various health related pharmacological activities.	(197)

metS, metabolic syndrome; T2DM, type II diabetes mellitus; NS, *Nigella sativa*; FBG, fasting blood glucose; FBS, fasting blood sugar; HbA1c, glycosylated hemoglobin; TG, triglyceride; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; HOMA, homeostatic model assessment of insulin resistance; BMI, body mass index; WC, waist circumference; SOD, superoxide dismutase;AST, aspartate aminotransferase; ALT, alanine Aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen.

(250). Recent work has shown that TQ, carvacrol, t-anethole, 4-terpineol, tannins, flavonoids, and alkaloids contribute to the radical scavenging properties of NSS with TQ and nigellone accounting for the majority of the activity (251). NS sustains the cellular microenvironment by increasing the body's antioxidant enzymes, SOD, GPx, and CAT, and enhancing ROS scavenging capacity (169) by increasing vitamin C and E levels (252). In addition to TQ, NS contains other antioxidants such as flavonoids, phenolics, ascorbic acids, and tocopherols (253). Because of its antioxidant capacity, TQ decreases tissue MDA levels, prevents DNA damage, reduces mitochondrial vacuolization and fragmentation, and maintains pancreatic β-cell integrity (252). Lipid peroxidation is a marker of significant stress, and TQ can reduce lipid peroxidation by its robust scavenging of ROS (254). TQ can be reduced to thymohydroquinone under normal intracellular physiological conditions, and to the pro-oxidant semiquinone under pathological conditions with excess of metal ions (255, 256). Based on examination of its molecular structure, the claim that reduced thymohydroquinone possesses any radical scavenging activity, let alone exceeding that of TQ, has been questioned (257). The non-enzymatic binding of TQ with intracellular antioxidants, GSH, NADH, and NADPH results in moieties whose scavenging potency far exceeds that of the free TQ and is at par with Trolox, a powerful antioxidant and vitamin E analog (258, 259). TQ can also be reduced to dihydro-thymoquinone by DT diaphorase, which induces oxidative stress from ROS, causing cytotoxicity and DNA damage (100). Dihydro-thymoquinone becomes a concern in specific circumstances, such as cancer, where DT diaphorase levels are elevated, or in animal trials where large

TABLE 2 Animal studies done in the last 5 years for assessing NS and TQ safety and dosage.

Sr. No.	Dose and form of NS	Duration of treatment	Trial/Test design	Outcome	References
1	TQ: 50 mg/kg in 0.1% DMSO	12 weeks (84 days)	60 male Wistar rats (age?) STZ-induced DM rats I: Healthy control II: DM untreated control II: DM TQ treated III: DM TQ-vehicle control Treatment via gastric gavage	Reduction in NO and MDA levels in testicular tissue • Exerted a protective effect against reproductive dysfunction induced by diabetes	(198)
2	NS powder: 300 mg/kg	8 weeks (56 days)	24 Albino rats (sex?) (age?) 8 weeks high-fat diet (HFD) I: Control II: HFD untreated control III: HFD virgin olive oil treated IV: HFD NS-treated Treatment via intragastric intubation	• Reduce the serum lipid profile, TC, TG, LDL, HDL, BGL and amylase • Significant increase in INS levels • Regeneration of the exocrine and endocrine parts of the pancreatic tissues	(199)
3	TQ: 80 mg/kg	7 weeks(49 days)	50 white male albino rats (*Rattus norvegicus*) (6–7 weeks) STZ-induced DM I: Control II: DM control III: DM TQ-treated IV: DM Met-treated V: DM Met+TQ-treated Treatment via p.o.	• Decreases the MDA levels and up-regulated the expression of Glut-2 • Enhance the antidiabetic activity of MET in STZ-induced diabetic rats	(200)
4	NS_1: 100 mg/kg NS_2: 200 mg/kg NS_4: 400 mg/kg	6 weeks (42 days)	70 male Wistar rats (10 weeks) STZ induced DM i.p. I: Control II: DM control III: DM NS_1-treated IV: DM NS_2-treated V: DM NS_4-treated Treatment via gavage	• Reduced serum glucose, lipids and improved AIP (Atherogenic index of plasma) • Significantly increased eNOS (endothelial nitric oxide synthase) • Decreased VCAM-1 and LOX-1 expression	(149)
5	NSO: (91 mg/100 ml) 1. 0.5 ml 2. 1 ml 3. 1.5 ml	40 days	30 Laboratory bred male albino Wistar rats (age?) STZ-induce DM i.p. I: DM control II: DM Met-treated III: DM NS-treated (1) IV: DM NS-treated (2) V: DM NS-treated (3) Treatment via p.o.	• Significant reduction in BGL • Partial regeneration of β islet cells of the pancreas by 1.5 ml of NS	(201)

(Continued)

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TABLE 2 (Continued)

Sr. No.	Dose and form of NS	Duration of treatment	Trial/Test design	Outcome	References
6	NSSP (NS seed polysaccharides) (0.1 ml/10 g) High dose: 140 mg/kg Med dose: 70 mg/kg Low dose: 35 mg/kg	4 weeks	60 male pathogen-free Kunming mice (4 weeks), 4 weeks on a high-fat diet (HFD) STZ-induced diabetes I: Control II: DM Met-treated III: DM NSSP high dose treated IV: DM NSSP med-dose treated V: DM NSSP low dose-treated Administration via intragastric tubing	• High-dose NSSP could significantly lower the levels of FBG, GSP, TG, TC, LDLc, MDA, TNF-α, IL-6, and IL-1β, and • Significantly increased INS, HDLc, T-AOC, SOD, CAT, p-AKT and GLUT4 • NSSP could improve the abnormal state of diabetic mice by regulating the PI3K/AKT signaling pathway with simultaneous changes in the gut microbiota profile.	(202)
7	TQ: 50 mg/kg BW	4 weeks (28 days)	18 male Sprague–Dawley rats (age?) STZ-induced DM I: Control II: DM III: DM TQ-treatment Treatment via gastric gavage	• Significantly lower levels of HbA1c, lipid peroxidase, and NO • Higher TAC • Attenuated the effect of STZ-induced diabetic nephropathy • TQ adjusts glycemic control and reduces oxidative stress without significant damaging effects on renal function.	(203)
8	TQ in corn oil TQ-10: 10 mg/kg TQ-20: 20 mg/kg	21 days	40 male Wistar rats (age?) STZ-induced T2DM I: Control II: DM untreated III: DM TQ-10 treated IV: DM TQ-20 treated V: DM TQ-10 + fluoxetine treated VI: DM TQ-10 + fluoxetine treated Treatment via p.o.	• TQ decrease in BGL, no further significant change was recorded with TQ + fluoxetine treatment. • Significantly decreased immobility time • Increased latency to immobility and locomotor activity • TQ and fluoxetine combination reduced TBARS level and increased GSH content but did not affect antioxidant enzyme activities. • Reduction in inflammatory markers (IL-1b, IL-6 and TNF-a) TQ + fluoxetine can be used to control depression	(204)
9	TQ: 50 mg/kg	21 days	Male ICR (CD1) mice (Envigo, IN, USA) (8–9 weeks) STZ induced DM I: DM met treated II: DM metf + TQ treated Treatment via p.o.	TQ showed a significant decrease in BGL compared to metf	(17)
10	TQ: 20 mg/kg 40 mg/kg 80 mg/kg	21 days	Wistar female albino (age?) Nicotinamide + STZ induced T2DM i.p I: Vehicle control II: DM control III: DM Met treated IV: DM Met-NCs treated V: DM TQ-20 treated VI: DM TQ-40 treated VII: DM TQ-80 treated VIII: DM TQ NCs-20 treated IX: DM TQ NCs-40 treated X: DM TQ NCs-80 treated XI: DM blank NCs Treatment via p.o.	• NCs showed a sustained release profile as compared to their pure forms. • TQ or Met and their NCs significantly decreased BGL and HbA_{1c} • improved the lipid profile • TQ-loaded NCs produced a dose-dependent antihyperglycemic effect comparable to TQ and Met. TQ NCs (containing half of the doses of TQ) produced a better antihyperglycemic effect in T2DM rats than TQ alone.	(205)

(Continued)

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TABLE 2 (Continued)

Sr. No.	Dose and form of NS	Duration of treatment	Trial/Test design	Outcome	References
11	TQ: 10 mg/kg TQ loaded NFs: 10 mg/kg	21 days	60 Wistar female albino rats (age?) Nicotinamide-STZ induced DM i.p. I: Control d. H2O II: Control oleic acid III: Diabetic control IV: DM Met treated V: DM GL treated VI: DM TQ treated VII: DM GL + TQ treated VIII: DM GL-NFs treated IX: DM TQ-NFs treated X: DM GL + TQ-NFs treated	• Significant decreases in BGL and HbA_{1c} • Significant improvements in BW and lipid profile Synergistic effect of combined NFs, leading to enhanced absorption of NFs and lesser cytotoxicity than pure bioactive compounds	(206)
12	TQ: 10 mg/kg 20 mg/kg	14 days	30 Wistar rats (8 weeks) STZ-indued T2DM in 4-week HFD I: Control II: DM control III: DM TQ-10 treated IV: DM TQ-10 treated V: TQ control Treatment via p.o.	• Significantly prevented hyperglycemia, hyperinsulinemia, hyperlipidemia, INS resistance, and inhibited DPP-IV • An alternative natural drug in the management of hyperglycemia-induced INS resistance	(176)
13	TQ?	1 h	Male Sprague–Dawley rats (age?) STZ induced T1DM 2-week daily pretreatment (via oral gavage) of T1DM rats with Sitagliptin I: TQ treatment in sitagliptin-pretreated II: TQ treatment in non-sitagliptin-pretreated Additionally, varying doses of exendin 9–39 were pretreated 30 min before TQ in DM with or without pretreatment with sitagliptin. Treatment via i.p.	• The direct effect of TQ on imidazoline receptors (I-Rs) was identified in CHO-K1 cells overexpressing imidazoline receptors (I-Rs). • Enhances GLP-1 secretion by intestinal NCI-H716 cells TQ may promote GLP-1 secretion through I-R activation to reduce hyperglycemia.	(207)
14	Methanol NS Plant extract: 500 mg/kg BW	120 min for glucose 240 min for sucrose	40 males + female Long Evan rats (age?) STZ induced T2DM	• The extract reduced postprandial glucose, • Improved glucose (2.5 g/kg, BW) tolerance in rats. • Significant improvement in GI motility • Reduced disaccharidase enzyme activity in fasting rats. • Potential hypoglycemic activity • Significantly improved INS secretion from isolated rat islets. Generate postprandial anti-hyperglycemic activity in T2DM animal models via reducing or delaying carbohydrate digestion and absorption in the gut and improving INS secretion in response to the plasma glucose.	(208)
15	Ethyl acetate fraction of Ethanolic NS plant extract: 200 mg/kg, 500 mg/kg and 1,000 mg/kg BW		25 male rats (age? Breed?) Alloxan induced T2DM	• Reduced blood glucose levels	(209)

(Continued)

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TABLE 2 (Continued)

Sr. No.	Dose and form of NS	Duration of treatment	Trial/Test design	Outcome	References
16	Hydroalcoholic extract of NS seed: 200 mg/kg and 400 mg/kg	Oral administration for 4 weeks	24 male Wistar rats (age?) STZ induced T2DM	• Reducing effect on FBS and oxidative biomarkers • Increases serum insulin levels	
17	Ethanolic extract of NS seed, using 20 and 40% wt/wt of feed	Oral administration for 15 days	35 Wistar albino rats (age?) (gender?) Alloxan monohydrate induced T2DM	• Significant decrease in blood glucose levels • Significant antioxidant activity (elevated SOD levels)	(210)
18	Methanolic extract of NS seed and NS oil; 2.5 ml/kg/day	Oral administration for 24 days	15 male rabbits (age?) Alloxan (150 m/kg) induced T2DM	• Both NSS methanolic extract and NSO were significantly hypoglycemic • NSO was more effective than methanolic extract of NSS in reducing serum catalase, ascorbic acid and bilirubin	(211)
19	Ethanolic extract of NS seed (100 mg/kg/BW) and TQ (10 mg/kg/BW)	Oral administration for 28 days	28 male Wistar rats (age?) STZ (90 mg/kg/BW) induced T2DM	• significant decrease in blood glucose, urea, creatinine, uric acid, total protein, total cholesterol, low-density lipoprotein, and very low-density lipoprotein, while high-density lipoprotein was increased. • Hepatic enzymes, alanine transaminase, aspartate aminotransferase, and alkaline phosphate were also normalized. • significantly increased body weight.	(212)
20	Ethanolic extract of NS seeds; 300 mg/kg/BW and 600 mg/kg/BW	Oral administration for 7 days	Male Wistar rats (200–250 g BW), number? age? gender? STZ (50 mg/kg/BW) induced T2DM	• Significant reduction in blood glucose, total cholesterol, triglycerides, • VLDL and non-HDL cholesterol comparable to metformin	(213)
21	NS oil; 2.5 ml/kg/BW	Oral administration for 56 days	30 Male Wistar rats (age?) STZ (45 mg/kg/BW) induced T2DM	• NS oil significantly normalize blood urea • Significantly nephroprotective and anti-DM	(214)
Safety/toxicity					
22	NS powder: 3 g/kg/day NSO: 2 g/kg/day NS ethanol extract: 0.5 g/kg/day	60 days	50 male Sprague–Dawley rats (age?) Cisplatin-induced nephrotoxicity I: Healthy control II: Diseased positive control (d. H2O) III: NS powder treatment IV: NSO treatment V: NS extract treatment Treatment via stomach tube	• Reduced serum levels of urea, creatinine, and K • Significant increase of Na, Na/K, vitamin D, nutritional markers, and antioxidant enzymes. All forms of NS contain potent bioactive components that help in cisplatin-induced renal toxicity in rats.	(93)
23	Aq. NS extract: 2 g/kg 6.4 g/kg 21 g/kg 33 g/kg 60 g/kg BW	6 weeks (42 days)	Subacute toxicity Female *Mus musculus* mice (6–8 weeks) I: Control II: NS 2 g/kg III: NS 6.4 g/kg IV: NS 21 g/kg V: NS 33 g/kg VI: NS 60 g/kg Antidiabetic effect female Wistar rats, *Rattus norvegicus*, Alloxan-induced DM i.p I: Control II: DM control III: DM NSE treated (2 g/kg) Treatment via an esophageal probe	• Aq. NS extract showed no variation in urea and albumin following the five doses administered • Significantly decreased glycemia, TG, TC, LDLc, and TBARS • Restored insulinemia and a significant increase in HDLc. • Liver indicated a decrease in lipids and possible glycogenesis.	(181)

(Continued)

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TABLE 2 (Continued)

Sr. No.	Dose and form of NS	Duration of treatment	Trial/Test design	Outcome	References
24	NS extracts: 1 g/kg 3 g/kg 5 g/kg 7 g/kg 10 g/kg BW NS fractions: 0.1 g/kg 0.3 g/kg 0.5 g/kg 0.7 g/kg	Signs of toxicity were observed after 2 h and every 24 h till 14 days	30 male + female Swiss albino mice (age?) I: Control II: NSE dose III: NSF dose Treatment via p.o.	• NS extracts were nontoxic up to a concentration of 10 g/kg.	(165)
25	NSOCS1: 1 ml/kg NSOCS2: 2 ml/kg	8 days	Healthy adult male+female albino rats (age?) I: Control II: Negative control: CMC p.o. + colistin III: NSOCS1: NSO + colistin IV: NSOCS2: NSO + colistin	• Dose-dependent improvement in tubular damage and reduced biochemical alteration. • NSO reduces colistin sulfate-induced nephrotoxicity, especially in a higher dose of 2 ml/kg.	(94)
26	NSO: 4 ml/kg	24–48 h	24 female Wistar-albino rats (age?) I: Control group II: NSO 48 before, saline 24 h before sacrifice III: Saline 48 h before, carboplatin 24 h before. IV: NSO 48 h before, carboplatin 24 h before Treatment via i.p.	• Reduce the degeneration in hepatocytes, fiber distribution, and density around the central vein and portal space • Hepatocyte cords preserved integrity, partial degeneration in hepatocytes, and decreased collagen fiber distribution around the central vein. • Insignificant lower apoptosis	(215)
27	TQ: 15 mg/kg	24 h	36 healthy male albino rats (age?) I: Control II: Acetaminophen (APAP) III: N-acetylcysteine (NAC) IV: α-Lipoic acid (ALA) V: TQ VI: ALA+TQ 3-doses, 1st before 24 h, 2nd after 2 h, and 3rd after 12 h of APAP dose. Treatment via p.o.	• Treatment with all antioxidants ameliorated most of the altered parameters • Treatment with the combination of ALA and TQ was the most effective therapy in the attenuation of liver injury • Marked improvement in hepatic degeneration • Natural antioxidants such as ALA and TQ may be considered as a potential antidote in combating liver injury induced by APAP	(216)
28	NSO-1: 1 ml/kg NSO-2: 2 ml/kg NSO-4: 4 ml/kg BW Subacute NSO: 4 ml/kg BW	360 min Subacute inflammation: 168 h	50 white female rats (Wistar-Bratislava) (age?) [Same animals were used for acute and chronic models with a wash-up of 2 weeks] Carrageenan-induced acute inflammation I: Control (saline) II: Positive control (Diclofenac sodium) i.p. III: NSO-1 IV: NSO-2 V: NSO-4 Oral route Freund's adjuvant-induced sub-acute inflammation I: Control (saline) II: Posivitve control (Diclofenac sodium) III: NSO-pre (7-day before FA) IV: NSO-treat (7 days after FA) V: NSO-adj (NS + Diclo) (7 days after FA)	Significant inhibitory effect of NSO on paw edema in all three doses In the acute phase, 1.5 h after administration, NSO (2 and 4 ml/kg) showed an anti-inflammatory effect comparable with diclofenac. In the sub-acute administration, • NSO had no anti-inflammatory effect. • Analgesic effect was observed only in the sub-acute inflammation • An antioxidant effect through the reduction of MDA and GSSG	(217)

(Continued)

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TABLE 2 (Continued)

Sr. No.	Dose and form of NS	Duration of treatment	Trial/Test design	Outcome	References
29	NSO-0.6% and 5% (w/w) TQ	90 days, single dose, acute and subchronic repeat dose	Acute toxicity study: 3 adult female Wistar rats given 5, 50, 300 and 2,000 mg/kb BW Subchronic repeated dose toxicity study: 5 male and 5 female adults Wistar rats per group as follows: 1 Control II: NSO 94 mg/kg BW (5% TQ) III: NSO 47 mg/kg BW (2.5% TQ) IV NSO 9.4 mg/kg BW (0.5% TQ)	Black cumin oil containing 5% (w/w) of TQ content was found to have a "no-observed-adverse-effect-level" NOAEL of 0.1 ml/kg or 94 mg/kg b. wt. in rodents, which also corresponds to a dose of 5 mg of TQ/kg b. wt. From this study, the safe human dosage may be derived as not more than 900 mg/kg b. wt. of BCO-5/day or 50 mg of TQ/adult/day.	(218)

NS, *Nigella sativa*; NSE, NS extract; NSFO, NS fixed oil; NSEO, NS essential oil; TQ, thymoquinone; DM, diabetes mellitus; STZ, streptozotocin; BW, body weight; CAT, catalase; GPx, glutathione peroxidase; GST, glutathione-S-transferase; GSH, reduced glutathione; NCs, nanocapsules; NFs, nano formulations; CPK, creatine phosphokinase; MDA, malondialdehyde; MDH, malate dehydrogenase; HbA1c, glycated hemoglobin; BGL, blood glucose level; p.o., per os; i.p., intraperitoneal; NO, nitric oxide; TC, total cholesterol; INS, insulin; TAC, total antioxidant capacity; GSP, glycosylated serum protein; GSSG, oxidized glutathione?; details unclear.

quantities of TQ are administered (260). TQ's lipophilic properties are similar to those of the mitochondrial electron transporter ubiquinone, and more research is needed to determine the precise link between thymohydroquinone and mitochondria (261).

NS and TQ improve insulin sensitivity by increasing MAPK pathway activation, muscle GLUT-4 levels, which helps to gradually normalize glycemia (Figure 2). TQ also in a dose-dependent manner inhibits COX and lipoxygenase (LOX) enzyme activities, consequently blocking the synthesis of inflammatory mediators, prostaglandins, thromboxane, and leukotrienes and reducing joint inflammation (262). TQ by suppressing pro-inflammatory cytokines, interleukin-1β (IL-1β), and tumor necrosis factor-alpha (TNF-α), as well as interferon-gamma (IFN-γ), and interleukin-6 (IL-6), mitigates disease severity. TQ also inhibits NO production from activated cells and macrophages, increasing inflammatory responses and promoting apoptosis (254). The antioxidant properties of NS and TQ were responsible for reversing streptozotocin-induced modifications in creatine kinase-MB and brain monoamines (263). It has been demonstrated that NS promotes AMPK in the liver and muscles, resulting in antioxidant and health-protective effects. Synthetic AGE inhibitors have largely failed to combat hyperglycemic and OS-related AGEs due to side effects, hence attention has switched to natural plant-based extracts (264). NS represents a promising natural candidate since both TQ and NSS extract have been found to inhibit AGE formation *in vitro* (265, 266). However, the NS-mediated AGE inhibition mechanism remains largely unknown (267). Recent computational studies have made significant advancements in our mechanistic knowledge of TQ's anti-glycation action on the eye lens crystallin proteins and its therapeutic promise in reducing DM-related ocular cataract (268). Furthermore, nothing is known about how NS and TQ interact with novel AGE forms such as melibiose-derived (MAGE), which is detected in high amounts in diabetics with microangiopathy (269). Because of their antioxidant content, both NSS and its alcohol or aqueous extract have effectively reduced diabetes-induced cytotoxicity *in vitro* (270, 271) and diabetic lab animals (272). However, it must be borne in mind that solvent extracts of Nigella seeds carry an array of antioxidants that can complicate establishing causality (90). Exogenous antioxidant supplementation for diabetic patients has some advantages, but the strategy was initially disregarded due to worries about a lack of clinical evidence and potential side effects (273), and it is still not used as a clinical strategy today (274), despite positive results from clinical trials using antioxidant supplementation for particular diabetic complications (274–276). It is broadly understood that any potential new antioxidant therapy for DM must target the disease and simultaneously effectively prevent the associated vascular complications (247).

TQ and its importance

Using the isolated and purified active principle of medicinal plants instead of the crude extract is advantageous because it circumvents the variability in the content of the bioactive compound in the natural materials and losses due to processing and preparation. Avoiding potential interactions among the constituents using a purified preparation allows for more accurate and reproducible dosage and better analytical assaying of safety and efficacy (36, 277). Several compounds derived from NS have therapeutic value, but none comes

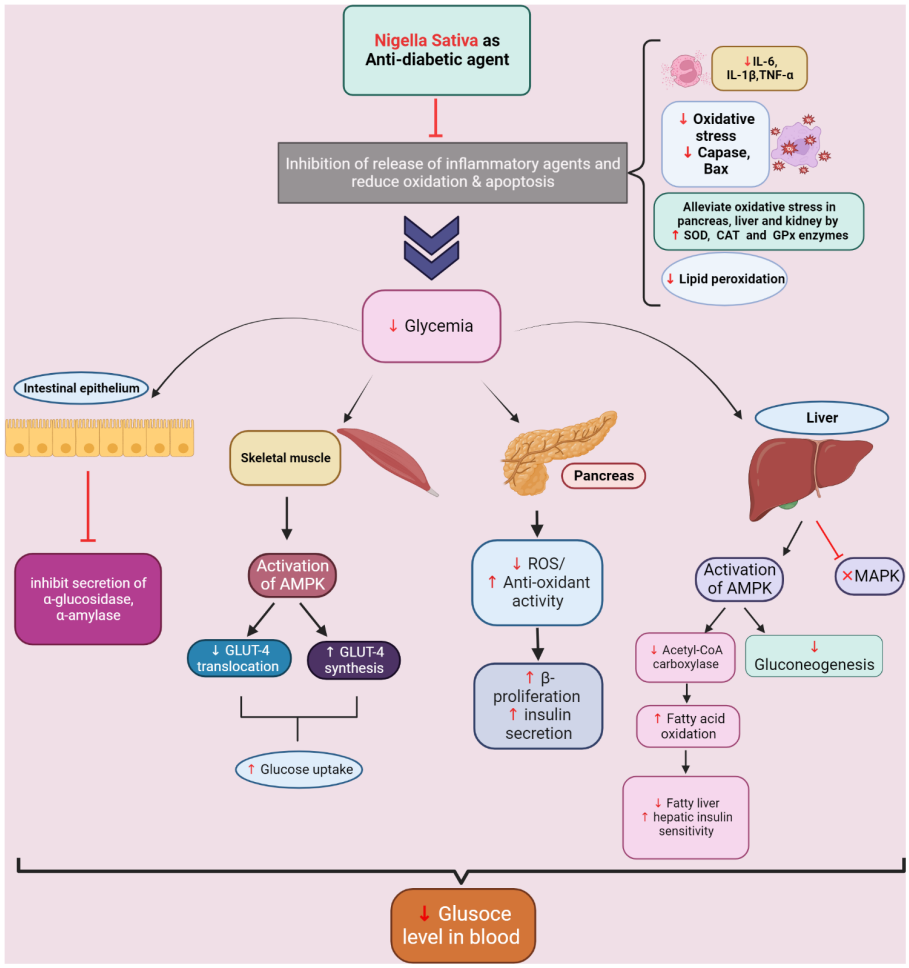

FIGURE 2
Antidiabetic action of *Nigella sativa*.

close to TQ as the main bioactive component with the most diverse pharmacological benefits (83, 278). It has been argued that the effectiveness of NS fractions in lowering glycemia and serum lipid levels might be a function of their TQ content, with the volatile oil outperforming the others (175). TQ is a monoterpene benzoquinone compound synthesized in plants from γ-terpinene during secondary metabolism (279). Reportedly first isolated from NSS in the 1960s (73, 280), it is a yellow crystalline substance chemically known as 2-isopropyl-5-methylbenzo 1,4 quinone having a molar mass of 164.20 g/mol with a molecular formula of $C_{10}H_{12}O_2$ (281) and CASRN: 490–91. Its tautomerism, where only the keto form of the molecule is thought to be pharmacologically active (162, 281), is disputed, and the reduced form of TQ has been misinterpreted as the enol form (282).

TQ has been shown in animal studies to effectively treat OS-related diseases with few or no side effects (36). It has been shown to have antioxidant, anti-inflammatory, antineoplastic, antimicrobial, analgesic, hypoglycemic, antihypertensive, and hepatoprotective properties (162, 254, 283, 284). TQ's oxidant-scavenging prowess has been ascribed to molecular quinone and the ease with which it passes through cell membranes to reach intracellular targets (285). TQ represents a relatively new class of compounds with antioxidant ability in CH bonds rather than phenolic OH groups (171, 286). The TQ molecule has specific CH groups whose bond dissociation values of the hydrogen atom transfer mechanism impart a free radical-based antioxidant activity comparable to potent antioxidants like ascorbic and gallic acids (171).

Depending on the cellular and physiological milieu, TQ can undergo both enzymatic and non-enzymatic redox reactions to generate either pro-oxidants (semiquinone) or antioxidants (thymohydroquinone); the former is associated with ROS generation, while the latter exert radical-scavenging activity (278). Animal studies have shown that TQ synergizes with metf to markedly reduce serum glucose, HbA1c, MDA, and TAC levels, which neither of them could do as well individually, substantiating its combinatorial role in conventional drug therapy (177). However, clinical trials using such combinations have reported minor health concerns that merit further investigation (287).

TQ has significant pharmacological and pharmacokinetic potential to be a strong drug candidate, as reflected by its compliance with Lipinski's "rule of five" (176, 288). One of the main problems in testing TQ in clinical trials has been a lack of standardized protocols to ensure uniform quality and dosage (182) (Table 1). Its high hydrophobicity, time-dependent aqueous solubility, aversion to alkaline pH, and significant photo-and thermolability have challenged pharmaceutical formulations (289). It is also poorly bioavailable and vulnerable to transformation by liver enzymes upon oral intake, which

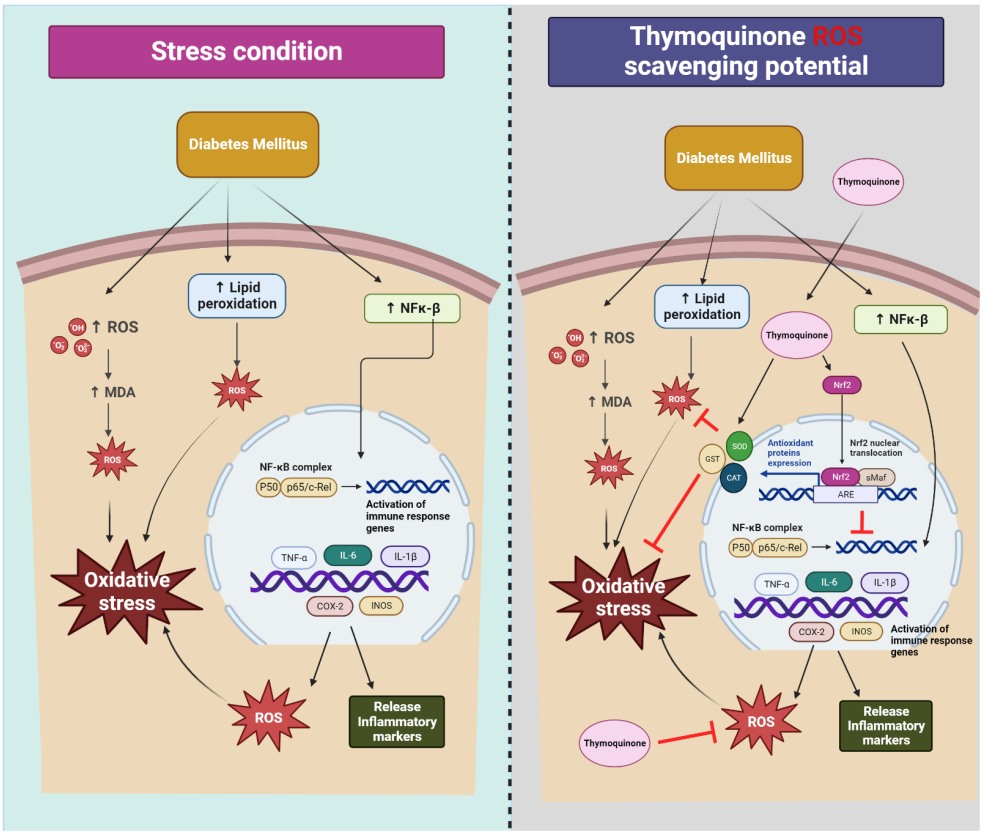

FIGURE 3
Mechanistic aspects of oxidative stress and its mitigation by thymoquinone.

necessitated the development of a version encapsulated in nanoparticles that have proven more effective than the non-encapsulated natural TQ as an anti-glycemic drug (103, 205, 290, 291). Recently nanosuspensions and gold nanoparticles phyto-formulated using NSS extract have demonstrated significant antioxidant and antidiabetic activity (292, 293). In addition, an array of nanotechnological carriers have come to the fore which could potentially overcome the poor solubility and bioavailability of orally administered TQ (280, 294), delivering a high payload of TQ via the oral route by mixing it with relatively non-toxic solvents like DMSO is also an option, and not just limited to diabetes treatment (295). Recently, synthetic analogs of TQ have come to the fore with greater efficacy and safety than the natural version, but these have been chiefly used against cancer and other diseases and not for diabetes treatment (284, 296, 297). TQ's chemical and biological transformation has yielded derivatives with enhanced antioxidant potential (298, 299). Given the limited supply of natural TQ (NS being the primary source), escalating future needs may have to be supplied through synthetic versions. The performance of synthetic TQ analogs, which surpass the natural version in oncology experiments, is very encouraging, but whether this holds true for diabetes remains to be seen (75).

Aspects of TQs mode of action

Although the high levels of TQ used in animal research have raised some safety concerns, its usage in mixtures by people for more than a millennium has been relatively incident-free (100) (Table 2). TQ's non-toxicity and safety makes it ideal for consideration as a pharmacological agent with substantial therapeutic and commercial potential (296). Increased oxidant levels and lipid peroxidation are hallmarks of diabetes (300), and TQ can directly scavenge ROS such as superoxide (O_2^-), hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2) that cause OS (87) (Figure 3). Its ability to quench free radicals matches that of SOD (179, 301), although it is not so effective against hydroxyl and 2,2′-diphenyl-p-picrylhydrazyl (DPPH) radicals (302, 305). TQ has been shown to suppress lipid peroxidation, reduce intracellular MDA (302, 303), and enhance antioxidant defenses by non-enzymatically augmenting the activity of antioxidant enzymes (304). Several studies have demonstrated that TQ increased the level and activity of both the primary antioxidant enzymes, SOD, CAT, and glutathione S-transferase (GST), and the secondary antioxidant enzymes like glutathione reductase (GR) and GPx (303, 305, 306). Its ability to react *in vivo* with such antioxidant enzymes, especially GSH, and form more potent quenching moieties that replenish and eventually replace the endogenous antioxidant system is critical in fighting OS-mediated pathogenesis (285). TQ is also able to check the auto-oxidation of glucose to prevent the runaway generation of NFκ-B-mediated ROS and proinflammatory cytokines typical of DM onset (307). Because of TQ's propensity to react with thiol-rich proteins and modulate powerful antioxidant enzymes like GSH, it is bundled with an exclusive group of therapeutic drugs called Michael reaction acceptors (308) that are associated with safeguarding overall cellular health (239, 309) and is a natural activator of Nrf2 signaling.

In the presence of OS, Nrf2 induced many of the cell's antioxidant enzymes while simultaneously repressing NF-κB, IL-1β, IL-6, TNK-z, COX-2, iNOS, TGF-β1, and NOX4, which decreased inflammation, DNA and mitochondrial damage (310). The possibility of using TQ to augment the new diabetic therapy based on the gut hormone incretin has gained much currency because of its few side effects compared to conventional drugs and the absence of any other herbal derivatives that demonstrably modulate incretin (GLP-1) (248, 311, 312).

NS produces ample amounts of TQ, but problems with the preparation of NS extract, non-standardization of testing parameters, disease severity, and duration of NS dosing could account for the conflicting claims where clinical trials have failed to find a role for NS in lowering MDA levels. Variable TQ content in commercially available NS products is another confusing issue in their varied efficacy, with requests for regulating them for TQ content (313, 314). A new study suggests that a TQ content of 30 mg per day be tested in a therapeutic context (314). Significant diversity in NSO chemotypes is also a concern, as those from Turkey and Egypt are classified as TQ phenotypes with the highest TQ content, whereas others, such as those from the subcontinent, are more mixed (p-cymene/TQ), and some may have phenylpropanoids instead of TQ, the so-called trans-anethole chemotype (315, 316). Similarly, care needs to be exercised when interpreting NS's *in vivo* antioxidant efficacy based on TAC measurements since it is an *in vitro* assay that measures only non-enzymatic antioxidant capacity, whereas NS has both (107, 253, 317).

Genetic impact of NS supplementation in DM

Genotype and environment both play a role in the etiology of T2DM (318, 319), and there has been strong interest in establishing the genetic triggers of OS in diabetes (276).

Insulin-like growth factor 1

NS has potent antidiabetic activity, as reflected by the up-regulation of several essential genes, such as insulin-like growth factor-1 and IGF-1. IGF-1 is widely present in mammalian tissues, and its functions include regulating metabolism and enhancing tissue development and growth (320). IGF-1 manifests its effects by binding specific receptors on target cells, stimulating glucose uptake, lowering blood glucose, and improving insulin sensitivity (321). Its mechanism of action appears independent of insulin receptor activation, but NSS has been shown to induce hypoglycemia by upregulating the IGF-1 gene and reducing DM-induced OS (322). IGF-1 improves insulin resistance in T2DM and in patients with more severe insulin resistance, where clinical trials have demonstrated the potential utility of IGF-1 in ameliorating clinical symptoms (157, 323, 324).

In diabetes, loss of insulin responsiveness can occur because some elements of insulin signaling pathways, such as insulin receptors, are disrupted. NSO upregulates insulin-signaling pathways and augments the expression of insulin-like growth factor-1 (IGF-1), inducing the signaling molecule, protein kinase B (Akt), and activating glucose transporter-4 (GLUT4). GLUT4 is then translocated to the plasma membrane, where it imports glucose into the cell (325). Dysfunctional GLUT-4 has been linked to insulin resistance (326). Thus, NS can decrease insulin resistance by improving tissue sensitivity to insulin action (327, 328), presumably in concert with its suppression of insulin clearance via inhibition of insulin-degrading enzymes (IDEs). Greater insulin sensitivity is also linked to NSO's ability to lower triglyceride levels (329).

DM-associated endothelial dysfunction

Endothelial dysfunction is described as an "impairment of the ability of the endothelium to maintain vascular homeostasis" properly and is the principal underlying reason for DM-associated vascular pathologies (330). DM is associated with reduced expression and activity of endothelial nitric oxide synthase (eNOS), which is central to maintaining cardiovascular tone and function (149, 331). TQ improves endothelial function by inhibiting OS and stabilizing the renin-angiotensin (RAS) system (332). The proliferation and migration of vascular smooth muscle cells (VSMCs) is a characteristic of endothelial dysfunction in diabetes, and inhibitory drugs are highly sought after. Animal studies show TQ's antiproliferative and anti-migratory effect on VSMCs through the AMPK/ Peroxisome proliferator-activated receptor gamma PPARγ/ Peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α) pathway (333). TQ reduces vascular inflammation by repressing the expression of vascular endothelial growth factor (VEGF) and monocyte chemo-attractant protein-1 (MCP-1), besides lowering levels of cytokines IL-6 and IL-8 in human vascular endothelial cells (HUVECs) (334). TQ has been shown to reverse endothelial dysfunction by increasing NO generation and bioavailability. Vascular cell adhesion protein-1 (VCAM-1) is involved in the adhesion of lymphocytes, eosinophils, and basophils to the vascular endothelium and is an essential mediator in developing atherosclerosis and DM. VCAM-1 can recruit monocytes to the sites of atherosclerotic lesions, initiating and developing vascular inflammation (335, 336). *Vcam-1* gene expression was upregulated in the aortic tissue of diabetic rats, while NS seeds significantly reduced *vcam-1* gene expression in the aorta, potentially reducing vascular inflammation and restoring endothelial function (149). TQ interfered with TNF-α signaling to modulate IL-6 and IL-8 expression and downregulated IL-8 and ICAM-1/VCAM-1 expression in rheumatoid arthritis (337). TQ also downregulated toll-like receptor-2 (TLR-2) and-4, which are crucial to the vascular inflammation of diabetic microangiopathy (338, 339), underscoring its potential for controlling and managing DM.

Effects on lectin-like oxidized low-density lipoprotein receptor-1

The LOX-1 LDL receptor is an essential element in the progression of atherosclerosis through its intimate relationship with CV dysfunction and DM pathogenesis. Experiments with diabetic rats showed upregulation of LOX-1 expression in the vascular endothelium of the aorta. LDL uptake by the LOX-1 receptor triggers many pathophysiological changes, such as decreased eNOS activity and stimulation of adhesion molecule expression (340, 341). NSS extract inhibited *lox-1* gene expression in aortic tissue (342). LDL binding to the LOX-1 receptor increased OS, decreased NO production, potentiated superoxide generation, and activated NF-kB-all of which exacerbate DM pathogenesis. Additional studies on human aortic endothelial cells demonstrated that high glucose levels increased *lox-1* gene expression; thus, down-regulating LOX-1 using NSS extract could potentially suppress the pathophysiological processes related to LOX-1 (343, 344), restore normal endothelial function and decrease vascular complications in DM (149).

NS and TQ suppression of cyclooxygenase-2

COX-2 continues to be a target for repression because of its central role in inducing the inflammatory processes associated with diabetes and metabolic syndrome (345, 346). The classical non-steroidal inflammatory drugs (NSAIDs) such as salicylates and the newer, more selective COXIBs (an NSAID sub-category of COX-2 inhibitors) have been used, but their side effects have initiated a search for safer, natural COX inhibitors (221). Culinary spices, such as NS, have garnered much attention because of their anti-inflammatory potential (347). Among the NS-derived benzoquinones, both TQ and its partially reduced form, hydroquinone, stand out as potent inhibitors of COX-2, while thymol selectively inhibited COX-1 (221). TQ demonstrated efficacy against autoimmune diseases, including T1DM, by repressing COX, LOX, cytokines, lipid peroxidation, and IL-1. ROS activates the Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (348), increasing DM-related inflammation (349, 350). Many inflammatory response factors such as adhesion molecules, inflammatory enzymes, proinflammatory cytokines, and chemokines are products of genes regulated by NF-κB (351, 352), including the inflammatory COX-2. COX-2 is a multiplex enzyme with cyclooxygenase and peroxidase activities that generate ROS and cause OS (353, 354). NS and TQ treatment of streptozotocin (STZ)-induced diabetic rats significantly suppressed the COX-2 expression in pancreatic tissue. The treatments that decreased COX-2 mRNA also reduced pancreatic tissue lipid peroxidation and MDA levels and increased SOD activity (355).

The synergy of NS and gut microbiota

The link between gut microbiota and the onset and incidence of type 1 and type 2 DM has been unequivocally established, triggered by the leaky gut condition arising from altered gut microbiota (356, 357). The microbial population in the gut is complex, unique to the individual, and plays a vital role in the body's physiological and metabolic processes. Extensive studies of the gut bacterial community have revealed species predominantly belonging to the phyla, Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Verrucomicrobia (10, 357). Gut bacteria are vulnerable to diet and stress (357), and a dysbiotic population contributes to the onset of T2DM through an altered synthesis of short-chain fatty acids, gut hormone levels, bile acids, and branched-chain amino acid metabolism (358). Metf, the mainstream glucose-lowering medication for DM, ameliorates gut dysbiosis (359, 360). Many treatments exist for diabetes, but its complexity has made curing it a challenge (202, 357), emphasizing the need for testing novel therapeutic approaches (358). Modulating the gut microbiota through pre-and pro-biotics for the complementary treatment and management of T2DM has recently gained much currency (361, 362). Several recent reviews have focused on the potential of next-generation probiotic candidates as adjuncts to conventional probiotics such as lactobacilli and bifidobacteria to offset the harmful consequences of DM (363). The antioxidant activity of a probiotic *Lactobacillus* species was identified as the reason for its hypoglycemic effect (364). Combining probiotics with existing allopathic antidiabetic medications and plant-based nutraceuticals is an exciting research avenue (357, 365). Co-administering NS with probiotics has yielded fruitful results in treating unrelated human disorders (366). One proposed mechanism of the antibacterial effect of NS and TQ against a variety of Gram-positive and Gram-negative bacteria is by targeting them with ROS while at the same time protecting host tissues from OS through antioxidants (284) and selectively allowing probiotic LAB species to flourish (367). This activity holds promise for T2DM patients once their gut becomes populated with opportunistic pathogens such as *Clostridium* spp., *E. coli,* and betaproteobacteria (368). Reversing diabetes-related gut dysbiosis through plant extracts such as those from NSS is of great scientific interest (202). High doses of NSS polysaccharides have been used to promote Bacteroidetes over Firmicutes to alleviate T2DM (202), and the use of beneficial yeast strains like *Saccharomyces boulardii* is also promising (369).

Conclusion

This review (1985 to January 2022) discusses the latest findings on diabetic pathogenesis and its treatment, emphasizing the herb *Nigella sativa* and its active constituent, thymoquinone. An obvious outcome of this narrative is that the different experimental designs used by various investigators mean that conclusions about its interventional potential should be interpreted with care. Despite many publications, the medically relevant dosage of TQ and NS supplements remains contentious. The multi-modal nature of NS fractions and TQ is minutely examined for their antidiabetic effects and verified in the lab, and clinical trials make their therapeutic potential as a complementary medication patently clear. However, the pharmacokinetic interaction of NS with conventional drugs raises some concerns which need addressing. Nevertheless, the review clarifies the need to assess NS and its products in clinical trials using diabetic patients with reasonable glycemic control but vascular complications.

Author contributions

AS: co-developed the concept, data collection, analysis, and preliminary draft writing. AZ: co-developed the concept, data collection and analysis, and improved and finalized the manuscript. HA: validated the data analysis. NK: did part of the data collection and analysis. 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.

Publisher's note

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Glossary

NS	*Nigella sativa*
DM	Diabetes mellitus
TQ	Thymoquinone
ID	Insulin-dependent
NID	Non-insulin dependent
T2DM	Type 2 diabetes mellitus
BPA	Bisphenol A
WHO	World Health Organization
USFDA	United States Food and Drug Administration's
CAM	Complementary and alternative medicine
ROS	Reactive oxygen species
Metf	Metformin
GLP-1	Glucagon-like peptide-1
TAIM	Traditional Arab and Islamic medicine
GRAS	Generally regarded as safe
NSS	NS seeds
NSO	NS oil
T1DM	Type 1 diabetes mellitus
MAPKs	Mitogen-activated protein kinase
AMPK	Adenosine monophosphate-activated protein kinase
SLGT1	Sodium-glucose linked transporter 1
SOD	Superoxide dismutase
CAT	Catalase
GPx	Glutathione peroxidase
LDL;	Low density lipoprotein
HDL-C	High density lipoprotein cholesterol
AST	Aspartate aminotransferase
ALT	Alanine transaminase
ALP	Alanine phosphate
APAP	Acetaminophen
GSSG	Oxidized glutathione
MDA	Malondialdehyde
GSH	Reduced glutathione
RNS	Reactive nitrogen species
NADPH	Nicotinamide adenine dinucleotide phosphate
OH	Hydroxyl radical
O-2	Superoxide anion
H_2O_2	Hydrogen peroxide
NO	Nitric oxide
TAC	Total antioxidant capacity
AGEs	Advanced glycation end product
RAGE	Receptor for AGE
DT	Dihydro-thymoquinone
GLUT-4	Glucose transport-4
COX	Cyclooxygenase

LOX	Lipoxygenase
IL-1β	Interleukin-1β
TNF-α	Tumor necrosis factor-alpha
INF-γ	Interferon-gamma
IL-6	Interleukin-6
MAGE	Melibiose-derived AGE
HbA1c	Glycated hemoglobin
DMSO	Dimethyl sulfoxide
DPPH	2,2′-Diphenyl-p-pierylhydrazyl
GST	Glutathione S-transferase
GR	Glutathione reductase
IGF-1	Insulin-like growth factor
eNOS	Endothelial nitric oxide synthase
RAS	Renin-angiotensin
VSMC	Vascular smooth cells
VEGF	Vascular endothelial growth factor
MCP-1	Monocyte chemo-attractants protein-1
HVECs	Human vascular epithelial cells
VCAM-1	Vascular cell adhesion protein-1
LOX-1	Lectin-like oxidized low-density lipoprotein receptor-1
CV, NSAIDS	Non-steroidal inflammatory drugs
NF-κB	Nuclear factor-kappa B
STZ	Streptozotocin
Akt	Protein kinase B
IDSs	Insulin degrading enzymes
LAB	Lactic acid bacteria

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From antiquity to contemporary times: how olive oil by-products and waste water can contribute to health

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Since antiquity, numerous advantages of olive oil and its by-products have been recognized in various domains, including cooking, skincare, and healthcare. Extra virgin olive oil is a crucial component of the Mediterranean diet; several of its compounds exert antioxidant, anti-proliferative, anti-angiogenic and pro-apoptotic effects against a variety of cancers, and also affect cellular metabolism, targeting cancer cells through their metabolic derangements. Numerous olive tree parts, including leaves, can contribute metabolites useful to human health. Olive mill waste water (OMWW), a dark and pungent liquid residue produced in vast amounts during olive oil extraction, contains high organic matter concentrations that may seriously contaminate the soil and surrounding waters if not managed properly. However, OMWW is a rich source of phytochemicals with various health benefits. In ancient Rome, the farmers would employ what was known as amurca, a mulch-like by-product of olive oil production, for many purposes and applications. Several studies have investigated anti-angiogenic and chemopreventive activities of OMWW extracts. The most prevalent polyphenol in OMWW extracts is hydroxytyrosol (HT). Verbascoside and oleuperin are also abundant. We assessed the impact of one such extract, A009, on endothelial cells (HUVEC) and cancer cells. A009 was anti-angiogenic in several *in vitro* assays (growth, migration, adhesion) and inhibited angiogenesis *in vivo*, outperforming HT alone. A009 inhibited cells from several tumors *in vitro* and *in vivo* and showed potential cardioprotective effects mitigating cardiotoxicity induced by chemotherapy drugs, commonly used in cancer treatment, and reducing up-regulation of pro-inflammatory markers in cardiomyocytes. Extracts from OMWW and other olive by-products have been evaluated for biological activities by various international research teams. The results obtained make them promising candidates for further development as nutraceutical and cosmeceutical agents or dietary supplement, especially in cancer prevention or even in co-treatments with anti-cancer drugs. Furthermore, their potential to offer cardioprotective benefits opens up avenues for application in the field of cardio-oncology.

KEYWORDS

olive oil, olive mill waste water, polyphenols, cancer prevention, cardiotoxicity, angiogenesis, health

Historical background

The domestication of the olive tree started in the Mediterranean region many thousands of years ago, and the production of oil from its fruit could date back as far as 2,500 BCE (1, 2). Although olives were appreciated on their own as a staple food, oil extracted from the fruit was possibly the main reason why the olive tree became so largely cultivated. Apart from its employment at the dining table, both for cooking and condiment, olive oil was used for many other purposes in ancient times, namely as lamp fuel, personal grooming, cosmetics, soap, and medicine. Perhaps because of this versatility, coupled with its extraordinary longevity (olive trees can live 3,000+ years, see the famous "olive tree of Vouves" in Crete), it also acquired a religious and symbolic role. While not so much employed for food, olive oil was used in Egypt as early as the New Kingdom period (1550–1,070 BCE) for some of the above-mentioned purposes, such as for lighting and as an ingredient in cosmetics, but it was also an offering to the gods. The Minoans used olive oil in religious ceremonies too. The oil became a principal product of the Minoan civilization, where it is thought to have represented wealth (3). It was used in the anointing of priests and in the preparation of offerings for the gods. Olive oil was also present in Minoan funerary practices, where it was believed to help the soul of the deceased on their journey to the afterlife.

In Ancient Greece, the olive tree was considered sacred and a symbol of peace, prosperity, and wisdom. According to Greek mythology, Athena, the goddess of wisdom and warfare, competed against Poseidon, the god of the sea, for the patronage of the city of Athens. As part of the competition, they were both asked to present a gift to the city that would be of the greatest benefit to its people. Athena won by planting an olive tree on the Acropolis, which was not only beautiful, but also provided a valuable source of food, oil, and fuel for the city's inhabitants. The Sacred Olive Tree can still be found in the Acropolis of Athens today. Of course, it is not the "original" one, but legend has it that it is a direct descendant, grown from propagation. The Olympic flame was lit using a concave mirror to focus the sun's rays, and a little olive oil, while a wreath made of twisted olive branches crowned victors in the athletic competitions. Amphoras filled with olive oil were among the prizes for the winners (4). Olive wreaths were common in Rome as well, as a symbol of victory in military campaigns, but also to announce the birth of a baby boy. An olive branch, of course, is found in the Bible (Genesis 8:11) as a symbol of peace, brought back to Noah's ark by a dove, and the recipe of the holy oil used by Moses to anoint priests and prophets includes olive oil in its ingredients. The olive tree and its fruits are also mentioned several times in the Quran. It is a symbol of strength, beauty, and prosperity, and is associated with blessings and divine guidance.

The curative powers of olive oil were already known in Ancient Egypt. The Ebers Papyrus is a medical text dating back to around 1,550 BCE. It contains recipes and remedies for various ailments, many of which involve the use of oils and ointments made from plants and other natural materials, including olive oil. There are recipes for ointments made with olive oil to treat skin conditions such as eczema and psoriasis, as well as to soothe insect bites and stings. It is also mentioned as a treatment for joint pain and eye infections. Even the Greeks believed that olive oil had medicinal properties, and it was recommended to treat skin conditions, digestive disorders and more. A mixture of olive oil combined with other oils was also used by Greek women as a form of birth control (5). Olive oil as a remedy for a number of ailments can be found in Traditional Persian Medicine (TPM). The Canon of Medicine (Al-Qanun fi al-Tibb), written by Avicenna (Ibn Sina) in the 11th century, mentions the use of olive oil for digestion, respiratory problems, skin diseases, joint pains, fevers, but also for mental health. Avicenna believed that olive oil had a calming effect on the nervous system and could be used to treat anxiety and depression. The Canon of Medicine is in many ways a synthesis of the medical knowledge of the ancient Greeks and the medical practices of the Islamic world. It drew extensively from the works of Hippocrates, Galen, and other ancient Greek physicians, as well as from the medical traditions of Persia, India, and other regions.

Review methodology

The research strategy included the definition of keywords and a search of online databases: Scopus, Web of Science, PubMed and Google Scholar. When sources were chosen in accordance with the criteria, they had to be available online in one of the listed databases. To provide distinct results for the same collection of terms, we chose to utilise keyword sequences in search engines.

The following word groups were used for the search as Boolean string: olive*[TI] AND (by-product* OR byproduct* OR wastewater* OR waste*) AND (Health OR cancer OR prevention OR cells).

The literature used in this review was found considering "All year" in the search criteria and was manually chosen for relevance to the Research Topic.

We accepted all articles written in English and we excluded book reviews, editorials, commentaries, opinion pieces, and topic overviews that did not explicitly identify as literature reviews.

Extra virgin oil production and waste water

Olive oil production normally encompasses several sequential stages, starting with harvesting and followed by cleaning and washing to eliminate leaves, twigs, dirt, and lingering impurities. The cleaned olives are then subjected to grinding, where they are crushed or ground to form a paste. Subsequently, the olive paste undergoes malaxation, a process where it is thoroughly mixed to facilitate the breakdown of oil droplets and the release of oil. After malaxation, the oil is separated from the paste either through a press or a centrifuge. Following this separation, the oil undergoes filtering to eliminate any remaining particles or impurities. Lastly, to preserve its flavor and quality, the oil is stored in a cool, dark environment. Extra virgin olive oil (EVOO) is a type of olive oil that is obtained mechanically, without the use of heat or chemicals, from olive fruits. It is considered the highest quality and most flavorful type of olive oil and is widely used as a condiment. EVOO has a rich, fruity taste and is high in monounsaturated fatty acids (MUFA), which are considered beneficial for heart health. It is also a good source of antioxidants, including polyphenols and vitamin E, which may help protect against oxidative damage in the body. Together with a wide variety of seasonal fruits, vegetables, whole grains, legumes, fish, nuts, and moderate amounts of cheese, meat, and wine, olive oil is an integral part of the Mediterranean diet (MD). This diet has been shown to have a range of health benefits, including a reduced risk of cardiovascular

pathologies, type 2 diabetes, neurodegenerative diseases, and some cancers (6, 7).

Olive trees provide numerous products and by-products rich in biologically active molecules (8). During the washing and processing of the olive fruit, large quantities of a dark, odorous effluent, known as OMWW, are generated. Interestingly, analyses of bioactive compounds distribution throughout the entire EVOO mill production chain evidenced the abundance of molecules in by-products such as OMWW and pomace, although the content is highly variable due to the characteristics of the olives and to the sample preparation steps used (9). The high concentration of organic matter contained in OMWW, which includes tannins, polyphenols, polyalcohols, proteins, organic acids, pectins, and lipids, may seriously damage the environment if not managed properly. It can contaminate the surrounding soil and water resources. It can also cause eutrophication, leading to increased plant and algae growth. Mediterranean regions produce about 97% of the worldwide olive oils, recently, olive tree products are increasing (30 million tons), approximately 5 kg of olives are needed to obtain 1 liter of oil; OMWW has been estimated at around 20 million m^3, and 1 m^3 of OMWW corresponds to 100–200 m^3 of domestic sewage (10–12). These figures can give us an idea of the extent of the problem. Many countries have been led to develop new technologies to deal with the issue to reduce pollution (13, 14). In recent years, intensive research in the field of OMWW management has suggested that these effluents may be a very valuable resource of chemical substances for a variety of purposes, from medicine to agriculture.

In Roman times the production of olive oil was already in the order of tens of millions of liters per year, so it was necessary to find ways to dispose of waste water (15). Simply dumping it into nearby rivers or lakes had serious environmental consequences, leading to water pollution and harm to aquatic ecosystems. A less environmentally damaging solution was to collect the OMWW in underground pits or pits lined with impermeable materials, such as clay, in order to allow the water to slowly evaporate or seep into the ground. This method was not yet ideal, as over time it could lead to soil and groundwater contamination. But not all the OMWW ended up being discarded. The dark, smelly, and bitter tasting water produced during the oil extraction process was known by the Mediterranean populations to have many useful qualities. Called amorge by the Greeks and amurca by the Romans, it had a number of applications (15, 16). It could be used as feed for animals, as fertilizer or pesticide for insects and weed, as lubricant for axles and belts (17–19). It was also employed in the making of plaster (20). In medicine, it was prescribed as a drink for various ailments, including gastrointestinal problems such as indigestion, constipation, and diarrhea. It was also used as a topical treatment for skin conditions like wounds, burns, and insect bites. Amurca was believed to have antiseptic and anti-inflammatory properties, which made it useful in treating these conditions (21).

The tradition of drinking OMWW for health reasons among farmers has continued almost to the present day, thus arousing the interest of both oil producers and scientific researchers (Figure 1).

What makes this by-product of olive oil production so powerful? OMWW contains a concentration of phytochemicals that is at least tenfold that of EVOO (22). Phytochemicals have been linked to various health benefits, such as reducing the risk of chronic diseases, including cancer, cardiovascular disease, and diabetes (Figure 1).

OMWW's exact composition can vary depending on factors such as olive variety, extraction process, and treatment methods, but usually includes:

- Phenolic compounds: aromatic compounds that can be found in various concentrations. They are responsible for the bitter taste of olive oil and have antioxidant properties.
- Fatty acids: organic acids that are found in high concentrations. They are the primary constituents of olive oil and are responsible for its characteristic taste and aroma.
- Carbohydrates: compounds that are found mainly in the form of sugars and are the primary source of energy for the microorganisms that degrade OMWW.
- Nitrogen-containing compounds: organic compounds that contain nitrogen atoms. They mainly occur in the form of proteins and amino acids, which are essential for the growth of microorganisms.
- organic acids: organic compounds characterized by acidic properties, which are found in varying concentrations in OMWW and are responsible for its low pH.

Polyphenols are a group of chemical substances found in plants. Their consumption may play an important role in maintaining health through the regulation of metabolism, obesity, chronic disease, and cell growth. OMWW contains at least 30 types of polyphenols in high concentration, among which (23–25):

- Hydroxytyrosol (HT): one of the most abundant polyphenols in OMWW. It is a potent antioxidant that has been shown to have anti-inflammatory, cardioprotective, and neuroprotective properties.
- Tyrosol (TYR): a phenolic compound with antioxidant, anti-inflammatory, and anti-microbial properties, precursor of HT.
- Oleuropein: a glycosylated secoiridoid with antioxidant, anti-inflammatory, and anti-microbial properties.
- Verbascoside: a phenylpropanoid glycoside with antioxidant, anti-inflammatory, and neuroprotective properties.
- Ligstroside: a polyphenol with antioxidant and anti-inflammatory effects and which may also support cardiovascular health.
- Luteolin: a flavone that has been shown to have antioxidant, anti-inflammatory, and anti-cancer properties.

OMWW can also contain vanillic acid, caffeic acid, p-coumaric acid, chlorogenic acid, ferulic acid and elenolic acid (23–26).

Treatment of waste water for use in various applications could contribute to sustainable water consumption and ecosystem conservation. Modern technology is making the process of extraction (23–26) much more efficient and economically and environmentally viable than in the past. This, paired with the increasing need to reduce and utilize agricultural waste, means that OMWW has now garnered much interest for its potential applications in many different industries (27). There are several new extraction processes that are being used, including:

- Membrane filtration: this process uses semi-permeable membranes to separate the different components of OMWW, such as water, organic acids, and polyphenols, based on their molecular weight and size.

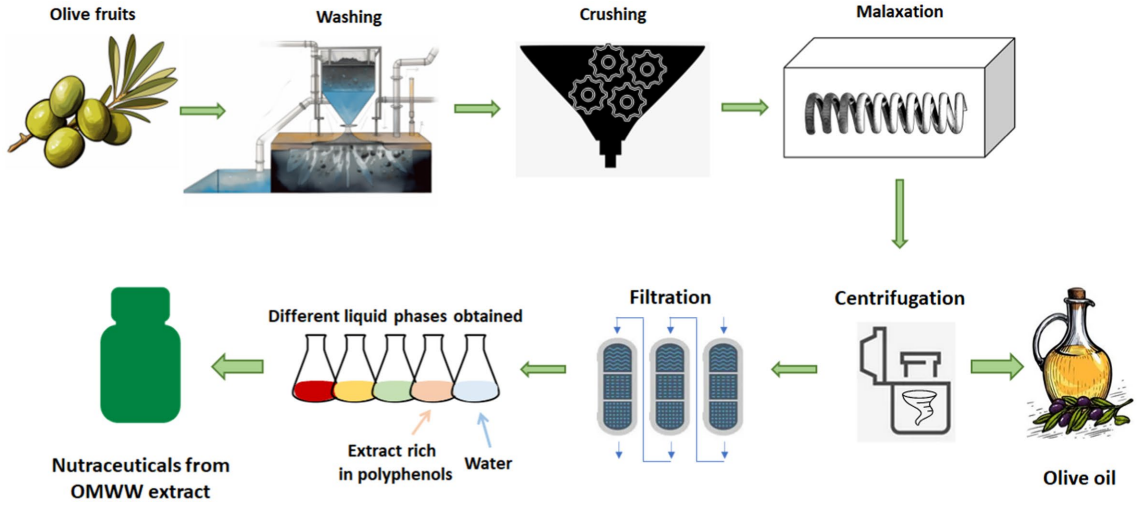

FIGURE 1
Olive oil production and valorization of olive mill wastewater products.

- Liquid–liquid extraction: this method involves using solvents to selectively extract specific compounds from OMWW, such as polyphenols, which can then be further processed and purified.
- Enzymatic hydrolysis: this process uses enzymes to break down complex molecules into simpler, more valuable compounds, such as glucose or fructose, which can be used in various industries.
- Supercritical fluid extraction: this method uses high-pressure and temperature conditions to extract compounds from OMWW, such as polyphenols and organic acids, using a supercritical fluid, such as carbon dioxide.
- Adsorption: this process involves the use of adsorbent materials, such as activated carbon, to selectively remove specific compounds from OMWW, such as polyphenols, while leaving other components behind (27).

Through the various processes, OMWW can find applications in several different fields. In agriculture it can be used as an irrigation source for non-food crops and as fertilizer, due to its high organic matter content. It can have industrial use in the production of biodegradable plastics, surfactants, and biofuels. It can be utilized as a source of energy through anaerobic digestion, which can produce biogas to generate electricity. Bioremediation, animal feed, bio-stimulants, and biopesticides are other useful applications (28).

OMWW for health supplements and cosmetics products

OMWW has been found to possess antioxidant and anti-microbial properties, making it a potential ingredient in health supplements and cosmetic products. Extracting valuable compounds from OMWW presents an opportunity to obtain sustainable ingredients for the production of functional and fortified foods. For instance, phenolic rich extracts from OMWW have been satisfactorily added to an olive spread (29) and to breadsticks (30). Supplementary Table 1 shows the polyphenol content of the batches of the OMWW extract, named A009, which was characterized and studied by our group.

The A009 phenol rich purified extract used by our team was obtained from Massimo and Daniele Pizzichini according to Patent formulation (Patent US 8,815,815 B2). Initially, a ceramic microfiltration (MF) was conducted using 2 tubular membranes made of alumina oxide with a 300 KDa cut-off (TAMI membranes, Nyons, France) and a filtration surface area of 0.35 m2. This step effectively removed solid particles, residual plant matter, and cells, all of which were subsequently discarded. The resulting MF permeate was then subjected to further concentration through reverse osmosis (RO) using a Polyamide spiral wound module (Microdyn Nadir, Wiesbaden, Germany) with a surface area of 7 m^2. The RO permeate was essentially purified water and was also discarded. Ultimately, the RO concentrate, achieved at a volume concentration ratio (VCR) of 3.6, constituted the olive extract, A009.

Today, a lot of consumers are interested in supplements containing vitamins, minerals, and other nutrients, and more than ever, research-based data is needed to properly advise customers, particularly about natural ingredients. A substantial obstacle for "clean label" ingredients has been created at the same time by the growing interest in the components used in food items.

A study revealed that when compared to the control, a fortified juice (as OliPhenolia®) with the addition of the phenols concentrate from OMWW did not exhibit off-flavor or off-odor. Additionally, the supplemented juice showed a stable phenol concentration after 60 days of refrigeration, results that could be applied in the production of orange juice that has a natural antioxidant concentration added as a "clean label" ingredient (31). In another investigation, Foti et al. (32) sought to produce a novel functional beverage beginning with OMWW that would have a health-promoting effect. The fermentation of OMWW, utilizing microbial pools in both single- and co-cultures resulted in an increase in HT and TYR concentrations as compared to the control sample. Fermented OMWW might be suggested as a new beverage and/or functional component that may also contain compounds as flavorings and probiotic microbes (32).

The potential for creating a novel nutraceutical product based on olive pâté (OP) and OMWW was investigated in a different study. In order to fulfill the European community's claims about a potential antioxidant effect on plasma lipids, researchers were able to produce a product that was high in trans resveratrol, OH tyrosol, and tyrosol. From both a commercial and nutraceutical point of view, the product had a promising market outlook due to its good palatability and stable results (29).

Cosmeceuticals are another interesting area of application for OMWW extracts. The strong antioxidant, anti-microbial, and anti-inflammatory properties of the OMWW fraction A009 offer protection from skin diseases (Table 1), improved skin health and beneficial effects on the skin ageing process (33, 42). An investigation of a biophenols extract, derived from an upcycling strategy using leftovers from the olive agri-food industry (from *Olea europaea* leaves and waste water), was prepared and tested as a cosmetic product and a dietary supplement (35). A considerable and progressive improvement in the state of the skin was seen after the combined action of the cosmetic and food supplement formulation, on 46 healthy volunteers, in a period of 8 weeks (35). This improvement was attributed to increases in collagen content, skin elasticity, and skin hydration (skin health indicators). Additionally, the therapy reduced the irritating effects of chemical agents and UV rays by acting as a skin protector (35). Drying OMWW polyphenols using a spray drying technique on human keratinocytes (HaCaT cell model) has been shown to improve cell repair and migration in scratch assays (43). Furthermore, a pro-oxidative and pro-apoptotic effect of a polyphenolic OMWW fraction on the UVA-damaged HEKa keratinocyte cells was observed (34). A009 has shown a potential to improve and prolong hair growth *in vivo*, and due to its antioxidant properties, it may help to maintain a healthy scalp and perhaps stop hair loss brought on by oxidative stress (36) (Table 1). OMWW's potential extends to the nutraceutical ophthalmic domain, where it could be utilized against various inflammatory conditions affecting the ocular surface. This is attributed to the presence of free radical scavengers in its composition. *In vitro* studies have already positioned OMWW for upscaling in ophthalmic nutraceutical applications (44). Several studies have suggested that the phenolic compounds in OMWW may have beneficial effects on cardiovascular health. For example, HT has been shown to reduce blood pressure and improve endothelial function, which could help to prevent atherosclerosis and other cardiovascular diseases (45–48). Hara et al. (41) investigated the effect of OMWW and HT on atherogenesis, OMWW (0.30% w/w) or HT (0.02% w/w) were added to a western-type diet for 20 weeks and fed to male apolipoprotein E-deficient mice. Without affecting body weight, plasma cholesterol levels, or blood pressure, OMWW and HT slowed the progression of atherosclerosis in the aortic arch, in a comparable manner. The aorta's generation of oxidative stress, as well as the expression of inflammatory molecules like IL-1 and MCP-1 and NADPH oxidase subunits like NOX2 and p22phox, were all reduced by OMWW and HT. (41)

A study showed that an HT enriched OMWW extract exerts a potent antioxidant and significant anti-microbial activity against two olive tree pathogens (*Pseudomonas savastanoi pv. savastanoi* and *Agrobacterium tumefaciens*) (49). Against therapeutically relevant Gram-positive and Gram-negative infections, resistant and multi-resistant to current antibiotic drugs, various OMWW samples demonstrated considerable antibacterial activity (50, 51).

TABLE 1 Effects of OMWW extracts on normal cells.

	OMWW extract		
Cells	*In vitro*	*In vivo*	References
Keratinocyte	Antibacterial effect against Gram-negative and Gram-positive bacteria. Antioxidant effect reducing ROS formation. Anti-inflammatory effect, reducing IL-8. Photoprotection UVA-damaged human keratinocytes	Improvement of skin hydration and collagen density, enhancement of skin elasticity and decrease of erythema index.	(33) (34) (35)
Human follicle dermal papilla	Positive influence on cell proliferation and release of growth factors IGF-1. Antioxidant effect reducing ROS formation and preventing oxidative stress.	Help in improving and extending hair growth.	(36)
Endothelial (HUVEC)	Suppression of proliferation, increase of apoptosis, inhibition of endothelial morphogenesis, migration, and invasion.	Inhibition of the angiogenic response induced by VTH: (VEGF, TNF-α, Heparin).	(37)
Cardiomyocytes	Enhancement of the proliferative-reducing effect of 5-FU and cisplatin. Reduction of IL-6 mRNA induced by 5-FU.	Reduction of mitochondria damage induced by chemotherapy cisplatin, 5-FU or doxorubicin	(38) (39)
Skeletal muscles		Functional amelioration of oxidative stress in 27-month-old rats	(40)
Aorta		Decrease of atherogenesis, IL-1β, CXCR2 levels, reduction of oxidative genes NOX2 and p22phox in Apolipoprotein E-deficient male mice fed on western-type diet.	(41)

The OMWW extract has positive effects on normal cell lines, both *in vitro* and *in vivo*, which are summarized in the table.

The increased phenolic levels or other ingredients present in the mixture, such as fatty acids, may be the cause of the enhanced antibacterial properties (52).

A recent review reported studies with bioactive compounds from olive by-products, that confirm ingesting olive-derived products promotes health. But little research has been done so far and further human studies are needed to confirm safety and health-promoting properties of olive oil by-products (53). An *in vivo* study showed for the first time that using OMWW as dietary supplementation can prevent cell death and tissue deterioration, and the harmful effects of oxidative stress in the cellular systems of rabbits (54).

In summary, due to its anti-inflammatory, antioxidant, and anti-microbial properties, both *in vitro* and *in vivo* evidence indicated the prospective use of OMWW as a food supplement and in skin cosmetic products.

OMWW effect on normal and tumor associated endothelial cells

A promising and perhaps less investigated aspect is the anti-angiogenic and angio-preventive potential of phenolic compounds. Angiogenesis is the process of forming new blood vessels from existing ones (55–58).

Under normal bodily conditions, cell multiplication is tightly regulated to balance programmed cell death (apoptosis), maintaining tissue size. However, tissue growth, like during increased metabolic demands, relies on angiogenesis. In healthy adults, angiogenesis is typically restrained, except during specific events like the female reproductive cycle (for endometrial regeneration and corpus luteum formation), pregnancy (for placenta development), and wound healing (granulation tissue formation) (55–59). Anti-angiogenesis is valuable in contexts like cancer treatment, where it curtails pathologic blood vessel growth, restricting tumor blood supply and inhibiting tumor growth (56, 57, 59). Achieving this balance between inhibiting pathological angiogenesis, often seen in diseases like cancer, and promoting physiological angiogenesis, crucial for tissue repair and development, is a complex challenge. This task involves targeted strategies that interrupt abnormal blood vessel growth in disease while enhancing functional vessel formation where necessary. One effective approach is targeting specific molecules or receptors overexpressed during pathological angiogenesis, such as growth factor (GF), GF receptors or integrins, effectively blocking the formation of abnormal blood vessels by inhibiting vascular endothelial growth factor (VEGF) signaling (55–59).

Our group carried out a study to evaluate the anti-angiogenic and angio-preventive potential of A009 on HUVECs proliferation, induction of apoptosis, migration, and network formation activities *in vitro*, and its ability to interfere with angiogenesis both *in vitro* and *in vivo* (37). To make sure that activity was not affected by potential changes in chemical composition and compounds present in the extract, such as those influenced by seasonal weather variations and the amount of time between olive oil extraction and extract preparation, experiments were conducted using two different batches of A009. HT, used as control, was from synthetic origin and with a purity of ≥98%. The anti-proliferative potential of A009 was evaluated by testing its ability to hinder endothelial cell proliferation, and then compared to the effect of HT alone at a similar concentration (Table 1).

A009 displayed the ability to suppress HUVEC proliferation after 24 h (dilution 1:1000), and at higher concentrations it completely arrested it (37). In contrast, HT alone, at a similar dilution, exhibited a comparatively lower effect. These results suggest that the presence of a diverse mixture of phenolics in the A009 extract enhances its anti-proliferative activity. The team then examined whether the cytostatic impact of A009 was linked to the induction of apoptosis, by performing a flow cytometry-based apoptosis assay. After 48 h of treatment, HUVECs showed increased apoptosis with A009 as compared to HT alone (37).

A009 was also shown to inhibit reactive oxygen species (ROS) production before and after H_2O_2 treatment (37). When there is an imbalance between the production of ROS and the antioxidant capacity of the host, it results in oxidative stress. ROS are generated as by-products during the mitochondrial electron transport of aerobic respiration, or by oxidoreductase enzymes and metal-catalyzed oxidations and are associated with several inflammatory conditions. Given the antioxidant compounds contained in A009, the group investigated its ability to scavenge ROS in HUVECs as compared to HT alone. A009 exhibited robust ROS scavenger effects in both pre- and post-treatment. Conversely, HT alone exerted little ROS scavenger activity (37).

In another study, the OMWW extracts showed antioxidant activity protecting both HUVECs and human pulmonary artery smooth muscle cells from oxidative stress-induced cell death (60).

In vitro, A009 can inhibit endothelial morphogenesis (37). When adding pro-angiogenic factors to endothelial cells plated on Matrigel, a reconstituted basement membrane matrix, HUVECs can form capillary-like networks (61, 62). A study showed that A009 was able to interfere with HUVECs morphogenesis in a dose-dependent manner, at a similar level to HT alone. A009 can also inhibit HUVECs migration and invasion. To form new blood vessels during angiogenesis, endothelial cells need to traverse basement membranes. Hence, the team's investigation focused on determining whether A009 and HT could impact the migration and invasion ability of HUVECs through Matrigel. A009 showed a significant decrease in the number of migrated and invaded endothelial cells in a dose-dependent manner, at dilutions of 1:500 and 1:250. Purified HT also exhibited a significant inhibitory effect on migration and invasion at the highest concentration, although to a lower extent (Table 1).

Additionally, the inhibitory effect of A009 on angiogenesis was assessed *in vivo* (37). The study investigated the effect of A009 and HT on *in vivo* angiogenesis using a subcutaneous Matrigel sponge assay. The results showed that A009 inhibited the angiogenic response induced by a pro-angiogenic cocktail (VTH: VEGF, TNF-α, heparin), as detected by macroscopic inspection, and quantified by Drabkin's assay for haemoglobin. HT showed limited effects on angiogenesis (Table 1). The inhibition of angiogenesis by A009 was confirmed by histological examination of the Matrigel pellets (37).

Bender et al. (63, 64) evaluated the bioavailability of HT and its metabolites, contained in food supplements obtained from OMWW (OliPhenolia bitter®, OliPhenolia®), in a randomized and controlled human trial. Bioavailability is a prerequisite for any compound effect, and they observed that after the ingestion of the food supplement, HT is absorbed and highly metabolized into its metabolites, which are likely the primary contributors to the positive effects observed (63, 64). In light of these findings, OMWW holds great promise as a food supplement for the prevention of oxidative stress *in vivo* and the resulting cardiovascular risk (63), as well as in preventing lipoxidation (64).

Exercise-induced aerobic metabolism increase is a well-known potential source of oxidative stress. Athletes typically use supplements made of plant-derived polyphenols to improve performance, hasten the recovery of muscle function, and lessen the adverse effects of exercise-induced oxidative stress (65, 66). Roberts and colleagues investigated the effect of OMWW (OliPhenolia®) on aerobic workout and acute recovery in healthy volunteers. They observed a modest antioxidant effect following the intake (16 days) of OMWW food supplements, rich in HT, with suppression of superoxide dismutase (SOD) activity and increased glutathione (GSH) in the 24 h after a 60 min intense aerobic exercise session (65, 66). An *in vivo* study demonstrated that treatment of 27-month-old rats with OMWW enriched in HT successfully alleviated skeletal muscle function decline originating from age-related oxidative stress (40).

There is some evidence to suggest that the phenolic compounds in OMWW may help to improve insulin sensitivity and regulate blood sugar levels, which could be beneficial for people with diabetes (45–48). Phenolic compounds in OMWW may have neuroprotective effects and could help to prevent or slow the progression of neurodegenerative diseases such as Alzheimer's (67–69) and Parkinson's (69). The effectiveness of a HT-rich OMWW extract to lessen Fe^{2+-} and nitric oxide (NO)-induced cytotoxicity in murine-dissociated brain cells was examined due to the detrimental effect that oxidative stress has on brain cell survival (70). Oral long-term HT consumption has the potential to protect against numerous oxidative stress pathways, according to *in vivo* and *ex vivo* findings (70). In TgCRND8 mice (double-mutant gene of APP695) it was observed that the effects of oleuropein aglycone on behavioral performance and neuropathology are not closely related to oleuropein aglycone by itself, in fact, a comparable neuroprotective effect was observed using a diet supplementation with the same dose of a mix of polyphenols found in the OMWW (71).

In summary, all the discussed studies provide evidence that bioactive compounds in OMWW can be valid allies in the treatment of various pathologies, with particular effect on angiogenesis, supporting the strong pharma-nutritional potential in cardiovascular, neurological and oxidative stress disorders.

Olive oil by-products and OMWW against cancer

OMWW extracts have shown promising results as candidates to prevention and treatment of cancer. Phytochemicals are particularly appealing as cancer chemopreventive agents due to their low toxicity and ability to modulate various signal transduction pathways in biological processes associated with cancer (72). Chemoprevention refers to the administration of bioactive molecules to block, revert, or delay the carcinogenic process. Chemopreventive agents can reduce cancer risk in several ways, such as proliferation inhibition, apoptosis induction, and angiogenesis inhibition. In recent years, studies have suggested that applying well-tolerated dietary supplements, such as carotenoids, green tea catechin, curcumin, fish oil fatty acids, and polyphenols, can reduce the risk of cancer development or progression through their antioxidant, anti-proliferative, anti-angiogenic, anti-inflammatory and pro-apoptotic effects in various types of cancers. HT and TYR, two main components of EVOO and OMWW, have been particularly associated with anti-proliferative and pro-apoptotic effects. The numerous biological properties of HT have been demonstrated *in vitro* and *in vivo* by a number of studies (73–77) and have also been recognized by the European Food Safety Authority (EFSA).

In cancer, angiogenesis plays a crucial role in tumor growth and metastasis. Indeed, tumors require a blood supply to provide oxygen and nutrients necessary for their growth and survival. While in normal tissue, angiogenesis is tightly regulated, in cancer, the balance between pro- and anti-angiogenic factors is disrupted, leading to the development of new blood vessels that feed the tumor (55–58). Cancer cells produce factors that promote angiogenesis, such as vascular endothelial growth factor (VEGF) and many others for review (56–58, 78), which stimulate the growth of new blood vessels. Anti-angiogenic therapy for cancer aims to inhibit the formation of new blood vessels and starve the tumor of its blood supply. This can be achieved by using drugs that target VEGF, CXCL8 or other pro-angiogenic factors, or their receptors. The ability of the OMWW extract A009 to affect cell proliferation and survival has been evaluated on colon, lung, prostate, breast, bladder, and melanoma human tumor cell lines (Table 2). Both functional and *in vivo* studies showed that A009 was able to inhibit tumor cell line growth in a dose dependent manner, showing a comparatively stronger inhibitory effect than HT alone.

Another olive tree by-product, leaf extracts, also exhibited anti-cancer and anti-inflammatory properties (84–87). These activities, in particular, are related to oleuropein, a main chemical compound of olive leaves. A recent study has demonstrated that oleuropein-rich leaf extracts (named ORLE), exert anti-tumor and anti-inflammatory activities in colon tumors, reducing cell proliferation and increasing cell apoptosis. Moreover, it is able to reduce nitric oxide synthase (iNOS) in colon tumor lesions and peritoneal macrophages of Apc-mutated PIRC rats (85). The extract helps to inhibit the pro-inflammatory signal generated by cancer cells or inflammatory cells of the tumor microenvironment, which is essential for the progression of colon cancer. Based on their results, the researchers suggest ORLE as a complementary therapy in combination with standard anti-cancer drugs (85).

Other studies have shown that the oil leaf extract has anti-proliferative and pro-apoptotic activity on both triple-negative breast and ovarian cancer cells (86). It is also effective on melanoma, reducing growth and inhibiting metastatic spread (87).

A diet rich in polyphenols, such as those found in olive oil and the Mediterranean diet or a by-product of olive oil production, can reduce the risk of developing colon cancer. Our study investigated the potential chemopreventive properties of A009 on colorectal cancer (CRC) cell lines (79). We used a murine xenograft model to test the effect of A009 on the growth of CT-26 CRC cells, using purified HT as a control. The results showed that A009 inhibited the proliferation, migration, invasion, adhesion, and sprouting of CRC cells, and also decreased the release of pro-angiogenic and pro-inflammatory cytokines (VEGF, IL-8) to a similar extent to HT alone. Moreover, *in vivo* experiments showed that A009 was more effective than HT alone in slowing down the growth of CT-26 tumors (79). The A009 enhanced the effect of the cisplatin and 5-fluorouracil (5-FU), two common chemotherapeutic drugs, on HT29 CRC cells (39). In human CRC-derived cell lines (HCT116 and LoVo) HT and the OMWW extracts decrease proliferation, normal (colonic CCD-841CoN; skin fibroblast WS1) cells are less responsive (80), OMWW extracts (88) increased apoptosis in CRC-derived cell lines. OMWW extracts negatively regulated NF-κB phosphorylation as TNF-α and IL-8, and PPARγ levels increase (80). OMWW extracts and HT promoted mitochondrial functionality involving the PPARγ/PGC-1α axis in HCT116 and LoVo CRC cells (89). Cardinali et al. (89) studied an

TABLE 2 Effect of OMWW extract on cancer cells.

	OMWW extract		
Cells	*In vitro*	*In vivo*	Combination with chemotherapy
Colon cancer	Suppression of proliferation, apoptosis, migration, invasion, adhesion, sprouting. Downregulation of VEGF and IL-8 (79). Negative regulation of NF-κB phosphorylation and TNF-α levels. Increase of PPARγ (80).	Slower growth of tumor mass (39).	Enhancement of the cisplatin and 5-FU drugs effect (39).
Lung cancer	Suppression of proliferation, induction of apoptosis, limitation of cell migration and invasion. Reduction in pro-angiogenic factors. Downregulation of CXCR4 and CXCL12 expressions (81).		
Prostate cancer	Reduction of cell viability, adhesion, migration, invasion, sprouting, and colonies formation (82).	Reduction of tumor size (39).	Enhancement of the cisplatin drug effect (39).
Breast cancer	Reduction of cell growth. Reduction size of breast cancer cell spheroids in combination with chemotherapy drugs (38).	Reduction of angiogenesis and enhancement of the T cell immune cell number (38).	Enhancement of the cisplatin, doxorubicin and 5-FU drugs effect (38).
Bladder cancer	Inhibition of growth and proliferation, both in chemo-sensitive and gemcitabine- and cisplatin-resistant tumour cells (83).		
Melanoma	Inhibition of A375 melanoma nodules growth in the melanoma skin model (33).		

The table lists the OMWW extract's effect on several cancer cell types, both *in vivo* and *in vitro*, as well as in combination with currently available chemotherapeutic drugs.

OMWW fraction rich in verbascoside and isoverbascoside, estimating their bioavailability both in *in vitro* digestion and Caco-2 human intestinal cell models. In the *in vitro* model to assess the bioaccessibility of phenolic compounds from OMWW, digestive recoveries were found to be 35.5% ± 0.55% for verbascoside and 9.2% ± 0.94% for isoverbascoside, underscoring the potential sensitivity of these phenolics to gastric and small intestine digestive conditions. Uptake of verbascoside and isoverbascoside was rapid, with peak accumulation occurring after 30 min, providing a rationale for subsequent *in vivo* studies on the bioavailability and bioactivity of OMWW components (89). In human colorectal carcinoma cells, an OMWW extract showed antioxidant effects against ileo-carcinoma cell line HCT8 cells modulating the intracellular ROS content (90).

Despite progress in targeted therapies, lung cancer remains the leading cause of cancer death worldwide. While avoidance of smoking is the most effective measure, chemoprevention could be useful, particularly for high-risk individuals. Our group evaluated the chemopreventive effects of A009 extracts on lung cancer cell lines (A549 and H1650) (81). A009 extracts inhibited cell proliferation, induced apoptosis, limited cell migration and invasion, and decreased the production of pro-angiogenic factors. The work demonstrated that the A009 extracts inhibited the growth of A549 and H1650 lung cancer cells in a time- and dose-dependent manner, and this was linked with increased apoptosis at 24 and 48 h, with higher induction in H1650 compared to A549 cells. A009 extracts also reduced the production of CXCR4 and CXCL12, which regulate cell migration and invasion, and inhibited the formation of invasive sprouts on Matrigel. Additionally, A009 extracts interfered with the production of pro-angiogenic factors, including VEGF, CXCL8, and CCL2, in both cell lines (81).

A009 can exert chemopreventive activities also for prostate cancer (PCa). Cell lines (PC-3, DU-145, LNCaP) were tested *in vitro* (82). Surface-Activated Chemical Ionization/Electrospray Ionization mass spectrometry (SACI/ESI-MS) was used to determine the polyphenol content in the extracts. The mass spectrometry analysis confirmed HT as the major component of A009, which was used as a reference compound to test the OMWW extract's chemopreventive properties *in vitro*. A009's chemopreventive activity was tested in proliferation assays and functional studies for cell adhesion, migration, and invasion. It was found to significantly reduce PCa cell viability up to 96 h in all cell lines investigated, similarly to HT. A009 inhibited PCa cell adhesion, migration, invasion, and sprouting, including interfering with LNCaP cell line's ability to form colonies/islets *in vitro* (82). Furthermore, A009 was able to inhibit VEGF production and CXCL8 release in all PCa cell lines investigated, and angiogenin only in LNCaP cells (82). *In vivo*, the A009 extract co-administered with cisplatin showed a synergistic effect in further reducing tumor size in mouse xenografts of PCa (39).

In a further study we investigated the anti-cancer effects of A009 on breast cancer cells (MDA-MB-231 and BT-549) when combined with chemotherapeutic agents such as doxorubicin, an anthracycline commonly used in breast cancer therapy, and 5-FU as the prototype fluoropyrimidine. *In vitro* experiments showed that A009 combined with doxorubicin or 5-FU effectively decreased breast cancer cell growth, and additive effects were observed in breast tumor spheroids (38). A009 was anti-angiogenic in the Matrigel sponge model containing breast cancer cells supernatants, and recruitment of T cells was increased by A009 (38).

Bladder cancer is a threatening tumor of the urinary system, approximately 90% of all bladder cancers worldwide are urothelial carcinoma. OMWW injection solution (Burg-Apotheke) was tested on resistant parental bladder cancer cell lines (T24, TCCSUP, and RT112) for 48 and 72 h proliferation (83). The clonogenic cell growth, number and size were significantly diminished in a concentration dependent manner. Cisplatin-resistant and gemcitabine-resistant tumor cells were exposed to OMWW, and their growth resulted significantly diminished. The OMWW treatment reduced pAkt (24 and 72 h), pRaptor (24 h), Rictor (24 and 72 h), pRictor, and pmTOR Akt–mTOR axis. It is assumed that OMWW's ability to inhibit growth and proliferation, both in

chemosensitive and gemcitabine- and cisplatin-resistant bladder cancer cells, is associated with cell cycling arrest through cyclin-CDK axis manipulation and suppression of Akt/mTOR pathway activation (83).

OMWW was also able to modulate the invasion of tumor cells in a 3D melanoma skin model, affecting both the growth and migration of melanoma cells and the melanoma nodes formation (33). A375 cells, human metastatic melanoma cells, were used and the treatment with A009 reduced A375 melanoma nodules growth *in vitro* compared to the untreated samples as determined by cell cluster size reduction (33).

The data collected in this review showed that olive oil industry by-products and their main bioactive compounds inhibited pro-inflammatory cytokines, as well as other molecules involved in inflammatory processes and in several cancer types as potential adjuvant therapy. Molecular targets that A009 extract decreased in several cancer cells are shown in Table 3.

TABLE 3 Molecular targets reduced by OMWW extract in cancer cells.

Cancer Cells	Molecular target	References
Colon	VEGF CXCL8 NF-kB TNFα Increase PPARγ	(79) (80)
Lung	CXCR4 CXCL12 VEGF CXCL8 CCL2	(81)
Prostate	VEGF CXCL8 Angiogenin	(82)
Bladder	pAkt pRaptor pRictor pmTOR Akt–mTOR axis	(83)

OMWW as cardioprotective during anti-cancer therapy

One of the most undesirable side effects in cancer patients receiving chemotherapy is cardiovascular toxicity, which can limit the effectiveness of treatment options (91–93), including combinations of different anti-cancer agents (38, 39). Chemotherapy drugs, commonly used in several neoplasms, are known for their cardiotoxicity (38, 39). A cardioprotective role was observed in mice co-treated with A009 extracts alongside cisplatin; the hearts of these mice showed reduced mitochondria damage compared to those treated with chemotherapy alone (38). *In vitro* we observed toxicity on rat cardiomyocyte H9C2 cells treated with chemotherapy drugs (doxorubicin, cisplatin, and 5-FU), while cell proliferation was not affected by A009 alone. However, co-treatment with A009 and either cisplatin or 5-FU did not further reduce cardiac cell proliferation induced by chemotherapy (39). When A009 was combined with 5-FU, it showed cardioprotective effects on neonatal murine cardiomyocytes. Co-treatment resulted in a smaller reduction in the number of cardiomyocytes compared to treatment with 5-FU alone (39). Additionally, A009 demonstrated cardioprotective effects in zebrafish embryos. Co-treatment with A009 reversed the doxorubicin-induced cardiotoxic effect, particularly in terms of cardiac area. Furthermore, it exhibited the ability to reduce the upregulation of the pro-inflammatory IL-6 and p16 mRNA induced by 5-FU in human cardiomyocytes. These findings indicate its potential as a preventative agent in cardio-oncology (39). Inhibition of cancer cells was extensive while effects on cardiomyocytes were limited (Table 1).

Cardiovascular toxicities are still one of the complications of chemotherapy (91–93). The studies indicate that the OMWW purified extract could exert cardiovascular protection (Table 1).

All of the above studies suggest that the polyphenol-rich OMWW extract is highly effective. Its polyphenols are more abundant and less expensive than purified single components, and the compounds can be assumed orally as a nutraceutical (61). Not only does A009 appear effective in preventing the growth of several tumor cells, but it can also prepare cells to respond better to chemotherapy (Table 1).

Mitochondrial damage is a key cause of cardio-toxicities brought on by chemotherapy because cardiomyocytes heavily rely on them for their energy needs. Polyphenols counteract the production of ROS that causes cellular and mitochondrial damage by acting as antioxidants.

Cardioprotective effect is reported in an ultrastructural analysis, mice treated with A009 extracts and cisplatin together showed less mitochondrial damage, displaying a rounder form, and having more, better-organized mitochondrial cristae, than mice treated with chemotherapy alone. Additionally, it was shown that animals treated with A009 and cisplatin together had more regular dispositions of muscular myosin and actin fibers in their hearts than animals treated with cisplatin alone. Finally, only hearts treated with cisplatin had inflammation and fibrosis visible under optical microscopy (39).

These data show that the use of OMWW extract could be a promising strategy to reduce chemotherapy-induced cardiotoxicity in oncological patients, influencing effect on the distribution of muscle proteins and mitochondrial function (39).

Scarce data about the OMWW mechanisms of action are found in the literature, so further investigation focused on fully understanding the mechanisms underlying OMWW cardioprotective effects is needed.

Environmental issues of OMWW

The circular economy is a system focused on reducing waste and promoting sustainability by prolonging the use of resources. It was included among the 17 United Nation Sustainable Development Goals adopted by all the Member States in 2015. These goals aim to globally implement strategies that enhance health and education, reduce inequality, stimulate economic growth, all while addressing climate change and preserving our oceans and forests.[1]

It is a model that aims to create a closed-loop system where materials, products, and waste are continually reused, recycled, or repurposed,

1 https://sdgs.un.org/goals

instead of being discarded. Food by-products are the leftover materials generated during the production, processing, and consumption of food. These by-products can include vegetable peels, fruit scraps, and animal bones, among others. In an enhancement of the circular economy, food by-products can be transformed into valuable resources, rather than being treated as industry waste. This can be achieved through a range of approaches, including:

- Upcycling: transforming food by-products into new products with a higher value, such as turning fruit waste into juices, jams, or supplements.
- Recycling: converting food by-products into new materials or products, such as compost or biofuel.
- Reusing: finding alternative uses for food by-products, such as using vegetable peels in animal feed.

Recycling olive oil by-products as nutraceuticals is a very promising proposition (43, 50, 94–97). The olive tree perhaps really is the first of all trees ("Olea prima omnium arborum est" is its Latin epithet), and EVOO, together with its powerful watery companion, OMWW, possibly are the "liquid gold" often mentioned throughout history.

Conclusion

The review emphasizes how crucial it is to recover phenolic compounds from OMWW and other by-products so that they can be used in the food and nutriceutical industries while also making a sustainable contribution to waste treatment. The findings from our studies and those of other research teams hold significant promise for the potential applications of purified extracts from both olive mill waste water and olive leaf extracts in the field of cancer prevention and treatment. The observed capacity of these extracts to not only reduce cancer cell growth but also to induce apoptosis and inhibit angiogenesis highlights their multifaceted potential as candidates for combating cancer. These attributes make them attractive prospects for further exploration in the realm of anti-cancer supplements.

Moreover, it is noteworthy that olive mill waste water extracts have demonstrated the ability to enhance the effectiveness of chemotherapeutic drugs while protecting the heart and cardiomyocytes. This synergy suggests that these extracts could serve as valuable adjuncts to conventional cancer treatments, potentially leading to more efficacious less toxic therapies.

Our studies reveal that while these extracts exhibit potent effects against cancer cells, they appear to have a comparatively lesser impact on cardiomyocytes. This selective action suggests a potential safety profile that could be advantageous in clinical applications.

Beyond their biomedical potential, the utilization of waste products from agricultural processing, such as these olive by-products extracts, carries a positive environmental impact. By repurposing what would otherwise be discarded, research into the utilization of olive oil by-products aligns with sustainable practices and contributes to the reduction of waste in the agricultural sector.

However, it is important to emphasize that while these initial findings are promising, further research is imperative. A comprehensive understanding of the underlying mechanisms behind the effects of olive production by-products is needed. This knowledge will enable the development of more targeted and effective strategies for their utilization in diverse fields, including nutraceuticals, cosmeceuticals, and cancer preventive extracts. This underscores the significance of continued investigations in order to unlock the full potential of these natural and processed compounds for the betterment of human health and the environment.

Author contributions

AA: Conceptualization, Writing – original draft. FA: Writing – original draft. PC: Writing – review & editing. LD: Writing – review & editing. LC: Writing – review & editing. DN: Conceptualization, 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

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1254947/full#supplementary-material

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Impact of omega-3 fatty acids supplementation on the gene expression of peroxisome proliferator activated receptors-γ, α and fibroblast growth factor-21 serum levels in patients with various presentation of metabolic conditions: a GRADE assessed systematic review and dose–response meta-analysis of clinical trials

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There is some debate about the effects of omega-3 fatty acids on the regulation of adipose tissue related genes. This systematic review and meta-analysis aimed to evaluate the effects of omega-3 fatty acids supplementation on the gene expression of peroxisome proliferator activated receptors (*PPAR-*α and *PPAR-*γ) and serum fibroblast growth factor-21 (FGF-21) levels in adults with different presentation of metabolic conditions. To identify eligible studies, a systematic search was conducted in the Cochrane Library of clinical trials, Medline, Scopus, ISI Web of Science, and Google Scholar up to April 2022. Eligibility criteria included a clinical trial design, omega-3 fatty acids supplementation in adults, and reporting of at least one of the study outcomes. Effect sizes were synthesized using either fixed or random methods based on the level of heterogeneity. Fifteen studies met the inclusion criteria. Omega-3 fatty acids supplementation significantly increased the *PPAR-*γ (10 studies) and *PPAR-*α (2 studies) gene expression compared to the control group (WMD: 0.24; 95% CI: 0.12, 0.35; $p < 0.001$ and 0.09; 95% CI: 0.04, 0.13; $p < 0.001$, respectively). Serum FGF-21 (8 studies) levels exhibited no significant change following omega-3 fatty acids supplementation ($p = 0.542$). However, a dose–response relationship emerged between the dose of omega-3 fatty acids and both *PPAR-*γ gene expression and serum FGF-21 levels. Overall, this study suggests that omega-3 fatty acids supplementation may have positive effects on the regulation of adipose tissue related genes in patients with various presentation of metabolic condition. Further research is needed to validate these findings and ascertain the effectiveness of this supplementation approach in this population.

Systematic Review Registration: https://www.crd.york.ac.uk/prospero/display_record.php?, CRD42022338344.

KEYWORDS

n-3 fatty acids, polyunsaturated fatty acids, PUFA, PPAR, fibroblast growth regulatory factor

Introduction

Omega-3 fatty acids have demonstrated a wide range of health benefits, including the capacity to reduce hypertriglyceridemia, adverse cardiovascular events, and the regulation of blood pressure, glucose tolerance, and nervous system functions. Moreover, omega-3 fatty acids have been associated with decreased insulin secretion, enhanced insulin resistance, and improved endothelial function. They also exhibit anti-inflammatory, antioxidant, and anti-thrombotic properties (1, 2). However, the precise mechanisms through which omega-3 fatty acids exert their metabolic effects remain incompletely understood. Previous studies have suggested that the metabolic effects of n-3 polyunsaturated fatty acids (PUFAs) involve the modulation of gene expression in adipose tissue (3). For example, omega-3 fatty acids are recognized as natural modulators of peroxisome proliferator activated receptors (*PPAR-α*, *PPAR-γ*, and *PPAR-δ*) and improvement of fibroblast growth factor-21 (FGF-21), but the whole mechanisms are not clear (4–8).

PPAR-γ and *PPAR-α* serve as nuclear receptors with pivotal roles in the regulating of lipid and glucose metabolism. PPAR-γ regulates adipocyte differentiation, lipid storage, and adipokine secretion. Defects in *PPAR-γ* function contribute to insulin resistance and obesity. Additionally, *PPAR-α* and FGF-21 promote fat oxidation and thermogenesis in adipose tissue, potentially counteracting metabolic abnormalities linked to obesity. The connection between *PPAR-γ*, *PPAR-α*, and serum levels of FGF-21 in the context of metabolic disorders such as type 2 diabetes (T2DM), non-alcoholic fatty liver disease (NAFLD), obesity, poly-cystic ovary syndrome (PCOS), gestational diabetes mellitus (GDM), cardiac disease, and dyslipidemia arises from their roles in regulating glucose and lipid metabolism, insulin sensitivity, inflammation, and oxidative stress. Modulating these pathways through PPARs activation or increased FGF-21 levels may hold therapeutic potential promise for these conditions.

Experimental studies showed that both fish oil and flaxseed oil up-regulate the expression of *PPAR*-α and *PPAR*-γ (9, 10). Rahmani et al. (11) observed a significant improvement in *PPAR*-γ gene expression following 12 weeks of fish oil supplementation in subjects with PCOS. Other studies investigated the impact of omega-3 fatty acids supplementation on the regulation of plasma FGF-21 levels and its role in modulating critical metabolic pathways in white adipose tissue.

FGF-21 is a novel metabolic regulator that is primarily produced by the liver (7, 12–14). Recently, it was described that omega-3 fatty acids can reduce circulating FGF-21 levels and enhance FGF-21 sensitivity, potentially through a *PPAR*-γ-dependent mechanism (15). Nevertheless, the outcomes of previous studies present conflicting findings (14, 16, 17). Consequently, the present study aimed to systematic review and meta-analysis clinical trials that evaluated the effect of omega-3 fatty acids supplementation on *PPAR-γ* and *PPAR-α* gene expression and serum FGF-21 levels in patients with various presentation of metabolic conditions. The results of this study could enhance our understanding of the metabolic actions of omega-3 fatty acids and offer insights into their potential therapeutic applications.

Materials and methods

This research followed the PRISMA statement for systematic reviews and meta-analyzes. The systematic review protocol was registered in PROSPERO under the code CRD42022338344. Ethical approval for the study methodology was obtained from the ethics committee of Isfahan University of Medical Sciences (IR.ARI.MUI.REC.1400.135).

Search strategy

Two researchers (BA and FS) independently conducted searches in various databases, including the Cochrane Library of clinical trials (CENTRAL), Medline, Scopus, ISI Web of Science, and Google Scholar for studies that investigated the effects of omega-3 fatty acids on the gene expression of *PPAR-γ, α* and serum levels of FGF-21 in individuals with different presentation of metabolic conditions. The search included all original papers published until April 2022. Various combinations of keywords and medical subject heading (MeSH) terms were used, including n-3 fatty acids, fish oil, n-3 oil, n-3 Polyunsaturated Fatty Acid, n-3 PUFA, alpha-Linolenic Acid, Docosahexaenoic Acids, Eicosapentaenoic Acid, DHA, EPA, ALA, omega 3, omega-3 fatty acids, peroxisome proliferator activated receptor, PPAR, Thiazolidinedione Receptor, NR1C3, FGF, fibroblast growth factor. There were no restrictions on publication year or language. Moreover, the reference lists of included studies were reviewed to identify any additional relevant studies. Two reviewers independently screened the titles and abstracts of the search results to select potentially relevant studies.

Study selection

Full texts of studies aligned with the objectives of the present study were examined, and those meeting the eligibility criteria were included. The inclusion criteria consisted of studies written in English or Persian, studies involving omega-3 fatty acids supplementation, studies evaluating the gene expression of *PPAR-γ, α* or serum levels of the FGF-21 as study outcomes, and clinical trial study designs. Due to limited number of clinical trials available, we were unable to include

a specific population group in our study. However, all human studies conducted in patients with metabolic conditions related to obesity, insulin sensitivity, and dyslipidemia were considered. The following reports were excluded: non-full-text articles, ecological studies, animal studies, observational studies, opinion articles, conference abstracts, review papers, editorials, studies not assessing relevant outcomes or populations, and studies using omega-3 fatty acids supplements in combination with other bioactive agents.

Data extraction

The data extraction was independently conducted by two researchers (AR and MA). In the case of discrepancies, consensus was reached through cross-examination by MS. The extracted study characteristics included the first author's name, year of publication, country, baseline age, body mass index (BMI), sample size, composition of the supplement and placebo, dose of omega-3 fatty acids, study duration, and study population. Additionally, mean ± SD values of serum FGF-21 and fold change of *PPAR-γ* and *PPAR-α* gene expression were derived from eligible studies at both the baseline and the end of the study.

Assessment of risk of bias

Two independent researchers assessed the quality of the trials using the revised Cochrane risk of bias tool for randomized trials (RoB 2). The RoB2 evaluates various aspects of trial design, conduct, and reporting. The quality of the studies was categorized as "Low risk," "High risk," or expressed as having "Some concerns."

Statistical analysis

The mean difference ± SD was used as the effect size and was pooled using fixed method meta-analysis (inverse variance). In the presence of significant heterogeneity, the random method (Dersimonian-Laird) was employed to pool effect sizes. Heterogeneity between studies was evaluated using the I^2 index and Cochrane's Q test. I^2 Interpretation is as follows: low if I^2 is <30%, moderate if I^2 is 30–75%, and high if I^2 is >75% (18).

Subgroup analyzes were conducted based on age (≤55 or > 55 years), source of omega-3 fatty acids (fish oil or plant-based oil), and different population groups (diabetic or non-diabetic/ obese or overweight/dyslipidemia or non-dyslipidemia) to explore potential variations in study results. Sensitivity analysis and meta-regression were performed to further investigate the effects of different variables on study outcomes. One-stage non-linear dose–response meta-analyzes were conducted using the DRMETA module developed by Nicola Orsini (19) to examine the effect of omega-3 fatty acids supplementation on the gene expression of *PPAR-γ* and serum FGF-21 levels. Publication bias was assessed using Begg's rank correlation, Egger's linear regression, and visual inspection of the funnel plot. If publication bias was detected, the Trim and Fill method was applied to adjust for intervention effects. All analyzes were performed using Stata, version 17 (Stata Crop, College Station, TX, United States) and a p-value of <0.05 was considered statistically significant.

Results

Characteristics of the studies

The initial electronic search resulted in 1783 studies after removing duplicates (see Figure 1). Following the title and abstract screening, 1712 studies, including review articles, study protocols, animal studies, or studies not related to the purpose of the present study, were excluded. A total of 71 studies underwent eligibility assessment, and 55 studies were excluded for various reasons, such as being single-arm studies, observational studies, studies not evaluating the gene expression of *PPAR-γ, α* or serum levels of FGF-21, studies not conducted in patients with metabolic diseases or insulin impairment (obesity, diabetes, dyslipidemia, polycystic ovary syndrome, and heart disease), and studies using a combination of omega-3 fatty acids with other nutrients or bioactive agents. Studies that reported outcomes other than gene expression (such as the activity of *PPAR-γ*) were also excluded. Ultimately, 15 studies met the inclusion criteria and provided sufficient data for meta-analysis (2, 5, 7, 8, 11–14, 16, 20–25). The general characteristics of the included studies are summarized in Table 1. These studies were conducted in various locations, including the United States (8, 21), Iran (5, 11, 20, 22–25), China (2, 12), Sweden (13, 14), Spain (7), and Poland (16). The intervention durations ranged from 3 to 24 weeks. Ten studies utilized fish oil as the source of omega-3 fatty acids (7, 8, 11–14, 16, 20, 21, 24), while five studies used plant-sources (flaxseed or perilla oil) (2, 5, 22, 23, 25). *PPAR-γ,α* gene expression were primarily assessed using peripheral blood mononuclear cells (PBMCs), with two studies using atrial myocardium and placental tissue samples (8, 21). The risk of bias assessment is summarized in Figure 2, with the most common issues related to allocation concealment (selection bias) and incomplete outcome data (attrition bias).

The effect of omega-3 fatty acids supplement on *PPAR-γ* gene expression

Ten studies, comprising 224 intervention and 218 control participants, evaluated the effect of omega-3 fatty acids supplementation on gene expression of *PPAR-γ* (2, 5, 8, 11, 20–25). The results of the meta-analysis (see Table 2) indicated that omega-3 fatty acids supplementation significantly increased *PPAR-γ* gene expression compared to the control group (difference in fold change: 0.24; 95% CI: 0.12, 0.35; $p < 0.001$; see Figure 3A). However, a high level of heterogeneity was observed among the studies ($I^2 = 93.65$; $p < 0.001$). A non-linear dose–response relationship was identified between the dose of omega-3 fatty acids and *PPAR-γ* gene expression ($p < 0.001$; see Figure 4A). Subgroup analysis revealed moderate, non-significant heterogeneity in populations with an average age over 55 years ($I^2 = 62.17$; $p = 0.071$) and in diabetic patients ($I^2 = 51.77$; $p = 0.101$). However, the between-subgroups heterogeneity test was not significant for age, presence of diabetes, weight status, and source of omega-3 fatty acids supplementation ($p > 0.05$). Sensitivity analysis did not lead to changes in the results when excluding one study at a time. The Galbraith plot (see Supplementary Figure S1) indicated five studies as potential sources of heterogeneity (2, 8, 11, 21, 24). Meta-regression analysis revealed a direct association between dose

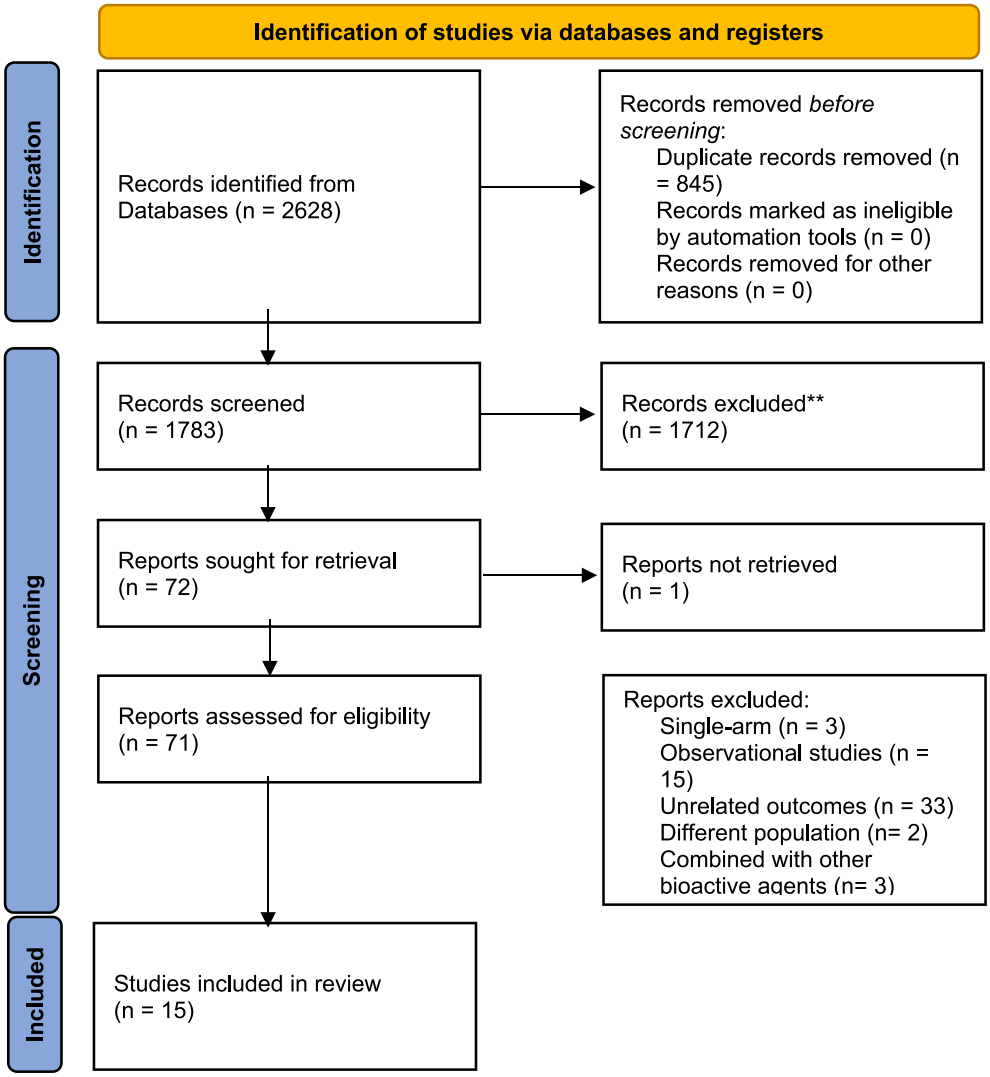

FIGURE 1
PRISMA Flow diagram of the study selection process.

($p = 0.002$) and the percentage of male participants in the study ($p = 0.033$) with changes in *PPAR-γ* gene expression following omega-3 fatty acids supplementation.

The effect of omega-3 fatty acids supplement on *PPAR-α* gene expression

Only two studies investigated the effect of omega-3 fatty acids supplementation on *PPAR-α* gene expression. It was demonstrated that omega-3 fatty acids supplementation significantly increased the *PPAR-α* gene expression compared to the control group (difference in fold change: 0.09; 95% CI: 0.04, 0.13; $p < 0.001$; see Figure 3B). There was no obvious heterogeneity between these studies ($I^2 = 0.0$, $p = 0.442$). Due to the limited number of studies, subgroup, sensitivity, meta-regression, and dose–response analyzes were not possible (Table 3).

The effect of omega-3 fatty acids supplement on serum FGF-21 levels

Five studies, with a total of 160 participants in the intervention group and 157 participants in the placebo group, provided eight effect sizes for evaluating the impact of omega-3 fatty acids supplementation on serum FGF-21 levels. The meta-analysis revealed no-significant difference in the change in serum FGF-21 between the omega-3 fatty acids and control groups (WMD: -21.13; 95% CI: −91.45, 48.08; $p = 0.542$; Figure 3C). However, a dose–response relationship was observed between the dose of omega-3 fatty acids and serum FGF-21 levels ($p = 0.042$; Figure 4B), with the highest FGF-21 level observed at a dose of 1,000 mg/day (WMD: 28.48; 95% CI:4.58, 52.37). A high level of heterogeneity was observed between studies ($I^2 = 85.38$, $p < 0.001$). The Galbraith plot (Supplementary Figure S2) identified the studies of Qin et al. (12) and Escoté et al. (7) as sources of heterogeneity. A significant reduction following omega-3 fatty acids supplementation was observed in patients with dyslipidemia and

TABLE 1 Characteristics of included studies.

First author, year	Country	Participants, *n* (% male)	Study duration, weeks	Age, years	BMI, kg/m^2	Omega-3 FA dose, mg/d	Omega-3 FA type	Placebo type	Population group
Anderson, 2014 (1)	United States	24 (66)	3	64.4	31.1	3,400	Fish oil	No treatment	Patients before elective cardiac surgery
Mansoori, 2015 (2)	Iran	68 (NR)	8	55.8	28.3	2,400	DHA-rich fish oil	Paraffin	T2DM patients
Qin, 2015 (3)	China	70 (73)	12	45.1	26.2	4,000	Fish oil	Corn oil	Patients with NAFLD characteristics associated with hyperlipidemia
Calabuig-Navarro, 2016 (4)	United States	33 (0)	24	27.3	33.4	2,000	Fish oil	Wheat germ oil	Obese and overweight woman
Zhao, 2016 (5)	China	26 (58)	24	48.9	32.5	8,000	Perilla oil	No treatment	Obese patients
Hashemzadeh, 2017 (6)	Iran	60 (NR)	12	59.6	30.4	1,000	Flaxseed oil	Paraffin	T2DM Patients with CHD
Nasri, 2017 (7)	Iran	60 (0)	12	26.8	27.1	2,000	Flaxseed oil	Paraffin	PCOS
Eriksson, 2018 (8)	Sweden	75 (71)	12	65.4	31.2	4,000	OM-3CA	NR	T2DM patients
Rahmani, 2018 (13)	Iran	40 (0)	12	26.6	26.4	2000	Fish oil	NR	PCOS
Jamilian, 2018 (11)	Iran	40 (0)	12	23.3	27.6	1,000	Flaxseed oil	Paraffin	PCOS
Jamilian, 2018 (10)	Iran	40 (0)	6	30.6	27.5	2,000	Fish oil	NR	GDM
Escoté, 2018 (9)	Spain	57 (0)	10	38.4	32.2	1,341	Fish oil	Sunflower oil	Obese and overweight woman
Oscarsson, 2018 (12)	Sweden	51 (59)	12	59.7	29.8	4,000	OM-3CA	NR	Overweight or obese individuals with NAFLD and hypertriglyceridemia
Jamilian, 2020 (14)	Iran	51 (0)	6	29.0	28.1	2,000	Flaxseed oil	Sunflower oil	GDM
Razny, 2021 (15)	Poland	64 (47)	12	41.3	32.9	1,800	Fish oil	Corn oil	Overweight or obesity (with abdominal obesity)

NR, not reported; BMI, body mass index; FA, fatty acid; T2DM, type 2 diabetes mellitus; NAFLD, non-alcoholic fatty liver disease; CHD, coronary heart disease; PCOS, polycystic ovary syndrome; GDM, gestational diabetes mellitus.

overweight (WMD: −92.38; 95% CI: −113.79, −70.98; $p < 0.001$). Heterogeneity was not significant in the older age (P 0.296) and diabetic patient (P 0.756) subgroups. Subgroup analysis identified the presence of dyslipidemia and weight status as sources of heterogeneity ($p < 0.001$). The meta-regression analysis suggested that study duration, sex, BMI, and dose as sources of heterogeneity. A direct association was found between BMI and the mean difference in serum FGF-21. Additionally, an inverse association was observed between the mean difference in serum FGF-21 and study duration ($p = 0.001$), the percentage of male participants in the study ($p < 0.001$), and dose ($p < 0.001$). Sensitivity analysis did not provide any further information.

Publication bias

Visual inspection of the funnel plot, Begg's non-parametric rank correlation test ($p = 0.07$ and 0.386, respectively), and the regression-based Egger test ($p = 0.06$ and 0.659) did not reveal significant

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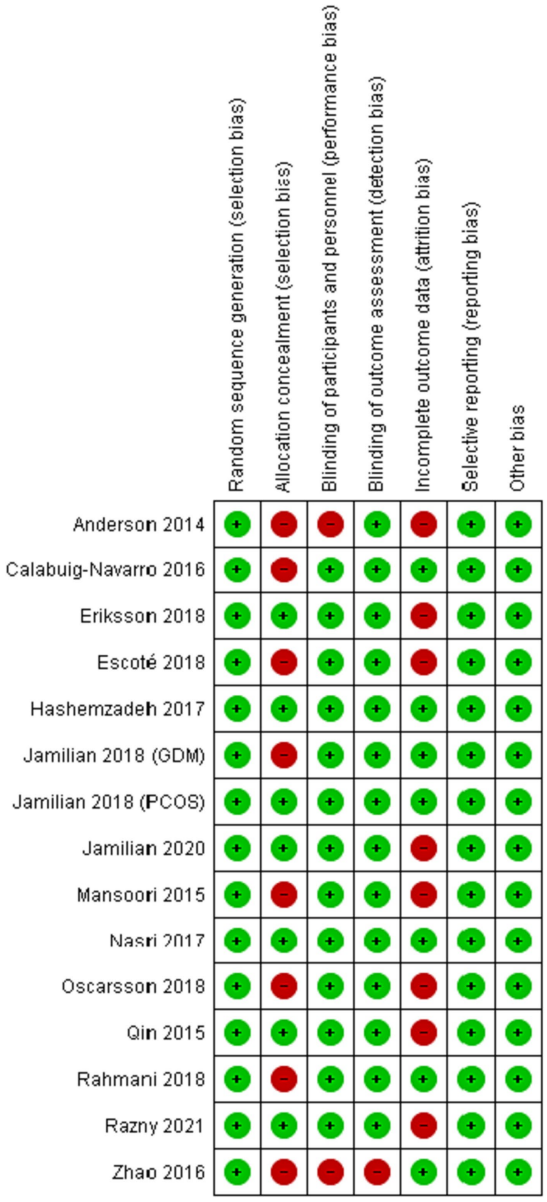

FIGURE 2
Risk of bias summary of included studies.

publication bias in the studies evaluating the effect of omega-3 fatty acids supplementation on *PPAR-γ* gene expression and serum FGF-21 levels (Supplementary Figures S3, S4).

The GRADE assessment

Table 4 provides the GRADE assessment profile of the study outcome. The evidence regarding the effect of omega-3 fatty acids supplementation on *PPAR-γ* gene expression was of "moderate" quality. The certainty of evidence was rated as "low" and "very low" for serum FGF-21 and *PPAR-α* gene expression, respectively.

Discussion

The primary objective of this research was to consolidate findings from existing clinical trials and assess the impact of omega-3 fatty acids supplementation on the expression of *PPAR-γ* and *PPAR-α* genes, and serum FGF-21 levels in patients with various presentation of metabolic conditions. The study included 15 clinical trials involving individuals with diverse health profiles, employing different doses and sources of omega-3 fatty acids as interventions, sometimes in conjunction with placebos as controls. The quality of evidence regarding the effect of omega-3 fatty acids supplementation on *PPAR-γ* gene expression was of "moderate" quality. However the level of certainty of evidence was "low" for serum FGF-21 and "very low" for *PPAR-α* gene expression, respectively.

The meta-analysis results indicated a significant elevation in *PPAR-γ* gene expression due to omega-3 fatty acids supplementation when compared to the control group. Two studies that explored the effect the impact of omega-3 supplementation on *PPAR-α* gene expression also reported significant increases compared to the control group. However, the meta-analysis did not reveal a significant difference in the change of serum FGF-21 between the groups receiving omega-3 fatty acids and control. A non-linear dose–response relationship was observed between the dose of omega-3 and serum FGF-21 levels, with the highest levels observed at a dose of 1,000 mg/day and declining in higher doses. Subgroup analysis showed a significant reduction in patients with dyslipidemia and overweight following omega-3 supplementation.

Previous experimental studies have demonstrated that omega-3 PUFAs activate members of the PPAR superfamily (26, 27), and increase *PPAR-α* mRNA expression in subcutaneous adipose tissues in obese adolescents after 12 weeks of of omega-3 fatty acids supplementation (28). *PPAR-γ*, a member of the nuclear receptor superfamily, plays a pivotal role in regulating glucose and lipid metabolism, immune function, and inflammation (11). It also influences adipocyte function, differentiation, insulin and lipid metabolism, and lipid storage (22). Down-regulation of *PPAR-γ* is involved in the pathological process of various diseases, including diabetes, atherosclerosis and cancer (11). The regulatory effect of *PPAR-γ* activity extends to genes like carboxykinase, glucose-6-phosphatase, and the fatty acid transporter-1, ultimately decreasing free fatty acids production and enhancing insulin sensitivity (29).

Previous research has indicated that that the intake of 1,000 mg of omega-3 fatty acids from flaxseed oil twice daily for 12 weeks can increase *PPAR-γ* gene expression in women with PCOS (23). Similarly, in a study (25) aimed at evaluating the impact of omega-3 fatty acids from flaxseed oil on genetic and metabolic profiles in women with GDM, a significant enhancement in *PPAR-γ* was observed. Moreover, supplementing GDM women with 1,000 mg/day of fish oil for 6 weeks was found to enhance *PPAR-γ* gene expression (24). Linseed oil was also shown to elevate *PPAR-γ* gene expression in goats (30). However, a study involving T2DM patients demonstrated that *PPAR-γ* gene expression did not respond to fish oil supplementation of 2,400 mg/day after 8 weeks (20). It appears that omega-3 fatty acids may affect metabolic conditions through pathways beyond PPAR regulation, including modulating cyclin-dependent kinase inhibitor 2A and telomerase activity (31). Additionally, no significant change in *PPAR-γ* gene expression was observed in the bovine uterus after exposure to

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TABLE 2 Overall estimates of meta-analysis for the effect of omega-3 fatty acids supplement on expression of *PPAR-γ,α* and serum FGF-21 levels in patients with metabolic risk factors.

Outcome	Subgroups	Studies, *n*	Reference	WMD (95% CI)	*p*	I^2 (%)	P heterogeneity	P heterogeneity between subgroups
PPAR-γ, fold change		10		0.24 (0.12, 0.35)	<0.001	93.65	<0.001	–
Age	≤55 years	7	(2, 5, 11, 21, 23–25)	0.25 (0.10, 041)	0.002	95.53	<0.001	0.587
	>55 years	3	(8, 20, 22)	0.20 (0.08, 0.31)	0.001	62.17	0.071	
Presence of diabetes	Yes	4	(20, 22, 24, 25)	0.14 (0.07, 0.20)	<0.001	51.77	0.101	0.154
	No	6	(2, 5, 8, 11, 21, 23)	0.29 (0.09, 0.50)	0.005	96.28	<0.001	
Weight status	Overweight	6	(5, 11, 20, 23–25)	0.18 (0.11, 0.24)	<0.001	56.35	0.043	0.339
	Obese	4	(2, 8, 21, 22)	0.31 (0.03, 0.59)	0.026	97.69	<0.001	
Source of omega-3	Fish oil	5	(8, 11, 20, 21, 24)	0.16 (0.04, 0.29)	0.007	88.32	<0.001	0.269
	Plant-based oil	5	(2, 5, 22, 23, 25)	0.30 (0.09, 0.52)	0.005	95.48	<0.001	
PPAR-α, fold change		2		0.09 (0.04, 0.13)	<0.001	0.0	0.442	–
Serum FGF-21, pg/ml		8	(7, 12–14, 16)	-21.13 (−81.84, 39.56)	0.494	85.38	<0.001	–
Age	≤55 years	5	(7, 12, 16)	−4.93 (−78.89, 69.03)	0.896	91.17	<0.001	0.333
	>55 years	3	(13, 14)	−64.41 (−159.28, 30.46)	0.183	17.90	0.296	
Presence of diabetes	Yes	2	(13)	−18.12 (−120.92, 84.68)	0.730	0.0	0.756	0.955
	No	6	(7, 12, 14, 16)	−21.69 (−91.45, 48.08)	0.542	89.46	<0.001	
Presence of dyslipidemia	Yes	2	(12, 14)	−92.38 (−113.79, −70.98)	<0.001	0.0	0.368	<0.001
	No	6	(7, 13, 16)	27.02 (−0.35, 54.4)	0.053	0.0	0.889	
Weight status	Overweight	2	(12, 14)	−92.38 (−113.79, −70.98)	<0.001	0.0	0.368	<0.001
	Obese	6	(7, 13, 16)	27.02 (−0.35, 54.4)	0.053	0.0	0.889	

FGF, fibroblast growth factor-21; PPAR, peroxisome proliferator activated receptors; WMD, weighted mean difference.

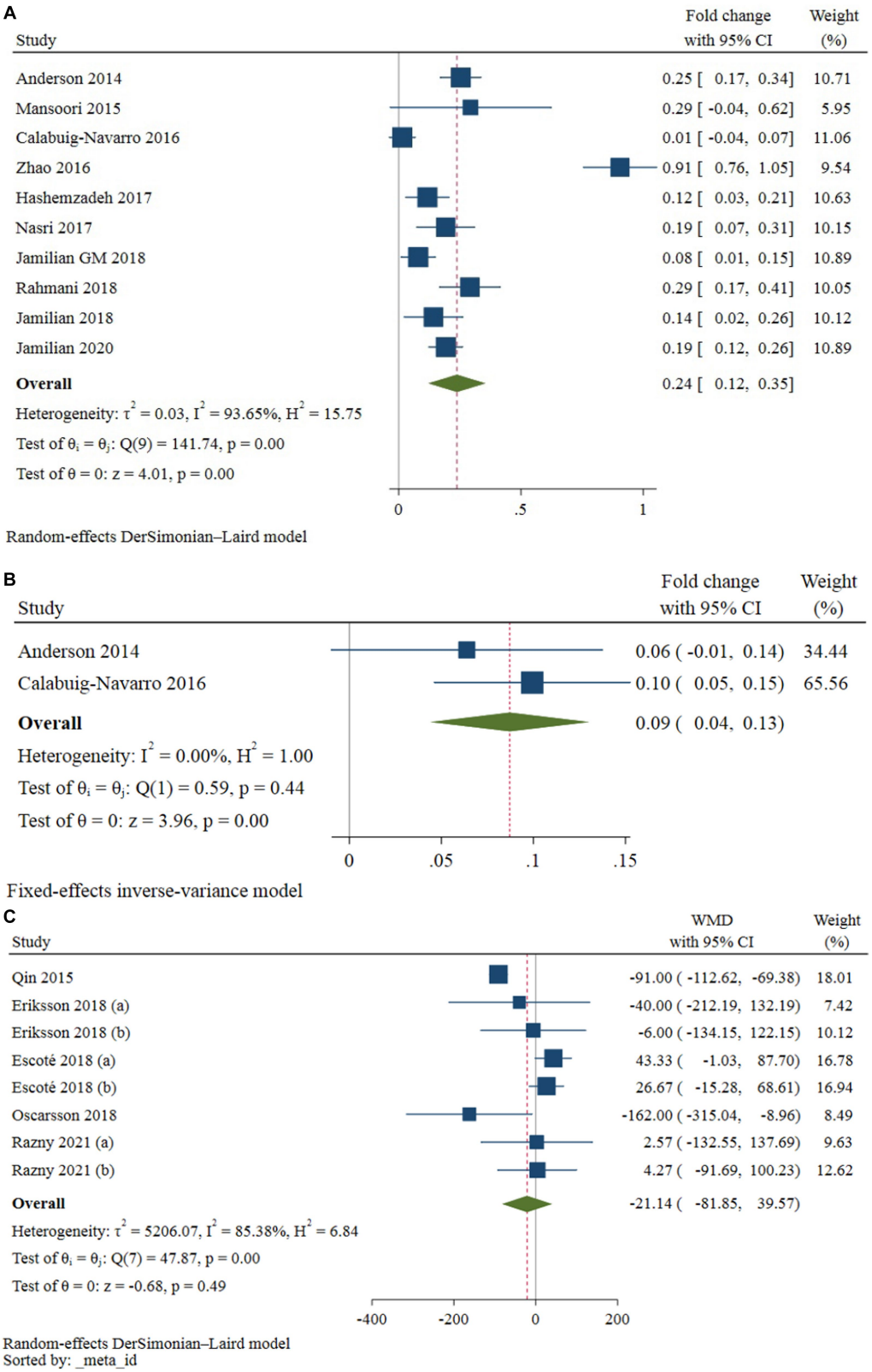

FIGURE 3
Forest plot of studies evaluating the effect of omega-3 fatty acids supplementation on the expression of *PPAR-γ* **(A)**, *PPAR-α* **(B)**, and serum levels of FGF-21 **(C)** in patients with metabolic risk factors.

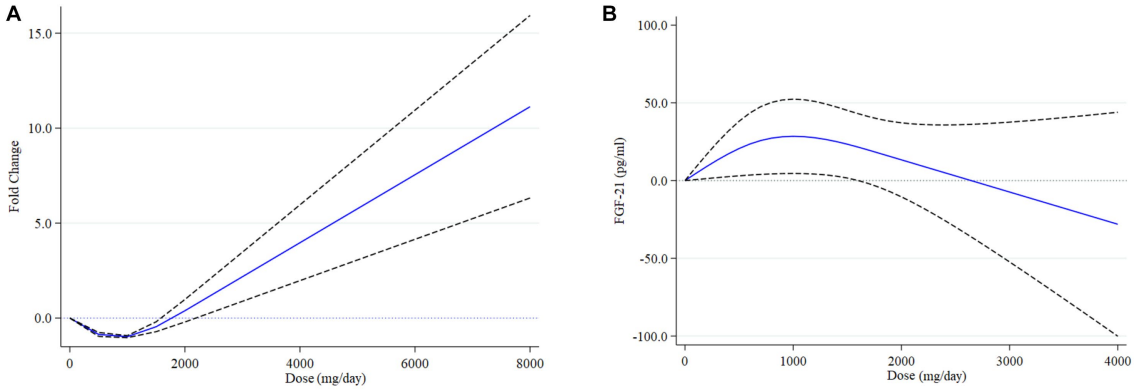

FIGURE 4
Non-linear dose–response relationship between omega-3 fatty acids supplement (mg/d) and difference of fold change of *PPAR-γ* expression **(A)** and mean difference of serum FGF-21 levels **(B)** in patients with metabolic risk factors.

TABLE 3 **Meta-regression for the effect of baseline characteristics on the association between omega-3 fatty acids supplementation and expression of *PPAR-γ* and serum FGF-21 levels in patients with metabolic risk factors.**

Variable	*N*	Coefficient	SE	*p*-value	I^2 (%)	P heterogeneity
PPAR-γ						
Age	10	0.005	0.004	0.228	93.53	<0.001
Study duration	10	0.012	0.009	0.201	94.21	<0.001
Male percent	10	0.004	0.002	0.033	92.56	<0.001
BMI	10	0.025	0.028	0.370	94.21	<0.001
Dose	10	0.357	0.114	0.002	90.65	<0.001
DHA/EPA ratio	5	0.053	0.073	0.471	90.31	<0.001
FGF-21						
Age	8	−3.12	2.74	0.256	80.38	<0.001
Study duration	8	−50.52	14.92	0.001	26.65	0.225
Male percent	8	−1.70	0.26	<0.001	0.00	0.526
BMI	8	18.80	2.90	<0.001	0.00	0.456
Dose	8	−0.05	0.006	<0.001	0.00	0.788
DHA/EPA ratio	8	3.98	16.18	0.806	87.31	<0.001

BMI, body mass index; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FGF, fibroblast growth factor-21; PPAR, peroxisome proliferator activated receptors; SE, standard error.

EPA (32). The variations in findings among studies may be attributed to differences in baseline characteristics of the study subjects, the varying dosages of fish oil supplements, and the study duration. The mechanisms and regulation of *PPAR-γ* signaling by fish oil remain largely unknown. It appears that omega-3 fatty acids are the natural ligands of *PPAR-γ*, and they are able to activate the production of *PPAR-γ* (11).

FGF-21 levels are typically reduced in patients with T2DM treated with anti-diabetes medications (33). However, animal studies have indicated that elevated FGF-21 levels or treatment with FGF-21 leads to improved glucose and lipid metabolism, weight loss, and NAFLD (34, 35). On the other hand, *in vivo* omega-3 PUFAs supplementation (mixture of EPA and DHA) induce the expression and release of FGF-21 (36). In mice, dietary omega-3 fatty acids prevent the increase in plasma FGF-21 levels induced by a high-fat diet (37). However, it was observed that EPA may prevent FGF-21 from declining during weight loss (7). In contrast, fish oil was found to reduce FGF-21 levels in patients with NAFLD, suggesting that fish oil may influence the amelioration of FGF-21 resistance (12). There is some evidence indicating that the elevated FGF-21 levels may not be the primary mechanism through which omega-3 PUFAs alleviate metabolic disorders. Omega-3 PUFAs and EPA alone have been reported to induce thermogenic activation, which in turn increases FGF-21 levels according to some investigations (7). Therefore, the impacts of omega-3 PUFAs on FGF-21 remain unclear and may depend on the specific tissue or metabolic status. Further studies are warranted to substantiate the beneficial impact of fish oil on the FGF-21 resistance in patients with impaired glucose metabolism, and to evaluate the underlying mechanisms for FGF-21 as a therapeutic target.

Strengths of this study include being the first systematic review and meta-analysis on the impact of omega-3 fatty acids supplementation on *PPAR-γ*, *α*, and serum FGF-21 levels in patients with metabolic conditions. The absence of publication bias and

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TABLE 4 GRADE profile of omega-3 fatty acids supplementation on expression of *PPAR-γ,α* and serum levels of FGF-21 in patients with metabolic risk factors.

No of studies	Certainty assessment						No of patients		Certainty	Importance
	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations	Omega-3	control		
PPAR-γ										
10	Randomized trials	Not serious	Very serious[1]	Serious[2]	Not serious	Strong association dose response gradient	224	218	⨁⨁⨁◯ Moderate	Critical
PPAR-α										
2	Randomized trials	Serious[3]	Not serious	Serious[4]	Serious[5]	None	29	28	⨁◯◯◯ Very low	Important
FGF-21										
8	Randomized trials	Not serious	Very serious[1]	Not serious	Serious[6]	Dose response gradient	160	157	⨁⨁◯◯ Low	Important

CI, confidence interval; FGF, fibroblast growth factor-21; MD, mean difference; PPAR, peroxisome proliferator activated receptors.
1. Downgraded because I-squared was > 75%; 2. More than 50% of the population are female; 3. Downgraded because 50–70% of the studies had a high risk of bias; 4. Studies are from different population groups; 5. There is a small number of studies; 6. There is wide 95% confidence interval.

inclusion of only clinical trials are also strengths. However, potential limitations of the study include the inherent variations among the original trials, such as different health conditions (such as NAFLD, T2DM, PCOS, GDM, overweight/obese with or without dyslipidemia, abdominal obesity), varying BMI ranges, divergent doses of omega-3 fatty acids, differing intervention durations, other concurrent interventions, and the relatively limited number of studies included in subgroup analyzes. Moreover, the omega-3 form animal and plant-based source were pooled together despite the structural difference. Therefore, it is imperative to interpret the results with caution and acknowledge that mentioned factors could have contributed to the observed heterogeneity. This heterogeneity could have affected the validity and generalizability of the findings. Although we attempted to evaluate the impact of these factors on our overall findings in subgroup analyzes, we acknowledge it is not possible to account for this heterogeneity directly in our analyzes. Overall, the presence of significant heterogeneity among studies is an important limitation that should be acknowledged and considered when interpreting the results. Additionally, most of the studies in dose–response meta-analysis had only two arms. Future research should consider using more biologically relevant exposure levels, such as absorbed DHA/EPA levels, and examining how intervention type may affect the result. Also, it should be aimed to minimize heterogeneity by employing consistent methodologies, standardizing the dose and source of omega-3 fatty acids, controlling for confounding variables, and ensuring a more homogeneous participant selection process.

Conclusion

Overall, omega-3 fatty acids supplementation in patients with various presentation of metabolic conditions significantly improved gene expression of *PPAR-γ, α*, but it did not affect serum FGF-21 levels. However, there was a dose–response relationship between the dose of omega-3 fatty acids and serum FGF-21 levels, with the highest level observed at a dose of 1,000 mg/day. Furthermore, a significant reduction was observed in patients with dyslipidemia and overweight following omega-3 fatty acids supplementation. This meta-analysis provides valuable insight into the therapeutic implications of omega-3 fatty acids in disorders related to metabolic conditions, but further research is needed to determine its effectiveness and safety on every specific disease, separately.

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.

Author contributions

AA, MS, and MA: study conception and design. AA, BA, and SH: data collection. MA, FS, and BA: analysis and interpretation of results.

AA, FS, SH, and MS: draft manuscript preparation. All authors reviewed the results and approved the final 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

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1202688/full#supplementary-material

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The metabolic effect of *Momordica charantia* cannot be determined based on the available clinical evidence: a systematic review and meta-analysis of randomized clinical trials

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Several studies have shown that *Momordica charantia* L. (Cucurbitaceae, bitter melon) has beneficial effects on metabolic syndrome (MetS) parameters and exerts antidiabetic, anti-hyperlipidemic, and anti-obesity activities. Since the findings of these studies are contradictory, the goal of this systematic review and meta-analysis was to assess the efficacy of bitter melon in the treatment of metabolic syndrome, with special emphasis on the anti-diabetic effect. Embase, Cochrane, PubMed, and Web of Science databases were searched for randomized controlled human trials (RCTs). The meta-analysis was reported according to the PRISMA statement. The primary outcomes of the review are body weight, BMI, fasting blood glucose, glycated hemoglobin A1c, systolic blood pressure, diastolic blood pressure, serum triglyceride, HDL, LDL, and total cholesterol levels. Nine studies were included in the meta-analysis with 414 patients in total and 4–16 weeks of follow-up. In case of the meta-analysis of change scores, no significant effect could be observed for bitter melon treatment over placebo on fasting blood glucose level (MD = –0.03; 95% CI: –0.38 to 0.31; I^2 = 34%), HbA1c level (MD = –0.12; 95% CI: –0.35 to 0.11; I^2 = 56%), HDL (MD = –0.04; 95% CI: –0.17 to 0.09; I^2 = 66%), LDL (MD = –0.10; 95% CI: –0.28 to 0.08; I^2 = 37%), total cholesterol (MD = –0.04; 95% CI: –0.17 to 0.09; I^2 = 66%,), body weight (MD = –1.00; 95% CI: –2.59–0.59; I^2 = 97%), BMI (MD = –0.42; 95% CI: –0.99–0.14; I^2 = 95%), systolic blood pressure (MD = 1.01; 95% CI: –1.07–3.09; I^2 = 0%) and diastolic blood pressure levels (MD = 0.24; 95% CI: –1.04–1.53; I^2 = 0%). *Momordica* treatment was not associated with a notable change in ALT, AST, and creatinine levels compared to the placebo, which supports the safety of this plant. However, the power was overall low and the meta-analyzed studies were also too short to reliably detect long-term metabolic effects. This highlights the need for additional research into this plant in carefully planned clinical trials of longer duration.

KEYWORDS

Momordica charantia, bitter melon, metabolic syndrome, insulin resistance, obesity, cardiovascular disease, dyslipidemia

1 Introduction

Metabolic syndrome (MetS) has been defined as a complex group of risk factors for cardiovascular disease and diabetes. These factors include elevated blood pressure (systolic blood pressure ≥ 130 mm Hg, diastolic blood pressure ≥ 85 mmHg), elevated triglyceride levels (TG; ≥150 mg/dL), low high-density lipoprotein cholesterol levels (HDL-C; male <40 mg/dL, female <50 mg/dL), hyperglycemia (fasting blood glucose ≥100 mg/dL), and obesity (waist circumference: male ≥94 cm, female ≥80 cm) (1, 2). It has been reported that approximately 30% of the world population is affected by MetS, making it a major global health challenge, and an important cause of mortality and morbidity (3, 4). The treatment of MetS is based on an improvement of lifestyle, promoting physical activity, and a balanced low-energy diet (2). Some medicinal plants may be useful tools in the treatment of several MetS components before beginning pharmacological therapy or to supplement medical treatment (2, 5).

Diabetes is one of the most common metabolic diseases and its prevalence is increasing steadily worldwide. According to International Diabetes Federation (IDF) data published in 2021, over 530 million adults are living with diabetes worldwide, and by 2045 their number will exceed 783 million (6). Direct complications from diabetes can lead to heart attack, stroke, blindness, kidney failure, and lower limb amputation (7). Many plants used in folk medicine were involved in clinical trials for assessing their potential as antidiabetic agents and for their positive effect on the treatment or prevention of MetS (2, 8–11). Among these are *Aloe vera* (L.) Burm.f. (10, 11), *Capparis spinosa* L. (12), *Cinnamomum cassia* and *C. zeylanicum* (13, 14), *Trigonella foenum-graecum* (15), *Coffea arabica, Theobroma cacao* (16), *Allium sativum* (2), *Gymnema sylvestre* (17, 18), *Curcuma longa* (19, 20), *Thea sinensis* (21), *Ilex paraguariensis* (22) and *Momordica charantia* (23).

Momordica charantia L. (Cucurbitaceae, bitter gourd or bitter melon) is a tropical and subtropical vine, the edible fruits, shoots, and leaves of which are widely used in the East Asian, South Asian, and Southeast Asian cuisines. Various parts of the plant, especially the fruits, are used in folk medicine in Asia and Africa. The medicinal use of the plant is preponderant in the treatment of diabetes. Various preclinical and clinical studies conducted so far showed the protective effects of *M. charantia* against metabolic syndrome and its associated disorders. The bitter gourd extracts were evaluated for numerous pharmacological activities, and most of them were performed on animals. Fruits and seed extracts reduced fasting glucose and glycosylated hemoglobin A1c in comparison to vehicle control when tested in animal models of type 2 diabetes (24). The water extract of leaves proved to have an anti-obesity effect on a high-fat diet (HFD)-induced obese mouse model through regulating lipid metabolism (25). Hypoglycemic and hypolipidemic effects of different fruit parts were tested on normal, hyperglycemic, and hyperlipidemic rats (26, 27).

The mechanism of action of *M. charantia* has not been fully elucidated. Charantin, a steroidal saponin mixture isolated from the plant is its main active constituent. Charantin has been shown to have insulin-like activity by augmenting insulin release, reducing gluconeogenesis, increasing hepatic glycogen synthesis, and increasing peripheral glucose oxidation (28). Antidiabetic activity could be confirmed for charantin, but not for steroidal saponin aglycones (29). The charantin-rich fraction of *M.charantia* reduced blood sugar levels in type 1 and type 2 diabetic animal models (30, 31). The treatment with the *M. charantia* extracts decreased plasma insulin and increased insulin sensitivity by increasing the expression of GLUT4 in the skeletal muscle and of IRS-1 in the liver of mice with type 2 diabetes. However, no effect on insulin sensitivity was detected in mice with type 2 diabetes (28).

Certain clinical trials suggested that bitter melon products may be promising phytomedicines to manage hyperglycemia (32, 33), ameliorating systemic complications of type 2 diabetes (33), including associated cardiovascular risk factors (34) and it can be considered in obesity management too (35, 36). In the last five years, numerous review articles have been published about bitter melon chemical compounds (37, 38), nutritional value (39, 40), and pharmacological actions (36, 41–43), but only a few are with meta-analysis. Jandari et al. performed a systematic review and meta-analysis of randomized clinical trials regarding the effect of bitter melon on blood pressure (44), Cortez-Navarrete et al. reviewed the metabolic effects of bitter melon reported in clinical trials (45), but without performing a meta-analysis, while Peter et al. evaluated the efficacy in lowering the elevated plasma glucose level in diabetes mellitus (46). The present systematic review and meta-analysis aimed to evaluate the effect of bitter melon on metabolic syndrome parameters.

2 Methods

2.1 Population, intervention, comparison, outcomes, and study design

The following PICO (patients, intervention, comparison, outcome) format was applied: P: patients in prediabetes or diagnosed with type 2 diabetes; I: *Momordica*; C: placebo; and O: change in metabolic parameters. We used PRISMA statement (47) to report the meta-analysis results.

2.2 Systematic review protocol

This systematic review and meta-analysis were registered in the International Prospective Register of Systematic Reviews (PROSPERO) *a priori* (ID: CRD42021293139).

2.3 Search strategy and data sources

We systematically searched PubMed/MEDLINE, Embase, Cochrane, and Web of Science databases for articles reporting randomized,

parallel-group, placebo-controlled clinical trials up to October 31, 2023, without limiting the language or publication year. The following main keywords and related terms were used: "momordica" AND "diabetes." For transparency purposes this meta-analysis relied on publicly available data. There was no need to contact the authors of the articles nor the manufacturers of the products under consideration for any additional information. We removed duplicate and records lacking an abstract and the final selection was based on article titles and abstracts. Two reviewers (EBK, BCL) independently reviewed the full texts of the remaining records. Any disagreement among reviewers was discussed and resolved, a third reviewer was available for consultation at any time (DC). We used Mendeley (version 1.19.8; Mendeley Ltd.) to manage references.

2.4 Study selection

Only randomized, placebo-controlled studies with adult prediabetes or with a type 2 diabetes mellitus diagnosis were included in the meta-analysis. A minimal follow-up duration of 4 weeks was determined as an inclusion criterion since this is the minimum period required for meaningful effects on glucose control as assessed by HbA1c concentration (48). Case series, case reports, non-randomized and open-label studies, and trials performed with patients with concomitant diseases affecting blood glucose levels or in which the intervention contained other active ingredients than *M. charantia* were not considered for analysis.

2.5 Data extraction

Data collection was executed following the PRISMA guidelines. The two independent reviewers (EBK, DC) extracted study characteristics and results. Any discrepancies in the extracted data were discussed and resolved. The following data items were selected from the included papers: study design, sample size and characteristics of the patient population, duration, intervention details, body weight, BMI, waist circumference, body fat, systolic and diastolic blood pressure, HbA1c, fasting glucose, total cholesterol, triglyceride, HDL, LDL, VLDL, creatinine, ALT, and AST levels. A statistical analysis of at least three clinical trials involving different patient populations was required for each outcome.

2.6 Statistical analysis

Papers included in this meta-analysis reported data in three ways:

A a pre-intervention value and a change score,
B a pre- and post-intervention value,
C a pre- and post-intervention value along with a change score.

Two distinct types of analysis were undertaken:

- Analysis of post-intervention values only (without using baseline or change information); this is unbiased in the case of randomized controlled trials, but is inefficient, however, the loss of efficiency is marginal if the correlation between the pre- and post-intervention values is less than 0.5 (49).
- Analysis of change scores; this is more efficient, especially if the correlation between the pre- and post-intervention values is higher than 0.5.

The first analysis requires the imputation of the post-intervention value in scenario A, the second requires the imputation of the change score in scenario B (no value must be imputed in all other combinations). Both imputation task requires the knowledge of correlation between the pre- and post-intervention values, this was obtained from studies of type C, i.e., studies with all data given were used to calculate the correlation which was then used to impute studies with partial information. The correlation coefficient was calculated as the sum of the variances of pre- and post-intervention values minus the variance of the change divided by two times the product of the standard deviation of the pre- and post-intervention values (50, 51). This was calculated separately for all studies, arms (i.e., placebo or active), and outcomes, and were then averaged across studies, i.e., an average was calculated for each outcome and arm after discarding impossible values (i.e., a correlation larger than 1 in absolute value). This average was used for imputation only if the range of the correlations for the given outcome and arm was less than 0.4, otherwise that outcome and arm's correlation was not imputed. After the value used for imputation was obtained, the post-intervention values were imputed using scenario A, and the change scores were imputed for scenario B.

Both types of meta-analysis (post-intervention and change score) were then run using the imputed datasets. The outcome measure was the mean difference. In case when median and lower/upper quartile was given, mean and standard deviation was estimated with the quantile estimation method of McGrath et al. (52). Common-effect meta-analysis, and random-effects meta-analysis (with restricted maximum likelihood estimation) was carried out (53).

Results are presented using standard forest plots, depicting both common- and random-effects results, together with the usual τ^2 and I^2 heterogeneity statistics and a test for the overall effect (both for the random-effects results).

Calculations were conducted under R statistical environment version 4.2.1 (54) using package metafor version 3.8–1 (55). Full source code is available at https://github.com/tamas-ferenci/MomordicaMetaAnalysis.

2.7 Risk of bias analysis

The Cochrane Collaboration tool was used to assess the risk of bias, which includes seven specific domains: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other bias scores. Studies were classified as having a high (red), unclear (yellow), or low (green) risk of bias in each domain. Disagreements about the quality of the studies were settled through discussion (TF, DC). The risk of bias summary table and figure were generated by the RevMan 5 software (56).

2.8 Ethics statement

Ethical approval was not needed because we only collect non-confidential information from which the patients' identities cannot be determined.

3 Results

3.1 Search results

The search resulted in 694 hits, after removing the duplicates 519 records remained and were potentially eligible for inclusion. In the full-text section stage, most articles were excluded due to the lack of placebo control (57, 58), lack of blinding (59), lack of numerically reported results (32, 60, 61), and since other plant parts (leaves) than *M. charantia* fruits were used as study (59, 62–65). We identified nine trials eligible for our review and meta-analysis (33, 66–73). The selection process is presented in Figure 1 (47).

3.2 Study characteristics

We analyzed nine randomized placebo-controlled trials (33, 66–73). Table 1 contains the summary of the main study and patient characteristics. We included only randomized double or single-blinded studies released between 2003 and 2023. Studies length varied between 4 and 16 weeks, the sample size was low (24–90 participants), the study drugs were inconsistent in quality (dry plant material and dry extracts) and in quantity (the daily dose was 2–6 g fruit or extract of fruit equivalent to 9 g fruit). The total number of participants was 414 and the locations of the studies were different Asian or North American countries. The minimum age of participants was 18 years (67) or 20 years (33), and the oldest patient was 80 years old (71), according to the available information.

3.3 Risk of bias assessment

Overall, the methodical quality of the trials included in our final quantitative analysis was reckoned to be good, only with low or unclear risk of bias for double-blind randomized trials (Supplementary Figures S1, S2).

Random sequence generation was described in six studies (33, 67–70, 72); however, the measures taken to ensure allocation concealment were given in only one trial (67). Performance and detection biases were unclear in five studies (33, 68–71) because the authors of these studies failed to report whether the intervention and the comparator were identical in size, shape, color, and odor; and it remained unclear whether the outcomes were assessed in a blinded manner or not. The study of Cortez-Navarrante (72) was judged to have low risk of performance and detection biases, because based on their article nor the patients neither the investigators were aware of the assigned treatment. Dans et al. stated that the treatment and the placebo capsules were identical, and the patients, the investigators, and the statistician were unaware of the treatments received until the end of the statistical analysis. Therefore, this study had low risk of performance and detection biases (67). However, in the study of John et al. (66) the investigators were not blinded and the tablets were dissimilar; therefore, this study had high risk of performance and detection biases.

All the included studies showed a minimal risk of attrition and reporting biases. The study of Dans et al. was funded by a company, but the authors stated that the sponsor had had no role in the data collection or the analysis of the study; therefore, the risk of other bias in this study remained low (67). However, in the study of John et al., the investigators were not blinded, and the tablets were dissimilar; therefore, this study had a substantial risk of performance bias (66).

3.4 Main findings

3.4.1 Effect of *Momordica charantia* fruits on glycemic indices

One primary outcome was fasting blood glucose level and relevant data were available from eight trials (Figure 2). Using the random effects model, the superiority of *M. charantia* over placebo on fasting blood glucose level could not be observed (MD = −0.03; 95% CI: −0.38 to 0.31; I^2 = 34%) with the analysis of change scores (Figure 2A). This finding was consistent when analyzing the data with the common-effect model. However, the assessment of the post-intervention suggests that *M. charantia* is more effective than placebo in decreasing fasting blood glucose levels (MD = −0.40; 95% CI: −0.76 to −0.03; I^2 = 0%) (Figure 2B). The primary reason of this contradiction is that the first analysis was based on 4 trials only (143 participants), whereas post-intervention values were available in 7 trials (296 participants) and missing values could not be imputed, because the calculated correlations were unacceptably dissimilar.

The analysis of HbA1c level resulted in somewhat similar results (Figure 3). The analysis of change scores was based on three studies (with data from 121 patients), whereas the analysis of post-intervention values on five trials (with data from 233 patients), and again, values could not be imputed. The meta-analysis of the post-intervention values using the random effects model indicated borderline efficacy for the *M. charantia* treatment with an MD = −0.24 (95% CI, −0.49 to 0.00, I^2 = 0%) (Figure 3B), however, the analysis of the changes scores definitely did not show superiority over placebo (MD = −0.12; 95% CI: −0.35 to 0.11; I^2 = 56%) (Figure 3A).

One of the recent trials (73) includes clinical indicators to assess insulin sensitivity and glucose metabolism, like HOMA-IR, Matsuda index, insulinogenic index. Because these parameters were not found in the other studies, they could not be evaluated in the meta-analysis.

3.4.2 Effect of *Momordica charantia* fruits on lipid profile

Regarding serum lipid levels, only the effects on HDL, LDL, and total cholesterol levels could be analyzed. Based on the changes in the mean differences data, as reported in three or four RCTs (Figure 4), *M. charantia* was not superior in any of these outcomes compared to placebo using the random effects model (MD = −0.04; 95% CI: −0.17 to 0.09; I^2 = 66%; MD = −0.10; 95% CI: −0.28 to 0.08; I^2 = 37%; and MD = −0.03; 95% CI: −0.15 to 0.22; I^2 = 0%, respectively). These results are consistent with those obtained using common effect model.

The assessment of the post-intervention values complemented these results with triglyceride data. There was no evidence of significant effects of *M. charantia* on HDL (MD = −0.02; 95% CI: −0.12 to 0.09; I^2 = 0%), LDL (MD = −0.01; 95% CI: −0.22 to 0.19; I^2 = 0%), total cholesterol (MD = −0.04; 95% CI: −0.27 to 0.19; I^2 = 0%) and triglyceride levels (MD = −0.09; 95% CI: −0.39 to 0.20; I^2 = 22%) (Supplementary Figure S3). The reliability of these results is reassured by low heterogeneity values and the consistency of common and random effects models.

The assessment of the VLDL data is not conclusive, as data from only two studies were available (68, 72).

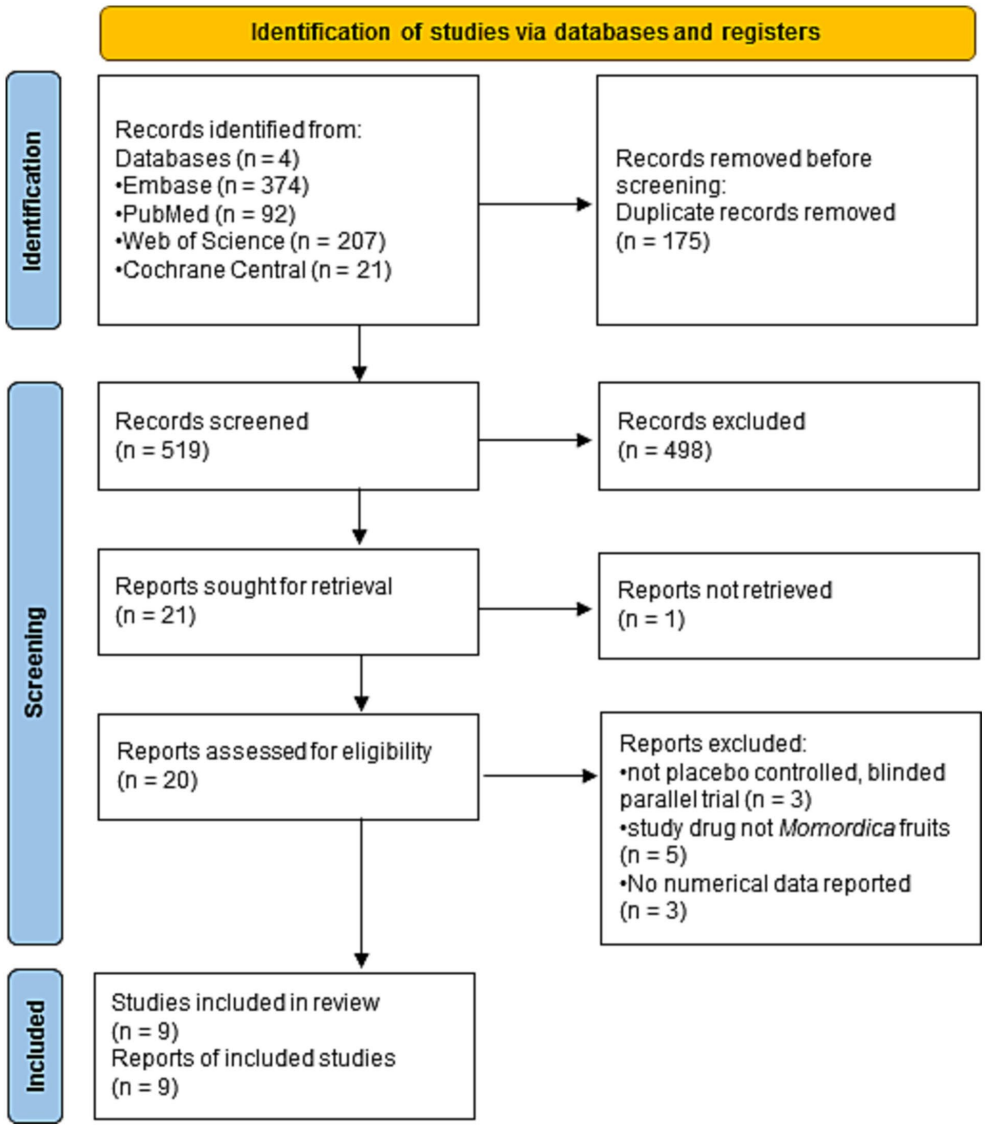

FIGURE 1
Flow diagram of study identification and selection by PRISMA 2020.

3.4.3 Effect of *Momordica charantia* fruits on anthropometric parameters

No significant effect on body weight (MD = −1.00; 95% CI: −2.59 to 0.59; I^2 = 97%), body fat (MD = −1.21; 95% CI: −2.62 to 0.20; I^2 = 0%) and BMI (MD = −0.42; 95% CI: −0.99 to 0.14; I^2 = 95%) was observed in the meta-analyses of change scores. The analysis of the post-intervention values yielded the same results (Supplementary Figure S4). The effect on waist circumference could only be assessed from post-intervention data (MD = 0.72; 95% CI: −3.36 to 4.80; I^2 = 13%) (Supplementary Figure S4). Common and random effects models had consistent results.

3.4.4 Effect of *Momordica charantia* fruits on blood pressure

Administration of *M. charantia* fruits did not demonstrate any effect on systolic blood pressure (MD = 1.01; 95% CI: −1.07 to 3.09; I^2 = 0%) or diastolic blood pressures (MD = 0.24; 95% CI: −1.04 to 1.53; I^2 = 0%) when comparing the change scores to that of the placebo group. This is supported by the examination of the post-intervention data (Supplementary Figure S5). Heterogeneity was very low and common and random effects model had similar results.

3.5 Adverse effects

Overall, there were no serious adverse effects reported by the studies included in our meta-analysis. Headache and gastrointestinal complaints were the most reported adverse events. In a double-blind RCT that used a special extract from fruits and seeds of *M. charantia* in addition to standard antidiabetic medication, adverse effects such as diarrhea and epigastric pain were reported after 1-month of administration (67).

The consumption of 6 g bitter melon pulp per day resulted in a significantly higher frequency of diarrhea and flatulence than in the placebo group (33).

TABLE 1 Baseline characteristics of studies included in the meta-analysis (RCT: randomized, controlled trial; DB: double blind; SB: single-blind).

Article	Country	Study design	Participants	Duration	Study drug	Daily dose
Cortez-Navarrete, 2018	Mexico	RCT, DB	24	12 weeks	dried powder of the fruit pulp	2 g
Cortez-Navarrete, 2022	Mexico	RCT, DB	24	12 weeks	Commercial herbal supplement *	4 capsules (2000 mg)
Dans, 2007	Republic of the Philippines	RCT, DB	40	13 weeks	special extract from fruits and seeds	2 capsules
John, 2003	India	RCT, SB	50	4 weeks	dried fruit	6 g
Kim, 2020	Republic of Korea	RCT, DB	90	12 weeks	dry fruit extract	2.38 g
Kim, 2023	Republic of Korea	RCT, DB	65	12 weeks	dry fruit extract	2.4 g
Kinoshita, 2018	Japan	RCT, DB	43	30 days	dry fruit extract	300 mg extract (equivalent to 9 g fruit)
Trakoon-osot, 2013	Thailand	RCT, DB	38	16 weeks	dried pulp	6 g
Yang, 2022	Taiwan	RCT, DB	40	3 months	mcIRBP-19** containing extract	600 mg

*500 mg capsules, each contains 200 mg Momordica extract (standardized to 2.5% bitter principle) and 300 mg dried fruit powder.
**Momordica charantia insulin receptor binding peptid-19.

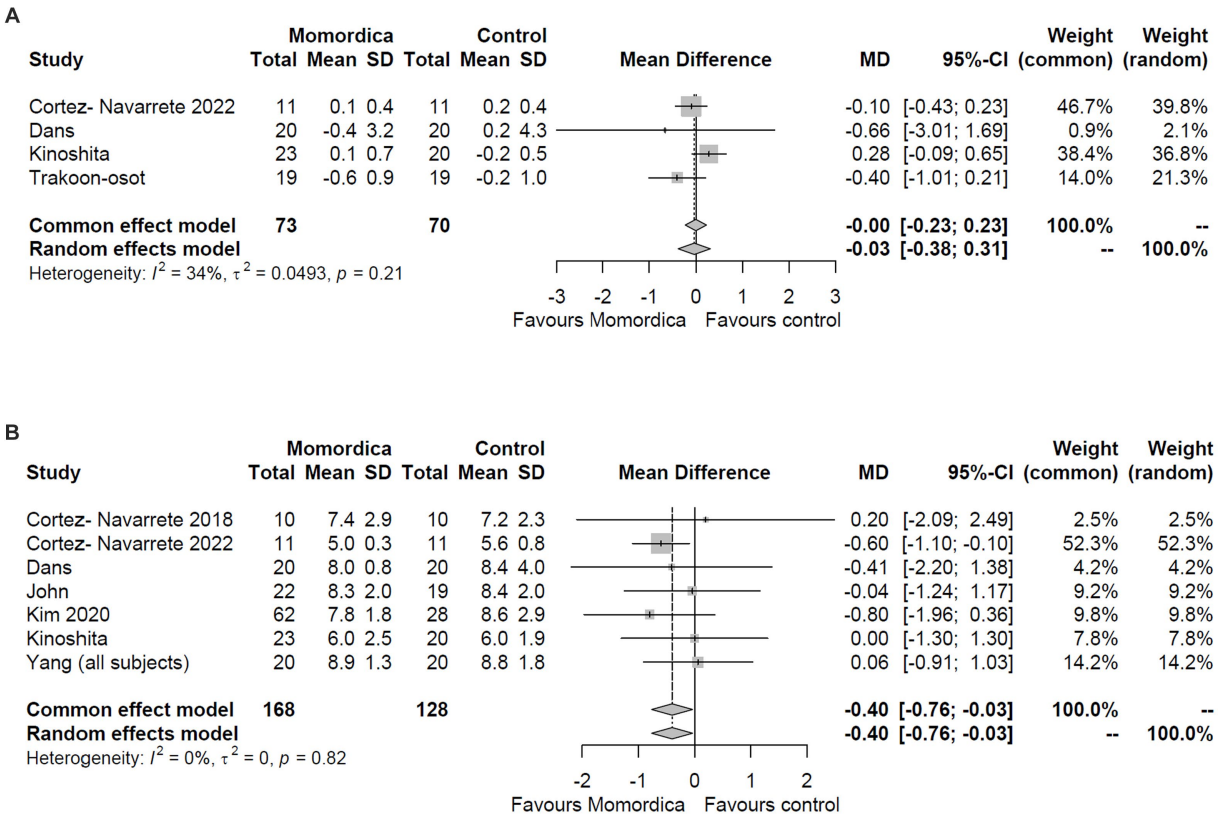

FIGURE 2
The effect of *Momordica charantia* on blood glucose level compared to placebo in the meta-analyses of the change scores **(A)** and post-intervention values **(B)** using the random effects and common effect models.

Based on data from four trials (33, 67, 68, 72), *M. charantia* administration exerted no significant effects on liver enzymes (ALT, AST) and creatinine levels (Supplementary Figure S6) compared to placebo, however confidence intervals were sometimes wide to draw reliable safety conclusions (i.e., power was low).

4 Discussion

4.1 Main findings

A vast number of studies have been conducted, in both animal and human subjects using the *M. charantia* plant or different extracts

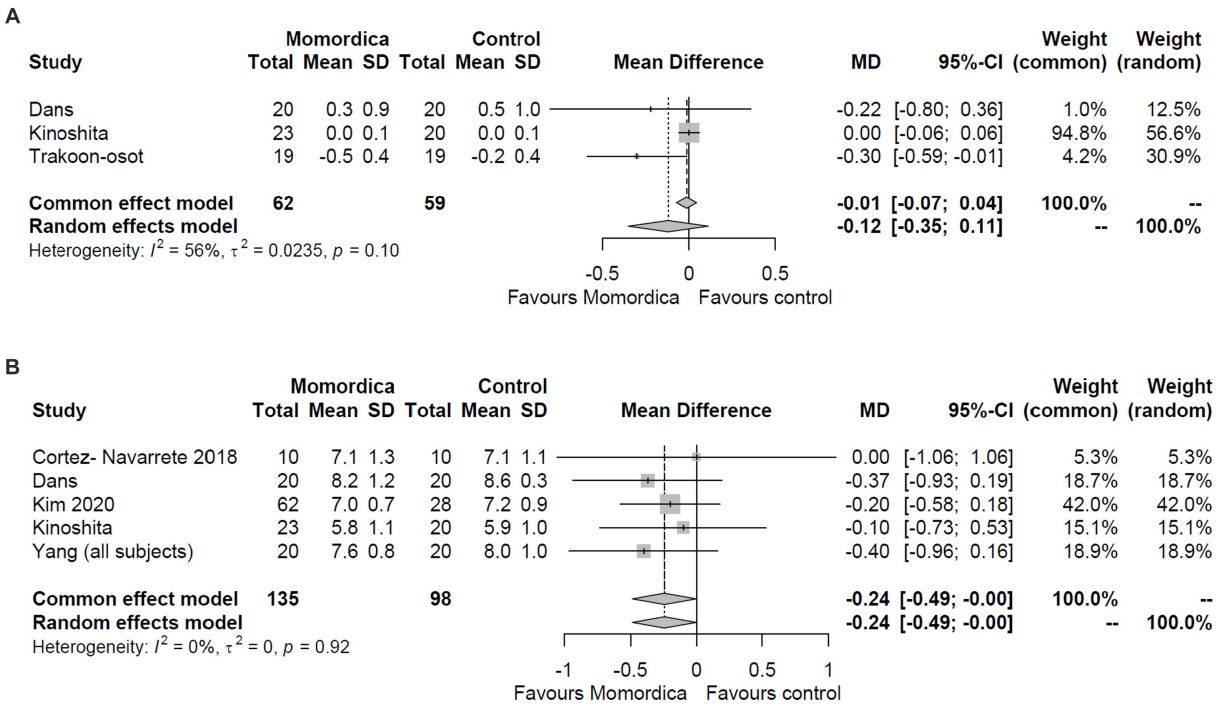

FIGURE 3
The effect of *Momordica charantia* on HbA1C level compared to placebo in the meta-analyses of the change scores **(A)** and post-intervention values **(B)** using the random effects and common effect models.

prepared with stems, leaves, and fruits of the plant. These studies have allowed the identification of a few health-promoting benefits, including hypolipidemic, hypoglycemic, and anti-obesity effects (25, 26, 32, 33, 35, 36). In this review and meta-analysis, we have systematically evaluated the existing evidence on the potential efficacy of bitter melon in the treatment of metabolic syndrome, based on randomized, parallel-group, placebo-controlled trials. All the included trials, except the studies of Cortez-Navarrete (68, 72) were carried out in different Asian countries. Based on the nine trials included in this study, our findings show that *M. charantia* mono herbal preparations do not have a significant overall positive influence on blood glucose levels and other cardiovascular risk factors associated with metabolic syndrome. However, *M. charantia* was found to be statistically significantly more effective than a placebo in terms of reducing HbA1C and (marginally) fasting glucose levels when post-intervention data were analyzed. Even this relatively week conclusion ($p = 0.032$ and $p = 0.050$ respectively) was dependent on the analytical approach used, as it vanished when change scores were used. The change score data set had smaller sample size and was much more heterogeneous. Overall, our confidence in this finding is therefore low. The absence of an impact on ALT, AST, and creatinine levels suggests that there were no potential hepato- or nephrotoxic consequences at the doses used, although, the small sample size limits power and does not allow the reliable assessment of safety.

A previous meta-analysis suggested that bitter melon alone or in combination with other herbal medicinal products can reduce the elevated fasting plasma glucose level (FPG), postprandial glucose (PPG), and glycated hemoglobin A1c (HbA1c) (46). Compared to the placebo, *M. charantia* significantly reduced FPG, PPG, and HBA1c with mean differences of −0.72 mmol/L, − 1.43 mmol/L, and − 0.26%, respectively. *M. charantia* also lowered FPG in prediabetes (mean difference − 0.31 mmol/L). As discussed above, our meta-analysis found only a very week evidence for the superiority over placebo when assessing the effect on blood glucose and HbA1c levels. The explanation for this discrepancy is that the dataset used for analysis was different. First, we included five trials that were published after the previous meta-analysis (69–73). Second, the positive outcome of the meta-analysis of Peter et al. (46) could be explained by the inclusion of a paper reporting a trial with a size effect in favor of *M. charantia* (58) that was excluded in the present investigation due to the lack of blinding.

Animal experiments suggest that *M. charantia* might have the potential for increasing insulin sensitivity in patients with type 2 diabetes (28). According to a recent meta-analysis of animal experiments with type 2 diabetic rats, fruit and seed extracts of *M. charantia* reduced fasting plasma glucose and after at least 3 months of treatment increased serum insulin level and reduced HbA1c, triglycerides, total cholesterol in comparison to vehicle control (24). However, it should be noted that although the dose of *M. charantia* applied in different experiments was diverse, the typical range of 150–600 mg dry extract/kg/day is several magnitudes higher than those in the clinical trials. These differences in dosing might be one explanation for the lack of efficacy observed in this meta-analysis. Furthermore, differences in the qualitative and quantitative composition may contribute to the outwardly unreliable efficacy. Although charantin is considered the major active constituent of the plant, *in silico* studies suggest the importance of further metabolites. Momordicoside D (ligand of Takeda-G-protein-receptor-5, TGR5), cucurbitacin (ligand of glucagon-like peptide-1 receptor, GLP-1r), and charantin (ligand of dipeptidyl peptidase 4, DPP-4) were identified as the antidiabetic constituents of bitter melon *in silico*. In subsequent animal experiments, these potential mechanisms of action were

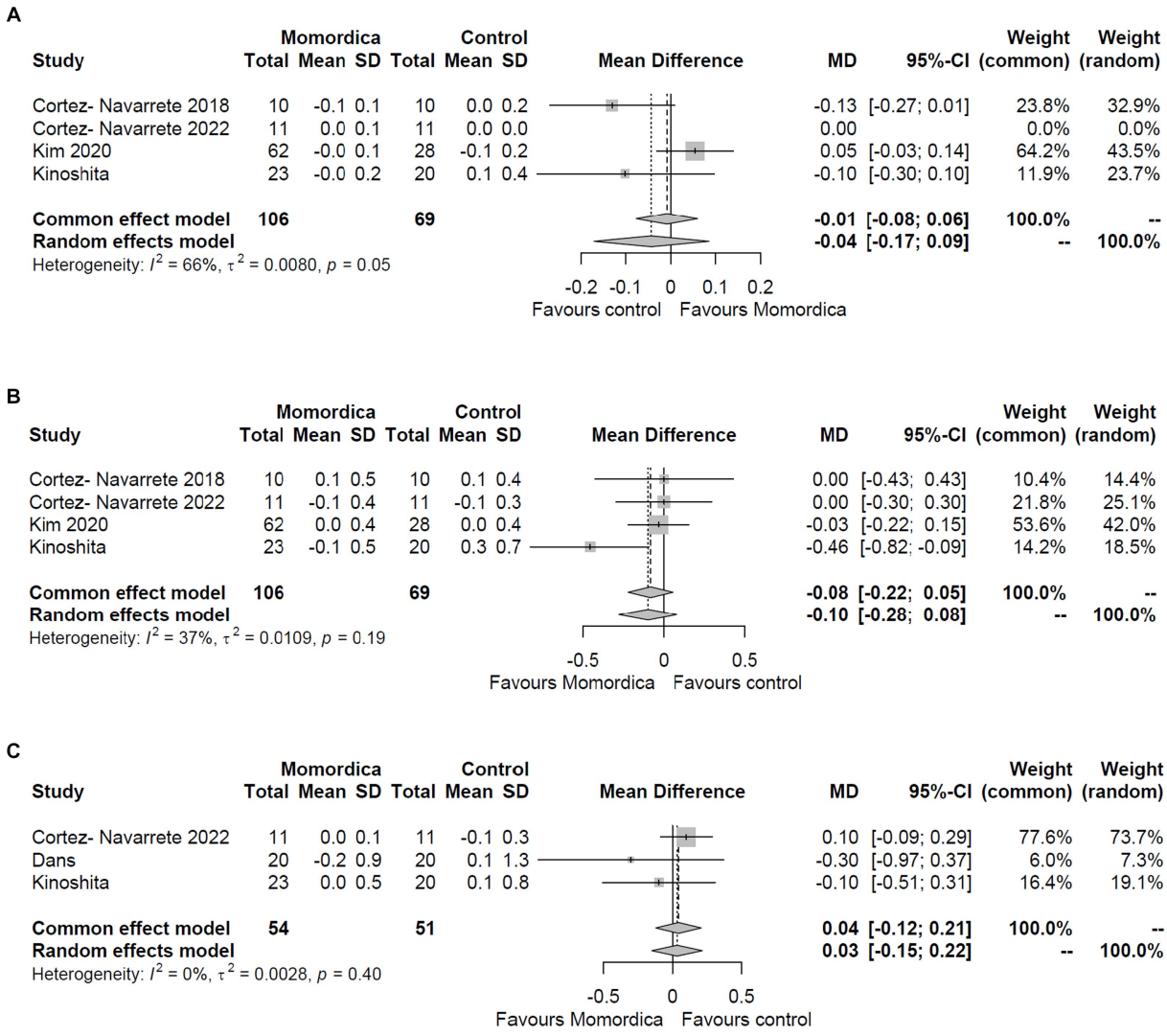

FIGURE 4
The effect of *Momordica charantia* on HDL **(A)**, LDL **(B)**, and total cholesterol levels **(C)** compared to placebo in the meta-analyses of the change scores using the random effects and common effect models.

reassured, since the extract of *M. charantia* significantly increased the expression of GLP-1r and TGR5 and decreased the expression of DPP-4 (74). The complex mechanism of action and the presence of multiple active components in this plant urge the need for the standardization of clinically studied products.

The lack of unambiguous efficacy on blood metabolic parameters might also be due to the short duration of the studies. HbA1c level reflects the cumulative glycemic history of the preceding two to three months (75). In the case of lipid levels, the efficacy of lifestyle changes can be expected within 3–6 months, and even in the case of a statin or combined therapy, the maximum percentage change will occur by 4 to 12 weeks after starting (76). Some of the studies meta-analyzed by us were only 30 days long (66, 69).

Regarding the effect on blood pressure, our findings are in accordance with the meta-analysis performed by Jandari et al. (44), which concluded that bitter melon preparations do not exert a significant antihypertensive effect. The effect of *M. charantia* on systolic and diastolic blood pressure was investigated in five trials (including 163 participants). The pooled effect size showed that neither systolic nor diastolic blood pressure changed following *M. charantia* supplementation. *M. charantia* seemed to be more effective in younger adults or when consumed for short durations, however, none of the subgroup analyses revealed significant efficacy compared to placebo. However, the duration of the included studies was 4–16 weeks, which does not allow the assessment of long-term antihypertensive effects (44). The potential long-term effect on blood pressure might be the result of the beneficial effect on blood lipid levels. For the effects on HDL, LDL triglyceride, total cholesterol levels, and ALT, AST, and creatinine concentrations, our meta-analysis is the first independent assessment of previously published clinical data. Our results do not support the hypothesis that the impact of bitter gourd on blood lipid levels might lead to antihypertensive effect.

The strength of our study is that we included only blinded, placebo-controlled studies that assessed the effect of *M. charantia* for

at least four weeks on prediabetic and diabetic patients. The analyzed studies were performed by different research groups in different countries. By excluding complex preparations, we aimed to assess the effect of this herbal component only.

4.2 Limitations

The most important limitation of our meta-analysis is that the number of included studies and the number of patients is low, leading to even a meta-analysis being underpowered, moreover, the applied doses were not uniform. Although we did not find unambiguous efficacy in any of the analyzed outcomes, bitter gourd was found to be effective in some clinical trials. The duration of the studies (4–16 weeks) was too short to reveal the potential effects of *M. charantia* on metabolic parameters. This highlights the need for additional research into this plant in carefully planned clinical trials.

5 Conclusion

Although bitter melon has been widely used by patients suffering from metabolic syndrome, the meta-analysis of randomized, placebo-controlled trials does clearly not support the rationale of this practice. In agreement with a previous meta-analysis, we did not find an effect on blood pressure. In contrast with the meta-analysis of Peter et al., our assessment did not reveal an unambiguous effect on the blood glucose level. The effect on HDL, LDL triglyceride, and total cholesterol levels was meta-analyzed for the first time by us, and we did not find any significant beneficial effect in any of the parameters. However, lack of efficacy in the short-term studies does not necessarily mean the lack of efficacy in the case of long-term treatment *M. charantia* use. The limited sample size should also be considered when interpreting this finding.

To assess the clinical efficacy of *M. charantia*, there is a call for long-term randomized controlled trials with larger sample sizes. The investigation of the dose-dependence of antidiabetic activity in humans should also be considered.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://github.com/tamas-ferenci/MomordicaMetaAnalysis.

Author contributions

EL-Z and DC: conceptualization. EL-Z, BC-L, and DC: methodology. E-BK and DC: data extraction and abstracts screening. E-BK and BC-L: full texts screening. TF: statistical analysis. EL-Z, MN, and DC: risk of bias analysis. EL-Z and MN: writing—original draft preparation. BC-L, TF, and DC: writing—review and editing. BT: risk of bias analysis and writing—review and editing. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered 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.2023.1200801/full#supplementary-material

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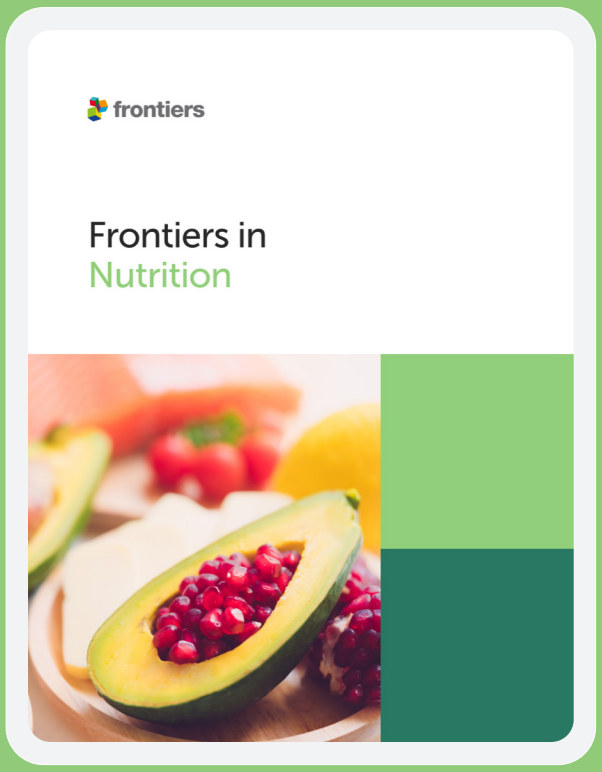

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