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Fruits, vegetables and herbs: Medicinal chemistry, metabolic and health effects

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Fruits, vegetables and herbs: Medicinal chemistry, metabolic and health effects

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Table of contents

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Editorial: Fruits, vegetables and herbs: medicinal chemistry, metabolic and health effects

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Editorial on the Research Topic
Fruits, vegetables and herbs: medicinal chemistry, metabolic and health effects

Non-communicable diseases (NCD), including cardiovascular diseases, cancers, chronic respiratory diseases and diabetes, are recognized as a major global challenge in the United Nations 2030 Agenda for Sustainable Development. Unhealthy lifestyles, namely physical inactivity, smoking, alcohol abuse, and unbalanced eating habits, are contributing to the spread of these chronic diseases (1).

A plant-based diet (PBD) is any dietary pattern that emphasizes the consumption of foods derived from plants and excludes or limits the intake of most or all animal products (2). Traditional Mediterranean and Eastern diets are good examples of healthful and sustainable plant-based dietary patterns. In fact, accumulating data strongly indicate that the high consumption of fruits, vegetables, herbs (medicinal and aromatic plants), legumes, whole grains, nuts, and olive oil is associated with a lower risk of morbidity, disability, and mortality by NCD (1, 2).

Fruits, vegetables and herbs play an important role in the quality of PBD once they are characterized by a nutrient and phytochemical profile that is low in calories and high in fibers, vitamins, flavonoids, phenolic acids, glucosinolates, terpenes, sterols, and methylxanthines, with strong antioxidant, hypoglycemic, anti-inflammatory, anti-hyperlipidemic, anti-hypertensive, neuroprotective, and anticarcinogenic properties (2–6). The evidence suggests that the health benefits of consuming these plant-based foods are due to additive and synergistic interactions between their different phytocomponents.

Considering that the selection of a healthy, well-balanced and sustainable dietary pattern by the general population is closely linked to their literacy in terms of nutrition knowledge, the main goal of our Research Topic "*Fruits, Vegetables and Herbs: Medicinal Chemistry, Metabolic and Health Effects*" is to present an overview of the possible role of fruits, vegetables and herbs (and/or their phytochemicals) in the prevention and management of NCD.

Our Research Topic is composed by nine papers (five original researches, one mini-review and three systematic reviews) covering several research aspects and recent advances related to the different classes of nutrients/phytochemicals commonly found in

fruits, vegetables and herbs, highlighting their chemical structures, occurrence, biological importance and mechanisms of action/interaction.

Globally, cardiovascular diseases are the leading cause of mortality, followed by cancers (1). Hypertension, hyperlipidemia, hyperglycaemia and oxidative stress are key metabolic changes that increase the cardiovascular risk. Herein, Feng et al. evaluated the effect of the oral administration of a hawthorn fruit hydroalcoholic extract in the prevention of the progression of hyperlipidemia in a high-fat diet rat model. In addition, Sookying et al. summarized and discussed the botanical aspects, phytochemical profile, antioxidant activity and toxicity of the leaves of tamarind, an African tropical food and medicinal plant.

Carotenoids are tetraterpene antioxidant pigments that are widely spread in colored fruits and vegetables, exhibiting yellow, orange, or red colors. Obesity is a triggering factor for several human diseases, namely for non-alcoholic fatty liver disease (NAFLD). In our Research Topic, Balbuena et al. evaluated and compared the effects of supplementation with orange carrots (carotenoid-rich) and white carrots (carotenoid-deficient) on NAFLD progression in a high-fat diet induced obese mice model.

Overweight/obesity is also an important risk factor for diabetes associated with insulin resistance. Considering that resveratrol is a polyphenol with antioxidant, hypoglycaemic, anti-inflammatory, and anti-hyperlipidemic properties, and the close relationship between insulin resistance and polycystic ovary syndrome (PCOS), Liang et al. reported the beneficial effect of resveratrol administration on ovarian insulin sensitivity in a PCOS rats model.

B-type procyanidins are polyphenolic compounds commonly found in Rosaceae family fruits, such as apples, pears and quinces (7). In our Research Topic, Osakabe et al. summarized the human intervention trials on the hormetic responses induced by this procyanidins type that may be responsible for the health effects of pome fruits intake. They also discussed the hypothesis of this hormetic pattern arises via neurotransmitter receptors expressed in the central nervous system.

Aging is the primary risk factor for most neurodegenerative diseases and Parkinson's disease (PD) is the second most common (after Alzheimer's disease) (3, 4). PD is characterized by the loss of dopaminergic neurons that causes involuntary/uncontrollable movements, rigidity, disequilibrium and lack of coordination. Li et al. reported the multiple mechanisms of action of Ping-wei-san plus herbal decoction (a traditional Chinese medicinal product composed by a mixture of several botanical species) against PD, by using multiomics analyses. Globally, low back pain is the principal cause of years lived with incapacity. Herein, Huang and Xie reported a Mendelian randomization study in order to evaluate whether dried fruit consumption prevents low back pain.

Cruciferous vegetables (CV), such as Portuguese cabbage, Brussels sprouts, cauliflower and broccoli, are essential components of PBD. Considering the intense debate on the association of consumption of CV with cancer prevention, Yu et al. summarized and discussed the evidence on the possible link between CV intake and bladder cancer risk.

Vitamin E is a fat-soluble antioxidant vitamin, commonly found in nuts, seeds, fruits, vegetables, and edible oils. Herein, Zhang and Yi present a systematic review on the multiple health benefits of this essential nutrient, namely in the prevention and management of several types of cancers, cardiovascular and neurodegenerative diseases.

We are currently facing an unprecedented level of diet-related diseases (1, 2). Yet, some clinicians seem to ignore the potential benefits of a healthy and well-balanced diet, rich in fruits, vegetables and herbs, and quickly prescribe drugs before giving patients a chance to correct their illnesses through shifts in their dietary habits and exercise regime. Therefore, "*Fruits, Vegetables and Herbs: Medicinal Chemistry, Metabolic and Health Effects*" is a timeless and crucial Research Topic, supported by novel and reviewed data analyzed in the papers included in this Research Topic. However, there are still many aspects to be clarified and understood in the amazing world of Fruits, Vegetables and Herbs.

Author contributions

BMS, LR, CVV, AV, and MBPPO participated in the design of the manuscript, analyzed the bibliographic data, collected them, and drafted the manuscript. All authors critically revised the manuscript, 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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Hawthorn fruit acid consumption attenuates hyperlipidemia-associated oxidative damage in rats

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Context: Hyperlipidemia is a highly prevalent risk factor for atherosclerosis and stroke. The currently available medications used to treat Hyperlipidemia cannot improve its oxidative stress damage. Consumption of hawthorn can regulate blood sugar and blood lipids, and its rich fruit acid is a natural antioxidant that can improve oxidative stress damage.

Objective: The present research aimed to investigate the protective effect of hawthorn fruit acid (HFA) on hyperlipidemia and to determine its potential molecular mechanism.

Materials and methods: Sprague-Dawley rats were fed a high-fat diet (HFD) to induce hyperlipidemia and treated orally with hawthorn fruit acids (HFA). Serum and liver levels of total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), superoxide dismutase (SOD), hydrogen peroxide (CAT), and malondialdehyde (MDA) were measured. Human hepatocellular carcinoma cell lines (HepG2) cells were treated with 0.1 mM oleic acid and HFA (0.125, 0.25 mg/mL), and intracellular TC, TG, HDL-C, SOD, CAT and MDA were measured. Changes in LDLR, HMGCR, Nrf2, HO-1, NQO1 protein and gene expression were analyzed by Western blot and qPCR.

Results: This study found that HFA treatment effectively reduced the level of triglyceride, cholesterol, and glucose, and attenuated hepatic steatosis in rats. Additionally, oxidative stress damage of rats was effectively reduced by treatment with HFA. Western blot and qPCR analysis indicated that HFA treatment inhibited fat accumulation in HepG2 cells by upregulating LDLR and downregulating HMGCR gene expression. HFA inhibits oleic acid (OA)-induced oxidative damage to HepG2 by activating the Nrf2 / HO-1 signaling pathway.

Conclusion: HFA administration can provide health benefits by counteracting the effects of hyperlipidemia caused by an HFD in the body, and the underlying mechanism of this event is closely related to the activation of the Nrf2/HO-1 signaling pathway.

KEYWORDS

hawthorn fruit acid, hyperlipidemia, cholesterol, anti-oxidation, atherosclerosis

Introduction

Hyperlipidemia is a metabolic disorder in which blood lipid levels are abnormally elevated (1–3). Due to exceptionally high lipid levels, excess lipids in the blood can accumulate in the walls of the arteries, thus inducing chronic diseases such as cardiovascular disease, stroke, and atherosclerosis (4, 5). An unbalanced diet is a cause of hyperlipidemia (3). High-fat diets (HFD) lead to oxidative stress damage by increasing reactive oxygen species (ROS) and decreasing antioxidant enzymes (6, 7). Prolonged exposure of cells to ROS leads to an imbalance in adipocytokine expression and impaired lipid metabolism (8, 9). Increased ROS production from accumulated fat leads to increased oxidative stress in the blood, which may severely affect other organs (9). Therefore, inhibition of oxidative stress in hyperlipidemic states is considered an essential therapeutic approach (10). Current anti-hyperlipidemic drugs include mainly statins, fibrates, and bile acid chelators; however, these drugs are not effective in reducing oxidative stress (6, 11, 12). In recent years, the use of synthetic antioxidants has been limited due to their possible toxicity and carcinogenicity, and natural antioxidant products have received increasing attention (13, 14).

Oral consumption of hawthorn can lower blood lipid levels, regulate blood glucose and antioxidants, etc (15–17). Hawthorn has recently become an influential component of nutrition and nutraceuticals due to its beneficial effects on the prevention of cardiovascular disease (18). More than 150 substances have been identified and isolated from hawthorn, including a large number of fruit acids, such as gallic acid, chlorogenic acid, ferulic acid, and P-coumaric acid (16, 18). Fruit acids that enable nutrient digestion and stimulate blood circulation are one of the quality indicators of hawthorn fruit (18). Many data suggest that the administration of fruit acids has strong antioxidant activity and hypolipidemic effects (8, 19–22). However, studies on the hypolipidemic and antioxidant mechanisms of hawthorn fruit acids are not clear. Therefore, we used an oleic acid-induced HepG2 cell model and an HFD-induced rat model to evaluate the therapeutic potential and mechanism of hawthorn fruit acids on hyperlipidemia.

Materials and methods

The hawthorn variety is *Crataegus pinnatifida Bunge* cv. "Waibahong," taken from Xinglong County, Chengde City, Hebei Province. The fresh hawthorn was freeze-dried in a vacuum freeze dryer (VaCo 2, Germany ZIRBUS Co., Ltd.), crushed with a crusher, and passed through a 100-mesh sieve to obtain hawthorn powder for later use.

Oxalic acid (OxA, $C_2H_2O_4$, CID:971), Tartaric acid (TA, $C_4H_6O_6$, CID:875), L-malic acid (L-MA, $C_4H_6O_5$, CID:222656), Ascorbic acid (AA, $C_6H_8O_6$, CID:54670067), Citric acid (CiA, $C_6H_8O_7$, CID:311), Gallic acid (GA, $C_7H_6O_5$, CID:370), Chlorogenic acid (ChA, $C_{16}H_{18}O_9$, CID: 1794427), Caffeic acid (CaA, $C_8H_9O_4$, CID:689043), P-coumaric acid (P-CA, $C_9H_8O_3$, CID:637542), Ferulic acid (FA, $C_{10}H_{10}O_4$, CID:445858) are all bought from Aladdin (Shanghai, China). Simvastatin ($C_{25}H_{38}O_5$, CID:54454) was purchased from Yuanye Bio (Shanghai, Guangzhou). β-actin (AC038), Nrf2 (A0674), HO-1 (A1346), NQO1 (A19586), HMGCR (A19063), LDLR (A14996) were purchased from ABclonal (Wuhan, China).

Hydroalcoholic extraction of hawthorn fruit

A certain amount of hawthorn powder was extracted with microwave assistance at 50% ethanol concentration, 28:1 (mL/g), 34 min, and 75°C. The supernatant was obtained by centrifugation and passed through 0.22 μm filter membrane for measurement.

Determination of hawthorn fruit organic and phenolic acids composition

Hawthorn organic acids and phenolic acids were separated using a high-performance liquid chromatograph (LC-20A) from Shimadzu, Japan. Organic acids were quantified on a Dima Platisil ODS C18 [4.6 × 250 mm ID, 5 μm] column. The

mobile phase was a 0.02 mol/L NaH_2PO_4 (pH 2.9): acetonitrile (98: 2) buffer solution. The sample (20 μL) was separated on the column at 37°C with a mobile phase flow rate of 0.5 mL/min; the absorbance was monitored at 213 nm with a UV detector. The peaks of organic acids were identified according to their retention times and quantified using an external standard curve. Phenolic acids were quantified on a Dima Platisil ODS C18 [4.6 × 250 mm ID, 5 μm] column. The mobile phases were solvent A (methanol: acetic acid: water = 10:2:88) and solvent B (methanol: acetic acid: water = 90:2:8). The samples (20 μL) were separated on a column at 37°C with a gradient elution program of 0% to 15% for B from 0 to 25 min, 15 to 50% for B from 25 to 45 min, and 50 to 0% for B from 45 to 53 min. The mobile phase flow rate was 1 mL/min; the absorbance was monitored at 280 nm with a UV detector. The peaks of phenolic acids were identified according to their retention times and quantified using an external standard curve.

In vitro lipid peroxidation analysis

A healthy male SD rat was sacrificed after fasting for 12 h and then the blood samples were taken in a heparin tube. Saline was added at 0–4°C, the supernatant was discarded after centrifugation, and the supernatant was repeatedly washed and discarded to prepare 0.5% erythrocyte suspension. Take 0.5 mL of erythrocyte suspension and add 1 mL of saline. Then add 0.1 mL of different concentrations of Hawthorn extract, 0.1 mL of deionized water for the blank control group, and 0.1 mL of Vitamin C (VC) corresponding to the concentration of Hawthorn extract for the positive control group, respectively. After mixing in a constant temperature water bath at 37°C for 60 min, 0.3 mL of 20% trichloroacetic acid was added in a water-cooled shake, and 2 mL of 0.1 mol/L hydrochloric acids were added. After centrifugation, 3 mL of the supernatant on the supernatant was added with 0.67% thiobarbituric acid 1 mL, boiling water bath for 15 min, and absorbance was measured at 532 nm after cooling. The obtained lipid oxidation inhibition rate was shown in equation (1).

$$\text{RBC lipid eroxidation inhibition rate \%} = \frac{A0-A1}{A0} \times 100\% \quad (1)$$

A healthy male SD rat was sacrificed after fasting for 12 h and then the blood samples were taken in a heparin tube. The livers were removed and ground into 5% liver homogenate in saline at 0–4°C. Take 1mL of liver homogenate, then add 0.1 mL of Hawthorn extract at different concentrations, 0.1 mL of deionized water for the blank control group, and 0. 1 mL of VC corresponding to the concentration of Hawthorn extract for the positive control group. After mixing in a constant temperature water bath at 37°C for 60 min, 1 mL of 20% trichloroacetic acid and 1 mL of 0.67% thiobarbituric acid were added after water-cooled shaking. After cooling in a boiling water bath for 15 min, the centrifuged clear solution was taken at 532 nm absorbance. The obtained lipid oxidation inhibition rate was shown in equation (2).

$$\text{Inhibition rate of liver lipid eroxidation \%} = \frac{A0-A1}{A0} \times 100\% \quad (2)$$

Cytotoxicity

HepG2 cells were obtained from the Institute of Medicinal Plants, Peking Union Medical College. The HepG2 cell culture method was reported in the literature (23). The cell viability assay was reported in the literature (24). 0, 0.125, 0.25, 0.5, 1, 2 mg/mL HFA and 0, 0.05, 0.1, 0.2, 0.25, 0.3 mM OA treatments were applied.

Cell culture and hawthorn fruit acid treatment

After the cells grew to 80% confluence, the medium was changed and drugs were added. The experiments were divided into five groups. The groups were as follows: (i) control group; (ii) OA treated group; 0.1 mM OA incubated for 18 h; (iii) OA + simvastatin group; 100 μM OA and 5 μM simvastatin incubated for 18 h; (iv) OA + HFA high dose group; 100 μM OA and 0. 25 mg/mL HFA incubated for 18 h; and (v) OA + HFA low dose group; 100 μM OA and 0.125 mg/mL HFA incubated for 18 h.

Oil red O staining

Stain lipids in HepG2 cells using Oil Red O solution. Observe the stained cells under a microscope (EVOSFL Color) and capture the images.

Cell biochemical index analysis

The intracellular accumulation of TC, TG, HDL-C, LDL-C, MDA, CAT, GSH-Px, GSH, and SOD was determined according to commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). Operation was conducted according to the commercial kit instructions.

Quantitative real-time polymerase chain reaction

The RT-qPCR method was reported in the literature (25). RT-qPCR was performed on the LightCycler 96 thermal cycler using a fluorescent PCR kit (SYBR Premix Ex TaqTM, Takara, United States). The primer sequences are shown in **Supplementary Table 1**.

Western blot analysis

Proteins were extracted using RIPA lysis buffer containing phosphatase and protease inhibitors (26). Aliquots containing the same number of proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred to a PVC membrane for immunoblotting. β-actin, Nrf2, HO-1, NQO1, HMGCR, and LDLR were all diluted 1000 times for use. A semi-quantitative analysis of the ratio of each target protein to the grayscale value of the internal β-actin reference was performed.

Animal experiments

Male rats were more sensitive to high-fat feeding and had more pronounced insulin resistance and decreased glucose tolerance than females. Therefore, we selected male rats to establish a hyperlipidemia model. Male Sprague-Dawley (SD) rats, weighing 230–270 g, from Hebei Medical University (Shijiazhuang, China), housed in an animal housing room at a constant temperature (25 ± 1°C) for 12 h with fixed darkness and light cycles (Permit No. SCXK2018-004, Laboratory Animal Quality Certificate No. 1804016).

In total of 48 male rats were randomly divided into the following 6 groups. Control group: normal diet + 0.9% saline solution treatment; model group: HFD + 0.9% saline solution treatment; simvastatin group: HFD + 1.8 mg/kg·d^{-1} simvastatin; high-dose group: HFD + 100 mg/kg·d^{-1} HFA extract; low-dose group: HFD + 30 mg/kg·d^{-1} HFA extract; Compound group: HFD + 100 mg/kg·d^{-1} HFA composite (HFA composites prepared from oxalic acid, tartaric acid, L-malic acid, ascorbic acid, citric acid, gallic acid, chlorogenic acid, caffeic acid, p-coumaric acid, and ferulic acid standards based on the results of HPLC assay). Except for the control group, the other 5 groups were fed with HFD for 2 weeks, and then administration started. These drugs were taken for 8 weeks, with each animal weighed every week, and blood collected from the tip of the tail. See **Supplementary Table 2** for dietary information.

Biochemical parameters

The cumulative amount of serum and liver homogenate of TC, TG, HDL-C, LDL-C, MDA, CAT, SOD, GSH-px, and T-AOC was measured using commercial kits. Operation was conducted according to the commercial kit instructions. The atherosclerosis index (AI) was calculated based on the content of T-CHO and HDL-C in the serum.

$$AI = (TC - HDL) \div HDL \quad (3)$$

Pathology

The liver tissue was cut into 4-μm slices and stained with hematoxylin and eosin stain. All sections were examined by light microscopy (Nikon Primo Star microscope, Carl Zeiss, Germany).

Statistical analysis

All data are expressed as the means ± standard deviation (SD) and analyzed by GraphPad Prism 8.0. One-way ANOVA was used to assess differences between multiple groups. Statistically significant results were indicated as$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ vs. control group; $^{\#}p < 0.05$,$^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$ vs. model group.

Results

Hawthorn fruit organic and phenolic acids composition

Figure 1A is a flow chart of the extraction of organic and phenolic acids from hawthorn. Based on the quantification of the standard curve of fruit acids determined by HPLC (**Supplementary Figure 1**), we determined that hawthorn extract contained 10 acids, namely OxA, TA, L-MA, AA, CiA, GA, ChA, CaA, P-CA, and FA (**Figure 1B**). Among them, citric acid had the highest content of 147.67 mg/g. The main components of phenolic acids were chlorogenic acid and ferulic acid with 1.04 mg/g and 0.84 mg/g, respectively (**Supplementary Table 3**).

The antioxidant capacity of HFA was initially verified by measuring its anti-lipid peroxidation level *in vitro*. **Figure 1C** shows that HFA administration inhibited lipid peroxidation in both liver tissue and erythrocytes. HFA treatment has a more stable anti-lipid peroxidation ability than VC and is a good antioxidant.

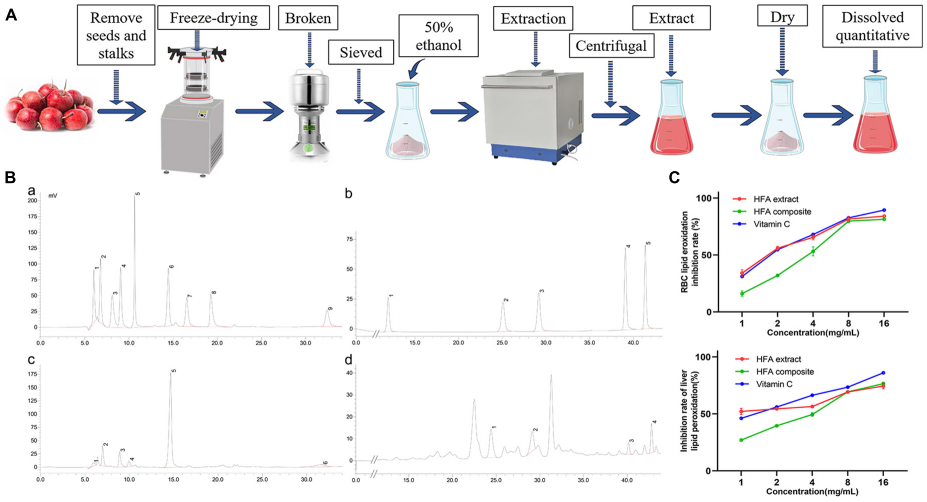

FIGURE 1
Identification of hawthorn fruit acids (HFA) and the *in vitro* antioxidant capacity of HFA. **(A)** Flow chart of HFA preparation; **(B)** high-performance liquid chromatogram; a. Peaks 1–9 are OxA, TA, PA, L-MA, AA, CiA, SA, D-MA, GA; b. Peaks 1–5 are GA, PA, ChA, CaA, P-CA, FA; c. Peaks 1–6 are OxA, TA, L-MA, AA, CiA, GA; d. Peaks 1–4 are ChA, CaA, P-CA, FA; **(C)** *in vitro* lipid peroxidation analysis. Data express mean ± SD (n = 3).

The lipid-lowering effect of hawthorn fruit acid on the HepG2 cells

According to the effect of OA on cell viability measured by CCK-8 (**Supplementary Figure 2A**), we chose 0.1 mM of OA to establish the HepG2 hyperlipidemic cell model. According to the effect of HFA on cell viability measured by CCK-8 (**Supplementary Figure 2B**), HFA of 0.25 mg/mL and 0.125 mg/mL were selected for the experiment. Compared with the control group, the TC, TG, and LDL contents of HepG2 cells were significantly increased and HDL contents were significantly decreased after adding 0.1 mM OA for 18 h (**Figure 2A**). Meanwhile, a significant increase in lipid droplets could be observed in the results of oil red O staining (**Figure 2C**). It proves that the cellular hyperlipidemia model was established successfully. In the HFA treatment group, a significant improvement of these indices could be observed; a significant decrease in the content of TC, TG, and LDL; and a significant decrease in intracellular lipid droplets and lipid accumulation. In conclusion, these results suggest that in OA-injured HepG2 cells, HFA administration reduces TC and TG content, increases HDL content, reduces the accumulation of lipid droplets in cells, and has a protective effect on HepG2 cells.

To determine the antioxidant effect of HFA on HepG2 cells, we measured the levels of MDA, GSH, SOD, and CAT in cells. MDA is cytotoxic as the end-product of lipid peroxidation by free radicals in living organisms, and it can lead to cross-linking and polymerization of macromolecules. SOD and CAT activities are important indicators to measure the ability to resist oxidation and scavenging free radicals. As shown in **Figure 2B**, compared with the control cells, MDA levels were significantly increased after OA injury ($P < 0.0001$). The levels of GSH, SOD and CAT were significantly decreased (P-values less than 0.0001, 0.001 and 0.01, respectively), indicating that the cellular antioxidant capacity was impaired and accelerated the hyperlipidemia process. HFA treatment could significantly reduce MDA levels ($p < 0.0001$) and increase the levels of GSH, SOD, and CAT (p-values less than 0.0001, 0.01, and 0.05, respectively). These indicators indicated the HFA has a good antioxidant capacity.

The effect of hawthorn fruit acid on lipid levels in hyperlipidemia rats

To investigate the ability of HFA on alleviating the symptoms of hyperlipidemia rats, the hyperlipidemia rat model was established by the rats which have been fed for 8 weeks by FAD. Before HFA treatment, the plasma levels of TC and TG of hyperlipidemia rats were 3.34 ± 0.51 mmol/L and 2.07 mmol/L, respectively, which were significantly higher than those of the blank control rats (1.49 ± 0.18 mmol/L and 0.76 ± 0.31 mmol/L). This demonstrates that the model was established successfully. The hyperlipidemia rats were further treated with HFA for 8 weeks. The results showed that the serum levels of TC (**Figure 3A**), TG (**Figure 3B**), and GLU (**Figure 3D**) decreased gradually, and the HDL-C levels increased gradually in the HFA-treated rats over time (**Figure 3C**). AI values calculated by TC and HDL-C decreased simultaneously (**Figure 3E**), suggesting a reduced risk of cardiovascular disease. Similarly, TC and TG were reduced in the liver of HFA-treated rats compared to hyperlipidemia

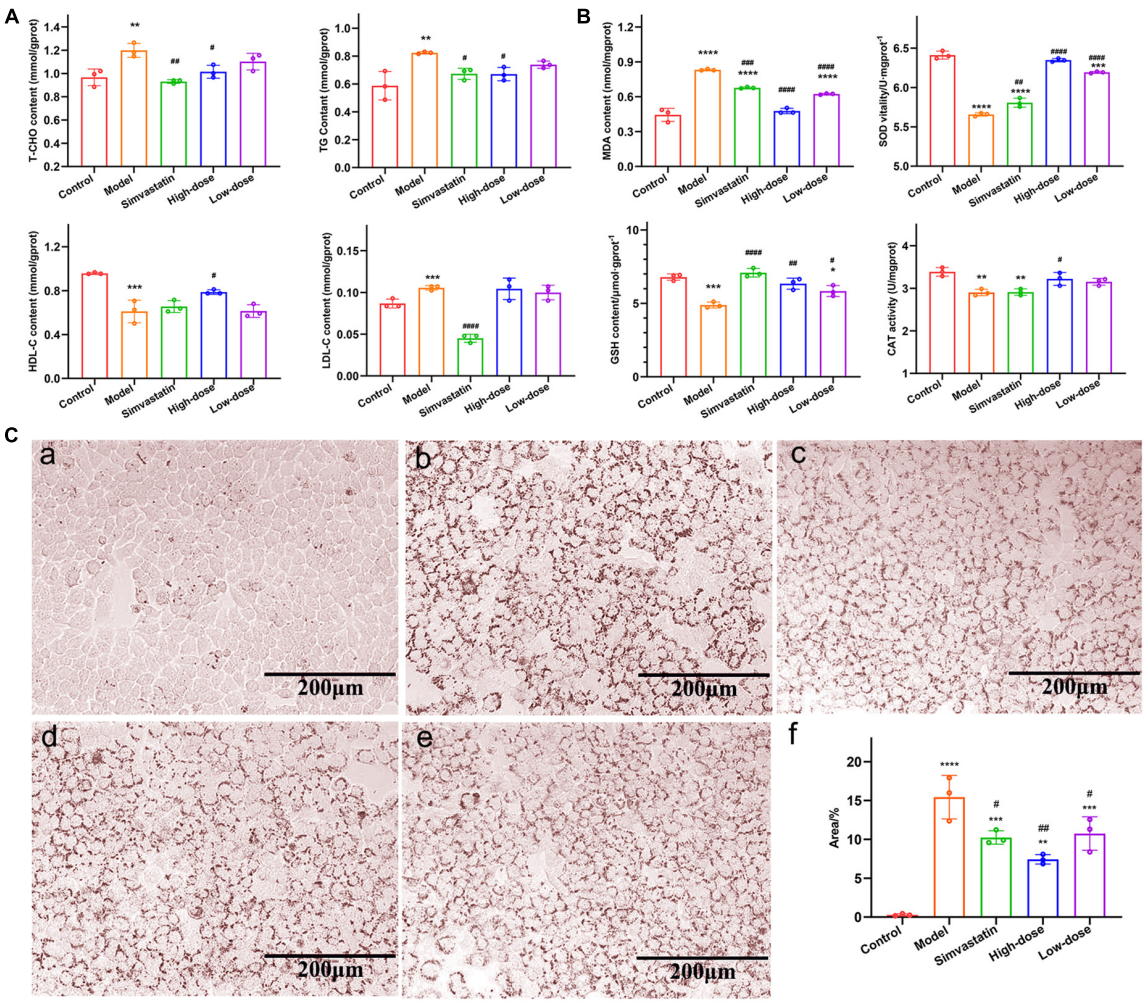

FIGURE 2
Effect of hawthorn fruit acid (HFA) on lipid accumulation and oxidative stress in HepG2 cells. **(A)** T-CHO, TG, HDL-C, and LDL-C in HepG2 cells treated with HFA and 0.1 mM OA; **(B)** MDA, GSH, SOD, and CAT levels in HepG2 cells treated with HFA and 0.1 mM OA; **(C)** Oil-red O staining (20×) of HepG2 cells treated with HFA and 0.1 mM OA: **(a–e)** for control, model, simvastatin, high-dose and low-dose groups, respectively; **(f)** Statistical results of oil-red O stained areas. Data represent mean ± SD (n = 3). Statistically significant results were indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ vs. control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$ vs. model group.

rats (Figure 3F). In conclusion, these results validated that HFA treatment effectively improved hyperlipidemia.

To observe the fat accumulation in the rat liver, we performed a pathological examination of the rat liver. Normal rats don't have fat accumulation in the liver. Its cells were intact and regularly arranged, and the nuclei of the cells were clear (Figure 4A). Hyperlipidemia rats (Figure 4B) showed severe changes in liver structure, such as hepatocyte necrosis, cytoplasmic vacuolization, cell degeneration, and loss of cell boundaries. Also, a large accumulation of fat in the hepatocytes was observed in the form of droplets. In the simvastatin group (Figure 4C), there was a reduction in the accumulation of intracellular fat and the cells were morphologically intact and relatively well aligned. Different degrees of fat accumulation were observed in the liver of the low and high-dose groups (Figures 4D,E). The morphology of the cells was changed and was irregularly arranged. In the high-dose group, the droplet-shaped fat particles were smaller and less numerous, and the cells were arranged more regularly. The condition of the liver in the compound group (Figure 4F) was between high and low. The results suggest that the administration of Hawthorn fruit acid extract can reduce the liver fat accumulation caused by HFD.

Antioxidant effect of hawthorn fruit acid on hyperlipidemia rats

To investigate the *in vivo* antioxidant capacity of HFA, we measured the enzymatic activities of CAT, GSH-px,

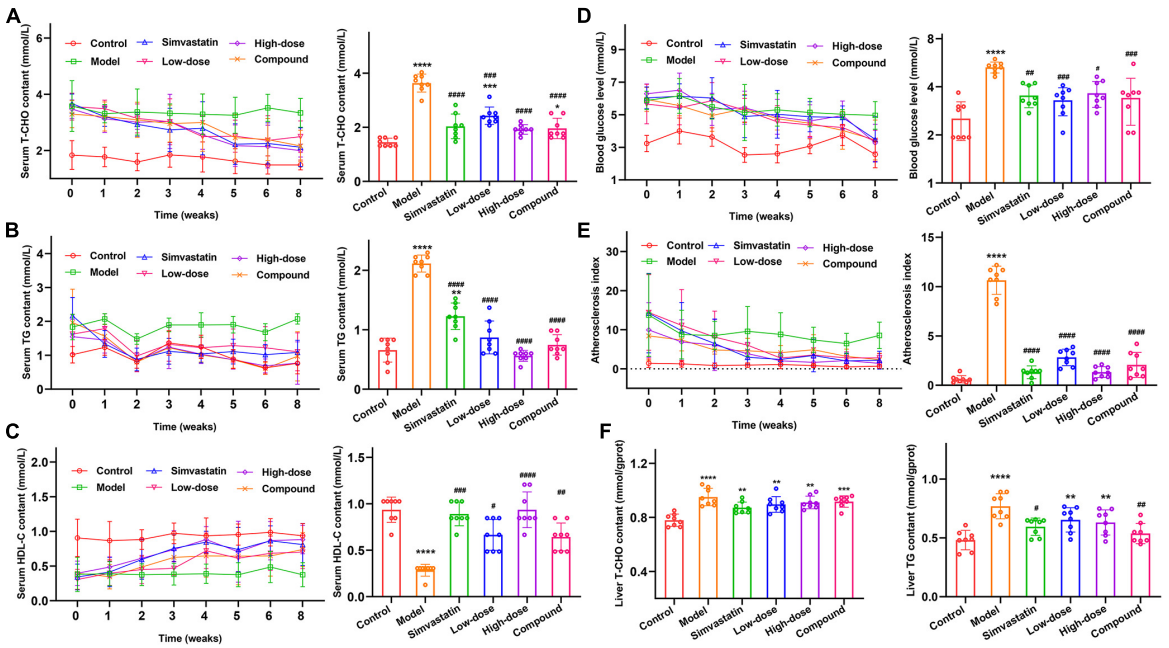

FIGURE 3
Effect of HFA on lipids in serum and liver of hyperlipidemic rats. **(A–E)** Changes in T-CHO, TG, HDL-C, GLU, and AI in rat serum during 8 weeks. **(F)** Changes in T-CHO and TG levels in rat liver homogenates. Data represent mean ± SD (n = 8). Statistically significant results were indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ vs. control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$ vs. model group.

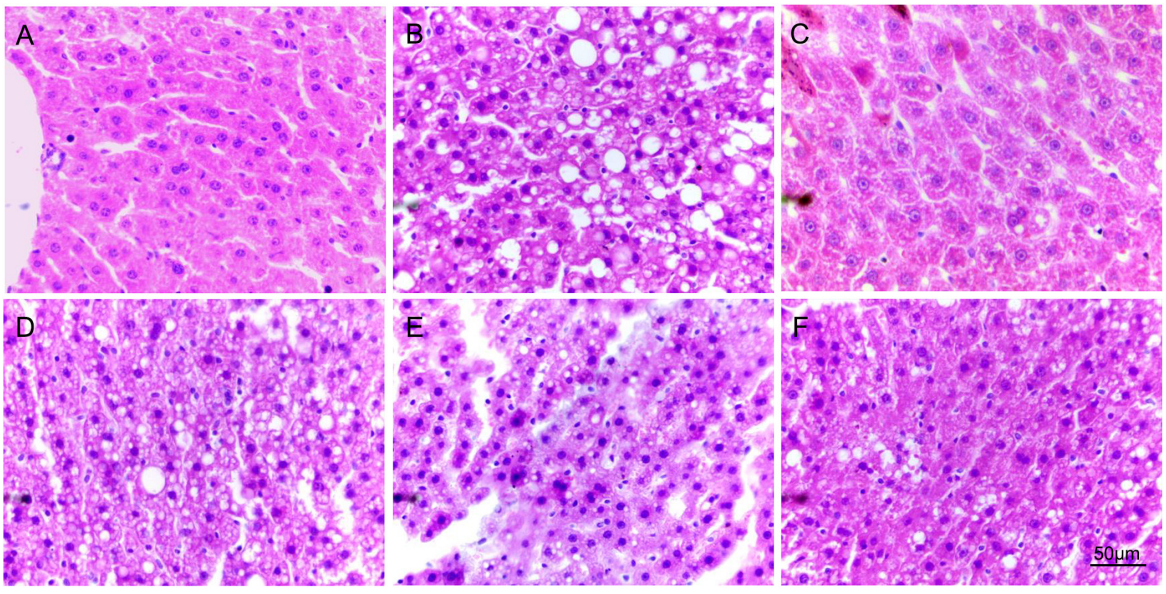

FIGURE 4
The images of H&E staining in the liver. **(A–F)** are the H&E staining pictures of liver slices in the control, model, simvastatin, low-dose, high-dose, and compound group, respectively.

SOD, the content of MDA, and T-AOC capacity in serum and liver homogenates of rats. The results showed that before HFA treatment, the serum levels of MDA, SOD, and CAT in hyperlipidemia rats were 13.78 ± 2.12 nmol/mL, 157.8 ± 10.96 U/mL, 36.91 ± 6.02 U/mL, respectively, which were significantly different from those in control rats

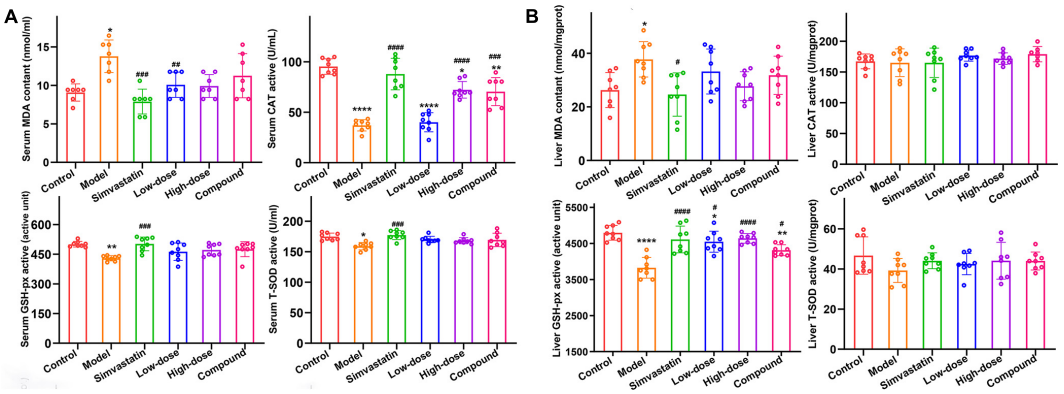

FIGURE 5
The effect of HFA on MDA and antioxidant enzymes in serum and liver of hyperlipidemia rats. **(A,B)** MDA, CAT, GSH-px and SOD levels in serum and liver, respectively. Data represent mean ± SD (n = 8). Statistically significant results were indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ vs. control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$ vs. model group.

(9.08 ± 1.12 nmol/mL, 172.7 ± 6.82 U/mL, 94.85 ± 7.81 U/mL). These phenomena were significantly improved after HFA treatment (**Figure 5A** and **Supplementary Figure 3**). A similar situation occurred in the liver (**Figure 5B** and **Supplementary Figure 3**). These results suggest that HFD leads to impaired antioxidant capacity and accelerated hyperlipidemia in rats. Administration of HFA reduces the accumulation of MDA in rats and increases the activity of antioxidant enzymes through antioxidant effects, thus reducing the damage caused by HFD in rats.

Effect of hawthorn fruit acid on HepG2 cell protein and gene expression

To clarify whether the hypolipidemic effect of HFA is related to its antioxidant activity, the protein and mRNA expression of Nrf2, HO-1 and NQO1 were measured in HepG2 cells in this study. As shown in **Figure 6**, the relative mRNA expression of Nrf2, HO-1, and NQO1 was significantly increased in HFA-treated cells compared to OA-treated cells, along with increased protein expression. Activation of Nrf2 resulted in increased expression of HO-1 and NQO1. These results suggest that the Nrf2/HO-1 pathway is activated to protect HepG2 cells from OA-induced oxidative stress.

To verify the possible mechanism of lipid reduction by HFA administration, we measured the protein and mRNA expression of LDLR and HMGCR in HepG2 cells. LDLR is a receptor for LDL-c and contributes to the transport and clearance of cholesterol from the plasma to the cytoplasm, and HMGCR acts as a key regulatory enzyme controlling endogenous cholesterol synthesis. Both LDLR protein expression and relative mRNA expression were significantly reduced and HMGCR protein expression and mRNA expression were increased in OA-induced cells compared to control cells. HFA treatment was able to significantly alter this phenomenon. These results suggest that HFA administration may regulate cholesterol biosynthesis and cholesterol metabolism by modulating the expression of HMGCR and LDLR.

Discussion

Hyperlipidemia is a highly prevalent risk factor for atherosclerosis and stroke, usually manifested by elevated TC and/or TG, and decreased HDL-C. In the present study, to investigate the hypolipidemic effect and mechanism of oral HFA, we first identified its main components as OxA, TA, L-MA, AA, CiA, GA, ChA, CaA, P-CA, and FA. Our results showed that HFA administration decreased TC and TG content and increased HDL-C content in HepG2 cells *in vitro*. In rats with HFD induced hyperlipidemia, after HFA treatment, the TC and TG contents gradually decreased with the duration of HFA treatment. At week 8, significant differences have been observed compared to the model group. These findings suggest that treatment with HFA administration can significantly improve the lipid levels in rats. Gallic acid, chlorogenic acid, and ferulic acid have been reported to have lipid-lowering effects (19, 20, 27); citric acid has a strong antioxidant capacity (28). The results of some studies have shown that in Sprague-Dawley rats fed with a hypercholesterolemic diet, chlorogenic acid significantly altered the increase in plasma total cholesterol and LDL (29); ferulic acid intervention significantly reduced TC, TG, and LDL-C and increased HDL-C in hyperlipidemia patients, while also significantly reducing the levels of MDA (10, 30); gallic acid alleviates hypertriglyceridemia and lipid accumulation by enhancing glycolytic and lipolytic pathways in the perirenal adipose tissue of hyperlipidemic rats (31). It is reported that the combination of weak organic acids has a synergistic function in inhibiting urinary path-ogens (32).

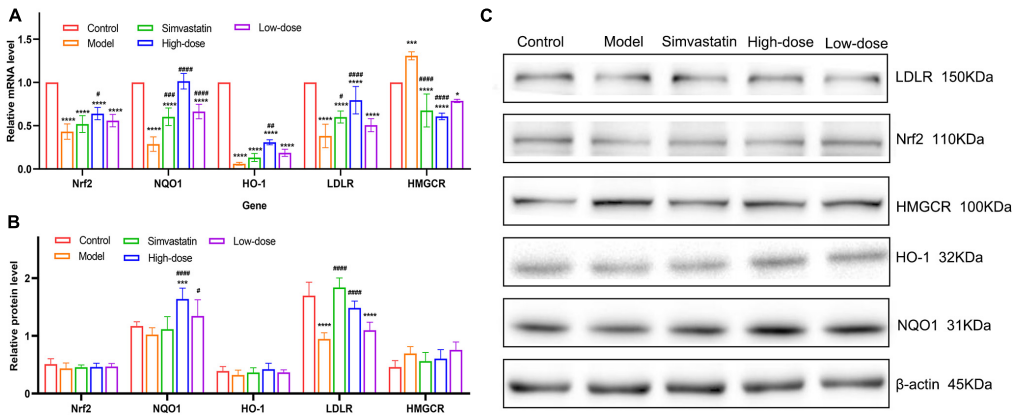

FIGURE 6
Effect of HFA on protein and gene expression in HepG2 cells. **(A)** Relative mRNA expression levels of Nrf2, NQO1, HO-1, LDLR, and HMGCR were quantified by RT-qPCR; **(B,C)** Protein expression levels of Nrf2, NQO1, HO-1, LDLR, and HMGCR. Data represent mean ± SD (n = 3). Statistically significant results were indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ vs. control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$ vs. model group.

We speculate that the good lipid-lowering activity of HFA may also be related to the synergistic effect of a variety of organic acids.

To verify the possible mechanism of oral HFA on lipid-lowering, we measured the expression of LDLR, HMGCR protein, and mRNA in HepG2 cells. In the results of this study, the expression of LDLR protein and mRNA in HepG2 cells showed an increasing trend after HFA treatment, while the expression of HMGCR protein and mRNA showed a decreasing trend. The ethanolic extract of Hawthorn was reported to inhibit the stimulatory effect of an HFD on HMGCR and p65 transcription and counteract the down-regulation of CYP7A1 and LDLR (33). Ferulic acid in the extract inhibited the expression of HMGCR, thereby decreasing TC and LDL-C (10). The results suggest that HFA administration can up-regulate LDLR expression and down-regulate HMGCR expression by regulating the metabolism of cholesterol.

Hyperlipidemia is associated with increased oxidative stress and increased lipid peroxidation (10). One study found that fat accumulation leads to an imbalance in adipocytokine production in response to oxidative stress and that selective increases in ROS produced in fat accumulation lead to elevated systemic oxidative stress (9). Many studies have shown that reducing ROS production improves insulin sensitivity, hyperlipidemia, and hepatic steatosis (34). In addition to lipid peroxidation, ROS can activate NF-κB and trigger inflammatory mediators (35). To clarify whether the hypolipidemic effect of HFA is related to its antioxidant activity, this study measured the protein and gene expression of Nrf2, HO-1, and NQO1 in HFA-treated HepG2 cells. After intracellular ROS production, the antioxidant response pathway is activated through nuclear translocation of Nrf2, which activates antioxidant response genes and induces phase II detoxification enzymes, such as HO-1 (36). HO-1 scavenges ROS, thereby preventing excessive lipid and protein oxidation, and plays an effective role in antioxidant, anti-inflammatory, and anti-apoptotic activities (37, 38). In this study, we observed that after HFA treatment of HepG2 cells both protein and mRNA expression levels in the cells were increased. Treatment with chlorogenic acid activates Nrf2 and increases the activity of antioxidant enzymes, thus antagonizing oxidative damage (37, 39, 40). Gallic acid protects against ethanol-induced gastric lesions in rats by activating the Nrf2/HO-1-associated pathway (41). Ferulic acid reduces oxidative stress, inflammation, and cell death by activating Nrf2/HO-1 signaling and PPARγ (35). These results may demonstrate that HFA administration increases the antioxidant capacity of HepG2 cells and reduces OA-induced hyperlipidemia by activating the Nrf2/HO-1 signaling pathway. Increased protein and mRNA expression of NQO1 was also observed in HepG2 cells.

In our findings, an HFD caused excessive accumulation of MDA in the rat liver. MDA is a product of lipid peroxidation. During fatty acid oxidation, the production of excessive ROS will disrupt the balance between antioxidants and oxidants in the liver (42). When ROS increase, CAT and GSH decrease, thus amplifying the effects of oxidative stress and creating a vicious cycle (43). Hyperlipidemia damages endothelial cells, triggering simultaneous macrophage invasion and lipid deposition, which are key steps in the development of atherosclerosis (44). ROS production serves as a key mediator involved in hyperlipidemia through these mechanisms: direct damage to cell membranes and nuclei and production of oxidized lipoproteins (45). Antioxidant enzymes reduce oxidant levels, thereby reducing lipid peroxidation. These enzymes known to be effective in atherosclerosis include SOD, GPx, CAT, and PON1 (45). In

the study, MDA levels were significantly reduced in rats treated with HFA. The total antioxidant capacity in serum and liver of HFA-treated rats was significantly increased, and the activities of CAT, GSH-px, and SOD enzymes were also found to be on the rise. These findings suggest that oral administration of HFA can protect rats from HFD-induced liver damage and reduce the risk of atherosclerosis by reducing oxidative damage.

In epidemiological studies in Asia and Europe, hyperlipidemia is commonly associated with diabetes (46). One study showed that a short-term HFD diet in hamsters resulted in severe hepatic steatosis, glucose intolerance, and a slight increase in fasting glucose, suggesting the development of type II diabetes (47). Another study showed that, compared with participants with normal lipids, subjects with mixed hyperlipidemia had a risk of developing type II diabetes that was more than three times higher in subjects with mixed hyperlipidemia compared to participants with normal lipids (46). To confirm whether HFA administration reduces the risk of atherosclerosis and hyperglycemia, we measured GLU and AI in hyperlipidemic rats. The results showed that feeding HFD to rats for 2 weeks could lead to the increase of Glu and the change of blood lipid level, thus increasing AI. After 8 weeks of HFA treatment, GLU and AI levels in rats decreased significantly. The results of this study show that oral administration of HFA to rats can reduce blood lipid and blood glucose levels and reduce the risk of atherosclerosis caused by HFD.

Conclusion

The results of this study showed that HFA administration was effective in preventing the progression of hyperlipidemia in a rat model, as demonstrated by its ability to attenuate the increase in serum lipid levels induced by HFD and to reduce lipid accumulation in the liver. HFA administration inhibited lipid accumulation in HepG2 cells by up-regulating LDLR and down-regulating protein and gene expression of HMGCR. HFA treatment protected hepatocytes by activating the Nrf2/ HO-1 signaling pathway to protect hepatocytes. In conclusion, this study demonstrates the potential of HFA as a therapeutic agent in the clinical treatment of hyperlipidemia.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the Experimental Animal Ethics Committee of Hebei Provincial Department of Science and Technology.

Author contributions

YF conducted most of the experiments under the guidance of XD and DM. SG and TZ helped in discussions about pathology. GS helped in data analysis. PZ, YH, and SQ carried out article correction. All authors discussed the results and commented on 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.2022.936229/full#supplementary-material

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Dietary consumption of cruciferous vegetables and bladder cancer risk: A systematic review and meta-analysis

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Objective: Previous studies on the association of cruciferous vegetables intake with bladder cancer risk have reported inconsistent results. We performed the present meta-analysis to summarize evidence on this association and to quantify the potential dose-response relation based on all available cohort studies.

Methods: A comprehensive literature search of relevant articles up to March 2022 was performed in PubMed and EMBASE. The summary risk estimates with 95% confidence intervals for the highest vs. the lowest intake of cruciferous vegetables were calculated. Dose-response meta-analysis was also performed for studies reporting categorical risk estimates for at least three quantitative levels of cruciferous vegetables intake.

Results: We found that the highest cruciferous vegetables intake was not significantly associated with a lower risk of bladder cancer, compared with the lowest cruciferous vegetables intake category (RR = 0.92, 95% CI 0.80–1.06). Linear dose-response meta-analysis indicated that the pooled RRs for 10 g/day or 1 servings/week increment of cruciferous vegetables intake was not significantly associated with a reduced risk of bladder cancer (P = 0.106 and P = 0.147, respectively). There was no evidence of significant publication bias either with Begg's test (P = 0.386) or Egger's test (P = 0.253).

Conclusion: The results of this study did not support the hypothesis that dietary cruciferous vegetables intake was associated with a lower risk of bladder cancer. Further large prospective cohort studies are warranted to confirm our preliminary findings.

KEYWORDS

cruciferous vegetables, bladder cancer, meta-analysis, cohort, risk

Introduction

Bladder cancer is a common disease, which ranks ninth in cancer incidence and is the 13th leading cause of cancer death among men and women worldwide (1). An estimated 573,278 new cases and 212,536 deaths from bladder cancer occurred in 2020 (2). Bladder cancer is classified as muscle-invasive bladder cancer (MIBC) and non-muscle-invasive bladder cancer (NMIBC) based on depth of tumor invasion. 75% of bladder cancers are non-muscle invasive (Tis, Ta, T1) (3, 4). Smoking is the most established risk factor for bladder cancer, among other risk factors including occupational exposure to arylamines and schistosomal infection (5). Emerging evidence indicates a significant influence of dietary factors [e.g., dairy product (6) and processed meat (7)] and dietary patterns [e.g., Western diet and Mediterranean diet (8)] on the risk of bladder cancer.

Cruciferous vegetables (e.g., broccoli, cauliflower, and cabbage) intake has been associated with multiple health outcomes (9). Epidemiologic studies investigating the association between cruciferous vegetables intake and bladder cancer risk, however, have yielded inconsistent results. Several case-control studies (10, 11), as well as a cohort study (12), reported a significant inverse association between cruciferous vegetables intake and bladder cancer risk. Nevertheless, many other studies found no association (13–15), including an international pooled study (15). Therefore, the aim of the present meta-analysis is to summarize the evidence on the association between cruciferous vegetables intake and bladder cancer risk based on all available cohort studies.

Materials and methods

Publication search

A comprehensive literature search of relevant articles was performed in the PubMed and EMBASE databases from their inception through March 2022 with the following search algorithm: (diet or nutrition or vegetable or cruciferous or broccoli or cauliflower or cabbage) and (bladder neoplasm or bladder cancer) and (cohort or prospective or nested case-control). The cited references from retrieved articles and reviews were also checked for additional relevant studies. No language restriction was applied. This systematic review and meta-analysis was planned, performed, and reported according to the standards of quality for reporting meta-analyses (16, 17).

Study selection

Studies included in this meta-analysis met all of the following criteria: (*i*) the exposure of interest was the cruciferous vegetables intake; (*ii*) the outcome of interest was bladder cancer incidence; (*iii*) the study design was prospective or cohort; and (*iv*) the risk estimates with their corresponding 95% confidence intervals (CIs) were reported or data were provided to calculate them. We excluded reviews/meta-analyses, editorials, correspondences, case reports, and non-human studies. Studies of other exposures and diseases were also removed. If multiple publications based on overlapping population were retrieved, the most informative one was included.

Data extraction

Two authors (PY and YL) independently extracted the data using a predefined extraction form. The following information was extracted from each study: the first author's name, year of publication, study region, study name, study population or source, sample size (number of cases and participants), participants' age and sex, follow-up time, method of diet assessment, method of outcome assessment, and adjusted confounders in the data analysis.

Quality assessment

The same two authors (PY and YL) independently completed the quality assessment using the Newcastle-Ottawa Scale (NOS).[1] NOS is an eight-item instrument and awards a maximum of nine points to each study. A higher score indicates better methodological quality. Any disagreements were resolved by consensus and discussion.

Statistical methods

The summary RRs and 95% CIs were estimated using a DerSimonian and Laird random effects model (18). Summary risk estimates were estimated by comparing the two extreme categories of the cruciferous vegetables intake related to bladder cancer risk.

Dose-response meta-analysis was performed using the method proposed by Greenland (19) and Orsini (20). Briefly, this method required that studies reported categorical risk estimates for at least three quantitative levels of cruciferous vegetables intake and the number of cases and person-years in each exposure category. The median/mean value or the midpoint of each category was regarded as the corresponding exposure dose. For upper, open-ended exposure categories, we assumed the width of the interval to be the same as the closest neighboring category. The lowest category of exposure was treated as the reference group. In addition, we tested a potential non-linear dose-response relationship between the cruciferous

1 http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp

vegetables intake and bladder cancer risk by modeling the cruciferous vegetables intake using restricted cubic splines with three knots at the 10th, 50th, and 90th percentiles of the distribution (21). A *P*-value for non-linearity was calculated by testing the null hypothesis that the coefficient of the second spline was equal to 0.

The heterogeneity across studies was assessed by the *Q* statistic and the I^2 score (22). The *Q* statistic was used to determine the presence of heterogeneity with a significance level set at $P \leq 0.10$. The value of I^2 was used to calculate the proportion of variation ($I^2 < 25\%$ low heterogeneity; $I^2 = 25$–50% moderate heterogeneity; $I^2 > 50\%$ high heterogeneity). The subgroup analyses were performed based on study region, study year, gender, number of cases, and number of participants. A sensitivity analysis was performed by repeating the meta-analysis after exclusion of each included study in turn. Potential publication bias was assessed by Begg's test (rank correlation method) (23) and Egger's test (linear regression method) (24). All of the statistical analyses were performed using STATA 11.0 (StataCorp, College Station, TX). A 2-sided *P*-value < 0.05 was considered significant unless stated otherwise.

Results

Literature search and study characteristics

The detailed process of the literature search and selection has been presented in a flow diagram (**Figure 1**). A total of seven prospective studies (12–15, 25–27) met the inclusion criteria for this meta-analysis evaluating the association between cruciferous vegetables intake and bladder cancer risk. Park et al.' study (13) reported the results separately by gender and thus was regarded as two independent cohorts. These cohorts were from the following regions: America ($n = 5$), Europe ($n = 2$), and International ($n = 1$). A total of 1,503,016 participants with 13,669 cases were included in this study. These studies were published from 1999 to 2021. Information on cruciferous vegetables consumption was obtained by self-reports or interviews with food-frequency questionnaires (FFQs). The outcome was collected from cancer registry, health insurance records or medical records. The study quality as assessed by the NOS was generally high. Only one study Larsson et al. (25) study had a score of 7, with all the other studies having a score of 8 (**Supplementary Table 1**). The detailed information of the studies at baseline are shown in **Table 1**.

High vs. low cruciferous vegetables intake

The multivariable-adjusted RRs of the highest vs. the lowest categories of the cruciferous vegetables intake in each study and for the combination of all of the studies are shown in **Figure 2**. The highest cruciferous vegetables intake was not significantly associated with a lower risk of bladder cancer, compared with the lowest category (RR = 0.92, 95% CI 0.80–1.06).

Dose-response meta-analysis

Two units, i.e., grams/day and servings/week, were used for cruciferous vegetables intake in the included studies and thus the dose-response analysis was performed separately. There was no evidence of a non-linear relationship between cruciferous vegetables intake and bladder cancer risk ($P = 0.169$ and

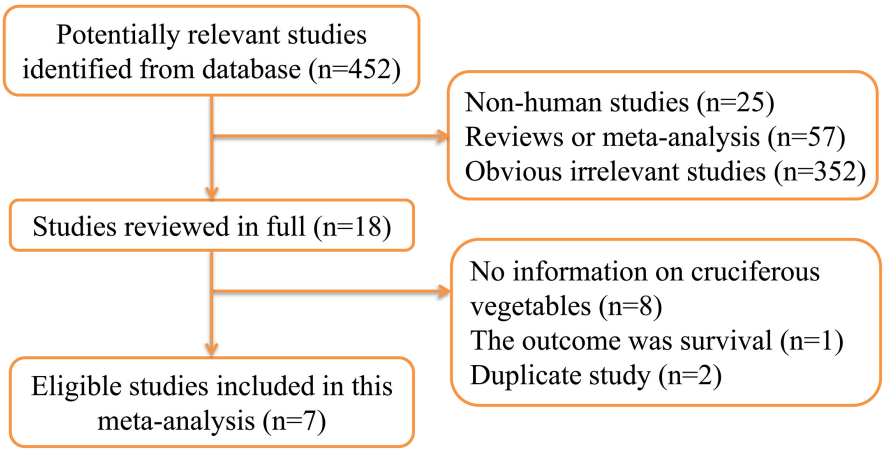

FIGURE 1
Flow diagram of literature search and study selection.

TABLE 1 Main characteristic of included studies.

References	Name	Region	Cohort	Case	Gender	Age (y)	Follow-up (y)	Expose	Outcome
Nguyen et al. (14)	NIH-AARP diet and health study	United States	515,628	8,567	Male and female	50–71	15	Self-administered FFQ	Cancer registry
Yu et al. (15)	BLEND	International	555,685	3,203	Male and female	NA	11 (Median)	Self-administered or trained interviewer administered FFQ	Cancer registries, health insurance records, or medical records
Park et al. (13)	Multiethnic cohort study	United States	83,694	429	Male	60.2 (8.9)	12.5 (Mean)	FFQ	Cancer registry
Park et al. (13)	Multiethnic cohort study	United States	102,191	152	Female	59.7 (8.9)	12.5 (Mean)	FFQ	Cancer registry
Larsson et al. (25)	Swedish mammography cohort	Sweden	82,002	485	Male and female	NA	9.4 (Mean)	Self-administered FFQ	Cancer registry
Holick et al. (26)	Nurses' health study	United States	88,796	237	Female	30–55	20	FFQ	Medical records
Michaud et al. (27)	ATBC cohort	Finland	27,111	344	Male	50–69	11 (Median)	FFQ	Cancer Registry
Michaud et al. (12)	HPFS	United States	47,909	252	Male	40–75	NA	Self-administered FFQ	Medical records

Y, year; FFQ, food-frequency questionnaire; NA, not available; BLEND, Bladder Cancer Epidemiology and Nutritional Determinants; HPFS, Health Professionals Follow-up Study; ATBC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention.

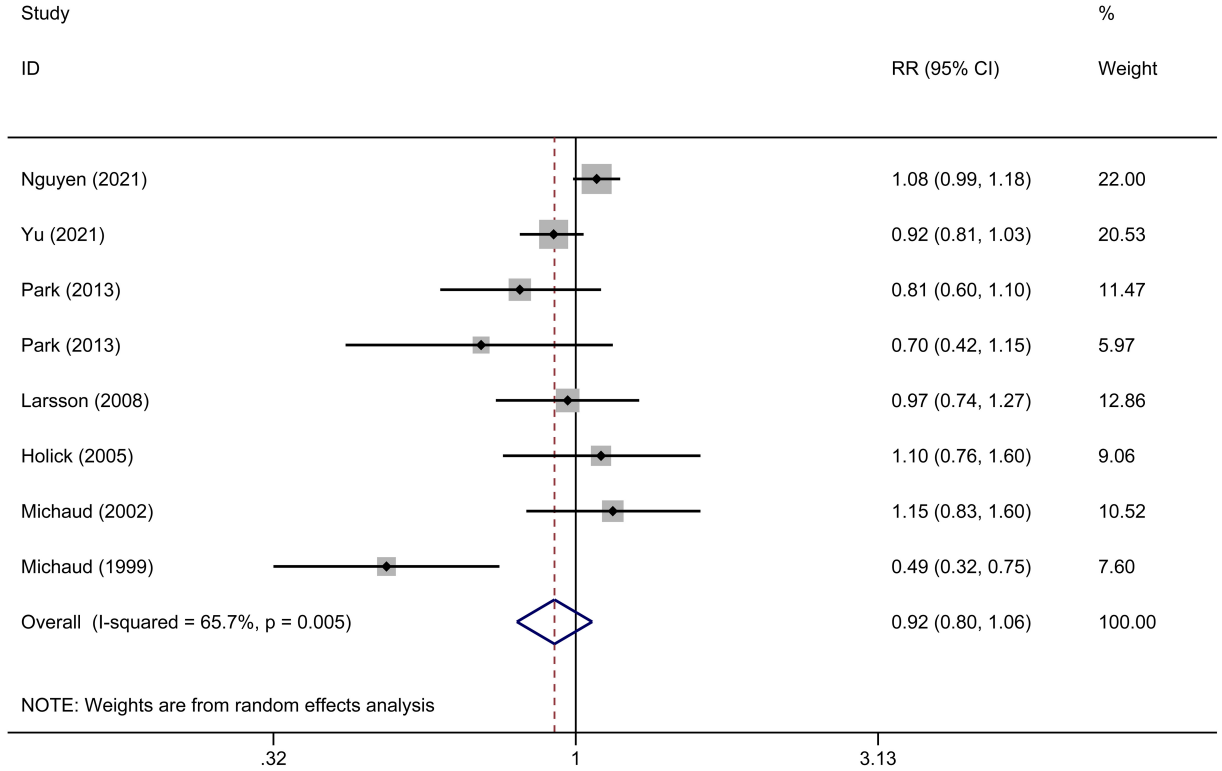

FIGURE 2
A forest plot of pooled relative risks (RRs) for cruciferous vegetables intake and bladder cancer risk (the highest category compared with the lowest category).

P = 0.708 for non-linearity, respectively). Linear dose-response meta-analysis indicated that neither 10 g/day or 1 servings/week increment of cruciferous vegetables intake was significantly associated with a reduced risk of bladder cancer (Figure 3, P = 0.106 and P = 0.147, respectively).

Evaluation of heterogeneity and subgroup analysis

There was some statistically significant heterogeneity among the studies, either assessed by Q statistic (P = 0.005) or I^2 index (I^2 = 65.7%). In the stratified analyses, no significant association was observed in any pre-specified subgroups (Table 2).

Sensitivity analysis and publication bias

In the sensitivity analysis, the impact of each study on the pooled RR was evaluated by repeating the meta-analysis after omitting one study at a time. As a result, exclusion of any single study did not substantially alter the pooled RR (Figure 4). There was no evidence of significant publication bias with Begg's test (Figure 5, P = 0.386) or with Egger's test (P = 0.253).

Discussion

This meta-analysis of prospective studies, including approximately 1,500,000 participants, showed that cruciferous

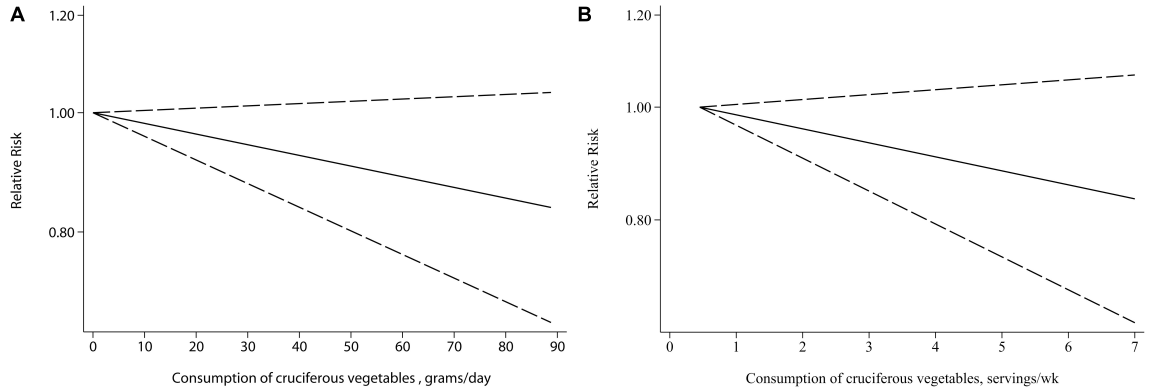

FIGURE 3
Relative risks (RRs) and the corresponding 95% confidence intervals (CIs) for the dose-response relationship between cruciferous vegetables intake and bladder cancer risk. The solid line and dash line represent the estimated RRs and their 95% CIs. **(A)** Grams per day; **(B)** servings/week.

TABLE 2 Subgroup analyses for the relationship between consumption of cruciferous vegetables and the risk of bladder cancer.

Factors stratified	No. of cohorts	RR (95% CI)	Q	*P*	I^2, %
All cohorts	8	0.92 (0.80–1.06)	20.40	0.005	65.7
Region					
Europe	2	1.04 (0.84–1.28)	0.62	0.432	0.0
United States	5	0.83 (0.63–1.11)	17.61	0.001	77.3
Publication year					
≥2010	4	0.94 (0.81–1.10)	8.67	0.034	65.4
<2010	4	0.90 (0.65–1.25)	11.17	0.011	73.2
Gender					
Male	4	0.84 (0.64–1.09)	10.47	0.015	71.3
Female	3	0.91 (0.76–1.08)	2.08	0.353	3.8
Participants, *n*					
≥100,000	3	0.97 (0.82–1.14)	6.66	0.036	70.0
<100,000	5	0.88 (0.68–1.14)	11.84	0.019	66.2
Cases, *n*					
≥1,000	2	1.00 (0.86–1.18)	4.53	0.033	77.9
<1,000	6	0.86 (0.68–1.08)	12.76	0.026	60.8

No., number; RR, relative risk; CI, confidence interval.

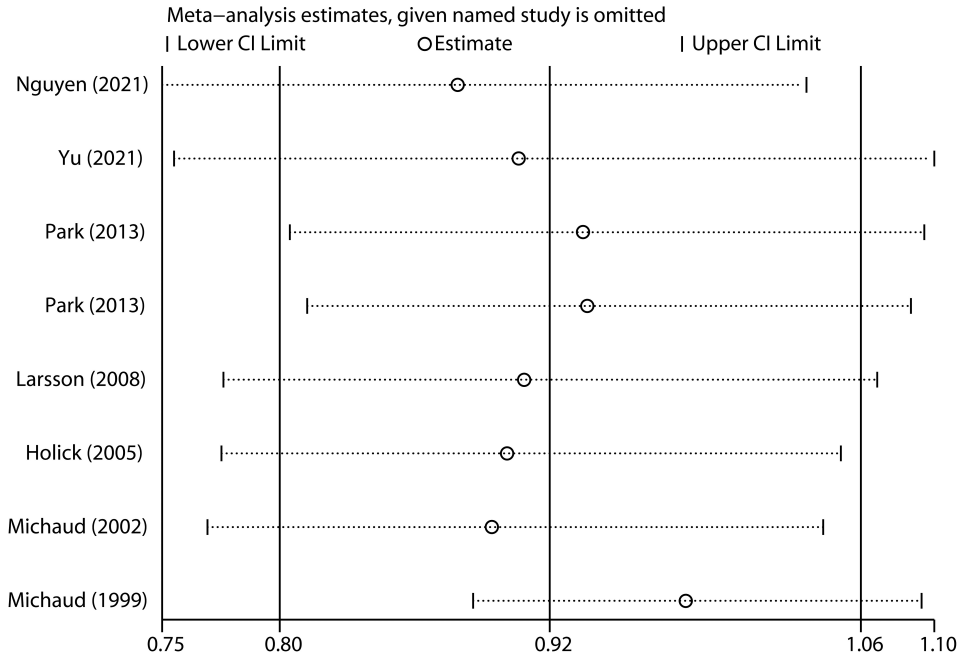

FIGURE 4
Sensitivity analysis of included studies.

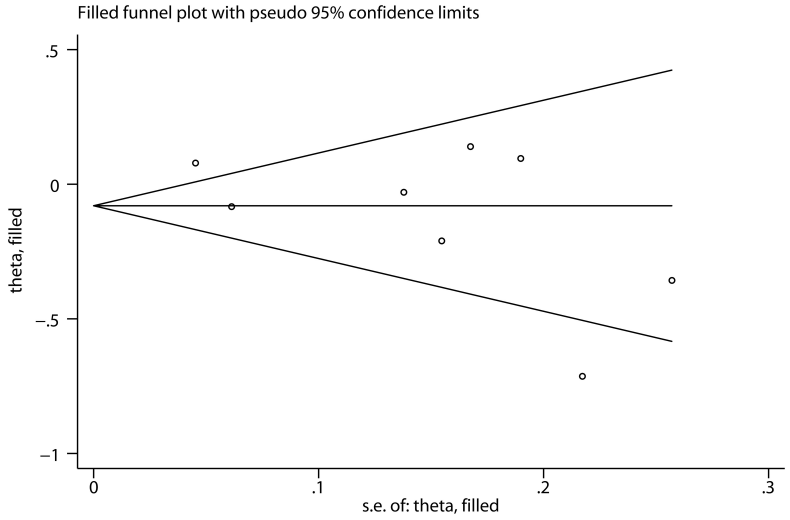

FIGURE 5
Publication bias as assessed by Begg's test.

vegetables intake was not significantly associated with the risk of bladder cancer, with consistent findings from the dose-response analysis and subgroup analysis.

A previous meta-analysis, published in 2013 by Liu et al. (28), exploring the association between cruciferous vegetables intake and bladder cancer risk was based on five cohort and five case-control studies. In the analysis of highest vs. lowest levels, cruciferous vegetables intake was significantly associated with a lower bladder cancer risk (RR 0.80, 95% CI 0.69–0.92). However, in a subgroup analysis of cohort studies the significant association did not hold, with a RR of 0.86 (95% CI, 0.61–1.11) which was pretty similar with our findings. Besides, the earlier meta-analysis did not examine the dose-response relationship and the sample size was relatively limited. By contrast, our meta-analysis included the recent studies, which further increased the sample size and improved the statistical power. In addition, only

prospective studies were included in our study, which avoided the select or recall bias from case-control studies.

Cruciferous vegetable intake has been associated with multiple health outcomes. Recently, Li et al. (9) performed an umbrella review of 41 systematic reviews and meta-analyses of 303 observational studies. It revealed that cruciferous vegetable intake might have beneficial effects on several outcomes, including gastric cancer, lung cancer, endometrial cancer, and all-cause mortality. Similarly, another umbrella review of meta-analyses and systematic reviews reported that consumption of cruciferous vegetable was associated with a reduced risk of death from any cause, cancers, and depression (29). The inverse association between cruciferous vegetable intake and mortality was also supported by a large prospective cohort study with a median follow-up of 16.9 years. HR (95% CIs) for all-cause mortality in the highest compared to the lowest quintile was 0.86 (0.80–0.93) for men ($P = 0.0002$ for trend) and 0.89 (0.81–0.98) for women ($P = 0.03$ for trend) (30).

Previously several mechanisms have been proposed to explain the potential relationship between dietary cruciferous vegetables intake and bladder cancer risk. Sulforaphane, an isothiocyanate, presents naturally in cruciferous vegetables and acts as a chemopreventive agent (31). Sulforaphane plays an important role in ROS (reactive oxygen species) and ROS-related pathways, which are associated with the initiation and progression of bladder cancer (32). He et al. (33) found that the inhibitory effect of Sulforaphane on bladder cancer cells also depends on GSH (glutathione, r-glutamyl cysteingl + glycine) depletion induced by Nrf2 translocation. Xia et al. (34) reported that Sulforaphane suppressed non-muscle-invasive bladder cancer cells proliferation through inhibition of HIF-1α-mediated glycolysis in hypoxia. Benzyl isothiocyanate, another isothiocyanate presented in cruciferous vegetables, also has been reported to prevent bladder cancer progression by suppressing IGF1R, FGFR3, and mTOR (35). Abbaoui et al. (36) proposed that cruciferous vegetable isothiocyanates, including sulforaphane (SFN) and erucin (ECN), may suppress bladder carcinogenesis *via* epigenetic modulation of gene expression associated with histone H1 phosphorylation. Although various isothiocyanates from cruciferous vegetables have been proved to exert a promising anticancer effect from a substantial amount of scientific research, our study, as well as many previous epidemiological studies, did not support that cruciferous vegetable intake was associated with the bladder cancer risk.

Our meta-analysis has several strengths. First, the present study had large sample size and statistical power and only prospective studies were included. Second, the methodological quality of the included studies was generally high as assessed by NOS. Third, both categorical analysis and dose-response analysis were performed with consistent results, indicating that the findings were robust and sound. However, several limitations should also be noted. First, the number of eligible studies was still limited and no Asian studies were available. Second, although no significant publication bias was detected as assessed either by Begg's test or Egger's test, some publication bias may still exist as small studies with null results were less likely to be published. Third, the methods of cruciferous vegetables assessment and the cut-off points used differed across the included studies, which might distort the pooled results. Finally, a significant heterogeneity was observed among the included studies, which may weaken the robustness of the conclusion.

In summary, the results of the current study did not support the hypothesis that dietary cruciferous vegetables intake was associated with a lower risk of bladder cancer. Further large prospective cohort studies are still warranted to confirm our preliminary findings.

Data availability statement

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

Author contributions

PY and YL contributed to the conception or design of the work and drafted the manuscript. PY, LY, and YL contributed to the acquisition, analysis, and interpretation of data for the work. YL critically revised the manuscript. All authors gave final approval and agreed to be accountable for all aspects of work ensuring integrity and accuracy.

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.2022.944451/full#supplementary-material

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Hormetic response to B-type procyanidin ingestion involves stress-related neuromodulation via the gut-brain axis: Preclinical and clinical observations

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B-type procyanidins, a series of catechin oligomers, are among the most ingested polyphenols in the human diet. Results of meta-analyses have suggested that intake of B-type procyanidins reduces cardiovascular disease risk. Another recent focus has been on the effects of B-type procyanidins on central nervous system (CNS) function. Although long-term B-type procyanidin ingestion is linked to health benefits, a single oral intake has been reported to cause physiological alterations in circulation, metabolism, and the CNS. Comprehensive analyses of previous reports indicate an optimal mid-range dose for the hemodynamic effects of B-type procyanidins, with null responses at lower or higher doses, suggesting hormesis. Indeed, polyphenols, including B-type procyanidins, elicit hormetic responses *in vitro*, but animal and clinical studies are limited. Hormesis of hemodynamic and metabolic responses to B-type procyanidins was recently confirmed in animal studies, however, and our work has linked these effects to the CNS. Here, we evaluate the hormetic response elicited by B-type procyanidins, recontextualizing the results of intervention trials. In addition, we discuss the possibility that this hormetic response to B-type procyanidins arises via CNS neurotransmitter receptors. We have verified the direction of future research for B-type procyanidins in this review.

KEYWORDS

B-type procyanidin, hormesis, sympathetic nervous system (SNS), central nervous system, hemodynamics, stress

Introduction

B-type procyanidins are characterized by a series of heteropolyflavan-3-ols, with a single interflavan bond between carbon-4 of the B-ring and either carbon-8 or carbon-6 of the C-ring (1–3). B-type procyanidins can be categorized by their degree of polymerization: monomers form linkages leading to oligomers. The most common

monomeric unit is (−)-epicatechin, and the C4–C8 bond (**Figure 1A**) is the most prominent. B-type procyanidins containing 2–7 monomeric units are defined as oligoprocyanidins which are abundant in cocoa (4–6), apples (7, 8), grape seeds (9, 10), and red wine (11–13).

Results of meta-analyses have suggested that intake of foods rich in B-type procyanidins is linked to reduced risk for cardiovascular disease, including coronary heart disease, myocardial infarction, and stroke (14–20). Randomized controlled trials and subsequent meta-analyses have confirmed that dark chocolate containing large amounts of B-type procyanidins can mitigate states related to the metabolic syndrome, including hypertension (21–23), dyslipidemia (24, 25), and glucose intolerance (25, 26). In addition, the latest large-scale randomized trial found a 27% reduction in cardiovascular death by ingestion of cocoa flavanol fraction, which is rich in B-type procyanidin monomer and oligomers, for 3.6 years (27). Recent studies have focused on the benefit of B-type procyanidin ingestion for the central nervous system (CNS). A few intervention trials have reported that B-type procyanidin might be effective in improving cognitive function (28–31).

Almost all B-type procyanidins ingested in food move into the colon, and some are degraded by the microbiome (32–34). Consequently, changes in the gut microbiome induced by ingestion of B-type procyanidins for a comparatively long period may alter the composition of metabolites in the colon (32, 35–38). One hypothesis is that these colon changes associated with gut microbiota contribute to the beneficial effects of B-type procyanidins.

Acute physiological changes have been reported to follow a single intake of foods rich in B-type procyanidins. These changes are related to hemodynamics (39–43), metabolism (44, 45), the autonomic nervous system (46), and cognitive function or cerebral blood flow (28, 47–54). These findings highlight the need to evaluate the acute and chronic physiological effects of B-type procyanidin ingestion.

In addition, the acute hemodynamic changes following ingestion of B-type procyanidin, such as flow-mediated dilation (FMD), do not show a monotonic dose response (55). Instead, these physiological changes follow a pattern of hormesis, with peak benefit at mid-range doses and less benefit at higher or lower doses. Comprehensive analyses of many earlier findings suggest that there is likely a mid-range optimal dose for the effects of B-type procyanidins on hemodynamics.

Polyphenols, including B-type procyanidins, elicit hormetic responses in cell culture (56–59). Cellular proliferation occurs at relatively low concentrations, but cytotoxicity is detected at high concentrations (60). *In vivo* animal and human dose-response findings for B-type procyanidins are relatively limited. Recently, however, results from animal studies confirmed that hemodynamics and metabolism show a hormetic dose-response to B-type procyanidin, and we found that these changes arise through sympathetic nerve activation, driven by CNS activation. Here, we review data from human intervention trials supporting a hormesis pattern of response to B-type procyanidins. Furthermore, we discuss the possibility that B-type procyanidins elicit this response via neurotransmitter receptors expressed in the CNS.

Hormesis

Bioactive compounds are expected to yield a monotonic dose-dependent response in terms of efficacy or toxicity (**Figure 1B**). In some cases, however, the pattern is characterized by an inverted U-shaped dose-response (**Figure 1C**). This pattern of hormesis can also reflect an adaptive response. For example, exposure to low amounts of a substance or stressor can induce resistance to higher doses of the same trigger. This exposure to mild levels of harmful factors can precondition a cell or an organism, inducing activation of stress resistance pathways and expanding maintenance and repair capacities (61, 62).

As an example, a moderate exercise program yields various benefits, such as decreased risk of cardiovascular disease, stronger bones and skeletal muscle, and longevity. An overly intense exercise program, however, can lead to harmful effects (63), so that the response to exercise "dose" shows a hormetic pattern. Exercise-related enhancement of cognition and mood also shows a hormetic response (64) that is reported to relate closely to adult hippocampal neurogenesis (65). Furthermore, moderate physical activity is generally accepted to be associated with cardiovascular (66–68) and metabolic benefits through sympathetic nervous system (SNS) (69–71). Recent evidence also links CNS plasticity to the effects of moderate exercise on SNS activity (72).

Polyphenols elicit a hormetic response in cell culture (73); curcumin (74), resveratrol (75) and B-type procyanidins (76). The hormesis effect of other polyphenols also has been confirmed *in vitro*, and these activities are considered to arise from modulation of a number of redox-based signaling pathways. Abundant evidence thus supports a hormesis effect of polyphenols in *in vitro* studies, but limited data illustrate these effects *in vivo*.

Hormetic response to B-type procyanidin in intervention and animal studies

As mentioned above, repeated ingestion of B-type procyanidins is reported to reduce the risk of cardiovascular diseases. Besides numerous intervention trials have been examined following the single ingestion of foods rich in B-type procyanidins. Regarding hemodynamics, a single ingestion

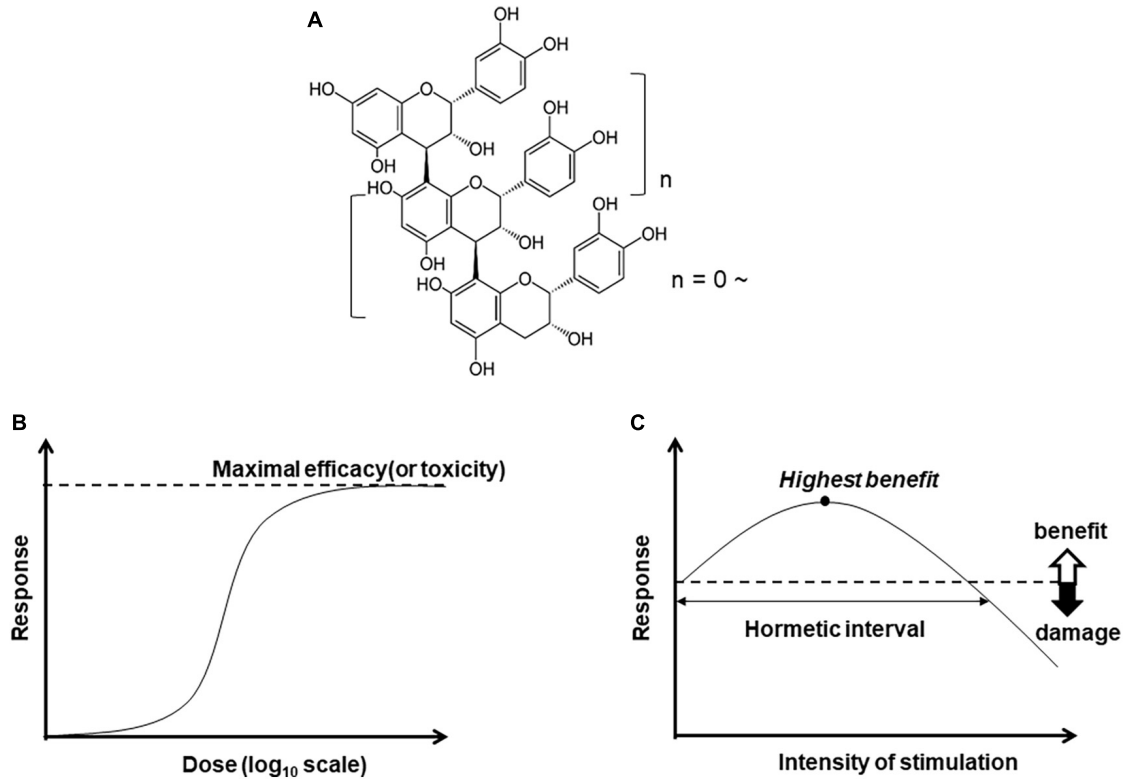

FIGURE 1
Chemical structure of B-type procyanidin (C4–C8) bond, **(A)**, and dose-response curves: **(B)** monotonic and **(C)** hormesis.

was associated with increased FMD at about 2 h following ingestion (23). These results indicated that a single treatment of B-type procyanidin might improve vascular endothelial cell function. Sun et al. assessed the dose-response pattern of human endothelial function to B-type procyanidin in cocoa (55). They concluded that cocoa flavanols could significantly improve endothelial function, with an optimal dose of about 710 mg. They also observed a non-linear association (inverted U-shape) between cocoa flavanols and FMD. There were no notable adverse effects in the intervention studies using 1.4 times (1,008 mg) or 1.76 times (1,248 mg) the effective dose (710 mg) shown by Sun et al. (55). Since these intervention studies used cocoa drinks or chocolate as the test food, it was limited intake amount. Therefore, the toxicity of type B-type procyanidins may not detect.

In addition, results of a recent intervention trial indicate that repeated supplements of B-type procyanidins are associated with improvement on memory tasks that depend on dentate gyrus functions (52). Intervention trials have been conducted in young adults, examining the effects on a memory task of a single ingestion of cocoa flavanols at doses from 172 to 994 mg (77). In almost all cases, cocoa flavanols were associated with enhanced working memory or mood and reduced fatigue, but evidence of dose-response in CNS studies is limited.

These results, taken together, suggest that a hormetic physiological response following a single intake of B-type procyanidins is likely. Studies of other polyphenols, such as curcumin and resveratrol, are too limited to allow for interpretations regarding dose response (78).

Repeated oral gavage with 10 mg/kg body weight (bw) of cocoa flavanol in rats resulted in significantly decreased blood pressure and markedly increased aortic endothelial nitric oxide synthase expression (eNOS), indicating this dose as optimal (79). On the other hand, a single oral administration of 10 mg/kg cocoa flavanol, resulted in a transient increase in mean blood pressure (BP) and heart rate (HR), along with a marked increase in blood flow in the cremaster muscle arteriole soon after treatment. A significant increase in eNOS phosphorylation was also observed in aorta dissected 60 min after this treatment. Similar but weaker alterations were observed at a dose of 1 mg/kg cocoa flavanol but 100 mg/kg cocoa flavanol did not trigger any changes in hemodynamics or eNOS phosphorylation.

We also compared B-type procyanidins such as the monomer [(-)-epicatechin; EC], dimer (procyanidin B2; B2), trimer (procyanidin C1; C1), and tetramer (cinnamtannin A2; A2) on hemodynamics (80). At a dose of 10 μg/kg, A2 and B2 were associated with a marked increase in cremasteric arteriole

blood flow, C1 was linked to a slight increase, and EC did not trigger any change. Based on these findings, a relative efficacy of B-type procyanidins on hemodynamics was suggested as follows: A2 > B2 > > C1 > > > EC (**Figure 2**). A dose-response study of A2 showed increased blood flow with a single dose of 10 μg/kg, but not with a dose of 100 μg/kg. In our dose-response study for A2 induction of thermogenic uncoupling protein (UCP)-1 expression in brown adipose tissue (BAT), we found that a single oral dose at 1 μg/kg was associated with significantly increased UCP-1 mRNA expression (**Figure 2**), but more than 1 μg/kg A2 (10 to 1,000 μg/kg) did not show any change (81).

Taken together, the results of animal studies of cocoa flavanol or the B-type procyanidins are consistent with those of intervention studies following a single intake of food rich in B-type procyanidin. The implication is that this polyphenol elicits an inverted U-shaped dose-response.

Target organ of B-type procyanidins from the perspective of bioavailability

B-type procyanidins show poor bioavailability, and intact forms in foods are hardly present in the blood (82). For this reason, how these polyphenols exert beneficial effects remains unclear. Recent studies suggest that the physiological changes following repeated B-type procyanidins ingestion may be related to alterations in gut microflora and/or their metabolites, but the mechanism for changes arising immediately after a single dose is unclear. Considering that most B-type procyanidins are present in the feces, the target organ of them is the gastrointestinal tract, including the oral cavity.

Single doses of B-type procyanidins do not draw a monotonic dose-response, and benefits are seen at the mid-range doses but not at lower or higher doses. Among various pharmacological agents, those that support social interactions or memory are reported to show biphasic reactions (83) and enhance memory (84). A single oral ingestion of cocoa flavanol has been also reported to improve cognition and mood in intervention studies. As noted, the primary target organ of B-type procyanidins appears to be the digestive tract, but activation of the CNS may be crucial to the mechanism of action.

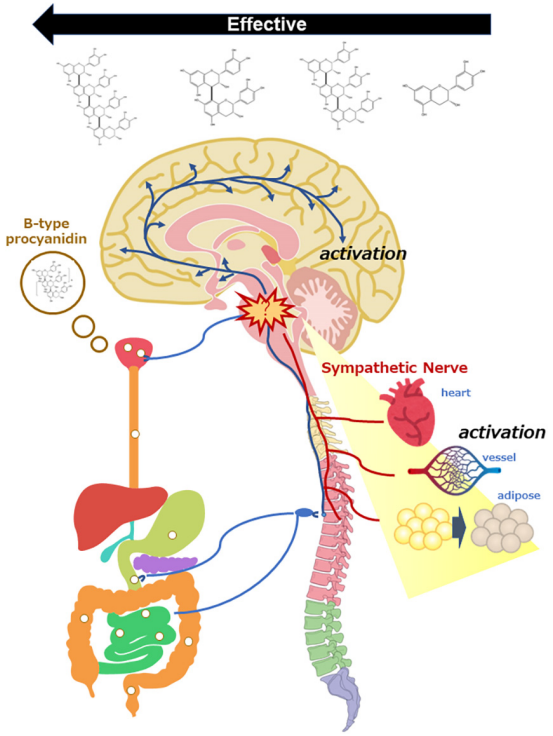

FIGURE 2
Scheme of sympathetic nerve activation by oral administration of B-type procyanidins, consequently activation of the central nervous system and peripheral organs.

Sympathetic nerve activation by B-type procyanidins

A single oral optimal dose of cocoa flavanol triggers an increase in blood flow in the cremaster arteriole soon after treatment in rats (85). Such a rapid response likely does not depend on absorption or distribution in the blood. The SNS is a well-known regulator of hemodynamic reflection, exerting its influence through adrenergic receptors (AdR) expressed in the myocardium, vascular smooth muscle, and vasomotor center in the medulla oblongata (86). Activation of myocardial β1 AdR, which are expressed predominantly in cardiac tissue, causes increased cardiac output and HR. Activation of the α1 AdR in vascular smooth muscle contracts blood vessels, leading to elevated BP (**Figure 3**) (87). For this reason, we used adrenaline blockers to examine whether the SNS is involved in the hemodynamic changes induced by B-type procyanidins. We found that a transient increase in HR caused by an optimal dose of cocoa flavanol could be markedly decreased by co-treatment with a β1 AdR blocker in rats. In addition, co-treatment with an α1 blocker inhibited the transient elevation in BP that a single oral dose of cocoa flavanol induced.

Sympathetic nervous system also regulates non-shivering thermogenesis through β3 AdR in BAT via UCP-1 (88). We have found that UCP-1 mRNA upregulation in BAT after an optimal dose of cocoa flavanol is markedly attenuated by co-administration of β3 blocker. These results implicate the SNS in the acute hemodynamic and metabolic changes following a single oral dose of B-type procyanidins.

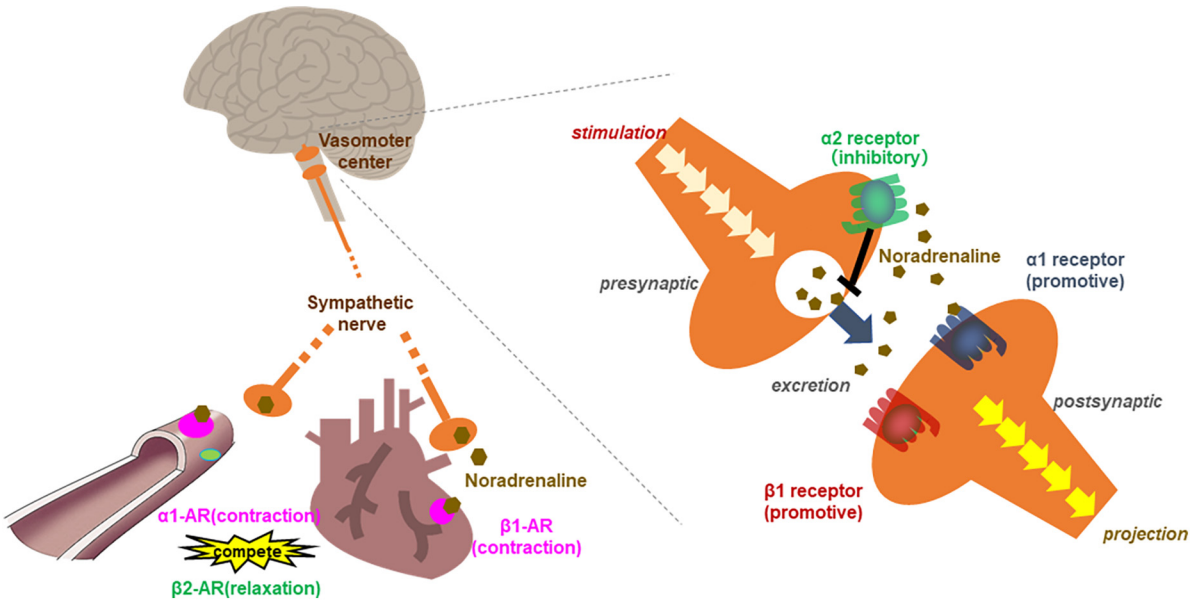

FIGURE 3
Scheme of the relationship between adrenergic receptors in the circulation system. β1 adrenergic receptors contracts the myocardium and increased cardiac output and heart rate. α1 adrenergic receptor in vascular smooth muscle contracts blood vessels, leading to elevated blood pressure. While β2 receptor relaxes vascular smooth muscle as a competitive effect against α1 adrenergic receptors. In the vasomotor center, presynaptic neurons release noradrenaline (NA) into the synaptic cleft upon stimulation. Noradrenaline binds the activating receptors of the postsynaptic neuron. NA also binds inhibitory autoreceptors of the presynaptic neurons, consequently, inhibited NA release from postsynaptic neurons.

In hormesis, effects at high doses can be less than effects at optimal doses. We evaluated the hormetic pattern of response to B-type procyanidins *in vivo* and found that a single oral administration of 10-fold the optimal dose of cocoa flavanol in rats yielded no transient hemodynamic alterations (79). In addition, as noted, an optimal dose of cocoa flavanol increased UCP-1 mRNA expression in BAT, but this change was markedly dampened at doses 10-fold the optimal level (89). Based on our findings linking the hemodynamic and thermogenic effects of B-type procyanidins to SNS activation, we focused on why optimal dose elicit these effects but not high doses.

Blood pressure and heart rate are regulated competitively by inhibitory and activating AdR. Activation of the β2 receptor relaxes vascular smooth muscle as a competitive effect against the vasoconstrictive action of α1 AdR and thus decreases BP (**Figure 3**) (87). In our co-administration study with a high dose of B-type procyanidins and α1 blocker in rats, although, we found no changes in BP (79). Besides, inhibitory α2 AdR, which are expressed in the preganglionic sympathetic fibers and vasomotor center in the CNS, down-regulate the SNS. Yohimbine is an α2 blocker that is reported to be more effective in CNS than SNS. Given this pattern, we conducted a co-administration study with a high dose of cocoa flavanol (100 mg/kg) and yohimbine. A single high dose of cocoa flavanol alone elicited no change in BP, but BP increased markedly and transiently by co-administered with yohimbine. Similar results were observed co-administration of B-type procyanidin tetramer A2 (100 μg/kg) and yohimbine (80).

As mentioned above, whereas a single oral dose of 1 μg/kg of A2 significantly increased UCP-1 mRNA expression in BAT, doses from 10 to 1,000 μg/kg A2 did not (81). In contrast, co-administration of a high dose (100 μg/kg) of A2 and yohimbine markedly increased UCP-1 mRNA expression. A recent report suggested that the premotor neurons controlling thermogenic effector activation lie primarily within the medullary rostral raphe pallidus (90). Non-shivering thermogenesis through the β3 receptor is inhibited by α2AR activation in this region (91).

α2 AdR are present on noradrenergic terminals in the peripheral nervous system and the CNS (92). α2 adrenergic autoreceptors lie in the presynaptic membrane of adrenergic neurons, inhibiting exocytosis of their neurotransmitters (mostly noradrenaline) as part of a negative feedback loop (93, 94) (**Figure 3**). Feedback inhibition of noradrenaline release from sympathetic nerves by α2-autoreceptors limits its exocytosis and protects heart under normal conditions (95). The reduced hemodynamic and metabolic output at a high dose of B-type procyanidins observed in our previous studies may cause activation of autoreceptor α2. Thus, SNS deactivation may be induced by a high dose of B-type procyanidins.

Stress and hormetic response to B-type procyanidins

The relationship between stress and hormetic responses is well known. Various factors induce the stress response, which involves rapid activation of the sympathetic–adreno–medullar (SAM) axis and the hypothalamus–pituitary–adrenal (HPA) axis (96). In the SAM, rapid physiological adaptation mediated mainly by noradrenaline results in transient responses, such as alertness, and appraisal of the situation, enabling a strategic decision. Sympathetic modulations induced by stressors rely on direct projections from the paraventricular nucleus of the hypothalamus (PVN), locus coeruleus, and rostral ventrolateral medulla to pre-ganglionic sympathetic neurons present in the dorsal intermediolateral cellular column of the spinal cord (97). As a result, noradrenaline is secreted from sympathetic nerve terminals, leading to activation of signaling pathways that evoke changes in blood vessels, glands, visceral organs, and smooth muscle. Considering the previous results following a single oral administration of B-type procyanidins, these changes may be induced by activation of the SNS.

The PVN, which also has a role in eliciting activation of the HPA, synthesizes oxytocin, vasopressin, and corticotropin-releasing hormone (CRH), depending on the target (98). CRH excreted from the PVN to the anterior pituitary induces release of adrenocorticotropic hormone, which drives the responses associated with release of cortisol (corticosterone in rodents) from the adrenal gland in the hours following stress. When blood cortisol exceeds a certain level, it exerts negative feedback on the hypothalamic release of CRH and the pituitary release of adrenocorticotropic hormone (99). Activation of these pathways results in adaptive conditions that mediate long-term memories of the experience. Therefore, HPA activation induced by optimal stress has a strong positive effect on memory, cognition, and stress resilience (100).

If the outcome following a single dose of B-type procyanidins arises as stress response, HPA activation is expected to occur at the same time as sympathetic hyperactivity. Therefore, we examined the activation of HPA following a single dose of B-type procyanidins. In mouse PVN, the optimal dose of cocoa flavanol (10 mg/kg bw) markedly upregulated CRH mRNA, as detected by *in situ* hybridization, 240 min after administration. A dose of 50 mg/kg cocoa flavanol also showed similar alterations 60 min after administration, with a significant elevation in plasma corticosterone (101). In addition, CRH mRNA in mouse PVN was increased significantly 60 min after administration of an optimal dose of A2 (10 μg/kg), and a similar change only 15 min after administration of a 10-fold oral dose of A2 (102). Few reports have described the relationship between stress intensity and the duration of response, but our results suggested that the reaction is faster with exposure to more severe stress. Taken together, these findings indicate that stimulation with an oral dose of B-type procyanidin might be a stressor for mammals, resulting in SNS activation (**Figure 2**).

Conclusion

Various stressors such as radiation, reactive oxygen species, calorie restriction, temperature, chemicals, and exercise elicit hormetic responses (103). Hormesis and the underlying biochemical pathways induced by the stressors confer protection against a range of pathological or aging processes (62). In this review, we especially focused on the hormetic alterations induced by B-type procyanidins, which are electrophilic compounds that easily cause redox reactions. The relationship between CNS activation and the chemical characteristics of B-type procyanidins remains unclear and requires further clarification. B-type procyanidins or related compounds may contribute to the beneficial effects of eating fruits and vegetables through hormetic responses induced by neuromodulation.

Author contributions

TF and YF collected the sources and drafted this manuscript. NO constructed the conception and finally approved of this 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.

Publisher's note

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Botanical aspects, phytochemicals, and toxicity of *Tamarindus indica* leaf and a systematic review of antioxidant capacities of *T. indica* leaf extracts

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Oxidative stress is a condition occurs when there is the imbalance between prooxidants and free radicals. It involves in cellular metabolism, aging, and immune response. Recently oxidative stress has been proved about its beneficial roles in human body. However, long term oxidative stress and high concentration of free radicals can lead to negative effects on organs, systems, and physiological conditions. Prooxidant or antioxidant, therefore, is one of the most important choices for the prevention of these anomaly. *Tamarindus indica* is a medicinal plant that has been reported as a source of antioxidants. The plants' leaves possess antioxidant effects according to many studies. However, these results have not yet been systematically summarized. The present systematic review summarizes and discusses about the *in vitro* antioxidant capacities of *T. indica* leaves. The plants' description and morphology, elements and phytochemical constituents, total phenolic and flavonoids contents and toxicity are also summarized and discussed here.

KEYWORDS

***Tamarindus indica*, antioxidant, *in vitro*, toxicity, phytochemicals, botanical aspects**

Introduction

Oxidative stress refers to the imbalance between the production of free radicals in the body and the capability of cells and tissues to clear them (1). Free radicals are generated from endogenous and exogenous sources by enzymatic and non-enzymatic reactions. They play crucial roles in human health. Free radicals, such as nitric oxide radical ($NO^{\bullet}$)

and superoxide radical ($O_2^{\bullet -}$), are involved in the defense mechanism to fight pathogens, the syntheses of some cellular structures, and cellular signaling pathways. In addition, they control blood flow by being cell-to-cell messengers, and they are required for non-specific host defense and induction of a mitogenic response (1). Thus, regular exposure to free radicals is one of the cellular homeostasis. Despite their benefits, free radicals can also contribute to the anomaly by being pro-oxidant. Long-term and high concentrations of free radicals are undesirable phenomenon (2). Oxidative stress occurs when there are excessive and rising levels of free radicals and oxidants in the body. Uncontrolled conditions lead to health problems and eventually increase the risk of metabolic, chronic, and degenerative diseases, such as cardiovascular diseases, neurodegenerative disorders, nephropathy, inflammation and immune-related diseases, sexual maturation and fertility disorders, and cancers (1, 3–7).

Oxidative stress is caused by excessive oxidants and a lack of antioxidants. Antioxidants refer to compounds able to impede or retard the oxidation of a substrate, acting at a lower concentration compared with that of the protected substrate (8). Antioxidants can be both endogenous and exogenous substances, similar to oxidants. Endogenous antioxidants are classified as enzymatic and non-enzymatic antioxidants. Exogenous antioxidants are introduced to the body in the form of a diet, and they act as oxidative defenses through different mechanisms and in different cellular compartments (6). Antioxidants such as vitamin C and E, coenzyme Q10, zinc and selenium, and polyphenols are sometimes inadequately consumed through routine diets. They have therefore sometimes been applied in the forms of dietary supplements or additive substances in foodstuffs.

Phytochemicals that are well-known as antioxidants are polyphenols, vitamins, carotenoids, minerals, and organosulfur compounds (9). There is plenty of research looking for the sources of powerful antioxidants due to their promising benefits for health from either their preventive or treatment perspectives. Thus, many plants that contain the aforementioned phytochemicals have been examined for their antioxidant activities and developed as sources of natural exogenous antioxidants.

Abbreviations: AAE, Ascorbic acid equivalent; ABTS, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); BCB, β-carotene bleaching; BHA, Butylated hydroxy anisole; BHT, Butylated hydroxytoluene; BW, Body weight; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DW, Dried weight; EDTA, Ethylene diamine tetraacetic acid; FE, Ferrous equivalent; FIC, Ferrous ion chelating; FRAP, Ferric ion reducing antioxidant power; GAE, Gallic acid equivalent; H_2O_2, Hydrogen peroxide; IC_{50}, Half maximal inhibitory concentration; N/A, Data not available; NO, Nitric oxide; $O_2^{\bullet -}$, Superoxide radical; QE, Quercetin equivalent; ROS, Reactive oxygen species; RUE, Rutin equivalent; SD, Standard deviation; TE, Trolox equivalent; Temp, Temperature; T_{room}, Room temperature.

Tamarindus indica L. (Fabaceae, Caesalpinioideae), or tamarind, is a tropical plant native to Africa. The plant has long been used as a food and herbal medicine. Its fruit pulp is well-known as a good source of vitamins, minerals, and organic acids. Tamarind fruit possesses several pharmacological activities, such as antifungal, antiasthmatic, hepatoprotective, and wound healing activities (10–13). Moreover, other parts of this plant, such as its leaf, stem bark, root bark, and seed, have also been reported as medicaments, e.g., antibacterial, antihyperlipidemic, antiulcer, anticancer, antifungal, wound healing, hepatoprotective and immunopotentiation agents (10, 11, 13–19).

There are various chemical constituents in *T. indica*. The fruit pulps contain furan derivatives, carboxylic acid, phlobatannin, grape acid, apple acid, flavonoids, pectin, sugars, and the like (20, 21). The seeds contain campesterol, β-amyrin, β-sitosterol, fatty acids, tannins, sugars, mucilage and polysaccharides, cardiac glycosides, and phenolics, among others (20, 22, 23). The components of the bark include tannins, saponins, glycosides, peroxidase, and lipids (20). The leaves contain orientin, iso-orientin, vitexin, iso-vitexin, glycosides, peroxidase, vitamin B_3, and vitamin C (20, 23). Polyphenols, e.g., flavonoids and phenolics, are present in almost every part of the plant, making *T. indica* an up-and-coming source of antioxidative agents.

In this review article, we describe original research on the antioxidant activities of *T. indica*, focusing on antioxidant effect of its leaves obtained from the *in vitro* experiments (antioxidant capacity) (24). The description and morphology of *T. indica*, major chemical constituents especially phenolic compounds and flavonoids, and toxicity of *T. indica* leaves were also summarized and discussed. *T. indica* leaf extracts possessed antioxidant capacity by free radicals scavenging, heavy metal chelating and transition. Total phenolic and total flavonoids contents might relate to antioxidant capacity of *T. indica* leaves. The elements in *T. indica* leaves might also be responsible for the antioxidant capacity. No toxic was reported from the using of *T. indica* leaves either *in vitro* or *in vivo* experiments. The limitations of this study are lack of *in vivo* antioxidant activity assay, standard compounds were applied in only some included studies, no specific chemical was reported as biomarker and no quantification analysis of active compounds was conducted, maturity level of raw materials used in the included studies was reported in only one study.

Methods

Data sources and search strategy

Two authors (SSa and SS) independently searched electronic databases (EMBASE, PubMed, Scopus, Thai Journal Online Database, Thai Thesis Database, Science Direct, and Clinical

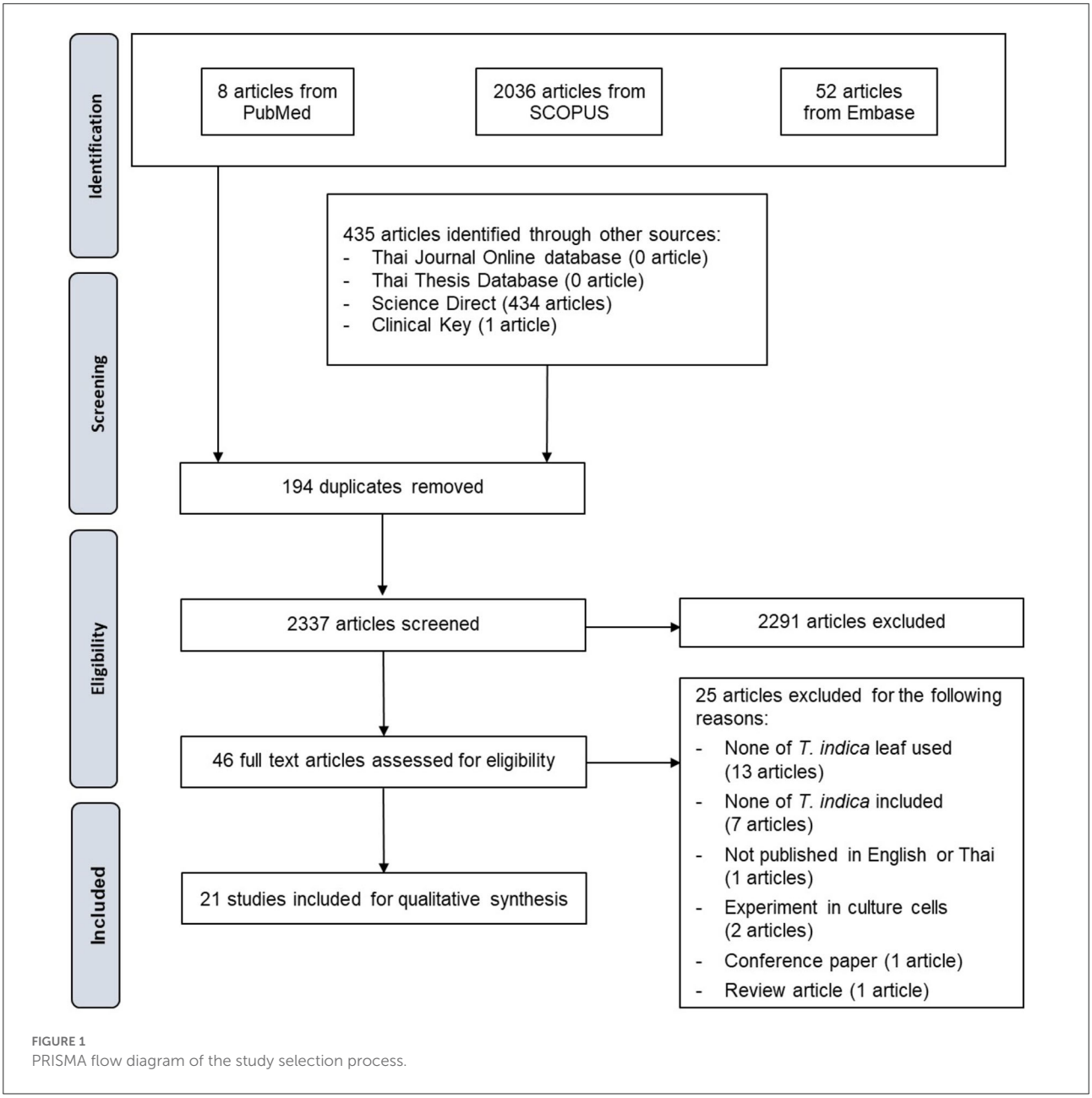

FIGURE 1
PRISMA flow diagram of the study selection process.

Key). Relevant articles were searched from inception to April 2022. The strategic search terms were "*Tamarindus indica*" AND [("leaves") OR ("leaf")] AND "antioxidant." We also searched references in literature reviews and manuscripts published in journals. No limitations were placed on language or study design. In addition, we contacted the related researchers and experts for details and explanations of the articles.

Study selection

The studies included in this systematic review were selected according to the PRISMA guideline (Figure 1). After searching for articles, we removed duplicates, screened titles, and abstracts, and obtained the full texts of each article. We included research classified as (1) studies of the antioxidant capacity of *T. indica* leaves and (2) studies reporting measured outcomes (antioxidant capacity). A bibliographic search was then performed to identify articles from conference proceedings for which the full text was available. We excluded articles whose data had been obtained from prior studies. Accepted articles were included in this systematic review. Two investigators independently conducted the assessments. Twenty-one research articles from 7 databases were included. In all studies, 10 assays were used for the determination of antioxidant capacity. Five studies reported the results of phytochemical screening tests and elemental analyses.

Seventeen studies revealed the quantity of total phenolics and total flavonoids, which are the major compounds responsible for *T. indica's* antioxidant capacity.

Outcome measures

The primary outcome of interest was measures of the antioxidant capacity of *T. indica* leaves. The secondary outcome was the total phenolic or total flavonoid content of *T. indica* leaves, and the correlation between the total phenolic or total flavonoid content of *T. indica* leaves and their antioxidant capacities, if applicable.

Data extraction

Two investigators independently reviewed each abstract and its associated full text. Each investigator also extracted data from each study for inclusion in the analysis. Data extraction was performed on study designs (part used, extract used, method and assay, and outcomes) and quality of studies Risk of bias was assessed using SciRAP with adaptation as a tool (25). The aspects of funding and competing interests were not focus in this study. In the report quality assessment, 1 item of test compound and controls, 2 item of test system, 3 items of administration of test compound, and 3 items of data collection and analysis were evaluated. In the methodological quality assessment, 3 items, 1 item, 1 item, and 3 items in the same aspects were evaluated, respectively. The results were reported as fulfilled, partially fulfilled, not fulfilled, and not determined. The latter was selected if the data was not available. Discrepancies were resolved by consensus.

Data synthesis and analysis

The statistical heterogeneity was analyzed using I^2 and X^2 tests. Percentage I^2 was identified based on the following equation: $I^2 = 100\%\ (Q\text{-}df)/Q$, where Q is Cochran's heterogeneity statistic and *df* is the degree of freedom. The heterogeneity was determined as "might not be important," "may represent moderate heterogeneity," "may represent substantial heterogeneity," and "considerable heterogeneity" by the ranges of 0–40, 30–60, 50–90, and 75–100%, respectively (26). For the X^2 test, a *P*-value of <0.1 (significant) was used to assess heterogeneity.

Results

Study selection

In all, 2,960 identified studies were systematically searched, and 435 studies were identified through other sources (434 from Science Direct and 1 from Clinical Key). No articles were identified through the Thai Journal Online database or the Thai Thesis Database. After 194 duplicates were removed, 2,033 studies remained. Of these, 2,291 were discarded based on a review of their titles and abstracts. Forty-six articles were then assessed for eligibility. Twenty-five were discarded (13 for none of *T. indica* leaf used, 7 for none of *T. indica* included, 2 for experiment conducted in cultured cells, 1 for not published in English or Thai, 1 conference paper, and 1 review article), leaving 21 for inclusion in the qualitative analysis (Figure 1). In the quantitative analysis, the included data had high levels of heterogeneity. The I^2 values of each data set classified as antioxidant capacity assay and outcome measure were all higher than 75% (92.0–99.4%). Therefore, a meta-analysis was not conducted.

Study characteristics

The characteristics of all 21 studies are summarized in Table 1. Risk of bias were shown in Figure 2. The results obtained from the evaluation of 4 aspects e.g., test compound and controls, test system, administration of test compound, and data collection and analysis. The plant materials were *T. indica* leaves with different pretreatments and untreated leaves before the extraction was manipulated. Fresh leaves were used in 9 studies, while the other studies used dried leaves that had been oven dried, air dried, shade dried or stir fried. The extraction solvents used were acetone, ethyl acetate, hexane, methanol, ethanol, and water. The extraction methods varied between studies. They were maceration, Soxhlet extraction, hot extraction, fluid extraction, cold percolation, and fresh preparation using a mortar and pestle. Five studies did not report their extraction method. Ten assays were used to determine the antioxidant capacity of *T. indica* leaf extracts. Even though the outcome measures differed between studies, the 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) radical scavenging and ferric ion reducing antioxidant power (FRAP) assays were the most commonly employed methods. Other assays used were metal chelating (ferrous [Fe^{2+}] ion chelating [FIC]), nitric oxide ($NO^{\bullet}$) radical scavenging, total antioxidant capacity (phosphomolybdenum), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical scavenging, hydrogen peroxide (H_2O_2) scavenging, superoxide radical ($O_2^{\bullet-}$) scavenging, hydroxyl radical ($HO^{\bullet}$) scavenging, and β-carotene bleaching (BCB) assays. No sample concentration or dose was provided in 2 studies (34, 45). The standard positive controls used in the assays were the universal antioxidants, i.e., ascorbic acid, quercetin, rutin, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT). However, a positive control was not determined in some studies. Phytochemical screening tests were conducted in 4 studies (Table 2). The total polyphenol and/or total flavonoid contents of *T. indica* leaf extracts were established in 17 studies,

TABLE 1 Study characteristics of the *in vitro* antioxidant capacity of *T. indica* leaf extracts.

References	Plant material	Solvent	Extraction method	Antioxidant capacity assay	Dose/ concentration	Outcome (unit)	Results (mean ± SD)	Remarks
Choudhary and Swarnkar (27)	Air-dried leaves (Temp: T_{room})	Methanol	Maceration	DPPH• radical scavenging	1,000 μg/ml	Scavenging capacity (%)	16.80*	Positive control: BHT = 68.20
				$O_2^{\bullet-}$ radical scavenging	1,000 μg/ml	Anion scavenging capacity (%)	31.86 ± 3.11	Positive control: BHT = 81.19 ± 3.43
Gomathi et al. (28)	Air-dried leaves (Shade dried) (Temp: N/A)	Acetone	Soxhlet extraction	DPPH• radical scavenging	N/A	IC_{50} (μg/ml)	171.00 ± 2.40	Positive control: BHT = 37.80 ± 0.80 BHA = 29.00 ± 1.20
		Methanol	Soxhlet extraction				124.70 ± 2.10	
		Water	Maceration				283.10 ± 1.10	
		Acetone	Soxhlet extraction	HO• radical scavenging	N/A	IC_{50} (μg/ml)	66.60 ± 2.10	Positive control: BHT = 7.80 ± 2.70 BHA = 12.30 ± 4.30
		Methanol	Soxhlet extraction				46.90 ± 2.20	
		Water	Maceration				79.20 ± 1.50	
		Acetone	Soxhlet extraction	FIC	N/A	Ferrous ion chelating capacity (mg EDTA Equivalent/g extract)	71.50 ± 0.60	Positive control: BHT = 143.07 ± 1.80 BHA = 192.10 ± 2.30
		Methanol	Soxhlet extraction				79.70 ± 1.20	
		Water	Maceration				64.30 ± 2.40	
		Acetone	Soxhlet extraction	BCB	250 μg	Peroxidation inhibitory capacity (%)	48.30 ± 0.70	Positive control: BHT = 67.8 ± 0.7 BHA = 80.9 ± 1.8

(Continued)

10.3389/fnut.2022.977015

TABLE 1 (Continued)

References	Plant material	Solvent	Extraction method	Antioxidant capacity assay	Dose/ concentration	Outcome (unit)	Results (mean ± SD)	Remarks
		Methanol	Soxhlet extraction				17.50 ± 0.10	
		Water	Maceration				11.30 ± 2.10	
Razali et al. (29)	Air-dried leaves (Temp: N/A)	Methanol	Maceration	DPPH$^{\bullet}$ radical scavenging		Antioxidant capacity (mmol TE/g dried weight)	3.17 ± 0.00	Positive control: Rutin = 3.32 ± 0.00 Quercetin = 3.60 ± 0.00
		Ethyl acetate	Maceration		25–100 μg/ml		2.76 ± 0.03	
		Hexane	Maceration				1.35 ± 0.04	
		Methanol	Maceration	FRAP	N/A	Ferric reducing capacity (mmol/g dried weight)	1.87 ± 0.09	Positive control: Rutin = 3.36 ± 0.003 Quercetin = 13.30 ± 0.002
		Ethyl acetate	Maceration				0.57 ± 0.9	
		Hexane	Maceration				0.12 ± 0.07	
		Methanol	Maceration	ABTS$^{\bullet+}$ radical scavenging	100–2,000 μg/ml	Antioxidant capacity (mmol TE/g dried weight)	1.65 ± 0.04	Positive control: Rutin = 1.72 ± 0.01 Quercetin = 4.18 ± 0.03
		Ethyl acetate	Maceration				0.70 ± 0.01	
		Hexane	Maceration				0.51 ± 0.03	
		Methanol	Maceration	$O_2^{\bullet-}$ radical scavenging	25–400 μg/ml	Anion scavenging capacity (mmol TE/g dried weight)	4.64 ± 0.003	Positive control: Rutin = 5.47 ± 0.01 Quercetin = 5.67 ± 0.004
		Ethyl acetate	Maceration				4.54 ± 0.14	
		Hexane	Maceration				3.99 ± 0.01	
Krishnaveni et al. (30)	Fresh leaves	Water	N/A	FRAP	Equivalent to 10 mg fresh leaves	Antioxidant capacity (mg AAE/g extract)	2.25*	

(Continued)

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TABLE 1 (Continued)

References	Plant material	Solvent	Extraction method	Antioxidant capacity assay	Dose/ concentration	Outcome (unit)	Results (mean ± SD)	Remarks
				FIC	Equivalent to 10 mg fresh leaves	Ferrous ion chelating capacity (mg EDTA Equivalent/g extract)	3.50*	
				NO• radical scavenging	Equivalent to 10 mg fresh leaves	Antioxidant capacity (mg QE/g extract)	1.22*	
				Total antioxidant capacity	Equivalent to 10 mg fresh leaves	Total antioxidant capacity (mg AAE/g extract)	29.40*	
Krishnaveni et al. (31)	Fresh leaves	Water	N/A	FRAP	N/A	Antioxidant capacity (mg AAE/g extract)	2.45*	
				FIC	N/A	Ferrous ion chelating capacity (mg EDTA Equivalent/g extract)	4.70*	
				NO• radical scavenging	N/A	Antioxidant capacity (mg QE/g extract)	1.10*	
				Total antioxidant capacity	N/A	Total antioxidant capacity (mg AAE/g extract)	27.30*	
Meher and Dash (32)	Air-dried leaves (Shade dried) (Temp: N/A)	Water	Hot extraction	DPPH• radical scavenging	50–500 μg/ml	IC50 (μg/ml)	346.63*	Positive control: Ascorbic acid = 56.70
		Ethanol	Maceration				301.83*	
		Water	Hot extraction	HO• radical scavenging	50–500 μg/ml	IC_{50} (μg/ml)	346.63*	Positive control: Ascorbic acid = 56.70
		Ethanol	Maceration				292.04*	

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10.3389/fnut.2022.977015

TABLE 1 (Continued)

References	Plant material	Solvent	Extraction method	Antioxidant capacity assay	Dose/ concentration	Outcome (unit)	Results (mean ± SD)	Remarks
		Water	Hot extraction	FRAP	500 μg/ml	Reducing power (FRAP value)	0.33 ± 0.03	Positive control: Ascorbic acid = 2.00
		Ethanol	Maceration				0.76 ± 0.08	
		Water	Hot extraction	NO• radical scavenging	50-500 μg/ml	IC_{50} (μg/ml)	339.35*	Positive control: Ascorbic acid = 77.31
		Ethanol	Maceration				279.90*	
Raghavendra et al. (33)	Air-dried leaves (Shade dried for 1 week) (Temp: N/A)	Methanol	Soxhlet extraction	DPPH• radical scavenging	N/A	IC_{50} (μg/ml)	210.00*	Positive control: Ascorbic acid = 6.80
				$ABTS^{\bullet+}$ radical scavenging	N/A	IC_{50} (μg/ml)	35.00*	Positive control: Ascorbic acid = 13.70
				Total antioxidant capacity	100-500 μg/ml	Total antioxidant capacity (μg/ml AAE)	72.00*	
Kaewnarin et al. (34)	Oven-dried leaves (Temp: 50°C) (Young leaves)	Ethyl acetate	Maceration	DPPH• radical scavenging	N/A	Inhibitory capacity (%)	23.40 ± 1.80	Positive control: N/A
		Ethanol	Maceration				17.60 ± 1.10	
Krishnaveni et al. (35)	Fresh leaves	Water	N/A	FRAP	N/A	Antioxidant capacity (mg AAE/g extract)	≈7.50–9.00	
				FIC	N/A	Antioxidant capacity (mg AAE/g extract)	≈4.50–5.50	
				NO• radical scavenging	N/A	Antioxidant capacity (mg QE/g extract)	≈7.00–11.50	
				Total antioxidant capacity	N/A	Total antioxidant capacity (mg AAE/g extract)	≈3.00–7.00	

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TABLE 1 (Continued)

References	Plant material	Solvent	Extraction method	Antioxidant capacity assay	Dose/ concentration	Outcome (unit)	Results (mean ± SD)	Remarks
Krishnaveni et al. (36)	Fresh leaves	Water	N/A	FRAP	N/A	Antioxidant capacity (mg AAE/g extract)	7.30*, 2.32*, 8.60*	Raw materials were obtained from 3 different sources
				FIC	N/A	Antioxidant capacity (mg AAE/g extract)	5.12*, 2.70*, 7.22*	Raw materials were obtained from 3 different sources
				NO• radical scavenging	N/A	Antioxidant capacity (mg QE/g extract)	8.68*, 6.90*, 13.80*	Raw materials were obtained from 3 different sources
				Total antioxidant capacity	N/A	Total antioxidant capacity (mg AAE/g extract)	5.60*, 6.76*, 6.08*	Raw materials were obtained from 3 different sources
Krishnaveni et al. (37)	Fresh leaves	Water	N/A	FRAP	N/A	Antioxidant capacity (mg AAE/g extract)	3.10 ± 0.05	
				FIC	N/A	Antioxidant capacity (mg AAE/g extract)	2.60 ± 0.27	
				NO• radical scavenging	N/A	Antioxidant capacity (mg QE/g extract)	4.60 ± 0.38	
				Total antioxidant capacity	N/A	Total antioxidant capacity (mg AAE/g extract)	2.50 ± 0.10	
Escalona-Arranz et al. (38)	Air-dried leaves	Water	Fluid extraction	DPPH• radical scavenging	N/A	IC_{50} (μg/ml)	44.36 ± 3.72	Positive control: Quercetin = 10.88 ± 0.81
				FRAP	N/A	IC_{50} (μg/ml)	60.87 ± 1.07	Positive control: Quercetin = 21.94 ± 0.80
				FIC	N/A	Estimated binding constant (mol/l)	1.09*	Positive control: Quercetin = 2.000

(Continued)

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TABLE 1 (Continued)

References	Plant material	Solvent	Extraction method	Antioxidant capacity assay	Dose/ concentration	Outcome (unit)	Results (mean ± SD)	Remarks
Krishnaveni et al. (39)	Fresh leaves	Water	Fresh preparation using mortar and pestle	FRAP	Equivalent to 10 mg fresh leaves	Antioxidant capacity (mg AAE/g extract)	2.81 ± 0.49	
				FIC	Equivalent to 10 mg fresh leaves	Antioxidant capacity (mg AAE/g extract)	3.33 ± 0.63	
				NO• radical scavenging	Equivalent to 10 mg fresh leaves	Antioxidant capacity (mg QE/g extract)	4.83 ± 2.45	
				Total antioxidant capacity	Equivalent to 10 mg fresh leaves	Total antioxidant capacity (mg AAE/g extract)	3.40 ± 1.12	
				H_2O_2 scavenging	Equivalent to 10 mg fresh leaves	H_2O_2 scavenging capacity (%)	2.13 ± 0.45	
Krishnaveni et al. (40)	Fresh leaves	Water	Fresh preparation using mortar and pestle	FRAP	Equivalent to 10 mg fresh leaves	Antioxidant capacity (mg AAE/g extract)	2.95 ± 0.08	
				FIC	Equivalent to 10 mg fresh leaves	Antioxidant capacity (mg AAE/g extract)	2.90 ± 0.34	
				NO• radical scavenging	Equivalent to 10 mg fresh leaves	Antioxidant capacity (mg QE/g extract)	3.41 ± 0.57	
				Total antioxidant capacity	Equivalent to 10 mg fresh leaves	Total antioxidant capacity (mg AAE/g extract)	0.98 ± 0.20	
				H_2O_2 scavenging	Equivalent to 10 mg fresh leaves	H_2O_2 scavenging capacity (%)	3.00 ± 0.48	
Krishnaveni et al. (41)	Fresh leaves	Water	Fresh preparation using mortar and pestle	FRAP	Equivalent to 10 mg fresh leaves	Antioxidant capacity (mg AAE/g extract)	3.00 ± 0.86	

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10.3389/fnut.2022.977015

TABLE 1 (Continued)

References	Plant material	Solvent	Extraction method	Antioxidant capacity assay	Dose/ concentration	Outcome (unit)	Results (mean ± SD)	Remarks
				FIC	Equivalent to 10 mg fresh leaves	Antioxidant capacity (mg AAE/g extract)	4.03 ± 0.98	
				NO• radical scavenging	Equivalent to 10 mg fresh leaves	Antioxidant capacity (mg QE/g extract)	3.55 ± 0.25	
				Total antioxidant capacity	Equivalent to 10 mg fresh leaves	Total antioxidant capacity (mg AAE/g extract)	2.10 ± 0.08	
				H_2O_2 scavenging	Equivalent to 10 mg fresh leaves	H_2O_2 scavenging capacity (%)	4.05 ± 0.66	
Kumar et al. (42)	Air-dried leaves (Shade dried)	Methanol	Cold percolation	DPPH• radical scavenging	50 μg/ml	Scavenging capacity (%)	28.58 ± 1.14	Positive control: Ascorbic acid = 96.50 ± 0.19
					100 μg/ml		39.43 ± 0.77	Positive control: Ascorbic acid = 96.45 ± 0.11
					200 μg/ml		61.70 ± 1.90	Positive control: Ascorbic acid = 96.67 ± 0.17
					300 μg/ml		77.36 ± 1.07	Positive control: Ascorbic acid = 96.25 ± 0.17
					400 μg/ml		87.56 ± 1.17	Positive control: Ascorbic acid = 96.25 ± 0.17
					500 μg/ml		91.39 ± 1.22	Positive control: Ascorbic acid = 96.49 ± 0.16
Leng et al. (43)	Fresh leaves	Methanol	Maceration	DPPH• radical scavenging	Equivalent to 2 mg fresh leaves	Inhibitory capacity (%)	16.458 ± 1.53	Positive control: N/A
	Oven-dried leaves (At 60°C for 3 h)	Methanol	Maceration		Equivalent to 2 mg oven-dried leaves		39.028 ± 0.25	Dose: Positive control: N/A

(Continued)

10.3389/fnut.2022.977015

TABLE 1 (Continued)

References	Plant material	Solvent	Extraction method	Antioxidant capacity assay	Dose/ concentration	Outcome (unit)	Results (mean ± SD)	Remarks
	Stir fried leaves (stir fried using kitchen stove at 180°C for 10 min)	Methanol	Maceration		Equivalent to 2 mg stir fried leaves		69.923 ± 0.11	Positive control: N/A
Muddathir et al. (44)	Air-dried leaves (Shade dried) (Temp: T_{room})	Methanol	Maceration	FRAP	1,000 μg/ml	Ferric reducing ability of plasma (mM FE/mg dried weight)	2.71 ± 0.06	Positive control: Quercetin = 3.96 ± 0.11 Ascorbic acid = 3.79 ± 0.10 BHT = 2.84 ± 0.03
Alrasheid et al. (45)	Air-dried leaves (Temp: N/A)	Ethanol	Maceration	$DPPH^{\bullet}$ radical scavenging	N/A	Scavenging capacity (%)	61.66*	Positive control: Ascorbic acid = 93.5
Chigurupati et al. (46)	Air-dried leaves (Mature and healthy leaves) (Shade dried)	Ethanol	Maceration	$DPPH^{\bullet}$ radical scavenging	1,000 μg /ml	IC_{50} (μg/ml)	1.42 ± 0.3	Positive control: Ascorbic acid = 1.09 ± 0.02
				$ABTS^{\bullet+}$ radical scavenging	1,000 μg/ml	IC_{50} (μg/ml)	1.62 ± 0.66	Positive control: Ascorbic acid = 1.02 ± 0.03
Ouédraogo et al. (47)	Air-dried leaves (Shade dried) (Temp: T_{room})	Water	Maceration	$DPPH^{\bullet}$ radical scavenging	3,750 μg/ml	Antioxidant capacity (μmol AAE/g extract)	360.02 ± 7.23	Positive control: Quercetin = 646.00 ± 0.00
				FRAP	100 μg/ml	Antioxidant capacity (μmol AAE/g extract)	677.26 ± 24.53	Positive control: Quercetin = 6034.64 ± 12.05
				$ABTS^{\bullet+}$ radical scavenging	100 μg/ml	Antioxidant capacity (μmol AAE/g extract)	7067.58 ± 0.00	Positive control: Quercetin = 14550.26 ± 281.08

AAE, ascorbic acid equivalent; ABTS, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); BCB, β-carotene bleaching; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EDTA, ethylene diamine tetraacetic acid; FE, ferrous equivalent; FIC, ferrous ion chelating; FRAP, ferric reducing antioxidant power; H_2O_2, hydrogen peroxide; N/A, data not available; NO, nitric oxide; QE, quercetin equivalent; SD, standard deviation; TE, Trolox equivalent; Temp, temperature; T_{room}, room temperature; * no SD available.

10.3389/fnut.2022.977015

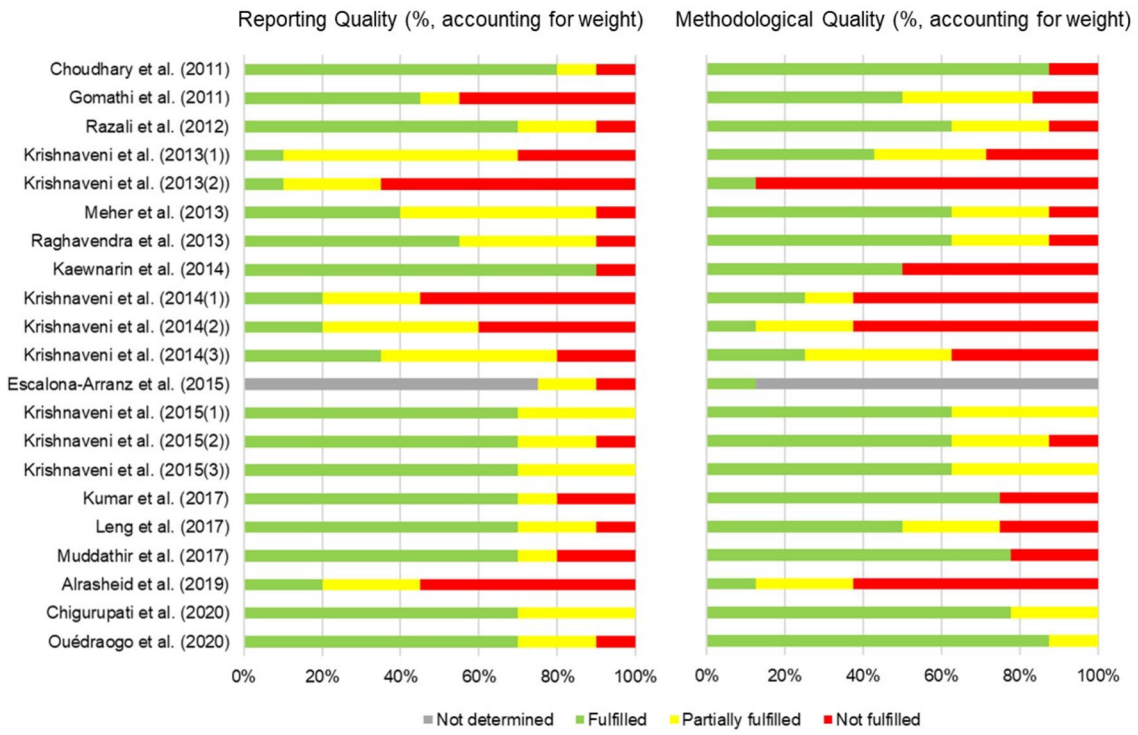

FIGURE 2
Risk of bias summary assessment of included studies. The bars represent the reporting quality and methodological quality of individual studies resulting from the average of the quality of (1) test compound and controls (2) test system (3) administration of test compound and (4) data collection and analysis. The evaluation used SciRAP with adaptation as a tool (25).

TABLE 2 Phytochemical screening of crude *T. indica* leaf extracts.

Phytochemicals	Raghavendra et al. (33)	Kumar et al. (42)	Alrasheid et al. (45)	Chigurupati et al. (46)	
	Methanolic extract	Methanolic extract	Ethanolic extract	Ethanolic extract	Methanolic extract
Flavonoids	–	N/A	–	N/A	N/A
Alkaloids	+	+	+/–	+	+
Tannins	N/A	N/A	+/–	+	+
Saponins	N/A	–	+	+	+
Steroids	–	N/A	+	+	+
Terpenoids	N/A	N/A	–	N/A	N/A
Coumarin	N/A	N/A	–	N/A	N/A
Glycosides	+	N/A	+/–	+	+
Phenolics	–	+	N/A	N/A	N/A
Monosaccharides	N/A	N/A	N/A	+	+
Carbohydrates	N/A	+	+	+	+
Reducing sugars	N/A	N/A	–	–	–
Non-reducing sugars	N/A	N/A	N/A	–	–
Amino acids	N/A	–	N/A	–	–
Proteins	N/A	–	N/A	+	+
Mucilage and gums	N/A	N/A	N/A	+	+
Lignins	N/A	N/A	+	N/A	N/A

+, positive; –, negative; +/–, negative and positive presented in different tests; N/A, data not available.

TABLE 3 Total polyphenol and total flavonoid contents of *T. indica* leaf extracts.

References	Total phenolic content	Total flavonoid content	Remarks
Choudhary and Swarnkar (27)	4.72 ± 0.08 mg GAE/g DW methanolic extract	1.06 ± 0.08 mg QE/g DW of methanolic extract	N/A
Gomathi et al. (28)	33.10 ± 4.00 mg GAE/g acetone extract 26.80 ± 2.10 mg GAE/g methanolic extract 16.01 ± 1.60 mg GAE/g aqueous extract	74.10 ± 1.10 mg QE/g acetone extract 24.30 ± 2.30 mg QE/g methanolic extract 7.30 ± 5.20 mg QE/g aqueous extract	Regression correlation coefficient: Total phenolic content with antioxidant capacity (r^2) in assays • DPPH$^•$ scavenging = 0.211 • HO$^•$ scavenging = 0.580 • Metal chelating = 0.720 • BCB = 0.482
Razali et al. (29)	309.00 ± 3.78 mg GAE/g methanolic extract 101.00 ± 12.26 mg GAE/g ethyl acetate extract 31.8 ± 3.70 mg GAE/g hexane extract	N/A	Regression correlation coefficient: Total phenolic content with antioxidant capacity (r) in assays • FRAP = 0.8899 • DPPH$^•$ scavenging = 0.8849 • ABTS$^{•+}$ scavenging = 0.8264
Krishnaveni et al. (30)	1.10 mg GAE/g water extract	9.70 mg QE/g water extract	N/A
Krishnaveni et al. (31)	0.10 mg GAE/g water extract	3.00 mg QE/g water extract	N/A
Raghavendra et al. (33)	20.00 mg GAE/g methanolic extract	410.00 mg QE/g methanolic extract	N/A
Kaewnarin et al. (34)	0.29 ± 0.00 mg GAE/g ethyl acetate extract	130.00 ± 3.90 mg QE/g ethyl acetate extract	Pearson correlation coefficient (r): • Total phenolic content with DPPH$^•$ scavenging capacity = 0.866 • Total flavonoid content with DPPH$^•$ scavenging capacity = 0.583
	0.15 ± 0.00 mg GAE/g ethanolic extract	69.30 ± 1.70 mg QE/g ethanolic extract	Pearson correlation coefficient: • Total phenolic content with DPPH$^•$ scavenging capacity = 0.779 • Total flavonoid content with DPPH$^•$ scavenging capacity = 0.796
Krishnaveni et al. (35)	≈5.00–5.50 mg GAE/g water extract	≈3.50–5.50 mg QE/g water	N/A
Krishnaveni et al. (36)	6.70 mg GAE/g water extract	8.00 mg QE/g water extract	N/A
Krishnaveni et al. (37)	6.10 ± 0.40 mg GAE/g water extract	6.60 ± 0.30 mg QE/g water extract	N/A
Krishnaveni et al. (39)	3.53 ± 2.02 mg GAE/g water extract	5.93 ± 2.36 mg QE/g water extract	N/A
Krishnaveni et al. (40)	7.23 ± 2.36 mg GAE/g water extract	2.20 ± 0.00 mg QE/g water extract	N/A
Krishnaveni et al. (41)	4.63 ± 2.19 mg GAE/g water extract	4.16 ± 0.05 mg QE/g water extract	N/A
Leng et al. (43)	39.31 ± 1.34 mg GAE/g methanolic extract of fresh leaves 47.74 ± 1.78 mg GAE/g methanolic extract of oven-dried leaves 139.87 ± 2.22 mg GAE/g methanolic extract of stir fried leaves	N/A	Regression correlation coefficient: Total phenolic content with antioxidant capacity (r^2) = 0.877
Muddathir et al. (44)	31.26 ± 0.38 mg GAE/g methanolic extract	N/A	N/A

(Continued)

TABLE 3 (Continued)

References	Total phenolic content	Total flavonoid content	Remarks
Chigurupati et al. (46)	1.80 mg GAE/g ethanolic extract (maceration) 1.01 mg GAE/g ethanolic extract (Soxhlet extraction)	1.44 mg RUE/g ethanolic extract (maceration) 1.04 mg RUE/g ethanolic extract (Soxhlet extraction)	N/A
Ouédraogo et al. (47)	202.40 ± 1.50 mg GAE/g water extract	99.00 ± 1.20 mg QE/g water extract	N/A

DW, dried weight; GAE, gallic acid equivalent; N/A, data not available; QE, quercetin equivalent; RUE, rutin equivalent.

and 4 investigations reported correlations between antioxidant contents and antioxidant capacities (Table 3).

Antioxidant capacity of *T. indica* leaves

A summary of the antioxidant capacities of *T. indica* leaf extracts is given in Table 1. Approximately 10 assays were used to determine antioxidant capacity. In each assay, some studies determined the antioxidant capacity using the same measurement, while some other investigations used different methods. The results obtained from each method are summarized in the following section.

DPPH (DPPH$^{\bullet}$) radical scavenging

The DPPH$^{\bullet}$ radical scavenging assay is a free radical scavenging antioxidant assay. The principle of the method is the reaction between antioxidant and an organic radical. The method has high sensitivity. The results are comparable to those of other free radical scavenging assays and are reproducible. The assay can be applied for the quantitative analysis of complex biological samples. Another advantage of DPPH$^{\bullet}$ radical scavenging assays is correlation with bioactive compounds (phenols, flavonoids) with regression factor (R) > 0.8. Although the DPPH$^{\bullet}$ radical scavenging assay can be performed easily, the DPPH$^{\bullet}$ radical is a synthetic radical that cannot represent the i*n vivo* system (48). More than this, the levels of antioxidants needed for scavenging these radicals are not physiologically possible nor relevant.

To determine the DPPH$^{\bullet}$ radical scavenging capacity of *T. indica* leaves, acetone, methanol, water, and ethanol were used to extract the air-dried leaves of *T. indica*. In the study of Gomathi et al. (28), it was found that acetone extract had better antioxidant capacity than methanol and water extracts (IC_{50} values of 171.00, 124.70, and 283.10 μg/ml, respectively). These values correlate with the finding of Meher and Dash (32) that ethanolic extract was more potent than water extract (IC_{50} values of 301.83 and 346.63 μg/ml, respectively). Aqueous extracts were used in the studies by Gomathi et al. (28), Meher and Dash (32), and Escalona-Arranz et al. (49). They reported that fluid extraction gave the highest antioxidant effect compared with maceration and hot extraction techniques. Ethanolic extracts obtained from maceration by Meher and Dash (32) and Chigurupati et al. (46) expressed IC_{50} values of 5.3- and 1.3-fold that of ascorbic acid, respectively, as a positive control. The methanolic extract obtained from cold percolation extraction by Kumar et al. (42) exhibited scavenging capacities of 28.6–91.4% in a concentration-dependent manner (50–500 μg/ml). The macerated-aqueous extract produced by Ouédraogo et al. (47) gave antioxidant capacity equivalent to ascorbic acid 360.0 mg/g extract.

A study by Leng et al. (43) compared the difference between raw material pretreatment methods before extraction using methanol by the maceration technique. The results showed that the extract obtained from the stir-fried, oven-dried and fresh leaves offered 69.9, 39.0, and 16.5% inhibitory capacity, respectively (dose equal to 2 g fresh leaves). Pretreatment by oven-drying in Kaewnarin et al.'s (34) study showed that extraction using ethyl acetate offered higher inhibitory capacity than ethanol (23.4 and 17.0%, respectively).

Razali et al. (29) compared the antioxidant capacity of the extract obtained from air-dried leaves and maceration extraction using methanol, ethyl acetate, and hexane. It was found that the methanolic extract presented the highest capacity, followed by the ethyl acetate and hexane extracts [3.2, 2.8, 1.4 mmol Trolox equivalent (TE)/g dried weight, respectively].

ABTS (ABTS$^{\bullet+}$) radical scavenging

The ABTS radical scavenging assay is a free radical scavenging antioxidant assay based on the same principle as the DPPH$^{\bullet}$ scavenging assay. The assay also provides reproducible results and regression factor (R) > 0.8 with bioactive compounds (phenols, flavonoids). However, the limitation of the assay is that ABTS$^{\bullet+}$ radicals do not exist naturally; thus, the result cannot represent the *in vivo* system as well as a DPPH$^{\bullet}$ radical scavenging assay (48). More than this, the levels of antioxidants needed for scavenging these radicals are not physiologically possible nor relevant.

The ABTS$^{\bullet+}$ radical scavenging assay was performed in 4 studies. The IC_{50} values of the methanolic and ethanolic extracts

were 35.0 μg/ml (ascorbic acid, 13.7 μg/ml) and 1.6 μg/ml (ascorbic acid, 1.0 μg/ml), respectively (33, 46). The results of the study by Razali et al. (29) showed that the methanolic extract obtained from maceration expressed antioxidant capacity close to that of the standard compound rutin (1.65 vs. 1.72 mmol TE/g dried weight), as ethyl acetate and hexane extracts possessed lower capacities (0.7 and 0.5 mmol TE/g dried weight, respectively). The $ABTS^{\bullet+}$ radical scavenging capacity determined in Ouédraogo et al.'s (47) study using aqueous extract was 7067.6 μmol AAE/g extract, which can be calculated as half of the positive control, quercetin (14550.2 μmol AAE/g).

Superoxide ($O_2^{\bullet-}$) radical scavenging

The superoxide radical scavenging assay is the assessment of antioxidants' ability to prevent $O_2^{\bullet-}$ radical generation. The generation of $O_2^{\bullet-}$ radicals generally occurs in the normal respiratory process. The $O_2^{\bullet-}$ radical is then converted into H_2O_2, which is further converted into O_2 and water. The assay resembles free radical production and quenching in the human body, and it is superior to the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical scavenging assays (48).

The $O_2^{\bullet-}$ radical scavenging capacity of methanol, ethyl acetate, and hexane extracts of *T. indica* dried leaves was determined by Razali et al. (29). The results revealed that the radical scavenging capacity of the methanolic extract was better than that of the ethyl acetate and hexane extracts (4.6, 4.5, 4.0 mmol TE/g dried weight, respectively). These results correlated with those of $DPPH^{\bullet}$ radical scavenging capacity in the same study. The methanolic extract used in Choudhary and Swarnkar's (27) study showed a scavenging capacity of 31.9% at 1000 μg/ml.

Hydroxyl ($HO^{\bullet}$) radical scavenging

The hydroxyl radical is the most harmful reactive oxygen species (ROS) in the human body. It can lead to cell damage, cell apoptosis, and cell mutation by reacting with polyunsaturated fatty acid moieties. Hydroxyl ($HO^{\bullet}$) radical scavenging assays have been developed to determine lipid peroxidation in cells and tissues by $HO^{\bullet}$ radicals. The method was also used to measure the radical capacity of $HO^{\bullet}$ and antioxidants with slight modification. This method offers accurate results in most cases (48).

The $HO^{\bullet}$ radical scavenging capacity of *T. indica* leaf extracts was investigated in 2 studies. It was found that the air-dried leaf aqueous extracts obtained from maceration and hot extraction exhibited IC_{50} values of $HO^{\bullet}$ radical scavenging capacity of 79.2 and 346.6 μg/ml, respectively (Gomathi et al. (28), Meher and Dash (32)). The methanolic and acetone extracts showed better capacity, with IC_{50} values of 46.9 and 66.6 μg/ml, respectively, in the study of Gomathi et al. (28), similar to the ethanolic extract in the study of Meher and Dash (32) (IC_{50} = 292.0 μg/ml).

Ferric ion reducing antioxidant power

Ferric ion reducing antioxidant power is a reducing potential antioxidant assay. It is referred to as the ferric reducing ability of plasma. The FRAP assay is a method in which antioxidants react with a ferrous (Fe^{3+}) complex, ferric-tripyridyltriazine $[Fe^{III}(TPTZ)]^{3+}$, forming an intense blue-colored ferrous complex $[Fe^{II}(TPTZ)]^{2+}$ under acidic conditions (pH 3.6). The strengths of the assay are its high sensitivity and reproducibility, its applicability to a broad spectrum of samples, and the correlation (R) with the H_2O_2 scavenging assay is > 0.8 (50). The limitation of the method is its non-specificity (48).

The FRAP assay was determined in 13 studies. One of these studies, Meher and Dash (32), reported the capacity as the μM ferric ion reduced to ferrous form per ml (FRAP value). The values were 0.3 and 0.8 for 500 μg/ml ethanolic and aqueous extracts, respectively, compared with 2.0 for ascorbic acid (positive control). Escalona-Arranz et al. (49) revealed an IC_{50} of 60.9 μg/ml water extract, while quercetin, the positive control, was 21.9 μg/ml. The ferric reducing ability of plasma was determined in the study of Muddathir et al. (44) using a methanolic extract, and the antioxidant capacity was 2.7 mM (ferrous equivalent FE)/mg dried weight. Another study described the reducing power in terms of ferric reducing capacity. The results were 0.1, 0.6, and 1.9 mmol/g dried weight for hexane, ethyl acetate, and the methanolic extract, respectively (29). The other studies focused on the capacity on the ascorbic acid equivalent. Eight studies conducted by Krishnaveni et al. resulted in 2.3–8.3 mg AAE/g extract (30, 31, 35–37, 39–41). Ouédraogo et al. (47) reported a value of 667.26 μmol AAE/g extract, which was ∼10% of standard quercetin (6034.6 μmol AAE/g).

Ferrous ion chelation

The principle of the method is based on the oxidative stress caused by ROS originating from transition or heavy metals. Even if the method gives good reproducibility and repeatability, there are still limitations. They are (1) non-specific reactions (the assay not only reacts with phenolic compounds but also reacts with peptides and sulfate in the test medium); (2) the result obtained from the assay sometimes does not correlate with the total bioactive assays; and (3) poor correlation with FRAP, $DPPH^{\bullet}$, and $ABTS^{\bullet+}$ radical scavenging assays (48).

The results from FIC assays are summarized herein. Krishnaveni et al. (30) and Krishnaveni et al. (31) reported the ferrous ion chelating capacity of aqueous extracts as 3.5 and 4.7 mg ethylene diamine tetraacetic acid (EDTA) equivalent/g extract, respectively, which is very different from the study of Gomathi et al. (28) (64.3 mg EDTA equivalent/g extract). The

acetone and methanolic extracts tested by Gomathi et al. (28) gave approximate results to the aqueous extract. The ferrous ion-chelating capacity of the aqueous extract determined by Escalona-Arranz et al. (49) was lower than that of the positive control quercetin (estimated binding constant = 1.1 vs. 2.0 mol/l). The antioxidant capacities of aqueous extracts obtained from fresh leaves determined in 6 studies by Krishnaveni et al. as the equivalent to ascorbic acid were in the range of 2.5–5.5 mg ascorbic acid equivalent (AAE)/g extract (35–37, 39–41).

β-carotene bleaching

The β-carotene bleaching assay determines the bleaching capability of antioxidants on β-carotene. The oxidized linoleic acid in an emulsion system is set to generate free radicals, leading to oxidative destruction of β-carotene. The rate of oxidative destruction is measured. The method can be applied to both lipophilic and hydrophilic samples. Nevertheless, it has some limitations similar to FIC assays (48).

There was only one study that investigated the β-carotene/linoleic acid peroxidation inhibitory capacity of *T. indica* leaf extracts (28). In this study, 250 μg of acetone, methanolic, and water extracts were applied. The peroxidation inhibitory capacities of the extracts were in the range of 11.3%−48.3%.

Nitric oxide ($NO^{\bullet}$) radical scavenging

$NO^{\bullet}$ radical is found in vascular endothelial cells. The radical is generated from an amino acid, L-arginine. The $NO^{\bullet}$ radical plays a vital role in the human body, and an excessive quantity of $NO^{\bullet}$ radicals can lead to several health complications. A nitric oxide radical scavenging assay was developed to determine the capability of antioxidants to scavenge $NO^{\bullet}$ radicals (48).

There were 9 studies that observed the $NO^{\bullet}$ radical scavenging capacity of *T. indica* leaf extracts. The IC_{50} values of ethanolic and aqueous extracts were determined by Meher and Dash (32). They were 3.6- and 4.4-fold that of the standard ascorbic acid, respectively (279.9 and 339.3 μg/ml vs. 77.3 μg/ml) (32). The scavenging capacities in the remaining 8 studies were determined by Krishnaveni et al. The values were in the range of 1.1–7.0 mg quercetin equivalent (QE)/g extract (30, 31, 35–37, 39–41).

Total antioxidant capacity

The total antioxidant capacity, or phosphomolybdenum assay, is the determination of the antioxidant capacity of the antioxidant sample to reduce molybdenum (VI) to molybdenum (V) or the formation of a phosphomolybdenum complex. The method can be applied to a wide spectrum of samples, but there are several limitations as well. They are (1) non-specific, (2) poorly correlated with bioactive compounds, and (3) poorly correlated with the results obtained from the $DPPH^{\bullet}$ radical scavenging assay (48).

The assays were performed in 8 studies by Krishnaveni et al. and one study by Raghavendra et al. Methanolic extract of *T. indica* leaf showed a total antioxidant capacity of 72.0 μg/ml calculated as ascorbic acid equivalent (33), while aqueous extracts showed capacities of 0.98–29.4 mg AAE/g extract (30, 31, 35–37, 39–41).

Hydrogen peroxide (H_2O_2) scavenging

Hydrogen peroxide is a major oxygen metabolite generated *in vivo* by activated phagocytes and oxidase enzymes. The H_2O_2 scavenging capacity of antioxidants is assessed based on a peroxidase system (48).

The H_2O_2 scavenging capacity of *T. indica* leaf extracts was determined in 3 experiments. The aqueous extracts obtained from 3 works of Krishnaveni et al. possessed H_2O_2 scavenging capacities of 2.1–4.1% at a dose of 10 mg fresh leaves (39–41).

T. indica description and morphology

T. indica belongs to the Fabaceae family and the Caesalpinioideae subfamily. The plant is an indigenous tropical evergreen tree up to 30 m in height, with a spreading crown up to 12 m in diameter. Leaves are unipinnate compound, 15 cm long, with an alternate arrangement. Young leaves are light green and become darker while maturing. Each leaf is composed of 10 to 18 pairs of opposite leaflets along the central axis, which close at night. Leaflets are narrowly oblong and sized 12–32 × 3–11 mm. The flowers are borne on inflorescences up to ∼20 cm in length. The floret is 2.5 cm wide and has a caesalpiniaceous pattern, 4 sepals, and 5 petals (3 pale yellow petals with pinkish to red veins and 2 tiny thread-like petals). The fruits are pod- or legume-like, indehiscent, 10–18 × 4 cm, and straight or curved. The raw fruits are brown, and the fleshy inside is green-soft. Ripe fruits are brown with a soft and sticky pulp. There are 3 to 10 seeds, which are ∼1.6 cm long, irregularly shaped, testa hard, shiny, and smooth (Figure 3) (51, 56).

Phytochemistry of *T. indica*

Four out of the included studies provided the phytochemical screening results of the crude extract obtained from *T. indica* leaves (33, 42, 45, 46) (Table 2). Alkaloids were detected in ethanolic and methanolic extracts in all studies. Tannins, saponins, steroids, glycosides, monosaccharides, carbohydrates, mucilage, and gums were detected in both the methanolic and ethanolic extracts by Chigurupati et al. (46), while reducing

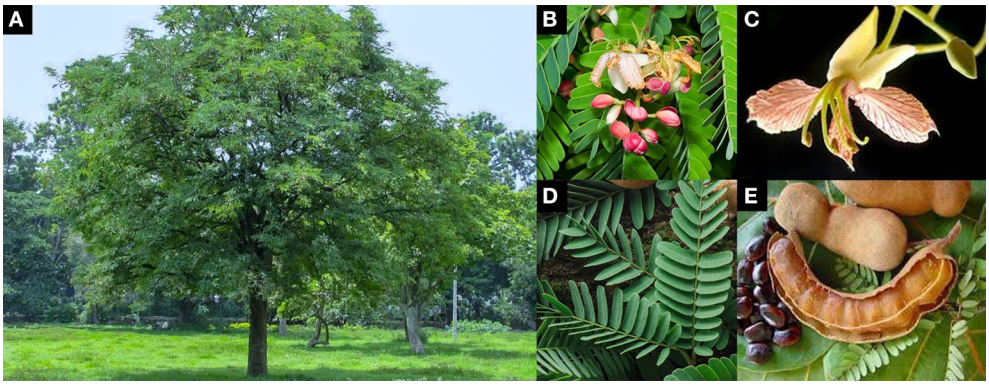

FIGURE 3
Characteristics of *T. indica* **(A)** habit **(B)** inflorescence **(C)** floret **(D)** leaves **(E)** ripe fruits and seeds [adapted from (51–55)].

and non-reducing sugars and amino acids were not present in either extract. Saponins and steroids were not detected in the methanolic extract of *T. indica* leaves by Raghavendra et al. (33) and Kumar et al. (42), which contrasts with those tested by Alrasheid et al. (45) and Chigurupati et al. (46). Proteins were not detected by Kumar et al. (42) but were detected by Chigurupati et al. (46). The absence of amino acids in the methanolic leaf extract tested by Kumar et al. (42) was confirmed by Chigurupati et al. (46). Although flavonoids and phenolics were not detected in the methanolic extract in the studies of Raghavendra et al. (33) and Alrasheid et al. (45), these compounds were quantitatively reported in the other studies presented in Table 3. Total phenolic and total flavonoid contents were quantitatively determined in 17 studies (Table 3). The phenolic contents were quantitatively determined as gallic acid equivalents, while the flavonoid contents were determined as quercetin or rutin equivalents. The total phenolic contents tended to be higher in the methanolic extract than in the samples extracted using ethanol, ethyl acetate, and hexane. However, the methanolic extract contained fewer phenolics and flavonoids than the acetone extract. The study of Leng et al. (43) indicated that the pretreatment method for plant materials significantly affects the total phenolic and flavonoid contents. The methanolic extract of stir-fried leaves had a significantly higher phenolic content than the methanolic extract of oven-dried leaves and fresh leaves. The difference in extraction methods, Soxhlet and maceration, in Chigurupati et al.'s (46) study showed non-significant results in total phenolic content. The results of Kaewnarin et al. (34) showed that either phenolics or flavonoids in ethyl acetate extract were 100% higher than those in ethanolic extract even though both samples were obtained with the maceration technique. The correlation between antioxidant capacities and total phenolic contents was analyzed in 4 studies, e.g., Gomathi et al. (28), Razali et al. (29), Kaewnarin et al. (34), and Leng et al. (43). Positive correlations were reported in all of them. Total flavonoid contents also had positive correlations with antioxidant capacities in the study by Kaewnarin et al. (34).

In addition to phenolic compounds and flavonoids, the chemical compositions of *T. indica* leaves and their relative abundance are detailed in Table 4. Some structures of chemical constituents of *T. indica* leaves are shown in Figure 4. The elements in *T. indica* leaf and leaf extracts investigated by Escalona-Arranz et al. (38) are presented in Table 5. Several classes of phytochemicals have been reported as constituents in *T. indica* leaves. Fatty acids, organic acids, terpenoids, tannins, flavonoids, and other organic compounds were reported to be found in *T. indica* leaf extracts. The type of solvent used in the extraction procedure provided different compositions and quantities.

Toxicity of *T. indica* leaves

An acute oral toxicity study of ethanolic leaf extract of *T. indica* was conducted by Livingston Raja et al. (62) in albino Wistar rats. After receiving the extract at 1,000, 2,000, and 4,000 mg/kg body weight (BW) orally for 14 consecutive days, a non-significant difference in blood chemical parameters and adverse effects was observed (62). In 2015, the acute oral toxicity and oral mucous irritability of *T. indica* leaf fluid extract were determined in rats by Escalona-Arranz et al. (49). In an acute oral toxicity test of 2000 mg/kg BW, the extract was reported to be a non-toxic substance within the scale of toxic class substances (OECD/OCDE 423 2012) (49). The extract did not cause significant changes in hair and skin, mucous and eye color, histopathology of visceral organs, behavior, or somatomotor capacity. The assays were completed with a survival rate of 100%. The extract also did not change the macroscopic characteristics of Syberian hamsters after exposure to the right malar bag.

TABLE 4 Major phytochemical compositions in *T. indica* leaves.

Major constituents	% Relative abundance	References
Oleic Acid	85.96 (ethanolic extract)	(57)
	39.00 (acetone extract)	
3-*O*-Methyl-d-glucose	43.09* (ethanolic extract)	(58)
4-C-methyl-myo-inositol		
2-C-methyl-myo-inositol		
9-Octadecenoic acid (*E*)-, methyl ester (Methyl oleate)	41.05 (acetone extract)	(57)
cis-Vaccenic acid	35.23* (aqueous extract)	(58)
trans-13-Octadecenoic acid		
Oleic Acid		
Benzyl benzoate	40.60 (leaf oil)	(59)
Limonene	24.40 (leaf oil)	(59)
	9.05 (chloroform extract)	(38)
3-Eicosyne	21.99 (n-hexane fraction obtained from ethanolic extract)	(38)
Tartaric acid	21.96 (chloroform fraction obtained from ethanolic extract)	(38)
	7.30 g/kg fresh weight (aqueous extract)	(60)
Octadecanoic acid	20.28* (aqueous extract)	(58)
Octadecanoic acid, 2-(2-hydroxyethoxy) ethyl ester		
Eicosanoic acid		
Hexadecanoic acid (Palmitic acid)	20.99 (n-hexane fraction obtained from ethanolic extract)	(38)
	18.39 (chloroform fraction obtained from ethanolic extract)	(38)
	8.14 (ethanolic extract)	(57)
7,10-octadecadienoic, methyl ester	16.13 (n-hexane fraction obtained from ethanolic extract)	(38)
Malic acid	15.95 (chloroform fraction obtained from ethanolic extract)	(38, 60)
	0.75 g/kg fresh weight (aqueous extract)	(60)
9,12,15-octadecatrienoic acid, methyl ester	13.57 (n-hexane fraction obtained from ethanolic extract)	(38)
10-Octadecenoic acid	12.74 (n-hexane fraction obtained from ethanolic extract)	(38)
	7.77 (chloroform fraction obtained from ethanolic extract)	(38)
Hexadecanol (Cetyl alcohol)	12.4 (leaf oil)	(59)
6,10,14-trimethylpentadeca-5,9,13-trien-2-one	9.70 (n-hexane fraction obtained from ethanolic extract)	(38)
Benzene-1,2-dicarboxylic acid (Phthalic acid)	9.45 (chloroform fraction obtained from ethanolic extract)	(38)
2,2-dimethoxy-propane	8.93* (ethanolic extract)	(58)
1,3-Dioxolane		
2-(1-methylethoxy)-ethanol		
Methyl-15-tricosanoate	8.39 (chloroform extract)	(38)
Pentadecanol	8.20 (leaf oil)	(59)

(Continued)

TABLE 4 (Continued)

Major constituents	% Relative abundance	References
4-hydroxy-4-methyl-2-pentanone (Diacetone alcohol) 2-methyl-2-hexanol N-methyl-ethanamine	7.87* (ethanolic extract)	(58)
n-Nonadecanoic acid	7.57 (chloroform fraction obtained from ethanolic extract)	(38)
Longifolene	7.51 (chloroform extract)	(38)
n-Hexadecanoic acid (Palmitic acid) L-Ascorbyl 2,6-dipalmitate Pentadecanoic acid	7.40* (aqueous extract)	(58)
Eicosane 1-Iodo-2-methylundecane 10-Methylnonadecane	7.34* (aqueous extract)	(58)
2,6-di-tert-butyl-4-methylphenol (Butylated hydroxytoluene)	7.24 (chloroform extract)	(38)
Methyl palmitate	6.41 (chloroform extract)	(38)
	7.09 (acetone extract)	(57)
Caryophyllene	5.56 (chloroform extract)	(38)
Diphenyl-ether	5.47 (chloroform extract)	(38)
Cryptopinone	5.28 (chloroform extract)	(38)
Linalool anthranilate	4.70 (leaf oil)	(59)
	3.96 (chloroform extract)	(38)
Oxalic acid	7.50 g/kg fresh weight (aqueous extract)	(60)
Citric acid	1.00 g/kg fresh weight (aqueous extract)	(60)
Caffeic acid	N/A (butanol fraction obtained from ethanolic extract)	(38)
Luteolin	N/A (ethyl acetate fraction obtained from ethanolic extract)	(38)
Luteolin-7-*O*-glucoside	N/A (ethyl acetate fraction obtained from ethanolic extract)	(38)
Apigenin	N/A (ethyl acetate fraction obtained from ethanolic extract)	(38)
Orientin	N/A (butanol fraction obtained from ethanolic extract)	(38)
	N/A (methanolic and chloroform extract)	(61)
Iso-orientin (Homo-orientin)	N/A (butanol fraction obtained from ethanolic extract)	(38)
	N/A (methanolic and chloroform extract)	(61)
Vitexin	N/A (butanol fraction obtained from ethanolic extract)	(38)
	N/A (methanolic and chloroform extract)	(61)
Isovitexin (Saponaretin)	N/A (methanolic and chloroform extract)	(61)
Quercetin	N/A (ethyl acetate extract)	(29)
Isorhamnetin	N/A (hexane extract)	(29)
Catechin	N/A (methanol extract)	(29)
Epicatechin	N/A (methanolic, ethyl acetate, hexane extract)	(29)
3-*O*-Caffeoylquinic acid (Chlorogenic acid)	N/A (methanolic and chloroform extract)	(61)
4-*O*-Caffeoylquinic acid (Chlorogenic acid)	N/A (methanolic and chloroform extract)	(61)

*More than 1 composition in the same peak determined by GC–MS.

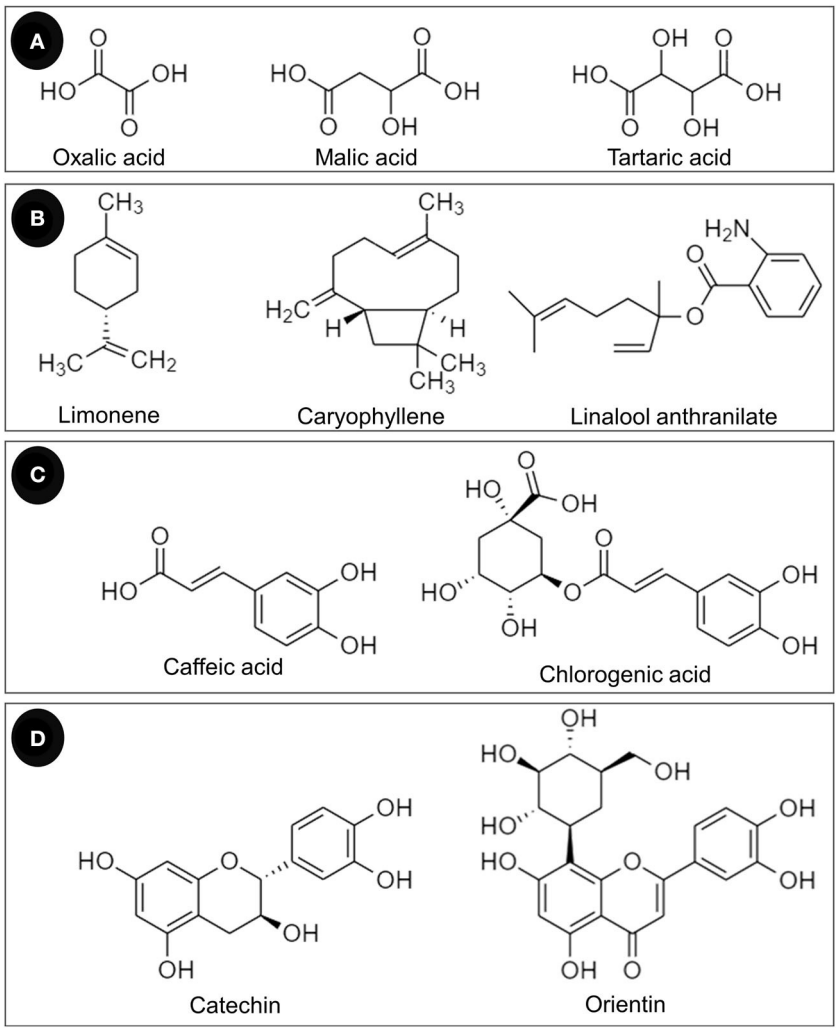

FIGURE 4
Chemical constituents of *T. indica* leaves classified as **(A)** organic acids, **(B)** terpenoids, **(C)** phenolic acids, and **(D)** flavonoids.

However, it did show degeneration of the epithelium and mild vascular congestion in muscular tissue. The results correlated with the results obtained by Amado et al. (63). Amado et al. (63) tested the acute oral toxicity of dry *T. indica* leaf extract in male Wistar rats using the limit dose of 5000 mg/kg BW. It was found that at day 14 after administration, no death was observed. There were no significant differences between the treatment and control groups. There were no changes in skin and pelage, mucous membrane and eyes, or color and morphology of visceral organs (63). Moreover, the acute oral toxicity of ethanolic extracts of *T. indica* leaves was also investigated in healthy Sprague Dawley rats by Chigurupati et al. (46). No lethality or abnormal behavior was observed over the 14-day period after the administration of 2000 mg/kg BW (46).

The intraperitoneal acute toxicity (50% lethal dose) of 566 mg/kg BW aqueous leaf extract was reported in the study of Akor et al. (64). The extract showed moderate toxicity in Wistar albino rats (64).

The toxicity in erythrocytes of ethanolic and aqueous extracts of *T. indica* leaves was conducted by Mehdi et al. (65). It was found that neither extract induced hemolysis, similar to normal saline solution (65). These results agreed with a previous study performed by Escalona-Arranz et al. (66). The study of *T. indica* leaf fluid extract on human blood cells was conducted, and the results revealed that the extract did not cause significant hemolysis at 20–100 mg/ml. The protein denaturation ratio after the application of plant extracts at 40–100 mg/ml was very low compared with the control; thus, it was proven to be less toxic. In addition, the extract showed a protective effect against

TABLE 5 Elements in *T. indica* leaf and leaf extracts (38).

Elements	*T. indica* leaf (μg/g)	Chloroform extract (μg/g)	Ethanolic extract (μg/g)
Al	5.27	0.013	1.181
Cd	0.0019	–	–
Co	0.880	–	0.108
Cr	0.250	–	0.079
Cu	7.900	0.196	0.857
Fe	16.160	0.241	1.107
Mn	2.500	0.027	0.750
Ni	0.461	–	0.052
Pb	0.700	–	0.050
Sr	0.325	–	0.051
Zn	7.990	0.031	0.292
Mo	0.260	–	–
V	–	–	–
Se	4.723	0.083	1.341

H_2O_2-induced oxidative damage in the human erythrocyte membrane at the same concentrations (66).

Discussion

This systematic review examined 21 *in vitro* studies of the antioxidant capacity of *T. indica* leaves. The samples used in antioxidant tests were prepared using water or organic solvents by different methods and with different pretreatments of the raw materials. The antioxidant capacity assays also differed between studies.

Oxidative stress is the disruption of redox signaling and control caused by the imbalance of free radicals and antioxidant defenses (8). Free radicals are found in human cells, animal cells, and other living organisms. They are generated by endogenous reactions and are caused by exogenous sources. In the human body, free radicals are produced by several biochemical processes. For example, H_2O_2 and $HO^{\bullet}$ radicals result from the reduction of molecular oxygen during aerobic respiration, and $O_2^{\bullet-}$ radicals and hypochlorous acid (HOCl) arise from the activation of phagocytes (67). Exogenous oxidants are caused by pollution and environmental stressors such as cigarette smoking, air pollution, radiation, and diet (8). Oxidative stress leads to a variety of health problems. It is both the primary cause of pathology and a secondary contributor to disease progression, e.g., cancers, cardiovascular disease, neurodegenerative disorders, diabetes, and metabolic syndrome (9). Thus, antioxidants are the first option to prevent and treat various health issues and anomalies, especially environmental pollution.

Antioxidants prevent oxidative stress-related damage by breaking radical chain reactions (9). Antioxidants are divided into endogenous and exogenous antioxidants. The latter class must be ingested through the diet. Endogenous antioxidants comprise enzymatic and non-enzymatic antioxidants, while exogenous antioxidants consist of water-soluble and lipid-soluble antioxidants. The well-known antioxidants obtained from natural sources are vitamin C and E, carotenoids, tannins, phenolic acids, and flavonoids (1, 8, 9).

Phenolics are strong antioxidants and are members of the "polyphenols," which are a large class of plant secondary metabolites. Flavonoids are natural compounds that contain hydroxyl groups that are responsible for metal chelation and free radical scavenging capacity. The compounds can react with $O_2^{\bullet-}$ and $HO^{\bullet}$ and are also able to chelate metal ions, so they regulate both iron homeostasis and redox state (9, 68). Pizzino et al. (1) summarized the antioxidant properties of flavonoids as ROS scavengers and ROS synthesis suppressors, antioxidant defense enhancers, enzyme inhibitors, and trace element chelators (1). Several flavonoids are present in *T. indica* leaf extracts, e.g., luteolin and its derivatives, apigenin, orientin, vitexin, quercetin, isorhamnetin, catechin, and epicatechin (Table 4). Non-flavonoid compounds such as caffeic acid and chlorogenic acid were also detected (Table 4).

Vitexin and iso-vitexin, which are apigenin derivatives, and orientin (the luteolin glycoside) were investigated for their *in vitro* antioxidant capacities and *in vivo* antioxidant activities. Khole et al. (69) studied the mechanism of vitexin and iso-vitexin for their antioxidant effects. They found that the compounds exhibited different capacities against ROS. Iso-vitexin scavenges $O_2^{\bullet-}$ radicals better than vitexin, while vitexin scavenges $NO^{\bullet}$ radicals better. Both compounds were active against short-lived radicals: $ABTS^{\bullet+}$ radical and $CO_3^{\bullet-}$ radical. These compounds protected HepG2 cells from H_2O_2–induced oxidative insult by modulating antioxidant enzyme levels and reducing intracellular ROS levels (69). In 2012, An et al. (70) observed the antioxidant activities of vitexin and orientin compared with vitamin E in an age mouse model. The results showed that vitexin and orientin had the capacity to improve the antioxidative system as well as to improve the levels of ATPase in the tissue and serum in aged mice induced by D-galactose. Furthermore, the compounds at 40 mg/kg BW were comparable to vitamin E for the improvement of neuronal cell structure and function in mice (70).

Catechin, epicatechin, rutin, and quercetin are ubiquitous polyphenols in herbs and food plants. These compounds showed better $DPPH^{\bullet}$ radical scavenging capacity than the analog of vitamin E, Trolox, in the study of Iacopini et al. (71). Similarly, in the $DPPH^{\bullet}$ scavenging, $ABTS^{\bullet+}$ radical scavenging, and FRAP assays performed by Tian et al. (72), quercetin manifested better antioxidant capacities than vitamin C and BHT in all assays. In a mechanistic study, quercetin exhibited several mechanisms against oxidative stress. It inhibited inducible nitric oxide

synthase in macrophages, so oxidative damage was inhibited. The compound also directly scavenges free radicals and inhibits the formation of oxygen free radicals through the chelation of ions of transition metals such as iron. Quercetin also inhibited xanthine oxidase and suppressed TNF-α modulated by oxidative stress, resulting in the decrement of oxidative injury and the modulation of immune response (73).

Luteolin and apigenin are plant flavonoids with a broad spectrum of biological activities. Both displayed superior $ABTS^{\bullet+}$ radical scavenging capacity to vitamin C and BHT in the studies of Tian et al. (72). In addition, luteolin showed surpassing results in $DPPH^{\bullet}$ radical scavenging and FRAP assays compared with both vitamin C and BHT (72). The *in vitro* mechanism of apigenin against oxidative stress includes oxidative enzyme inhibition, modulation of redox signaling pathways (NF-κB, Nrf2, MAPK, and P13/Akt), reinforcement of enzymatic and non-enzymatic antioxidants, free radical scavenging, and metal chelation (74). An *in vivo* experiment in arterial aging mice conducted by Clayton et al. (75) revealed that apigenin could increase NO bioavailability; normalize ROS, antioxidant expression, and oxidative stress; and abolish the inhibitory effect of ROS (75). The mechanisms of the antioxidant action of luteolin have been summarized as ROS scavenging, ROS-generating oxidase inhibition, enhancement and protection of endogenous antioxidants, direct inhibition of oxidative-catalyzed enzymes, and chelation of transition metal ions (76).

Chlorogenic acid and its major metabolite, caffeic acid, are classified as phenolic acids. Chlorogenic acid is hydrolyzed into caffeic acid in the intestine after ingestion. The antioxidative effect of caffeic acid has been evinced using different *in vitro* assays by Gülçin (77), i.e., the ferric thiocyanate method, total reduction capability, $ABTS^{\bullet+}$ radical scavenging, $DPPH^{\bullet}$ radical scavenging, $O_2^{\bullet-}$ radical scavenging, and ferrous metal chelating capacity (77). The *in vivo* antioxidant assay of chlorogenic acid and caffeic acid was performed using the 2-methyl-6-p-methoxyphenylethynylimidazopyrazynone method to emit $O_2^{\bullet-}$ radical scavenging capacity. The IC_{50} values of chlorogenic acid and caffeic acid were 41.0 and 10.1 μM, respectively, whereas allopurinol provided an IC_{50} of 15.0 μM (78). Caffeic acid exerted its cytoprotective effect in ischemia/reperfusion injury in the rat small intestine caused by ROS. The compound decreased lipid peroxidation and reduced DNA damage in UV radiation-induced oxidative stress. In addition, caffeic acid showed *in vivo* antioxidant activity against chemical-induced toxicity (such as cisplatin-, carbon tetrachloride-, and cadmium-induced toxicity of the liver and kidney) in various animals (79).

Not only polyphenols but also other phytochemicals, vitamins and elements found in plants have also been reported to be responsible for antioxidant capacity. *T. indica* leaves contain the sugar acid form of ascorbic acid and some elements that possess antioxidant effects, e.g., selenium, copper, zinc, and manganese. These constituents might exhibit antioxidative effects via different mechanisms, and the overall antioxidant capacity might be caused by antagonistic, synergistic, or additional effects of these compounds and elements.

Most of the 21 studies that were reviewed executed antioxidant capacity by using more than one assay. This might be due to the differences in method principles and their strengths and limitations regarding cost and facility requirements, difficulty of operation, time spent, sensitivity and specificity, reproducibility and repeatability, correlation with phytochemical content, coverage spectrum of biological samples, and representativeness of the *in vivo* system. As a consequence of these factors, the results obtained from each study did not correlate with others.

Furthermore, the results still differed even when the same antioxidant assay was used. These occurrences were attributed to variations in the sources of the raw materials, their pretreatments, the extraction methods, and the solvents used for sample preparation. Furthermore, the phytochemical screening results differed between studies in that some classes of plant constituents were detected by some but not others. Other reasons for discrepancies in the screening results are that the quantity of the compounds was below the detection limit of the particular screening method employed and interference from other chemicals. Both factors might cause false-positive and false-negative results.

Considering toxicity, several studies performed the acute oral toxicity of *T. indica* leaf extracts, and no death was observed at the maximum single dose of 5,000 mg/kg BW and a 14-day repeated dose of 4,000 mg/kg BW. However, it was found that after exposure to *T. indica* leaf fluid extract in the right malar bag in Syrian hamsters, signs of mucous irritation were observed. These findings were explained by the presence of organic acids and polyphenols in *T. indica* leaves, which could slightly irritate the mucous membrane. Hence, the extract is considered a light irritant to the mucous membrane and could be a very mild irritant to the skin (49).

Conclusions and future recommendations

In the present study, the antioxidant capacity of *T. indica* leaves was reviewed. *T. indica* leaf extracts exhibited *in vitro* antioxidant capacity through free radical scavenging capacity and transition and heavy metal chelating capacity. There is a high possibility that the antioxidant capacities are responsible for the polyphenols and the elements. The polyphenols found in *T. indica* leaves are flavonoids and phenolic acids such as catechin, vitexin, orientin, apigenin, and luteolin. In addition, elements such as selenium, copper, manganese, and zinc are

present. These chemicals and elements are well-known as strong antioxidants, which makes *T. indica* leaves a promising natural antioxidant mixture. The safety of *T. indica* leaves was investigated in erythrocytes and animals. The extracts were found to be safe after oral administration of 4,000 mg/kg BW for 14 days, and no death was observed after the ingestion of 5,000 mg/kg BW. The 50% lethal intraperitoneal dose was 566 mg/kg BW.

The limitations of this systematic review are as follows:

- All studies were *in vitro*-based experiments.
- Positive controls were used only in some studies. Therefore, comparisons between the studies and the reported potency of *T. indica* leaf extracts are difficult to make.
- The extracts used in the included studies were not quantified for each active constituent or each biomarker of antioxidant capacity. The total phenolic and total flavonoid contents were shown in 17 studies.
- The maturity level of leaves and technology used in the treatment and extraction method were reported in only some studies.

To apply *T. indica* leaf extract as a source of antioxidant, confirmed results from an *in vivo* study and a clinical trial should be considered. Standardization of the extract with regard to its active constituents or total phenolic and total flavonoid content should be performed, especially if the extract is prepared using a different method and solvent. The effective dose should be taken into account to avoid excessive intake and antioxidative stress. The toxicity might also be a concern. An intensive *in vivo* study of subacute, subchronic, and chronic toxicity should be performed.

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/s.

Author contributions

SS, AD, and SSa: conceptualization. SSo and SSa: data curation, formal analysis, and writing—original draft. SS, AD, and SSa: methodology. SSa, AD, and PP: supervision. AD and PP: validation. SSo, AD, SSa, and PP: 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.977015/full#supplementary-material

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Carotenoids in orange carrots mitigate non-alcoholic fatty liver disease progression

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Background: Carotenoids are abundant in colored fruits and vegetables. Non-alcoholic fatty liver disease (NAFLD) is a global burden and risk factor for end-stage hepatic diseases. This study aims to compare the anti-NAFLD efficacy between carotenoid-rich and carotenoid-deficient vegetables.

Materials and methods: Male C57BL/6J mice were randomized to one of four experimental diets for 15 weeks (n = 12 animals/group): Low-fat diet (LFD, 10% calories from fat), high-fat diet (HFD, 60% calories from fat), HFD with 20% white carrot powders (HFD + WC), or with 20% orange carrot powders (HFD + OC).

Results: We observed that carotenoids in the orange carrots reduced HFD-induced weight gain, better than white carrots. Histological and triglyceride (TG) analyses revealed significantly decreased HFD-induced hepatic lipid deposition and TG content in the HFD + WC group, which was further reduced in the HFD + OC group. Western blot analysis demonstrated inconsistent changes of fatty acid synthesis-related proteins but significantly improved ACOX-1 and CPT-II, indicating that orange carrot carotenoids had the potential to inhibit NAFLD by improving β-oxidation. Further investigation showed significantly higher mRNA and protein levels of PPARα and its transcription factor activity.

Conclusion: Carotenoid-rich foods may display more potent efficacy in mitigating NAFLD than those with low carotenoid levels.

KEYWORDS

phytochemicals, nutrition, beta-oxidation, nuclear receptors, lipid metabolism

Abbreviations: ACCα, acetyl coenzyme A carboxylase alpha; ACOX-1, acyl-CoA oxidase; AMPK, AMP-activated protein kinase; CD36, cluster of differentiation 36; CPT-I, carnitine palmitoyltransferase-I; CPT-II, carnitine palmitoyltransferase-II; DGAT2, diacylglycerol *O*-acyltransferase 2; FAS, fatty acid synthase; HFD, high-fat diet; HFD + OC, high-fat diet w/20% orange carrot powder; HFD + WC, high-fat diet w/20% white carrot powder; IACUC, Institutional Animal Care and Use Committee; LFD, low-fat diet; MTP, microsomal triglyceride transfer protein; NAFLD, non-alcoholic fatty liver disease; PPARα, peroxisome proliferator-activated receptor alpha; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; SCD-1, stearoyl-CoA desaturase-1; SREBP-1, sterol regulatory element-binding protein 1; TG, triglyceride.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by a 5–10% excessive lipid deposit in hepatocytes without significant alcohol intake (1). It is the most common form of chronic liver disease in the USA, affecting 80–100 million individuals (2). Obesity has been shown to be the principal risk factor for NAFLD development (3). Although isolated hepatic steatosis is considered a benign condition or minimal rate of progression (4), NAFLD patients generally have a higher risk of developing end-stage liver diseases and increased morbidity and all-cause mortality than healthy individuals (5, 6). Currently, there is no FDA-approved medication for NAFLD, and the optimal method of alleviating NAFLD is the adoption of lifestyle changes, including weight management and embracing healthy eating habits (7).

Carotenoids are naturally occurring pigments synthesized by plants, algae, and photosynthetic bacteria (8–10). They can scavenge reactive oxygen species and deactivate electronically excited sensitizer molecules (11). Accumulating evidence has shown health benefits of carotenoids in various organs (12–15). Recently, a randomized, double-blinded trial showed that compared to consuming vegetable pastes containing low levels of carotenoids, the consumption of carotenoid-rich vegetable pastes promoted the decrease of visceral fat and waist circumference among obese subjects (16), indicating that carotenoids from whole foods may display an independent beneficial efficacy against obesity. However, the role of fruits or vegetables against NAFLD was less evident. A cross-sectional study with middle-aged subjects failed to observe a significant association between fruit or vegetable intake and NAFLD (17). Consistently, a meta-analysis with six cross-sectional studies and two case-control studies found the association between fruit intake and the likelihood of developing NAFLD to be not beneficial (18). A potential explanation for the negative results might be the misclassification of the food groups by combining the low-carotenoid food with those enriched with carotenoids. Therefore, the major knowledge gap is whether whole foods containing high carotenoid levels (such as orange carrots) are more efficient in preventing NAFLD than low-carotenoid foods (such as white carrots).

The efficacy of β-carotene in ameliorating NAFLD has been shown in multiple studies (19–21). However, realizing that single nutrient theories were inadequate to explain the efficacy of daily dietary changes on non-communicable diseases (22), an increasing number of nutritional studies shifted their focus from single nutrients to whole foods. Specifically, scientists in the carotenoid studies found that whole food enriched with β-carotene, such as apricots and Campari tomatoes, were effective in mitigating diet-induced NAFLD (23, 24). Nevertheless, these foods contain a wide arrange of compounds such as fiber, so whether the anti-NAFLD efficacy was mainly from carotenoids or other compounds remains unknown. Another class of food-derived compounds known as flavonoids have been documented to provide health benefits through anti-hyperlipidemic, anti-inflammatory, and anti-diabetic effects against high-fat and high-fructose conditions (25).

From the mechanistic point of view, NAFLD results from imbalanced hepatic lipid homeostasis, which can be caused by increased hepatic triglyceride (TG) uptake, enhanced fatty acid synthesis, or decreased β-oxidation. Peroxisome proliferator-activated receptors (PPARs) belong to a ligand-activated transcription factor superfamily comprising three isoforms: alpha (α), beta/delta (β/δ), and gamma (γ) (26). PPARα is ubiquitously expressed while highly expressed in the liver (26, 27). Over the last several decades, various studies have focused on investigating PPARα due to its critical role in lipid and lipoprotein metabolism (28–30) by regulating a wide spectrum of target genes such as acyl-CoA oxidase 1 (ACOX1) (30) and carnitine palmitoyltransferase-I (CPT-I) (31). AMP-activated protein kinase (AMPK) is a master regulator of metabolism, which coordinates metabolic pathways and therefore maintains energy homeostasis (32). Interestingly, genetic liver-specific AMPK activation in mice alleviated the diet-induced obesity and NAFLD, suggesting that AMPK could be a potential target for preventing NAFLD (33). Since several publications reported that dietary carotenoids and their metabolites were effective in upregulating PPARα and activating AMPK (34–36), we performed further studies focusing on these targets.

To conclude, the major gap of knowledge in the carotenoid and NAFLD research relates to the failure of introducing a positive control that contain the similar compounds from the food source other than carotenoids. Therefore, the objective of this study was to compare the anti-NAFLD efficacy between carotenoid-rich vegetables (orange carrots) and carotenoid-deficient vegetables with similar concentrations of other compounds (white carrots). We hypothesize that orange carrots are more efficient in mitigating NAFLD in HFD-induced obese mice compared with white carrots.

Materials and methods

Animals

All animal protocols for the study were approved by the Institutional Animal Care and Use Committee (IACUC) at North Carolina State University. C57BL6J mice (male, n = 48) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Four mice were co-housed in one cage in a controlled temperature and humidity room with a 12-h light/dark cycle and fed with a standard chow diet. At 11 weeks of age, mice were randomized to four dietary groups (n = 12 in each group): low-fat diet (LFD, D12450J, 10% calories from fat), high-fat diet (HFD, D12492, 60% calories from fat), HFD with 20% w/w

white carrot (HFD + WC), and HFD with 20% w/w orange carrot (HFD + OC). We utilized 20% (w/w) carrots due to the limited bioaccessibility of carotenoids (37). Moreover, the cleavage efficiency of carotenoids to produce apocarotenoids is much higher in mice than in humans (38, 39). Therefore, although 20% carrots seem to be a supraphysiological dose in human beings, such dosage serves the purpose of this study (our research interests focus on carotenoids, not carotenoid metabolites). Food and water were administered *ad libitum*. Compositions of dietary pellets and vitamin mix (V10001) have been provided in **Supplementary Table 1**. The dietary intervention lasted for 15 weeks. Carrots were purchased from local grocery stores and lyophilized at NC Food Innovation Lab in Kannapolis, NC. All diet pellets were made at Research Diets Inc. (New Brunswick, NJ, USA). Body weight for each mouse and the food consumption amount were recorded weekly. Submandibular blood collection was performed every month; one mouse in the LFD group was deceased during the submandibular blood collection, making LFD $n = 11$ for subsequent studies. At the end of the study, mice were anesthetized with isoflurane, followed by cardiac puncture. Blood was transferred to a capillary blood collection tube and placed at room temperature for 30 min. Serum was collected by centrifuging the blood at 1,500 x *g* for 10 min at 4°C. Livers were weighed and washed with saline. A piece of liver tissue was fixed in 10% formalin (Thermo Fisher Scientific, Waltham, MA, USA) for histopathological examination. All the tissues were harvested, snap-frozen in liquid nitrogen, then stored at −80°C for further analysis.

Body composition analysis

EchoMRI-100 (EchoMRI, Houston, TX, USA) was used to measure body composition measurements of fat and lean masses in mice at baseline and the end of the study. Briefly, non-anesthetized mice were placed in a restraining cylinder, locked using the Velcro attachment. The tube was subsequently inserted to the chamber unit of the equipment, and mice's fat mass, lean mass, free water, and total water mass were determined.

Liver and serum triglyceride analysis

Liver and serum TG levels were assessed using a commercialized colorimetric kit (Abcam, Waltham, MA, USA). Weighed liver samples (approximately 100 mg) were homogenized in an extraction buffer (5% Tween-20 in deionized water). Serum samples were 1:1 diluted with the extraction buffer. Both liver and serum mixtures were incubated at 90°C for 3 min and centrifuged at 24,532 x *g* for 2 min. Then the supernatants, lipase, TG probe and TG enzyme mix were successively added to the 96-well plate according to the manufacturer's protocol. The output was measured on a microplate reader at OD 570 nm.

Histology

Liver tissues were fixed in 10% formalin and washed with 70% ethanol. Then, the livers were placed in a tissue processor for dehydration, clearing, and paraffin wax infiltration, followed by paraffin embedding and sectioning. Five-micrometer sections of liver tissues were stained with hematoxylin and eosin and examined using a ZEISS Axio Observer microscopy platform (Carl Zeiss Microscopy, White Plains, NY, USA) with AxioVision software. Liver steatosis was assessed according to the percentage of the macro- and micro-vesicular fat vacuoles at 20 X magnification in four fields, using Image J software (NIH, Bethesda, MD, USA), as was described previously (40). Briefly, the original images were converted to 8-bit black-and-white, followed by black and white inversion. Then, an upper threshold of the grayscale was applied to the converted images. Subsequently, a particle analysis was conducted by using the "Analyze Particles" function of Image J. A circularity parameter of 0.5–10 was set to remove any potential noise that are not lipid droplets.

RNA isolation, cDNA synthesis, and quantitative polymerase chain reaction

Liver mRNA extraction was performed using the PureLink RNA Mini Kit (Thermo Fisher Scientific) as reported previously (13, 41). Weighted liver tissues (approximately 100 mg) were homogenized in a lysis buffer with 1% 2-mercaptoethanol and centrifuged at 2,600 x *g* for 5 min. Then, the supernatant was transferred to an RNase-free tube and washed with 70% ethanol. The entire mixture was filtered by the spin cartridge and washed with two different wash buffers. A total of 100 μL RNase-free water was added to the center of the spin cartridge at three separate times; the mixture was incubated at room temperature for 1 min, followed by centrifugation at 12,000 x *g* for 2 min to elute the liver mRNA. Liver cDNA synthesis was performed by using Novo cDNA Kit (BioVision, Minneapolis, CA, USA) according to the manufacturer's instructions. Each reaction contained 500 ng mRNA samples, 1 μL random primer, 1 μL dNTP, 5 μL RT buffer, 0.5 μL RNase inhibitor, and 1 μL RTase in a Biometra TAdvanced 96G Thermal Cycler (Analytik Jena, Jena, Germany). The program conditions were 25°C for 10 min, 42°C for 50 min, and 85°C for 5 min. The quantification of liver cDNA was carried out by mixing the 10-fold diluted cDNA with 10 μL 2X PowerUp SYBR Green Master Mix, 2 μL of 10 μM primer mix that includes forward and reverse primers, and 3 μL PCR water. The cycling conditions

were described previously (13). Primer sequences were listed in **Supplementary Table 2**.

Protein extraction and western blot

Protein was extracted from whole cell lysates of liver tissue (approximately 50 mg) by homogenizing the tissues in a radioimmunoprecipitation assay buffer (Thermo Fisher Scientific) containing 1% protease inhibitors. Subsequently, the homogenates were centrifuged at 25,200 x *g* for 30 min at 4°C, and the supernatants were collected for further analyses. Liver nuclear protein was extracted by using the Nuclear Extraction Kit (Abcam). Briefly, 100 mg frozen liver tissues were homogenized in a pre-extraction buffer and incubated on ice for 15 min. After centrifugation for 10 min at 16,128 x *g* at 4°C, an extraction buffer containing 1% dithiothreitol (DTT) and a protease inhibitor cocktail was added. The entire mixture was placed on ice for 15 min, followed by centrifugation at 4°C for 10 min. The concentration of the proteins was quantified by using the Pierce Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific).

The detailed western blot protocol was described in our previous publications (13, 41). The primary antibodies including acetyl coenzyme A carboxylase alpha (ACCα), diacylglycerol *O*-acyltransferase 2 (DGAT2), fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD-1), acyl-CoA oxidase 1 (ACOX1), carnitine palmitoyltransferase-II (CPT-II), sterol regulatory element-binding protein 1 (SREBP-1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), and AMP-activated protein kinase (AMPK) were mouse obtained from Santa Cruz Biotechnology (Dallas, TX, USA); phospho-AMPK (Thr172, p-AMPK), and peroxisome proliferator-activated receptor alpha (PPARα) were obtained from ABclonal (Woburn, MA, USA). The antibodies from Santa Cruz Biotechnology were 1:1000 diluted with 5% bovine serum albumin (BSA, Thermo Fisher Scientific). The antibodies from ABclonal were 1:2000 diluted with 3% non-fat milk (Research Products International, Mt Prospect, IL, USA). An anti-mouse secondary antibody (1:1000 5% BSA) or anti-rabbit secondary antibody (1:500 3% non-fat milk) was applied to the membranes that were blotted with primary antibodies from Santa Cruz Biotechnology or ABclonal, respectively.

Briefly, 50 μg protein was loaded to the gel. We utilized 4–12% Bis-Tris gels with MOPS running buffer for proteins with a low to medium molecular weight (DGAT2, SCD-1. ACOX-1, CPT-II, p-AMPK, AMPK, and PPARα), while 3–8% tris-acetate gels with tris-acetate SDS Running Buffer were utilized to separate high molecular weight proteins (MTP, FAS, ACCα, SREBP-1, and PGC-1α). Gels and running buffers were purchased from Thermo Fisher Scientific. Electrophoresis was conducted per manufacturer's instructions. A dry transfer system (iBlot2) was employed to transfer proteins from gels to nitrocellulose membranes. This was followed by non-specific blocking with 5% bovine serum albumin (BSA) for 1 h. After three washes, the membranes were incubated in the primary antibodies at 4°C overnight. If the primary antibodies were not conjugated with horseradish peroxidase (HRP), a secondary antibody was applied. Membranes were developed for visualization with the addition of chemiluminescent reagents and the signals were extracted with a UVP ChemStudio Imaging System (Analytik Jena). The signal intensity of all blots was analyzed by using Image J software. β-Actin was used as a loading control. The signal intensity ratios between the target proteins and β-actin were calculated and further analyzed.

Extraction of carotenoids and high-performance liquid chromatography

β-Apo-8′-carotenal (purity ≥ 96%, catalog number: 10810, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in HPLC-grade acetone and added to the samples as an internal control before carotenoid extraction. A total of 80 mg dietary pellets were placed in the ZR Bashing Bead Lysis Tubes (Zymo Research, Irvine, CA, USA). HPLC-grade acetone (500 μL) was added to each tube, followed by a vibrant vortex and centrifugation for 5 min at 16,000 x *g* at 4°C. Individual liver tissue (approximately 60 mg) was homogenized in 1 mL HPLC-grade acetone and centrifuged at 16,000 x *g* at 4°C for 10 min. For serum carotenoid extraction, 60 μL pooled serum from the co-housed mice was extracted with a mixture of petroleum ether (PE), acetone, and butylated hydroxytoluene (BHT) (PE: acetone = 3:1:1% BHT), followed by centrifugation at 1,792 x *g* for 1 min. All the supernatants were collected and dried under nitrogen gas. Carotenoids in the dietary pellets were reconstituted in 80 μL acetone, whereas liver carotenoids and serum carotenoids were reconstituted in 200 and 60 μL acetone, respectively.

The reconstituted carotenoids (5 μL) were detected by an Ultimate 3000 HPLC (Thermo Fisher Scientific). Varying concentrations of pure α-carotene (purity ≥ 97%, catalog number: 50887, Sigma-Aldrich) and β-carotene (purity ≥ 97%, catalog number: 217538, Millipore Sigma, Burlington, MA, USA) were used as external controls. The channels used for β-apo-8′-carotenal was 459 nm, for β-carotene was 455 nm, and for α-carotene was 449 nm. The HPLC conditions were described previously (42). Briefly, the column was Acclaim C30, 5 μm, 4.6 × 150 mm. The gradient of the mobile phase was set as follows: acetonitrile: methanol: methyl tert-butyl ether (v/v/v: 25:75:0 at time 0–20 min, 15:35:50 at time 20–25.5 min, 25:75:0 at time 25.5–30 min). The flow rate for the mobile phase was 1.0 mL/min.

PPARα transcription factor activity assay

The activity of PPARα was measured using a commercialized kit (Abcam) according to the manufacturer's protocol. Following nuclear protein extraction and protein concentration quantification, 90 μL Assay Buffer was added to a 96-well plate containing a specific double-stranded DNA probe with the peroxisome proliferator response element (PPRE) immobilized at the bottom. Subsequently, 10 μL liver nuclear proteins were added. The plate was sealed and incubated overnight at 4°C without agitation. Then, a diluted PPARα primary antibody (1:100 with Assay Buffer) was added to the plate, followed by incubation for 1 h at room temperature. After washing the plate with the supplied wash buffer, we added a secondary antibody, followed by an anti-rabbit HRP conjugate. Following an hour of incubation, 100 μL Stop Solution was added to end the reaction, and the plate was read at OD 450 nm.

Statistical analysis

Normality of distribution of the data was assessed using the D'Agostino-Pearson omnibus normality test with $p > 0.05$ considered normally distributed. Equality of data variance was examined by using an F test with $p > 0.05$ considered equal variances. One-way ANOVA with *post hoc* Tukey HSD was utilized to compare the differences between multiple groups. Kruskal-Wallis test with *post hoc* Tukey HSD was used to compare the differences of non-parametric parameters between multiple groups. Two-way mixed ANOVA with *post hoc* Tukey HSD was employed to depict the difference between multiple groups by including time as a factor. Statistical significance was set as $p < 0.05$. All statistical analysis was performed using GraphPad Prism 9 (San Diego, CA, USA). Values are listed in the text and figures as mean ± standard error of the mean (SEM).

Results

Carotenoid composition-diet pellets, serum, and liver

Carrots are the main dietary sources of α-carotene and β-carotene (43); thus, we measured the concentration of these carotenoids using HPLC as described in the methods section. LFD and HFD pellets did not contain either α-carotene or β-carotene (**Table 1A**). The incorporation of the white carrots essentially acted as a carrot carotenoid control group as they were integrated into the dietary pellets at the same percentage. Therefore, the components of the white carrot and orange carrot pellets are similar, except for these carotenoids. Consistently, the HFD + WC pellet consisting of 20% white carrot powder contained no detectable α-carotene and a negligible amount of β-carotene (0.002 μmol/g). The HFD + OC pellet consisting of 20% orange carrot powder was determined to contain a similar amount of α-carotene and β-carotene at concentrations of 0.12 and 0.11 μmol/g, respectively.

In the circulation, α-carotene and β-carotene were not detected in the LFD and HFD groups since these compounds were not present in the diets (**Table 1B**). Notably, neither α-carotene nor β-carotene was detected in the serum of the HFD + WC fed group. Both α-carotene and β-carotene were detected in the serum of the HFD + OC fed group with a higher concentration of β-carotene (1.4 ± 0.1 μM) compared to the concentration of α-carotene (0.5 ± 0.2 μM). We measured the hepatic concentration of α-carotene and β-carotene since liver is the main storage of carotenoids including α-carotene, β-carotene, lycopene, lutein, zeaxanthin, and β-cryptoxanthin (44, 45). We observed neither α-carotene nor β-carotene in the LFD, HFD, and HFD + WC groups (**Table 1C**), which was in line with the minimal carotenoid content in the diet pellet and circulation. α-Carotene and β-carotene were measured in HFD + OC group at concentrations of 3.3 ± 1.9 and 8.0 ± 3.0 μmol/g, respectively, in line with earlier reports that β-carotene was found in higher concentrations than α-carotene in liver (44, 45). The chromatograms of α-carotene and β-carotene within the diet pellet, serum, and liver samples are provided in **Supplementary Figure 1**.

Carotenoids in orange carrot significantly reduced body weight

We monitored the weight gain throughout the study. The body weights of the HFD + OC became significantly lower than the HFD group from Week 2, which persisted for the duration of the study ($p < 0.05$), except for Weeks 12 and 13 (**Figure 1A**). There was a continuous trend of lower body weight changes in the HFD + OC group compared to HFD + WC that reached statistical significance at Weeks 4, 5, and 6 ($p < 0.05$). Food consumption is reported in **Supplementary Figure 2**. Following Week 15 and prior to necropsy, body weight was recorded a final time (**Figure 1B**). On average, mice in the HFD gained significantly more body weight than the LFD group (25.8 ± 3.4 g vs. 5.8 ± 2.5 g, $p < 0.01$); HFD + WC was not significantly different from HFD. HFD + OC was significantly lower than HFD (20.0 ± 4.0 g vs. 25.8 ± 3.4 g, $p < 0.05$) and was trending to be lower than HFD + WC, but this difference was not statistically significant.

Consistent with the pattern of body weight gain, the Echo-MRI results depicted a significantly higher fat mass increase in the HFD than in the LFD group (19.9 ± 0.7 g vs. 2.9 ± 0.7 g, $p < 0.01$). At the same time, the HFD + WC was not significantly different from the HFD group. HFD + OC was significantly lower than HFD (15.4 ± 0.9 g vs. 19.9 ± 0.7 g, $p < 0.01$) and

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TABLE 1 α-Carotene and β-carotene concentrations in panel (A) diet pellets (n = 3), (B) serum (LFD: n = 11; HFD, HFD + WC, HFD + OC: n = 12), and (C) liver (LFD: n = 11; HFD, HFD + WC, HFD + OC: n = 12).

Treatment groups	(A) Diet pellets (μmol/g)		(B) Serum (μ M)		(C) Liver (μ mol/g)	
	α -Carotene	β -Carotene	α -Carotene	β-Carotene	α-Carotene	β-Carotene
LFD	0.0	0.0	0.0	0.0	0.0	0.0
HFD	0.0	0.0	0.0	0.0	0.0	0.0
HFD + WC	ND	0.002	ND	ND	ND	ND
HFD + OC	0.12 ± 0.01	0.11 ± 0.01	0.52 ± 0.20	1.43 ± 0.10	3.30 ± 1.90	8.04 ± 3.00

ND, not detected. Values are means ± SD. Diet pellets were administered *ad libitum*.

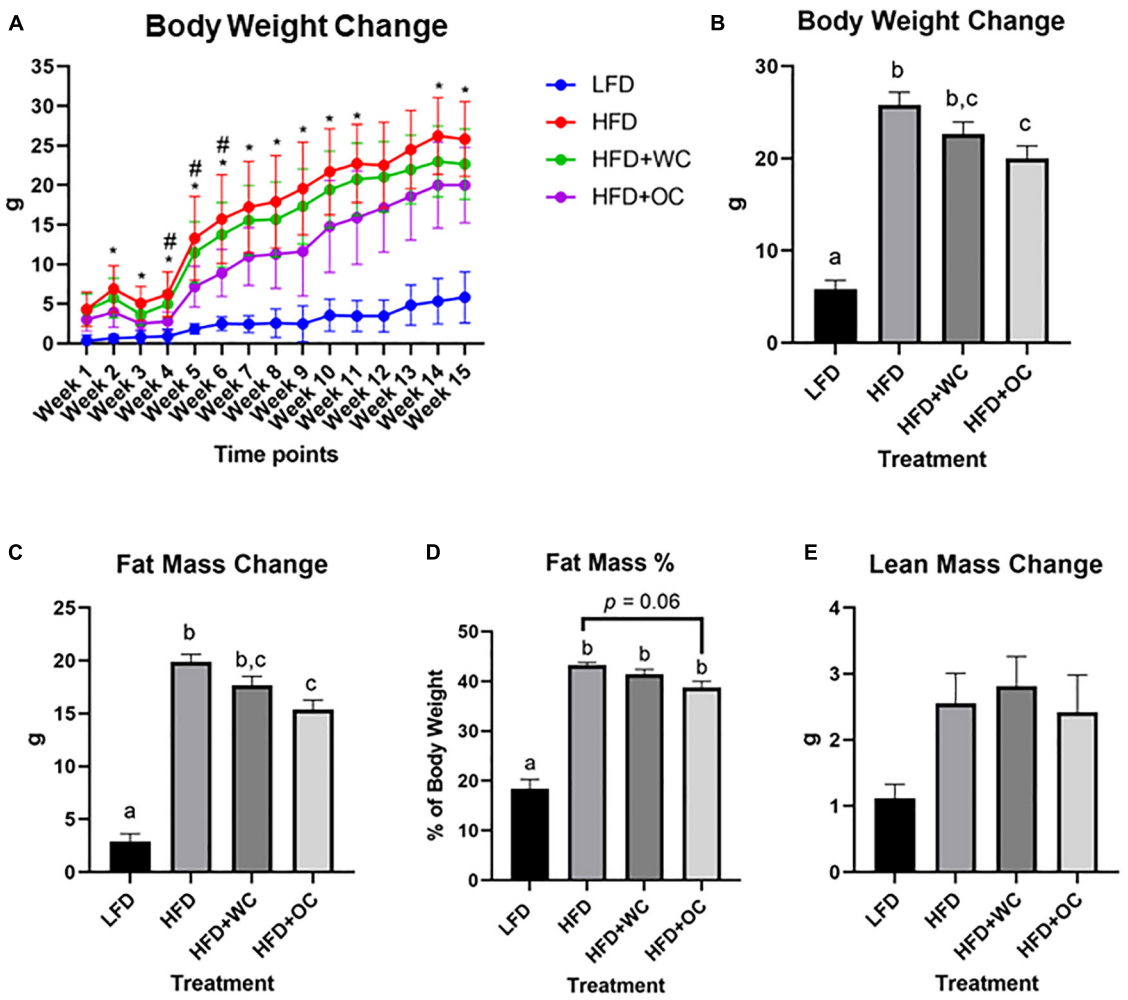

FIGURE 1
Orange carrot-rich diet inhibited HFD-induced changes in body composition. LFD: n = 11; HFD, HFD + WC, HFD + OC: n = 12. **(A)** Weekly changes in body weight compared to baseline (week 0), *HFD vs. HFD + OC, $p < 0.05$; #HFD + WC vs. HFD + OC, $p < 0.05$. **(B)** Body weight change at endpoint (week 15) compared to baseline. **(C)** Change in fat mass determined by EchoMRI. **(D)** Fat mass percentage of Week 15 body weight. **(E)** Change in lean mass determined by EchoMRI. Values are means ± SEM. Body weight change was analyzed by two-way mixed ANOVA with *post hoc* Tukey HSD. Changes of body weight, fat mass and lean mass were analyzed by one-way ANOVA with *post hoc* Tukey HSD. Letter difference indicate statistical significance ($p < 0.05$).

was trending to be lower than HFD + WC, but this difference did not reach statistical significance (Figure 1C). The fat mass percentage of total body weight was measured, of which the HFD, HFD + WC, and HFD + OC groups were significantly higher than the LFD group (Figure 1D). The decrease in fat mass percentage from HFD conditions seen in the HFD + OC

group nearly reached significance ($p = 0.06$). The changes in the lean mass amongst the groups were not statistically significant (Figure 1E).

Carotenoids in orange carrots inhibited NAFLD severity

Hepatic steatosis

Liver weights depicted a similar trend to the overall body weight (Figure 2A). The HFD treatment group had a significantly higher liver weight-to-body weight (LW/BW) ratio than the LFD group. The LW/BW ratio of the HFD + WC group decreased from the HFD group but was not significantly different; additionally, this decrease reached a level statistically comparable to the LFD group. Finally, the LW/BW ratio of the HFD + OC group was significantly lower than the ratio of the HFD group, though not significantly different from the HFD + WC ratio. Notably, orange carrot supplementation sufficiently decreased the liver weight/body weight ratio in the HFD + OC group to a point comparable to LFD, like the HFD + WC group but to a greater degree.

H&E staining depicted the extent of fat deposits within the livers (Figure 2B). The LFD liver histology showed a very minimal presence of fat. The HFD livers were riddled with fatty deposits that were large in both size and number, which covered a significantly larger percentage of area than the LFD livers ($23.7 \pm 2.9\%$ vs. $1.2 \pm 0.2\%$, $p < 0.01$). The HFD + WC livers still contained fat throughout the tissues ($13.8 \pm 2.6\%$), but not to the extent of the HFD livers ($p < 0.01$). However, in the HFD + OC group, the area of livers covered by lipid droplets ($3.2 \pm 0.6\%$) was significantly reduced compared to both HFD and HFD + WC ($p < 0.01$) and to a level comparable to LFD.

Triglycerides and free fatty acids in the liver and the circulation

Based on the differential presence of fatty deposits within the livers throughout the treatment groups, hepatic triglyceride (TG) levels were measured (Figure 3A). The HFD livers contained a significantly higher content of TG than the LFD livers (28.3 ± 1.5 mM/g tissue vs. 14.3 ± 2.4 mM/g tissue, $p < 0.01$), and a significant reduction in TG content was observed in the HFD + WC livers (17.6 ± 0.9 mM/g tissue) compared to the HFD group ($p < 0.01$), which was further reduced in the HFD + OC livers (12.9 ± 0.1 mM/g tissue, $p < 0.01$). However, the difference in TG content between the HFD + WC and HFD + OC groups was not significant. Circulatory TG levels were investigated by measuring triglyceride content within the serum (Figure 3B). As expected, TG content in the HFD group was significantly higher than in the LFD group (0.7 ± 0.1 mM vs. 0.3 ± 0.1 mM, $p < 0.01$). Serum TG in the HFD + WC group (0.6 ± 0.1 mM) was significantly higher than LFD ($p < 0.01$) and comparable to the HFD group. Notably, the serum TG content was significantly reduced in the HFD + OC (0.4 ± 0.04 mM) compared to both HFD and HFD + WC groups ($p < 0.01$, $p = 0.04$, respectively), and such decrease was brought down to a level comparable to the LFD group.

To investigate how serum TG was associated with the TG content in the liver, fatty acid transport cluster of differentiation 36 (CD36) and microsomal triglyceride transfer protein (MTP) were examined. As a result, the LFD livers contained the least mRNA amount of *cd36* compared to HFD, HFD + WC, and HFD + OC ($p < 0.01$) (Figure 3C). This trend of higher *cd36* content is expected in these HFD due to the increased composition (60%) of fat within the diet compared to LFD (10%). The protein expression of MTP within the liver was quite consistent across the groups with no significant differences (Figure 3D). These data indicate that the higher lipid accumulation in the HFD liver might be due to the increased hepatic fatty acid intake, not disturbed fatty acid output.

Fatty acid synthesis

In order to look into the changes observed in the histology and triglyceride experiments, genes and proteins related to fatty acid synthesis were investigated. Fatty acid synthase (FAS) is a major player in this process as the enzyme catalyzes the *de novo* synthesis of fatty acids (46). Protein expression of FAS in the LFD and HFD were comparable, which were slightly higher than the carrot groups. However, there were no significant differences between all the treatment groups (Figure 4A). Additionally, this study did not lead to significant changes in *fas* at the mRNA level (Figure 4F).

Stearoyl-CoA desaturase-1 (SCD-1) is involved in fatty acid synthesis by catalyzing the generation of monounsaturated fatty acids (MUFAs), such as oleate and palmitoleate formed *via* desaturation of stearoyl-CoA and palmitoyl-CoA, respectively (47). Firstly, the LFD and HFD groups did not differ significantly (Figure 4B). HFD + WC led to a drop in SCD-1 expression, but this was non-significant compared to both LFD and HFD groups. Notably, the HFD + OC significantly decreased SCD-1 compared to the HFD ($p < 0.01$), which was not achieved by the HFD + WC. While the difference between the carrot-fed groups was not significant, a lower SCD-1 expression can be observed in the HFD + OC. The mRNA levels of SCD-1 depicted similar trends as the protein expression. Analysis achieved *via* qPCR portrayed that mRNA levels of *scd-1* were differentially expressed across the treatment groups ($p < 0.01$), and the pattern was consistent with the protein expression (Figure 4F).

Protein expression of acetyl coenzyme A carboxylase alpha (ACCα) was also investigated due to its role in catalyzing the carboxylation of acetyl-CoA to form malonyl-CoA (48). ACCα did not appear to be highly expressed in the liver, and no significant trends were observed amongst the treatment group (Figure 4C). Finally, protein expression of diacylglycerol O-acyltransferase 2 (DGAT2) was investigated due to its role in

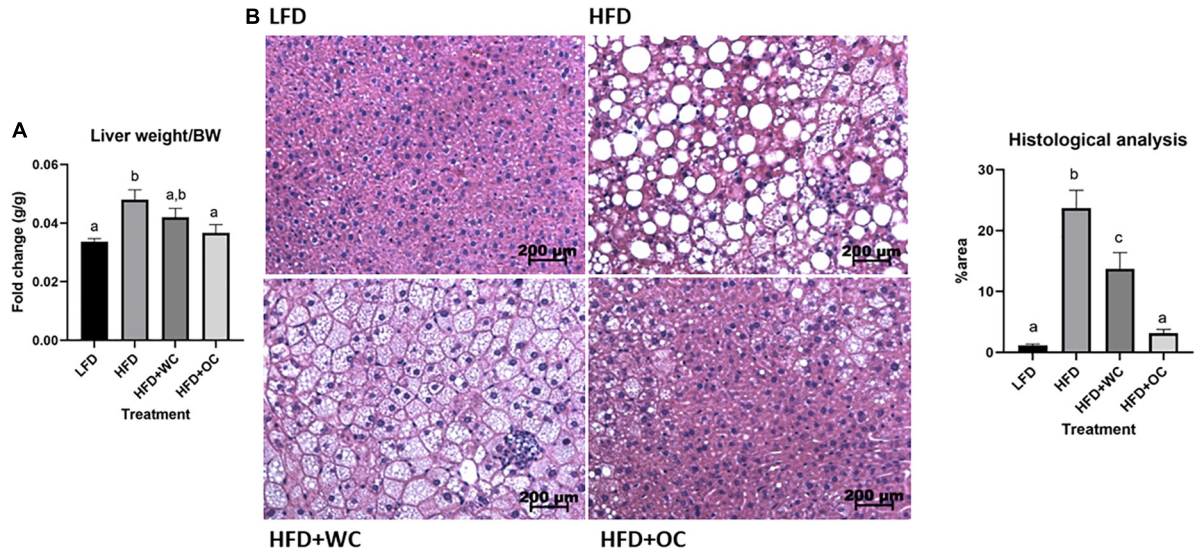

FIGURE 2
Orange carrot-rich diet improved status of HFD-induced hepatic steatosis. **(A)** Graphical representation of liver weight-to-body weight (BW) ratio. LFD: $n = 11$; HFD, HFD + WC, HFD + OC: $n = 12$. **(B)** Hematoxylin and eosin (H&E) staining of livers, percentage of area covered by lipid deposits at 20 X magnification. Liver weight was analyzed by one-way ANOVA with *post hoc* Tukey HSD. Liver histology was analyzed by using the Kruskal-Wallis test with *post hoc* Tukey HSD. Letter differences indicate statistical significance ($p < 0.05$).

synthesizing triglycerides by covalently binding diacylglycerol to long-chain fatty acyl-CoAs (49). Most of the groups did not have differential expression of DGAT2 as the changes between LFD, HFD, and HFD + WC were not significant (Figure 4D). Surprisingly, the HFD + OC group expressed a significantly higher content of DGAT2 compared to all the other dietary groups ($p < 0.01$).

Sterol regulatory-element binding proteins (SREBPs) are a family of transcription factors responsible for regulating lipid biosynthesis, and adipogenesis *via* enzymes involved in cholesterol, fatty acid, triacylglycerol, and phospholipid synthesis (50). The activity of SREBP-1 is dependent on the cleavage of its precursor compound; thus, the ratio of cleaved SREBP-1/SREBP-1 portrays the relative activity within that group. This ratio was comparable in the LFD, HFD, and HFD + WC groups (Figure 4E). The HFD + OC group had the lowest ratio (HFD + OC vs. LFD, $p < 0.01$; HFD + OC vs. HFD, $p = 0.09$), indicating the least amount of SREBP-1 activity. At the same time, this change did not reach a significant decrease compared to the HFD and HFD + WC groups; this ratio was significantly lower than the LFD group. There were no observed significant differences in *srebp-1* between the groups at the mRNA level (Figure 4F).

β-Oxidation

To further investigate hepatic lipid regulation, we explored β-oxidation related targets, including acyl-CoA oxidase 1 (ACOX1) and carnitine palmitoyltransferase-2 (CPT-II) within the fatty acid β-oxidation pathway. The HFD + OC group had the most robust ACOX1 protein expression, significantly higher than the LFD group ($p < 0.01$) (Figure 5A). Despite the non-significant difference, there was a pattern of higher ACOX1 in the HFD + OC group compared to other groups. The protein content of ACOX1 portrayed a slightly different story than the mRNA levels. Most of these treatment groups did not exhibit differential expression of *acox1* mRNA levels as the changes between LFD, HFD, and HFD + WC were not significant (Figure 5B). Notably, the HFD + OC group expressed a significantly higher content of *acox1* compared to all the other treatment groups ($p < 0.01$).

The LFD and HFD livers expressed comparable levels of CPT-II protein content. HFD + WC had a slightly higher but insignificant increase in expression than HFD (Figure 5C). HFD + OC livers had the strongest expression of CPT-II, significantly higher than both LFD and HFD groups ($p < 0.01$, $p = 0.0149$, respectively). Despite the lack of significance, the HFD + OC CPT-II expression trended higher than the HFD + WC group. The *cpt* mRNA content was consistent throughout most groups as the LFD, HFD, HFD + WC levels were comparable (Figure 5D). Notably, there was a significant increase in *cpt* within the HFD + OC group compared to the HFD and HFD + WC groups ($p = 0.02$, $p < 0.01$, respectively).

Potential underlying pathways

As phosphorylation of AMPK is required for activation of downstream regulation (51–53), protein levels of the

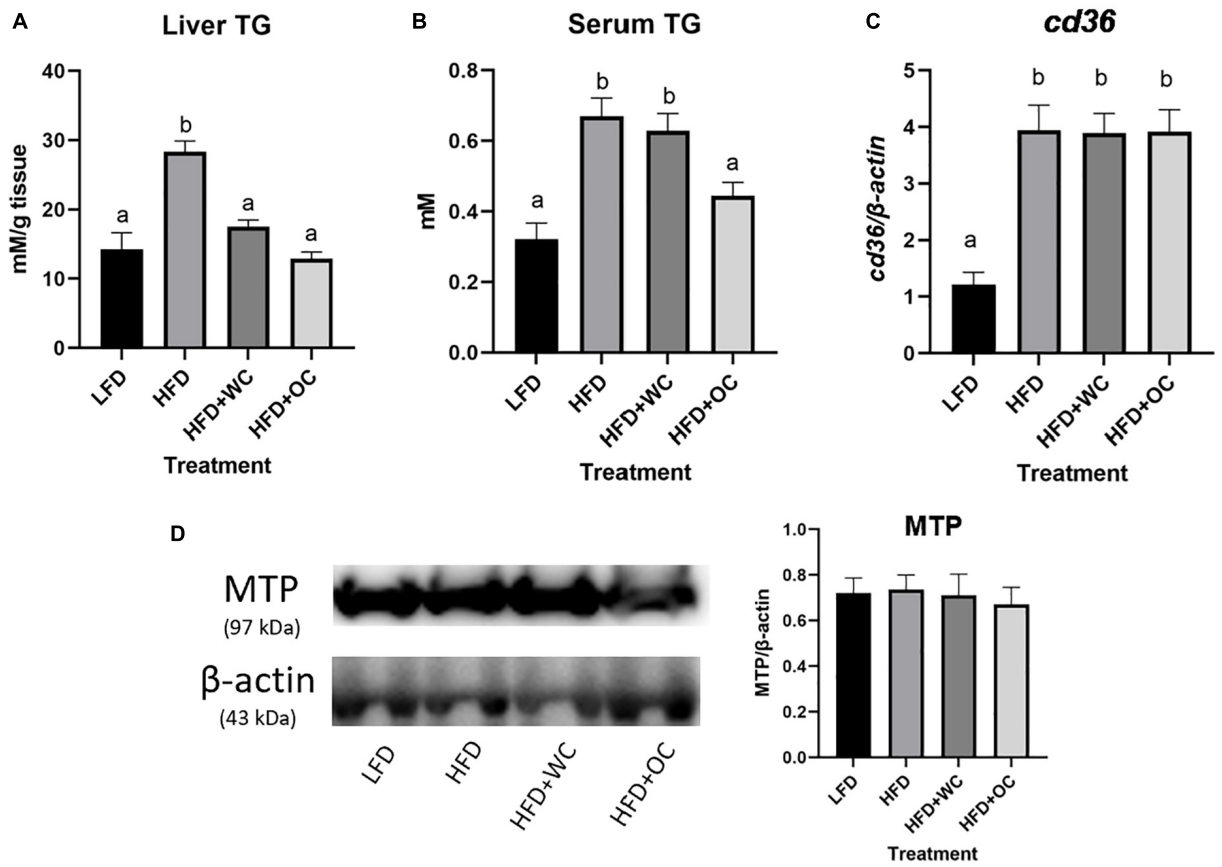

FIGURE 3
Hepatic and circulatory fat content. **(A,B)** Triglyceride (TG) content in panel **(A)** liver and **(B)** serum. For both liver and serum TG assay, LFD, HFD, HFD + WC, HFD + OC: $n = 10$. **(C)** Changes in mRNA levels in hepatic *cd36* generated from qPCR. **(D)** Western blot of hepatic MTP protein expression performed with 3–8% tris-acetate gel, graphical changes between the dietary groups. For both western blot and qPCR assays, LFD: $n = 11$; HFD, HFD + WC, HFD + OC: $n = 12$. Values are means ± SEM. Data were analyzed by one-way ANOVA with *post hoc* Tukey HSD. Letter differences indicate statistical significance ($p < 0.05$).

p-AMPK/total AMPK ratio within the liver were assessed (Figure 6A). The LFD and HFD groups expressed comparable levels of the p-AMPK/AMPK ratio. The HFD + WC group exhibited a slight increase in p-AMPK/AMPK compared to the LFD and HFD groups, but this difference was not significant in measure. The HFD + OC group had a further increase in the p-AMPK/AMPK ratio that was significantly higher than the LFD, HFD, and HFD + WC groups ($p < 0.01$, $p < 0.01$, $p = 0.03$, respectively).

AMPK activity can promote the activity of PPARs by upregulating peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (54). The PGC-1α protein expression was consistent throughout most groups as the LFD, HFD, HFD + WC levels were comparable (Figure 6B). Notably, there was a significant increase in PGC-1α within the HFD + OC group compared to the HFD and HFD + WC groups ($p < 0.01$). The mRNA levels depicted similar trends to protein expression as *pgc-1α* in the HFD and HFD + WC groups were significantly decreased from LFD ($p < 0.01$) (Figure 6C). The HFD + OC group restored the *pgc-1α* to a statistically comparable level with the LFD group.

Consistently, the protein content of PPARα within the LFD and HFD groups were similar (Figure 6D). The HFD + WC group exhibited a slightly greater expression of PPARα than the LFD and HFD groups, but this difference was not significant in measure. Finally, PPARα expression was the strongest in the HFD + OC group; this change was significant compared to the LFD and HFD groups ($p < 0.01$), but not against the HFD + WC group. At the mRNA level, *pparα* was similar across the LFD, HFD, HFD + WC groups but significantly higher in the HFD + OC group ($p < 0.05$) (Figure 6E). To further determine the influence of PPARα within the liver, PPARα transcription factor activity was investigated within nuclear protein extracts (Figure 6F). PPARα transcription factor activity within the HFD + OC was comparable with the HFD + WC group, but statistically higher than those within the LFD and HFD groups ($p = 0.04$, $p = 0.03$, respectively).

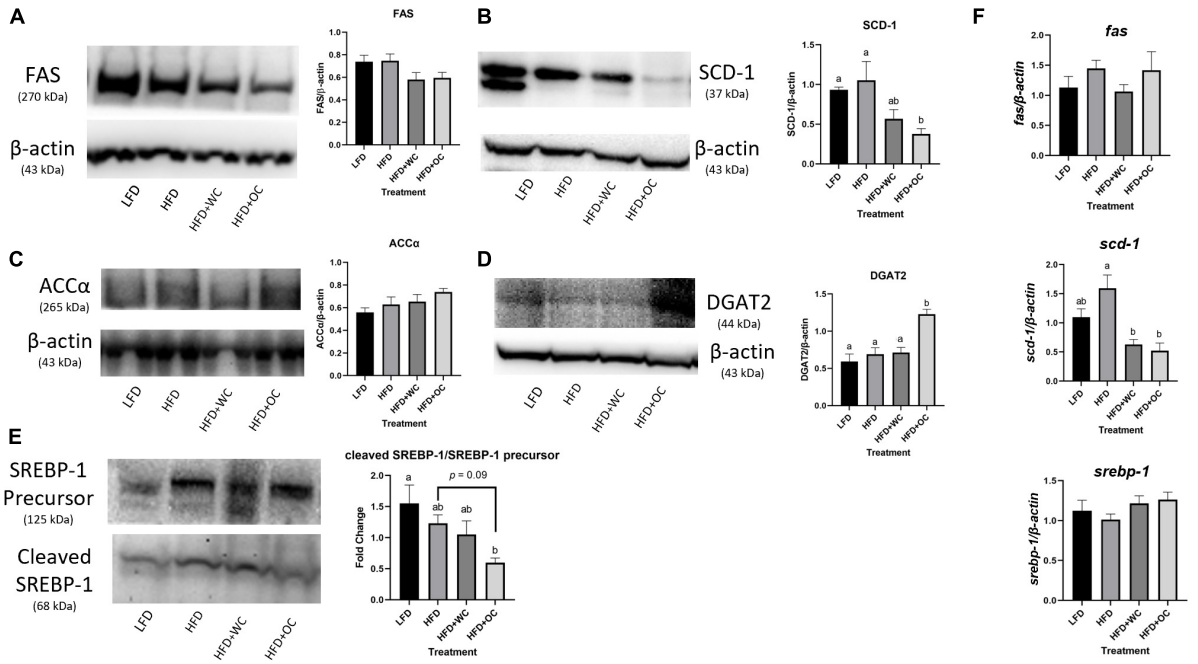

FIGURE 4
Carotenoids minimally inhibit hepatic fatty acid synthesis. Western blot of hepatic **(A)** FAS, **(B)** SCD-1, **(C)** ACCα, **(D)** DGAT2, and **(E)** ratio of cleaved SREBP-1 to SREBP-1 precursor protein expression; blots in panels **(A,C,E)** performed with 3–8% tris-acetate gels; blots in panels **(B,D)** performed with 4–12% Bis-Tris gels; graphical changes between the dietary groups. **(F)** Changes in mRNA levels in hepatic *fas*, *scd-1*, and *srebp-1* generated from qPCR. For both western blot and qPCR, LFD: n = 11; HFD, HFD + WC, HFD + OC: n = 12. The fold change of mRNA and protein levels were analyzed by one-way ANOVA with *post hoc* Tukey HSD. Letter differences indicate statistical significance ($p < 0.05$).

Discussion

Throughout the study, the orange carrot diet displayed more potent efficacy in reducing the HFD-induced body weight gain than the white carrot diet. In a clinical study by Takagi et al. (16), subjects were randomly assigned to one of the four diets: high lycopene + high lutein, high lycopene + low lutein, low lycopene + high lutein, and low lycopene + low lutein. At the end of the study, the visceral fat level was significantly decreased in all the dietary groups. However, a significant decrease in waist circumference was only observed in the high lycopene + high lutein (16). Such data were consistent with the results of our study, showing that the dietary carotenoids in the vegetables may add another layer of protective effect against lipogenesis. However, the study by Takagi et al. employed lycopene and lutein. To our best knowledge, till date, no study has used the same methodology to explore the effects of alpha- and beta-carotene, in whole food.

α-Carotene and β-carotene are among the most frequently consumed dietary carotenoids in North American diets (55). Liver is the major storage organ for dietary carotenoids, including α-carotene, β-carotene, lycopene, β-cryptoxanthin, lutein, and zeaxanthin, while β-carotene is one of the most abundant carotenoids, other being lycopene (44, 45, 56). In this study, the average serum and hepatic β-carotene concentrations of the orange carrot supplemented mice were 1.4 ± 0.1 μM and 8.0 ± 3.0 μmol/g tissue, respectively. The average hepatic α-carotene concentration of the orange carrot supplemented mice was 3.3 ± 1.9 μmol/g tissue, and the serum α-carotene concentration was not detected due to the extremely low circulating α-carotene concentration that was expected at a nanomolar level (57). These concentrations fall within the range of typical β-carotene concentrations in human serum (0.04–2.26 μM) and livers (0.39–19.4 μmol/g tissue), and human hepatic α-carotene concentration (0.075–10.8 μmol/g) (55). Oral β-carotene supplementation or the consumption of a high-β-carotene diet may lead to a higher circulating β-carotene concentration at 0.68–2.26 μM (58, 59). We recently engineered *S. boulardii* that could synthesize high doses of β-carotene, so the intake of such engineered probiotics may lead to an even higher β-carotene concentration in the circulation (42), indicating that the dosage of supplemented dietary carotenoids through carrots was of physiological relevance.

As provitamin A carotenoids, α-carotene and β-carotene can be enzymatically cleaved at the 15-, 15′-double bond by β-carotene dioxygenase 1 (BCO1) to produce all-trans retinal (vitamin A aldehyde) (9). In the liver, all-trans retinal can be either reversibly reduced to all-trans retinol and then esterified to retinyl esters for storage, or irreversibly oxidized to all-trans retinoic acid (ATRA), the biologically active form of

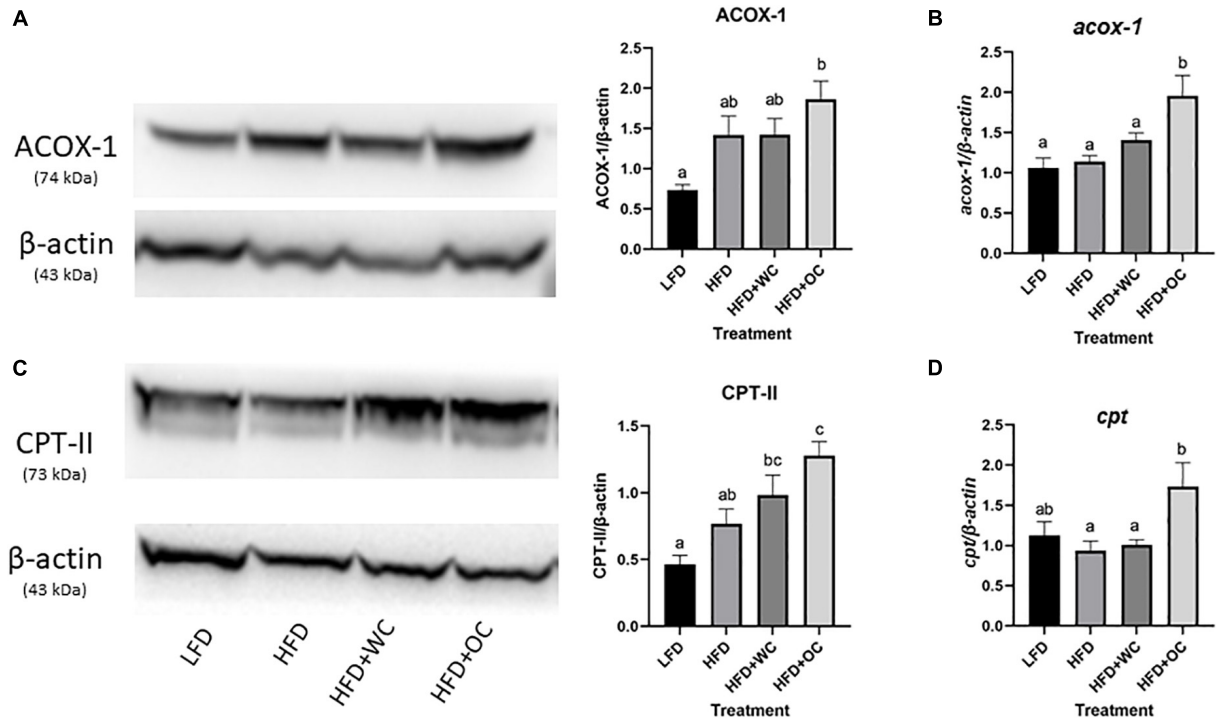

FIGURE 5
Carotenoids in HFD + OC diet promote hepatic β-oxidation. Western blot of **(A)** ACOX-1 and **(C)** CPT-II protein expression; performed with 4–12% Bis-Tris gels; graphical changes between the dietary groups. Changes in mRNA levels in hepatic **(B)** *acox-1* and **(D)** *cpt* generated *via* qPCR. For both western blot and qPCR, LFD: $n = 11$; HFD, HFD + WC, HFD + OC: $n = 12$. The fold change of mRNA and protein levels were analyzed by one-way ANOVA with *post hoc* Tukey HSD. Letter differences indicate statistical significance ($p < 0.05$).

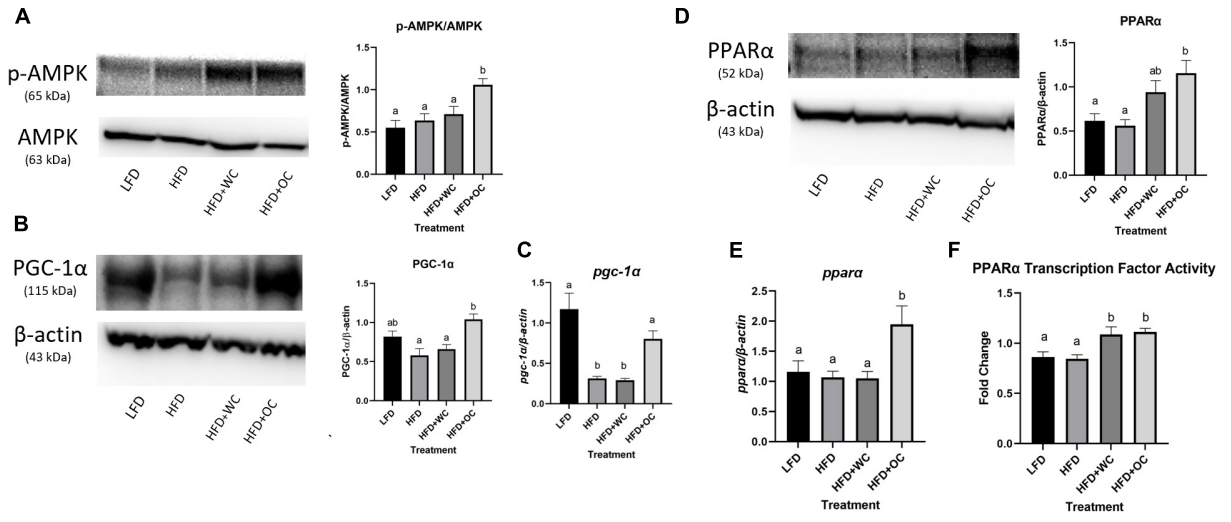

FIGURE 6
Carotenoids in HFD + OC diet enhance regulators of hepatic lipid metabolism. **(A)** Western blot of phosphorylated AMPK (p-AMPK) and total AMPK protein expression, performed with 4–12% Bis-Tris gel; statistical analysis of p-AMPK/AMPK ratio. **(B)** Western blot of PGC-1α protein expression, performed with 3–8% tris-acetate gel. **(C)** Changes in mRNA levels in hepatic *pgc-1α*. **(D)** Western blot of PPARα protein expression, performed with 4–12% Bis-Tris gel. **(E)** Changes in mRNA levels in hepatic *pparα*. For both western blot and qPCR, LFD: $n = 11$; HFD, HFD + WC, HFD + OC: $n = 12$. **(F)** Graphical changes in nuclear PPARα transcription factor activity. LFD, HFD, HFD + WC, HFD + OC: $n = 10$. Values are means ± SEM. The fold change of mRNA, protein, and PPARα transcription factor activity were analyzed by one-way ANOVA with *post hoc* Tukey HSD. Letter differences indicate statistical significance ($p < 0.05$).

vitamin A (60). It has been reported that the hepatic ATRA and retinol concentrations in mice livers were at the pmol/g tissue range (61), which was beyond the limit of detection of our carotenoid quantification method. Therefore, it is unknown whether orange carrot supplementation results in higher levels of hepatic vitamin A and ATRA. As the biologically active form of vitamin A, ATRA is a high-affinity ligand for retinoic acid receptors, while its isomer, 9-*cis*-retinoic acid (9cRA) is an agonist of retinoid X receptors (62–64). However, we were unable to detect 9cRA in livers and serum of the animals. Since we did not observe a significant change in hepatic RXRα and RARβ expressions (**Supplementary Figure 3**), and the hepatic RXRβ concentration was undetectable (data not shown), the beneficial effects of orange carrots may stem from α- and β-carotene as parent compounds, not ATRA.

Hepatic steatosis may result from excessive fatty acid or TG uptake, reduced TG output, increased *de novo* lipogenesis, decreased β-oxidation, or a combination. In the HFD group, we observed promoted levels of serum TG, which was intriguingly significantly correlated with higher liver TG levels ($p < 0.01$) (**Supplementary Figure 4**). CD36 mediates long-chain fatty acid uptake in liver (65). In this study, the expressions of *cd36* mRNA were higher in mice fed on the HFD diet, despite white carrot or orange carrot supplementation, indicating that the increased hepatic uptake of FFA from the circulating system played a vital role in developing HFD-induced liver steatosis. MTP is rate-limiting for the assembly of apoB-containing lipoprotein and hepatic TG secretion (66, 67), so the comparable MTP protein expressions across the dietary groups suggested that hepatic TG output minimally contributed to the development of NAFLD.

The orange carrot supplementation was more efficient in alleviating HFD-induced NAFLD than the supplementation of white carrots. Such result is in line with a previous publication that the consumption of spinach and tomato (both containing high levels of carotenoids) efficiently ameliorated NAFLD in rats fed with the HFD (68). However, the positive control of that study was a lower percentage of carotenoid-rich vegetables, leading to a proportionally lower concentration of other beneficial compounds, such as fiber. Therefore, it is unknown whether the anti-NAFLD efficacy of that diet was mainly attributed to carotenoids or fiber. The advantage of the current study is that we employed white carrot as a positive control, which contains an extremely low level of carotenoids, but an equivalent amount of fiber, compared to the orange carrot. Thus, we are confident that the NAFLD-preventive efficacy of the orange carrot diet was primarily from α-carotene and β-carotene, not fiber. ACCα is the rate-limiting enzyme in regulating fatty acid synthesis (48, 69). In the current study, the ACCα protein levels did not differ among the groups. Although we observed a pattern of decreased FAS and significantly reduced SCD-1 in the HFD + OC group, DGAT-2 significantly increased in the HFD + OC group. Therefore, it is inconclusive to address whether orange carrot supplementation ameliorated NAFLD through modulating fatty acid synthesis. ACOX1 catalyzes the first and rate-determining step in peroxisomal fatty acid oxidation (70), and the mutation of ACOX1 was shown to induce NAFLD progression and exacerbate hepatocellular damage (71). In the current study, ACOX1 was significantly improved in the mice with orange carrot supplementation, which was in line with the report that the consumption of foods high in β-carotene and other carotenoids increased hepatic ACOX1 in rats (68). CPT-II is one of the key enzymes in the mitochondrial β-oxidation of fatty acids (72). A significantly enhanced CPT-II protein in the HFD + OC group indicates that promoted fatty acid β-oxidation may be the primary mechanism of an orange carrot-rich diet that prevented HFD-induced NAFLD development.

By further exploring the molecular targets involved in β-oxidation, we found a significantly increased p-AMPK expression in the HFD + OC group compared with the HFD group. AMPK is a heterotrimeric complex, and its α subunit is the main catalytic domain (53). Although the α subunit can be phosphorylated at Thr172, Thr258, and Ser485 sites, phosphorylation of Th172 is the hallmark of AMPK activation (51–53). Previous studies have shown that the activation of AMPK could protect against diet-induced NAFLD and NASH (33, 73). AMPK phosphorylation can inhibit the cleavage and maturation of SREBP-1, subsequently attenuating hepatic steatosis through regulating lipogenic genes (74), so it is possible that the dietary carotenoids in the orange carrot could reduce SRBEP-1 cleavage through promoting the phosphorylation of AMPK. In addition to AMPK activation, we observed significantly higher hepatic levels of *pparα* mRNA, PPARα protein, and PPARα transcription factor activity in the mice supplemented with orange carrots, indicating that the dietary carotenoids in orange carrots might prevent the development of NAFLD by targeting the PPARα pathway. PPARα, PPARβ/δ, and PPARγ are three PPAR isoforms ubiquitously expressed in various tissues, but PPARα is mainly present in liver (75). In NAFLD patients, the hepatic PPARα expression was negatively correlated with occurrence of NASH, severity of NAFLD, ballooning of the hepatocytes, and NASH activity score and fibrosis (76). Ip et al. found that in mice, PPARα knockout resulted in significantly more severe steatohepatitis, while the administration of Wy-14643, a potent PPARα agonist, substantially prevented diet-induced NAFLD and liver injury (77). Furthermore, the injection of Wy-14643 promoted expression of acyl-CoA oxidase (77), which was in line with our finding that the increased PPARα was associated with an elevated level of ACOX1 in the HFD + OC group. PGC-1α acts as a coactivator of PPARα and promotes PPARα-mediated transcriptional activity in modulating its target genes, such as genes involved in β-oxidation (78). Interestingly, in this study, we found a more potent increase in PGC-1α protein expression in the HFD + OC group, compared with the HFD + WC group, indicating that the dietary carotenoids in the orange carrots may

activate the PGC-1α-PPARα pathway. In summary, our study reveals that the dietary carotenoids in the orange carrots rich in dietary carotenoids, specifically α-carotene and β-carotene, may regulate the fatty acid synthesis and β-oxidation-related genes by activating AMPK and PPARα. However, how these compounds promote the phosphorylation of AMPK and activate the PGC-1α-PPARα pathway remains enigmatic. Another potential target of interest in future studies can be epoxide hydrolase (sEH) as previous studies have shown that inhibiting sEH may be involved in alleviating HFD-induced hepatic adiposity and inflammation (79, 80).

One major limitation of the current study is the number of the examined proteins. Since a large variety of proteins with various functions orchestrates the lipid metabolism process, we could not analyze all these participants. With regard to this, we are planning to utilize proteomics, a powerful tool that characterizes a large scale of proteins by their expressions, functions, structures, and protein-protein interactions (81), in our future studies to acquire a broader perspective. Another limitation is that we only examined liver, although the development of NAFLD could be a joint result of changes in several organs. Therefore, we cannot conclude that carotenoids alleviate NAFLD by directly targeting the liver. Previous studies have reported that carotenoids may mitigate NAFLD *via* the gut- and adipose-liver crosstalk (14). For example, one study showed that disrupted free fatty acid mobilization from mesenteric adipose (MAT) tissue to liver significantly exacerbated NAFLD in mice (82). A potential brain-liver axis involving melanocortin-4 receptor, neuropeptides like neuropeptide Y and agouti-related peptide together regulates food intake, energy expenditure and the pathogenesis of NASH (83, 84). In addition, we failed to provide precise measurement of food consumption of each dietary group as the mice portrayed a behavior of tearing up their food and placing it within their bedding. Although we observed a consistently higher food consumption in the HFD + OC group, compared with other dietary groups, it is difficult to explain whether the orange carrot diet motivated the food intake or promoted physical activity that led the mice to tear more food for bedding. Last but not the least, the β-actin bands were not unanimous in this article. This was due to the different types of gels used. For the high molecular proteins such as MTP, FAS, ACCα,

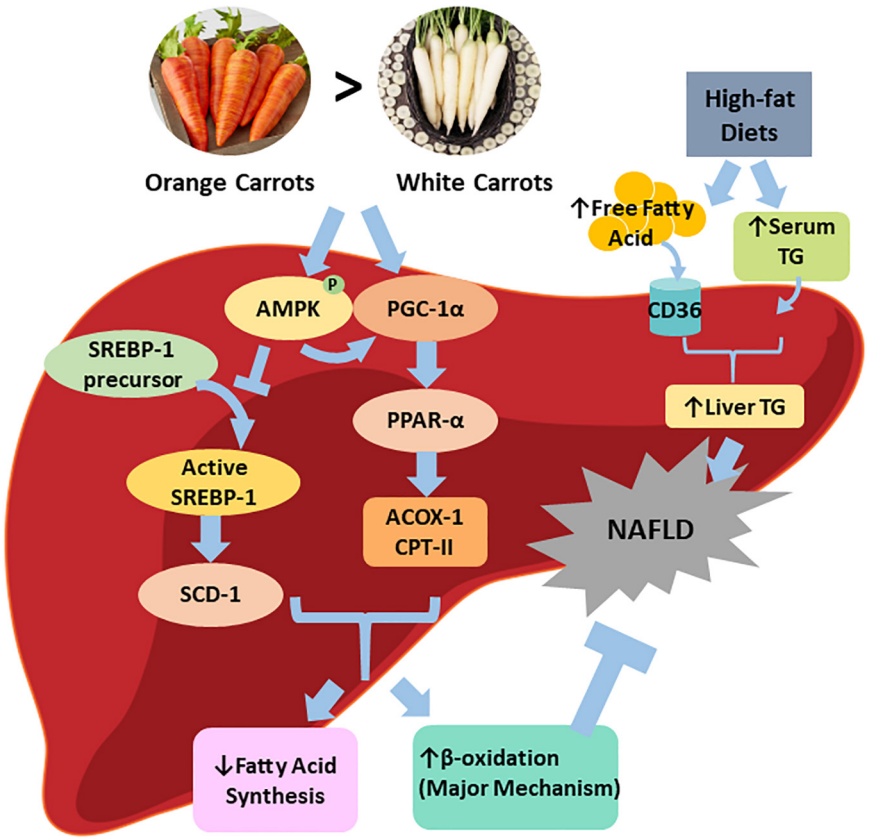

FIGURE 7
Graphical representation of proposed pathway in which carotenoid-rich orange carrots alleviate NAFLD severity. Orange carrots rich in α-carotene and β-carotene combated the severity of hepatic steatosis brought on by a high-fat diet by partially inhibiting fatty acid synthesis and significantly enhancing β-oxidation proteins, due to promotion of master regulators of hepatic lipid metabolism (i.e., p-AMPK, PGC-1α, PPARα).

and PGC-1α, we used tris-acetate gels for optimal separation, which impaired the resolution of β-actin. However, the β-actin expressions were identified within each blot, so our statistical analysis was not affected.

Conclusion

Our results showed that orange carrot supplementation was more effective in preventing HFD-induced NAFLD than white carrots, potentially by increasing hepatic β-oxidation through upregulating PPARα (**Figure 7**). Such data indicate that carotenoid-rich fruits and vegetables may be more efficient in alleviating NAFLD than those with a low carotenoid level. Further clinical trials are warranted to confirm the findings before providing any dietary suggestions to NAFLD patients.

Data availability statement

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

Ethics statement

The study was approved by the Institutional Animal Care and Use Committee (IACUC) at North Carolina State University (protocol code #21-004 and date of approval: 02/26/2021).

Author contributions

AE: supervision, project administration, and funding acquisition. All authors conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing—original draft preparation, writing—review and editing, visualization, read, and agreed to the published version of the manuscript.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.987103/full#supplementary-material

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Mechanisms of the *Ping-wei-san plus* herbal decoction against Parkinson's disease: Multiomics analyses

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Introduction: Parkinson's disease is a neurodegenerative disorder involving loss of dopaminergic neurons. Multiple studies implicate the microbiota-gut-brain axis in Parkinson's disease pathophysiology. *Ping-wei-san plus* Herbal Decoction, a traditional Chinese medicine composition with beneficial effects in Parkinson's disease, may have a complex array of actions. Here we sought to determine whether gut microbiota and metabolic pathways are involved in *Ping-wei-san plus* herbal therapy for Parkinson's disease and to identify functional pathways to guide research.

Methods and results: The model of Parkinson's disease were induced with the rotenone. The *Ping-wei-san plus* group received the PWP herbal decoction for 90 days, after which all groups were analyzed experimentally. PWP herbal treatment improved motor behavior and emotional performance, balanced gut microbiota, and benefited dietary metabolism. Tandem Mass Tags mass spectrometry identified many differentially expressed proteins (DEPs) in the substantia nigra and duodenum in the PWP group, and these DEPs were enriched in pathways such as those involving cAMP signaling, glutamatergic synapses, dopaminergic synapses, and ribosome-rich functions in the gut. The PWP group showed increases in recombinant tissue inhibitors of metalloproteinase 3, and nucleotide-binding oligomerization domain, leucine rich repeat, and pyrin domain containing proteins 6 in the substantia nigra and decreased parkin, gasdermin D, recombinant tissue inhibitors of metalloproteinase 3, and nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing proteins 6 in the duodenum.

Discussion: In conclusion, this study combined gut microbiota, metabolomics, and proteomics to evaluate the mechanism of action of

Ping-wei-san plus on Parkinson's disease and revealed that PWP herbal treatment modulated gut microbiota, altered metabolite biological pathways, and affected functional pathway protein expression in Parkinson's disease mice, resulting in therapeutic effects.

KEYWORDS

Parkinson's disease, microbiome, metabolomics, tandem mass tag labeling, herbal, *Ping-wei-san plus*

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by bradykinesia, resting tremors, muscle rigidity, and postural instability (1). The loss of dopaminergic neurons and the formation of alpha-synuclein (α-synuclein, AS)-containing Lewy bodies in the substantia nigra of the midbrain are considered to be the main pathophysiological characteristics of PD (2). Methods for the prevention of α-syn spread in the gut-to-brain axis are required to prevent neurodegeneration and behavioral deficits. More and more works have implicated the microbiota-gut-brain axis in Parkinson's disease (3, 4). This axis is gaining traction in fields that investigate its biological and physiological basis. Communication along the gut-brain axis is a fundamental aspect of the synergy between microbiota and the host accessing gut-brain signaling pathways to modulate the host brain and behavior. The microbiota and the brain communicate with each other *via* various routes mediated by microbial metabolites such as short-chain fatty acids and branched-chain amino acids, including the immune system, tryptophan metabolism, the vagus nerve, and the enteric nervous system (5, 6). Recently, two live bacterial drugs [*Parabacteroides distasonis* MRx0005 and *Megasphaera massiliensis* MRx0029 (7)] for the treatment of PD received FDA approval for clinical use and marked the expansion of microbial therapy into the area of neurological disorders. Some doubt that intervention through the gut microbiome alone will be effective in treating PD, considering that, even if the disease does originate in the gut, microbial therapy is unlikely to restore dopamine neurons that have already been lost from the brain. To date, we believe that herbal medicine is a more promising complementary medicine than active bacterial replacement therapy because of its potential to regulate gut microbiota, reduce nervous system inflammation, protect neurons from oxidative stress damage, and restore intestinal barrier integrity (8).

Traditional Chinese medicine (TCM) is one of the most important components of complementary and alternative medicine (CAM). Traditional Chinese medicinal compounds (TCMCs) are a traditional combination of Chinese herbal medicines. According to historical records, multiple Chinese herbal medicines have been combined to exert more therapeutic effects by acting on multiple pathogenic mechanisms in the treatment of various diseases. In our clinical work, we prescribed medicines according to the symptoms of patients with PD and found that a classic TCMC prescription *Ping-wei-san* plus medicine (*PWP*, containing *Rhizoma Atractylodis* 12 g, *Magnolia officinalis var.* 9 g, *Pericarpium Citri Reticulata* 9 g, *Anemarrhena asphodeloides Bunge* 12 g, *Rhizoma Coptidis* 6 g, *Aucklandia lappa DC.* 9 g, *Cistanche deserticola Y.C.Ma* 15 g, *Radix Glycyrrhizae* 3 g, components are classified)[1] [2] can not only methods for the prevention of α-syn spread in the gut-to-brain axis are required to prevent neurodegeneration and behavioral deficits. More and more works have implicated the microbiota-gut-brain-axis in Parkinson's disease effectively improve the symptoms of patients with PD, but also improve the prognosis of patients with PD. According to previous studies, TCMCs play a therapeutic role in the treatment of neurological diseases such as PD and depression through the hypothalamic-pituitary-adrenal (HPA) axis or antioxidative, ferroptosis and autophagy, etc. (9–12). Because of the broadly effective active components of TCMCs, their mechanism of action is extremely complex, which is undoubtedly also the advantage of TCMCs in treating diseases. To better understand the complex effects of PWP, we used proteomic ("tandem mass tags" or TMT mass spectroscopy, microbial community analysis (16s RNA sequencing), and metabolomic techniques to gain mechanistic

Abbreviations: DEPs, differentially expressed proteins; PD, Parkinson's disease; TCM, traditional chinese medicine; CAM, complementary and alternative medicine; TCMC, traditional chinese medicinal compound; PWP, Ping-wei-san plus; HPA, hypothalamic-pituitary-adrenal; TH, tyrosine hydroxylase; ELISA, enzyme-linked immunosorbent assay; WB, western blotting; IHC, immunohistochemistry; DAB, diaminobezidin; LC–MS, liquid chromatography mass spectrometry; OPLS-DA, orthogonal partial least squares-discriminant analysis; VIP, variable importance in projection; HPLC, high performance liquid chromatography; TIMP3, tissue inhibitors of metalloproteinase 3; casp-1, caspase-1; ITIH3, inter alpha-globulin inhibitor H3; NLRP6, nucleotide-binding oligomerization domain,leucine rich repeat and pyrin domain containing proteins-6; HRP, horseradish peroxidase; OD, optical density; PCoA, principal coordinate analysis; GO, gene ontology; BP, biological processes; CC, cellular component; MF, molecular function; PPI, protein-protein interaction; OTU, operational taxonomic unit; MAPK, mitogen-activated protein kinase; IL18, interleukin 18; GSDMD, gasdermin D.

1 www.theplantlist.org

2 http://herb.ac.cn/

insight into the pathogenesis of PD and how TCMCs exert their therapeutic effects.

Therefore, our study focused on understanding the mechanisms by which herbs regulate the microbiota-gut-brain axis and sought to elucidate the effectiveness of microbial-based intervention and therapeutic strategies from multiple perspectives.

Materials and methods

Materials

Female C57BL/6 mice (12–14 weeks, 22–26 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., for use in experiments. Herbal medicated feed and AIN-93 standard feed were purchased from Nantong Trofi Feed Technology Co., Ltd., China. Most reagents used in this study were purchased from MedChemExpress. cn and Abcam.cn. Herbal medicine were purchased from Shanghai Pharmaceutical Co., Ltd.

Experimental design

The PWP herbal decoction was prepared by the Pharmacy Department of the Affiliated Hospital of Qingdao University. All mice were raised in an environment with a 12-h light/dark cycle at a temperature of 20 ± 1°C with available food and water. All experiments were conducted according to the principles established for the care and use of laboratory animals by the National Institutes of Health and approved by the Medical Professional Committee of the Affiliated Hospital of Qingdao University (QYFY WZLL 26506). All mice were randomly divided into the following groups (n = 10–12 per group): control, PD model, PWP, L-Dopa therapy and solvent comparison groups. Mice in the control group were fed American lnstitute of Nutrition-93 standard feed daily at a dose of 6-7 gram per mouse. Mice in the PWP group were given herbal medicated feed at a dose of 6-7 gram per contained the herbal dosage of 975 mg/kg every day for 30 consecutive days. Then, mice in the PD group and PWP group were given rotenone solvent gavage at a dose of 0.75 mg 7.2 mg, 10% DMSO 360 μl, PEG300 1,440 μl, Tween-80 180 μl/kg, once a day in the morning. Rotenone Solvent, containing rotenome 7.2 mg, 10% DMSO 360 μl, PEG300 1,440 μl, Tween-80 180 μl, normal saline 1,620 μl (MedChemExpress, China). Mice in the solvent comparison group were gavaged a solvent solution in the morning once daily. The groups were processed for two months. After treatment, we performed substantia nigra tyrosine hydroxylase (TH) immunohistochemical staining and behavioral tests to detect whether the PD model was successful. Next we removed 4 mice from the model group as the L-Dopa treatment group and performed L-Dopa (MedChemExpress, China) gavage in the afternoon once daily at a dose of 20 mg/kg, dissolved in PBS at a concentration of 1 mg/mL and dissolved by ultrasonic. Intestinal permeability tests were performed before death. Fecal samples were collected from all mice and used for 16S rRNA gene sequencing and metabolomic analysis. After behavioral testing, the mice were deeply anesthetized with isoflurane inhalation followed by 10% chloral hydrate and euthanized by cervical dislocation. The substantia nigra and duodenum were isolated for TMT, enzyme-linked immunosorbent assay (ELISA), and western blotting (WB) (**Figure 1A**).

Immunohistochemistry

Mice were transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde for tissue fixation. The corpus striatum was dissected from brain according to the mouse brain atlas, and immediately frozen in liquid nitrogen until further use. Striatal tissues were fixed in cold 4% paraformaldehyde and embedded in paraffin. For IHC, the brain tissue slide was deparaffinized. Sections were incubated with primary anti-body: anti-Tyrosine Hydroxylase (1:500; Abcam) for 60 min followed by goat-anti mouse IgG (1:1,000; Abcam) for 30 min at room temperature and visualized with diaminobezidin (DAB) reagent. After counterstaining with hematoxylin, all slices were detected using an optical microscope(CX31;Olympus, Tokyo, Japan).

Behavioral testing

Open field test

Mice were acclimated 7 days prior to the experiment to familiarize them with the investigator, and then weighed and placed in the testing room before testing. The test mouse was placed onto the central zone of the open field box and started on the timer to allow the mouse to explore the test area for five min. The entire area of the box was cleaned before proceeding to the next test mouse.

Rota-rod test

After completing the open field test, the Rota-Rod test was performed the following day. The Rota-Rod speed was set to accelerate from 5 to 50 rpm over 150 s, and the time and speed (in rpm) required for the animal to fall from the Rota-Rod were measured. Before starting the Rota-Rod study, all mice were allowed to walk for 1 min at the same rod speed (5 rpm). After 1 min, the Rota-Rod speed was gradually increased to 50 rpm for 2 min, and the rod speed was maintained until the mice fell off the bar. The trials were performed three times and were conducted every 20 min.

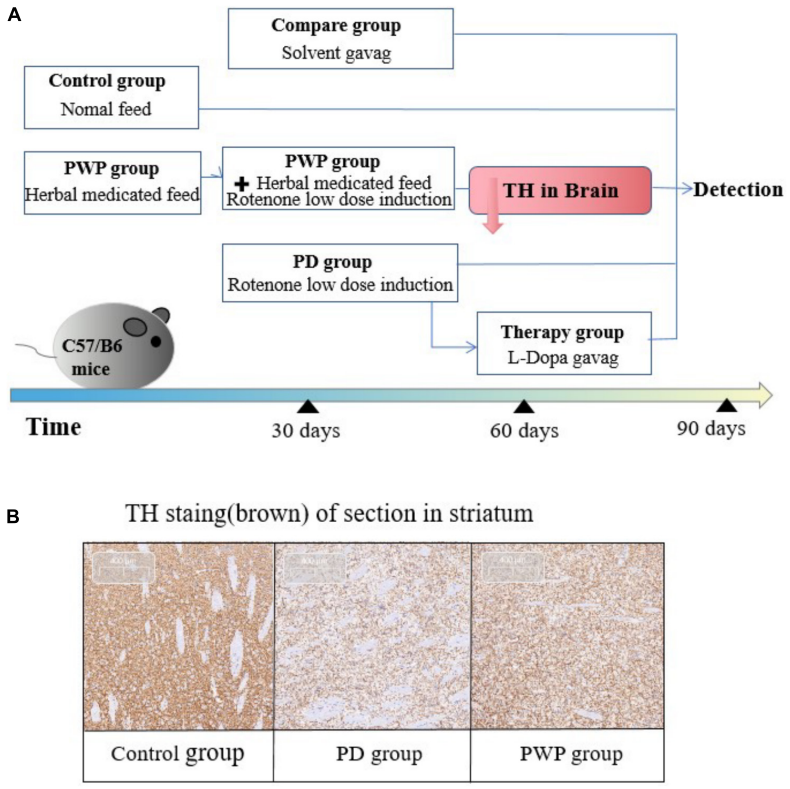

FIGURE 1
Experimental process. **(A)** Mouse experiment study design. **(B)** Confirmation of PD animal model establishment: TH immunohistochemical staining in striatum on the 60th day.

16S rRNA microbial community analysis

To analyze the taxonomic composition of the microbial community, 16S rRNA sequencing analysis was performed. Briefly, PCR amplification was performed, the purified amplicons were pooled, and paired-end sequencing was performed. The raw data were then analyzed. Sequencing of the 16S rRNA amplicon (V3-V4 region) was performed using MiSeq 300PE (Illumina MiSeq System), resulting in a total of 1715085 sequence reads (723701476 bases) and an average sequence length of 421 bp. Bioinformatics was performed using Mothur and QIIME2.0 software and included taxonomic annotation, taxonomy-based comparisons at the genus level, β-diversity analysis (principal coordinates analysis), and dissimilarity analyses. Detailed sequencing analysis procedures are available in the **Supplementary materials** and Methods.

Fecal metabolomic analysis

Liquid chromatography mass spectrometry (LC–MS) analysis was performed by Majorbio Bio-Pharm Technology (Shanghai, China). The fecal samples of the control, PD, and PWP groups were subjected to liquid chromatography for component separation; a single component entered the ion source of the high vacuum mass spectrometer for ionization and was separated according to the mass-to-charge ratio (m/z) to obtain a mass spectrum. Finally, the mass spectra of the samples were analyzed. The qualitative and quantitative results of the samples were obtained.

The LC–MS data from fecal pellets were processed using the Majorbio Cloud Platform (Shanghai, China), and metabolites were identified. Normalized data were visualized by orthogonal partial least squares-discriminant analysis (OPLS-DA) using the ropls package in R. The ellipses in the OPLS-DA plots were employed to characterize metabolic perturbations among groups in a Hotelling T2 region with a 95% confidence interval threshold.

The variable importance in projection (VIP) was calculated based on the OPLS-DA model to identify significant metabolites with a VIP $>$ 1.0 and P-value $<$0.05. The KEGG[3] database was used to explore related metabolic pathways.

3 https://www.kegg.jp/kegg/

Tandem mass tags labeling, high performance liquid chromatography fractionation and LC-MS/MS analysis

After trypsin digestion of the protein samples, the peptide was desalted using a Strata X C18 SPE column (Phenomenex) and vacuum-dried. The peptide was reconstituted in 0.5 M TEAB and processed for the 6-plex TMT kit according to the manufacturer's instructions. Briefly, the labeling reagent was thawed, dissolved in acetonitrile, mixed with the peptides, and incubated at room temperature for 2 h. The labeled peptides were mixed, desalted, and lyophilized under vacuum.

The labeled peptides were fractionated into 60 fractions by high pH reverse-phase HPLC using an Agilent 300Extend C-18 column (5 μm particle size, 4.6 mm ID, 250 mm length) with a gradient of 8% to 32% acetonitrile (pH 9.0) over 60 min. Then, the 60 fractions were combined into 18 fractions, and each fraction (volume of 800 μL) was dried by vacuum centrifugation pending MS analysis. LC-MS/MS analysis of labeled peptides was performed as previously described (13). The related technical support and bioinformatics analyses were provided by Jingjie PTM BioLabs (Hangzhou, China).

Western blot

The protein expression of gasdermin D and parkin in the substantia nigra and duodenum was detected by western blotting. Total protein was extracted from the substantia nigra and duodenum tissues in RIPA lysis buffer containing protease inhibitors. Protein concentrations were determined by a BCA assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Protein samples were mixed with loading buffer (Beyotime, Shanghai), and then the mixtures were boiled at 98°C for 5 min. Equal amount of protein (40 μg/lane) was loaded on an 4-10% of sodium dodecyl sulfate-polyacrylamide gel electrophoresis at a constant voltage of 90 V. After 180 min, the gels were transferred to a poly (vinylidene difluoride) membrane (Millipore Corp., MA) and blocked with 5% non-fatty milk or BSA in Tris-buffered saline Tween-20 (TBST) solution at room temperature for 1 h. The membranes were then incubated with the and incubated with primary antibodies respectively anti-GSDMD (1:2,000; Abcam), anti-Parkin (1:1,500; Abcam), anti-β-actin (1:1,500; Abcam) at 4°C overnight, washed three times with TBST. The membranes were further incubated with 1:5,000 dilution of the horseradish peroxidase-conjugated secondary antibody for 1 h and then washed three times with TBST again. Specific protein bands were then detected using ECL reagent (Millipore Corp., MA) and quantified using Tanon 5,200 Chemiluminescence Imaging System (Shanghai, China),quantified by Image J software (Rawak Software Inc., Germany).

ELISA

The protein expression of recombinant tissue inhibitors of metalloproteinase 3 (TIMP3), caspase-1(Casp-1), inter alpha-globulin inhibitor H3 (ITIH3), and nucleotide-binding oligomerization domain,leucine rich repeat and pyrin domain containing proteins-6 (NLRP6) in the substantia nigra and duodenum was detected using ELISA kits. Briefly, 100 μl of horseradish peroxidase (HRP)-labeled detection antibody was added to each well of standard and sample 96-well plates, and they were then sealed with a sealing film and incubated at 37°C for 60 min. After five washes, 50 μl of substrate A was added to each well, followed by 50 μl of substrate B, shaken gently, mixed, and incubated at 37°C for 15 min in the dark. 50 μl of stop solution was added to each well to terminate the reaction. The optical density (OD) of each well was measured using a microplate reader at a wavelength of 450 nm within 15 min of reaction termination.

Intestinal permeability evaluation

Mice fasted for four hours were gavaged with 0.5 ml of fluorescein isothiocyanate conjugated-dextran (FITC-dextran; FD-4, 22 mg/ml, molecular mass 4.4 kD, Sigma-Aldrich). Blood samples were collected after 3.5 hours and diluted 1/100 in PBS to measure fluorescence intensity using a fluorospectrophotometer with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. FITC-dextran concentrations were determined from a standard curve generated using serial dilutions of FITC-dextran.

Statistical analysis

Statistical analysis was conducted using SPSS for Windows (version 19.0; SPSS Inc., Chicago, IL, USA). All data are presented as the mean ± SD. The significance of the differences between the two groups was analyzed using Student's unpaired *t*-test, and multiple comparisons were analyzed using one-way ANOVA followed by Dunnett's *post hoc* test. The differential abundances of genera and metabolites were determined using non-parametric tests, including the Wilcoxon rank-sum and Kruskal-Wallis H tests. Correlations among fecal metabolites, 16S levels, and physiological and biochemical indices were evaluated using both the Pearson correlation coefficient and Spearman rank correlation. *P*-values <0.05 was considered statistically significant.

Results:

Establishment of the PD disease model and behavioral responses related to motor and dopaminergic activities

The IHC results showed that the TH content in the striatum of the PD group decreased significantly after 30 days of low-dose rotenone induction, which confirmed the establishment of the PD disease model (Figures 1A,B). By the way PWP attenuated these effects. The pole test results showed that the PD mouse model exhibited exercise fatigue and bradykinesia, whereas PWP-treated mice partially recovered their motor skills. The mice treated with PWP recovered from movement disorders within 90 days, close to the control group condition (Figure 2A). Behavioral recovery does occur in this animal model with PWP intervention. Mice in the control and PWP groups spend more time in the central, open area of the box. Mice in the PD group that were stressed showed less activity in the open field, increased stereotypical behavior, preferred staying close to the walls, and traveled more in the periphery (Figure 2B).

Ping-wei-san plus herbal medicines alter the fecal microbiota of Parkinson's disease mice

16S rRNA analysis revealed 317 distinct taxa, and we further analyzed 243 taxa. Principal coordinate analysis (PCoA) was used to identify the significance of the differences and highlight within-replicate variations (Figures 3A–C). LEfSe analysis (LDA threshold of 2) revealed that *Actinobacteria* and *Verrucomicrobiota* were significantly enriched in the feces of PWP-fed mice compared to the PD group. Conversely, *Bacteroidota* and *Campilobacterota* were enriched in the PD group (Figure 4A). Species difference analysis revealed significant differences in the microbial taxa at the family level

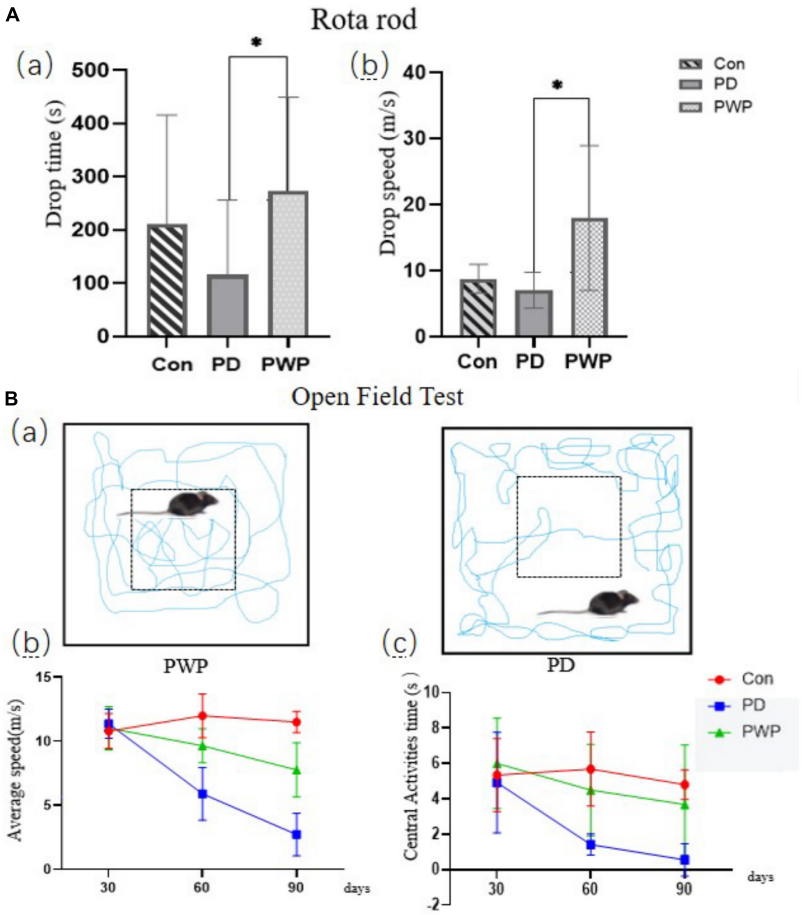

FIGURE 2
Behavioral testing. **(A)** Rota-Rod test performed on the 90th day, including drop time (s) and drop speed (m/s). **(B)** Open field testing performed on the 30th, 60th, and 90th days; motion track simplified diagram showing PD mice (right) and PWP mice (left) with movement (blue trails) in the peripheral or central regions; test data included average speed and central activities time. *Means that $p < 0.1$ for significant differences.

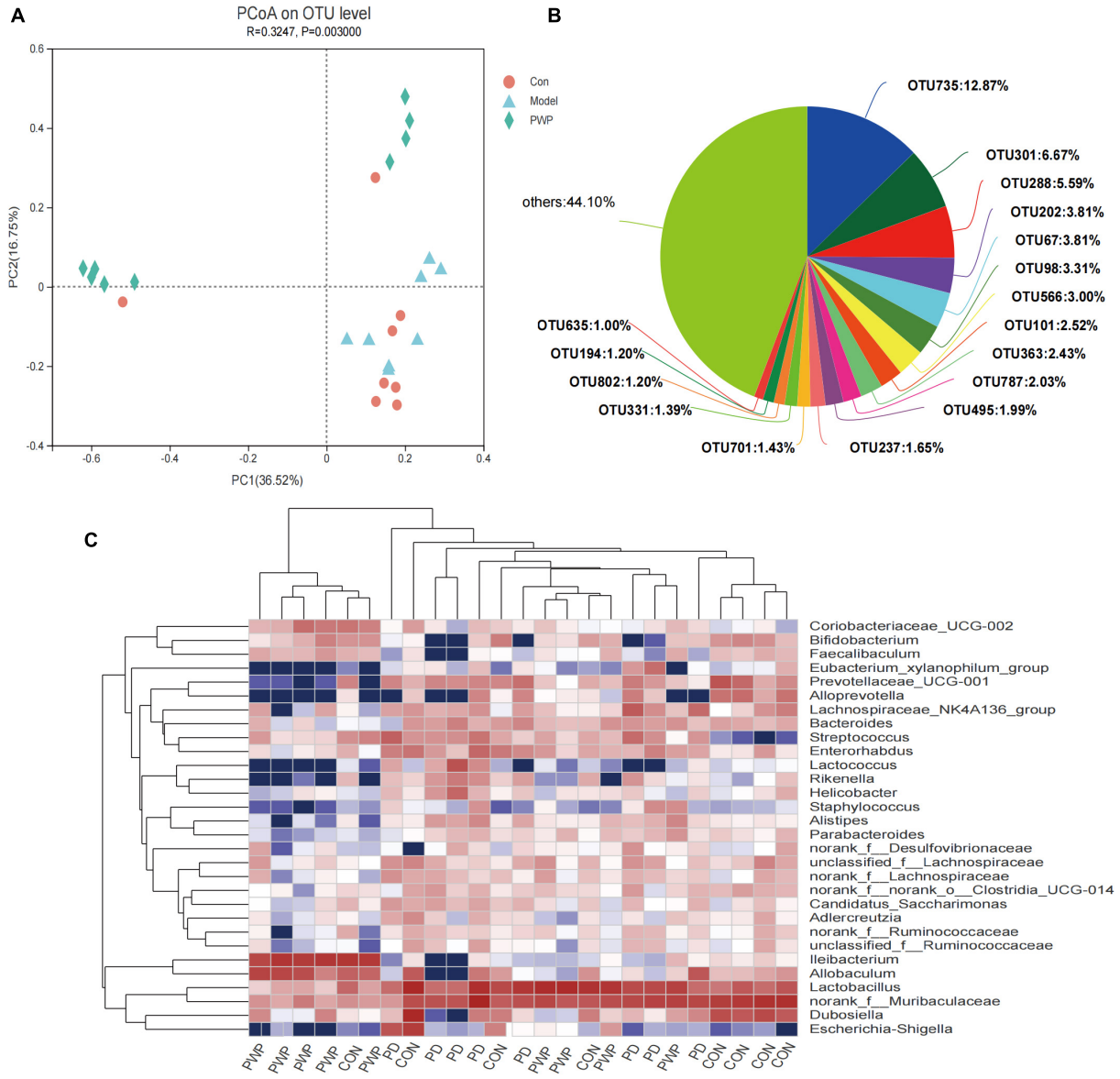

FIGURE 3
The result of 16S microbiome analysis. **(A)** PCoA on OTU level on identified β-microbial diversity in the control, PD, and PWP groups. **(B)** The microbial diversity on OTU level of the control, PD, and PWP groups. **(C)** Community heatmap analysis by Genus.

among the three groups. The results showed that the abundance of *Firmicutes* and *Verrucomicrobiota* in the PWP-fed group was significantly higher than that in the PD group ($^{*}P < 0.05$, $^{**}P < 0.01$, Wilcoxon rank-sum test), while *Bacteroidota*, *Proteobacteria*, *Campilobacterota*, and *Patescibacteria* in the PWP-fed group were significantly lower than those in the PD group ($^{*}P < 0.05$, $^{**}P < 0.01$, Wilcoxon rank-sum test). The abundances of *Deferribacterota*, *Campilobacterota*, *Patescibacteria*, and *Desulfobacterota* in the PD group were significantly higher than those in the control group ($^{**}P < 0.01$, $^{*}P < 0.05$, Wilcoxon rank-sum test) (Figure 4B).

Ping-wei-san plus herbal medicines alter the fecal metabolomic of Parkinson's disease mice

The metabolite profiles of fecal samples were analyzed using LC-MS. The metabolites in the control, PD, and PWP groups were well separated, with fractions 1 and 2 explaining 51.8 and 12% of the variance, respectively (Figure 5A). Based on the HMDB and KEGG database, the 97 molecules compounds were identified including fatty acyls, prenol lipids, flavonoids, carboxylic acids and derivatives

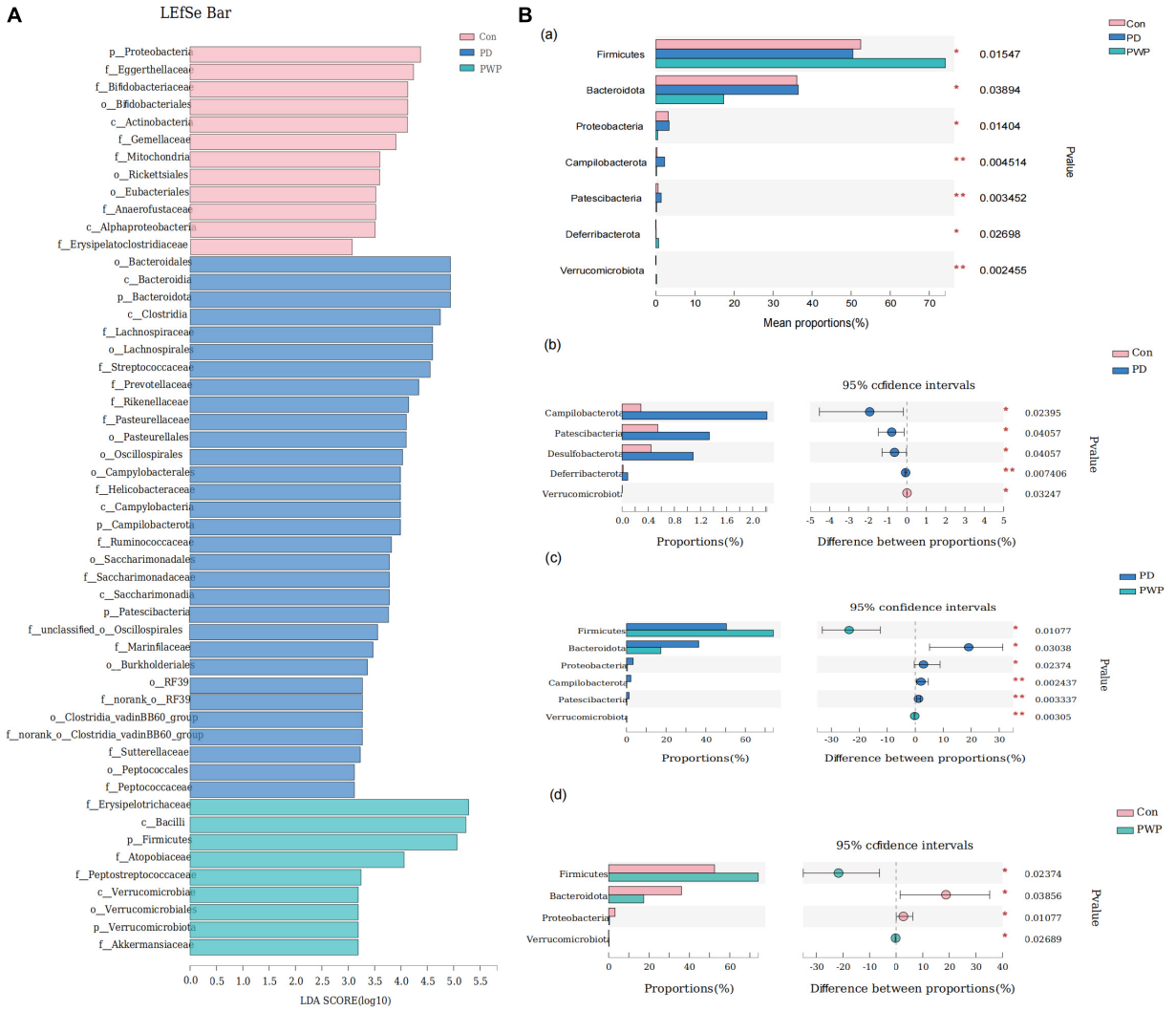

FIGURE 4
The result of 16S microbiome analysis. **(A)** LEfSe bar of control, PD, and PWP groups. **(B)** Results of Kruskal-Wallis H test, showing difference between groups.

(Figure 5B). Among the seven categories in KEGG metabolic pathway, most abundant metabolites were annotated in amino acid metabolism, lipid, biosynthesis of other secondary metabolites, digestive system, cancer: overview, chemical structure transformation maps, xenobiotics biodegradation and metabolism, carbohydrate metabolism, signal transduction (Figure 5C). The content of Lucyoside N, Arginyl-Glutamine, Niacinamide, 1,3-Disopropylbenzene, 9(S)-HODE of fecal metabolites were higher in the PD group than that in the PWP group, and the content of VPGPR Enterostatin and dihydrowyerone acid of fecal metabolites were higher in the PWP group than that in the PD group (P<0.001,Figure 5D).

We identified the top 30 differential metabolites in the PD group versus the PWP group, as well as the PD group versus the control group by VIP based on the OPLS-DA model (Figure 6A). The results of KEGG pathway enrichment showed that PWP mainly plays a role in the treatment of PD through the biosynthesis of alkaloids, such as ornithine, lysine, and nicotine. The metabolic pathways involved include acid, biotin metabolism, phytohormone signaling, tropane, piperidine, pyridine alkaloid biosynthesis, tryptophan metabolism, and vitamin digestion and absorption (Figure 6B). The results of the KEGG functional pathway analysis showed that PWP treatment of PD mainly exerted its therapeutic effect through three metabolic pathways: organic system, metabolism, and environmental information processing (Figure 6C). Concomitantly, we found the top five significant difference of KEGG pathway enrichment were cholinergic and anticholinergic drugs, plant hormone signal

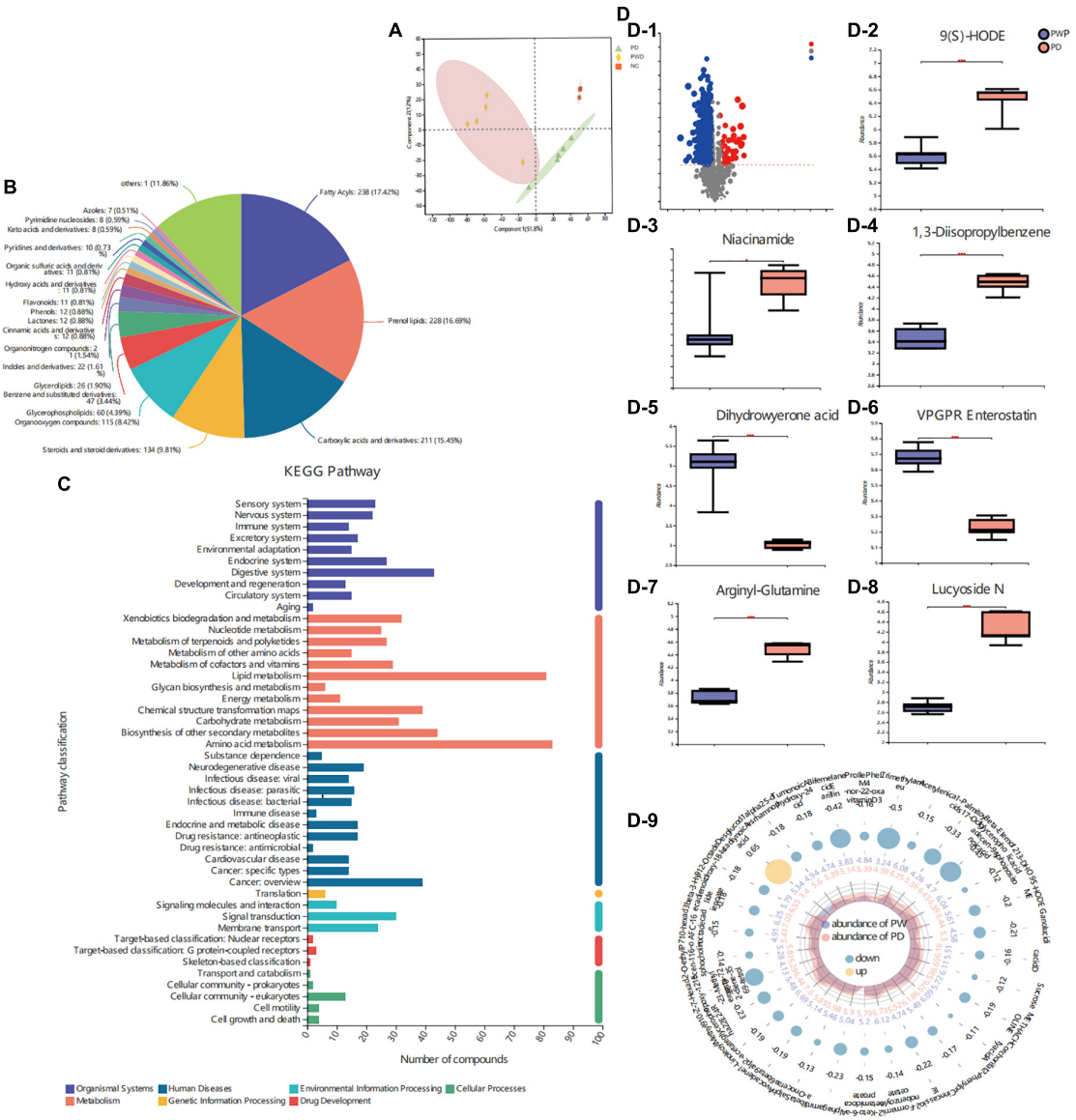

FIGURE 5
The result of Fecal Metabolomics. **(A)** Principal component analyses (PCAs) on identified metabolites in control, PD, and PWP groups. **(B)** Pie chart based on counts of HMDB chemical taxonomy (class) for all metabolites detected in this study class. **(C)** KEGG pathway classification of metabolites detected and annotated. **(D)** Discrepant metabolites in comparison of the PD group and PWP group.

transduction, dopaminergic synapse, axon regeneration and endocrine and other factor-regulated calcium reabsorption ($P = 0.12$,Figure 6D).

Combined analysis of fecal metabolomic with gut microbes

Fecal metabolomics combined with 16S analysis showed that the content of Bacteroides in the PD group was higher than that in the PWP group, and the VIP differential metabolite 9-hydroxy-10E, 12Z-octadecadienoic acid (9-HODE, 9(S)-HODE) increased the content of Bacteroides (Figure 7). The 9 (S)-HODE content in the feces of the PD group was higher than that in the PWP group (Figure 5D-2).

Brain and gut proteomic bioinformatics

We first obtained data regarding differentially expressed proteins (DEPs) involved in PWP-treated PD in the substantia

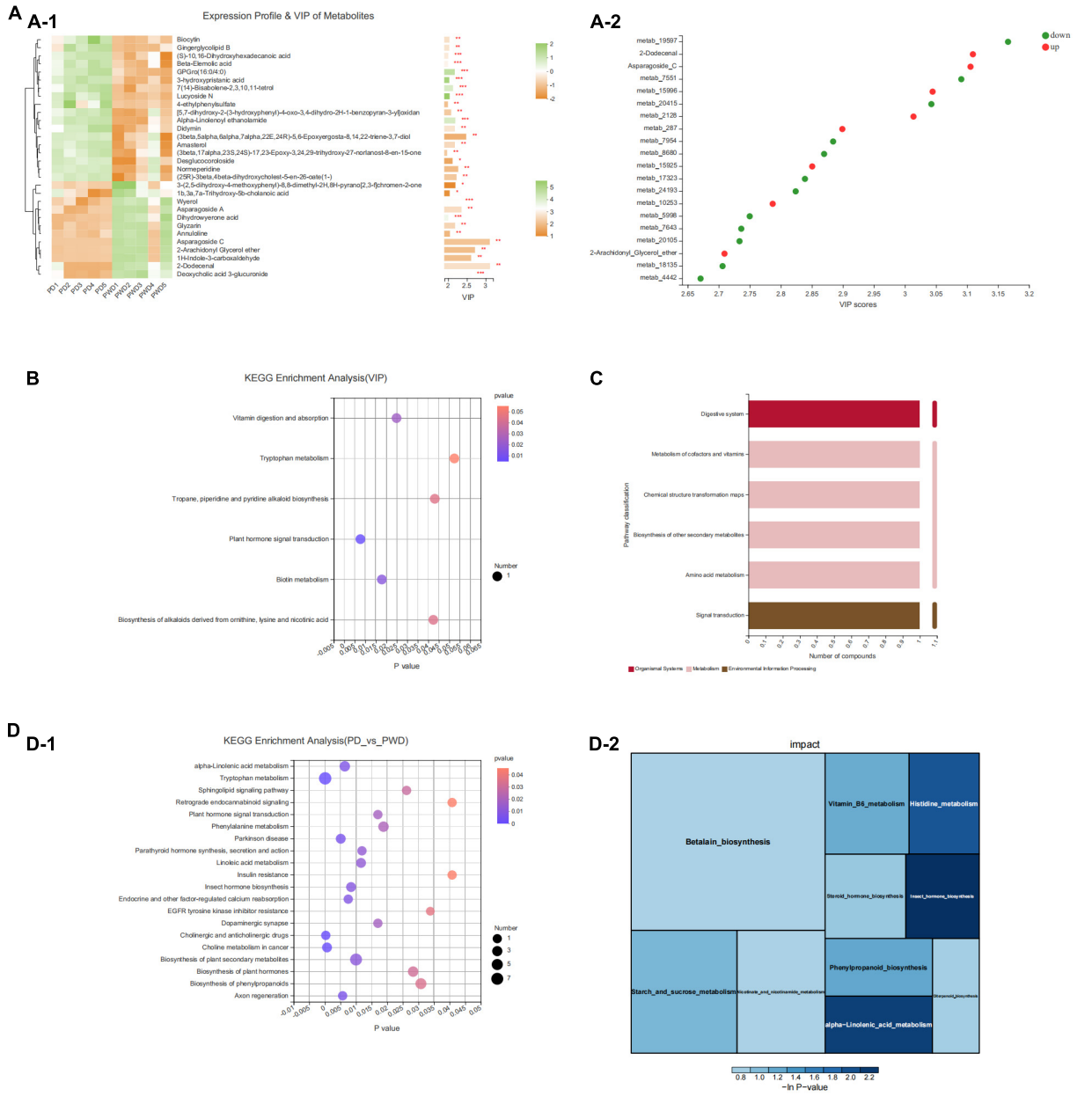

FIGURE 6
The result of Fecal Metabolomics. **(A)** A heatmap of differentially expressed metabolites between the PD and PWP groups showing the top 30 significantly differentially expressed metabolites. **(B)** Bubble map showing the results of KEGG pathway enrichment analysis. **(C)** Secondary metabolic classification of KEGG functional pathway. **(D)** KEGG enrichment analysis of the PD and PWP groups.

nigra and duodenal tissues by differential analysis. In the substantia nigra tissue, we identified 98 upregulated and 98 downregulated DEPs (Figure 8A-1). In duodenal tissue, we found 624 upregulated DEPs and 591 downregulated DEPs (Figure 8A-2). We then analyzed the biological effects and signaling pathways involved in PWP treatment of PD using Gene Ontology (GO) enrichment analysis and KEGG enrichment analysis. In biological processes (BP), DEPs in the substantia nigra tissue and duodenal tissue are enriched in multiple identical BP, such as cellular processes, biological regulation, response to stimulus, and localization. In the cellular component (CC), DEPs in the substantia nigra and duodenal tissues were enriched in three identical CC, including cell, intracellular, and protein-containing complexes. In molecular function (MF), DEPs in the substantia nigra tissue and duodenal tissue were enriched in multiple identical MF, such as binding, catalytic activity, molecular function regulator, and structural molecule activity (Figure 8B). In the KEGG pathway database,

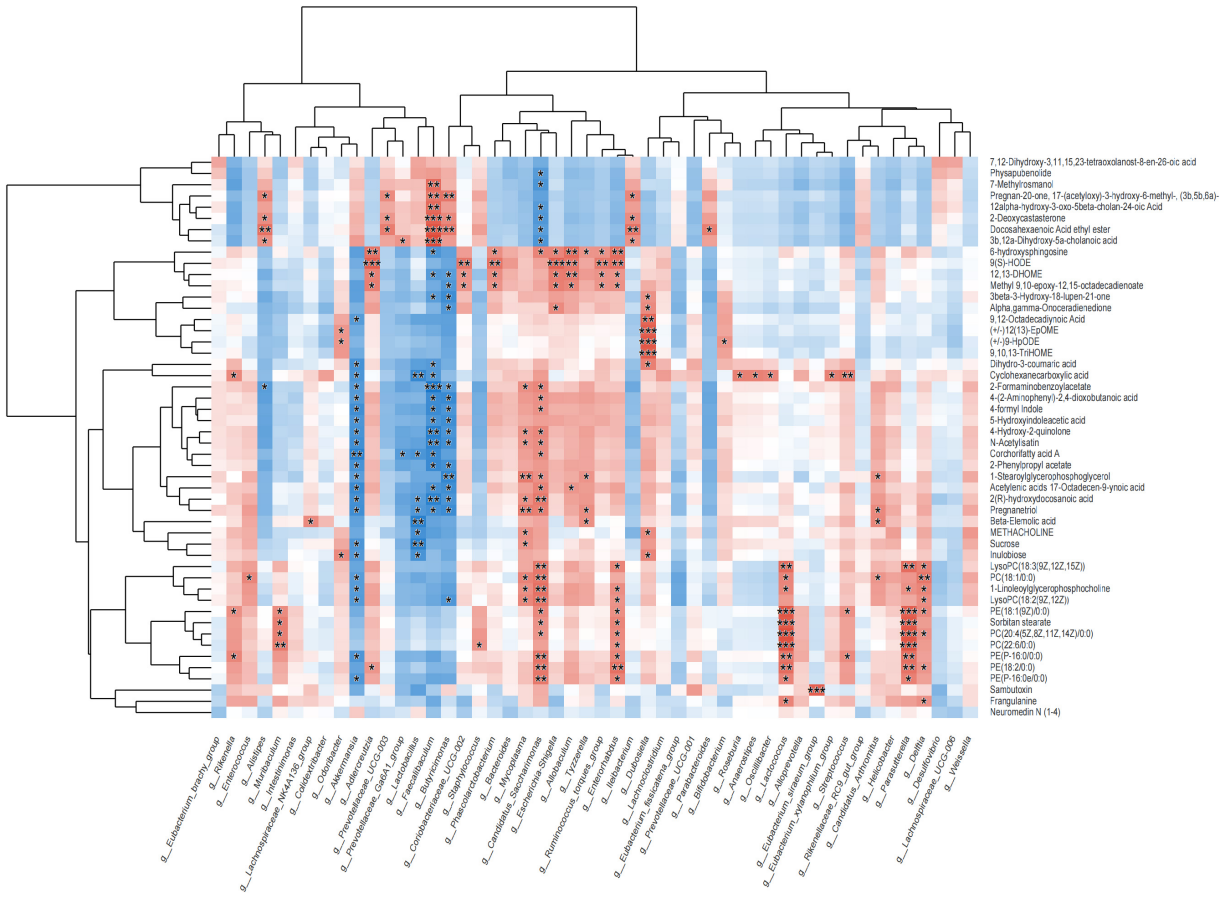

FIGURE 7
Correlation between metabolics and 16S microbiome analysis.

the DEPs in the substantia nigra tissue were mainly enriched in the calcium signaling, cAMP signaling, glutamatergic synapse, dopaminergic synapse, and alcoholism pathways (**Figure 8C-1**). The DEPs in duodenal tissue were mainly enriched in the ribosome, coronavirus disease (COVID-19), and focal adhesion pathways (**Figure 8C-2**). Finally, we constructed protein-protein interaction (PPI) networks of DEPs in different tissues using the STRING database, with a confidence score >0.7 as the filter condition. We demonstrated PPI network of DEPs in the substantia nigra tissue and in the duodenal tissue (**Figure 9**).

Protein expression in brain and gut by western blotting and ELISA

After identifying the differentially expressed proteins in the substantia nigra and duodenum by TMT, we performed WB and ELISA detection of some of these proteins to evaluate the pathway by which PWP promotes PD motility and gastrointestinal function through gut microbes. We found that in the substantia nigra, PWP increased the expression of TIMP3, and NLRP6, while in the duodenum, PWP decreased the levels of Parkin, GSDMD, TIMP3, and NLRP6 (**Figures 10A,B**).

Intestinal permeability test

Differences in the expression of inflammatory factors were observed between the groups in the TMT study. To verify whether inflammatory injury caused changes in intestinal permeability in PD mice, the FITC-dextran flux assay was used to measure permeability and evaluate intestinal epithelial function. PWP herbal stimulation resulted in an increase in intestinal permeability, which was reflected by an increase in the transmembrane flux of FITC-dextran (**Figure 10C**).

Discussion

Due to the multi-component and multi-target properties of TCMCs, TCMCs are also recognized as one of the most important treatments in the clinical setting, not only playing the

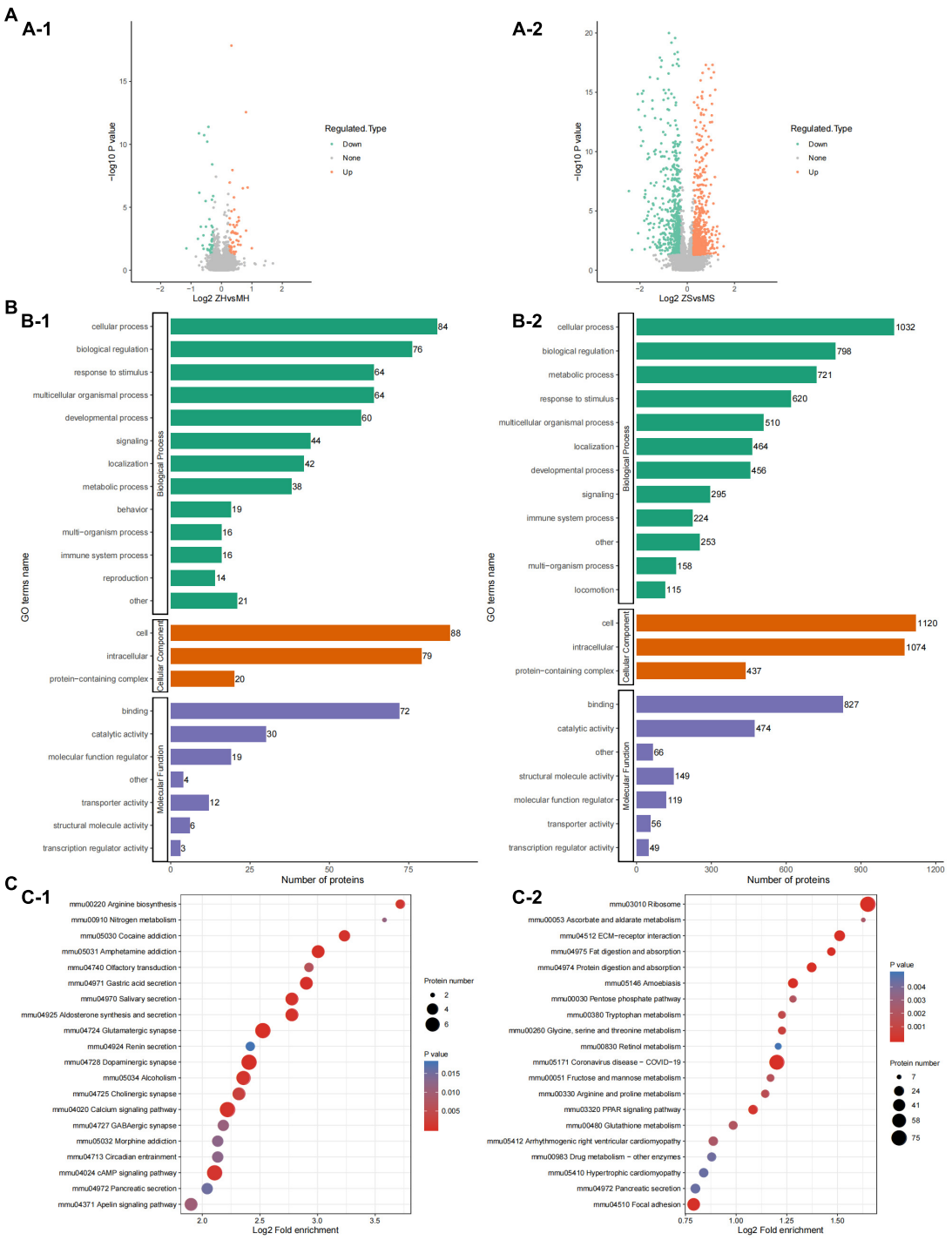

FIGURE 8
The results of TMT labeling studies. **(A)** Volcano plot of differential proteins between the PD and PWP groups. **(A-1)** Differential proteins in substantia nigra tissue. **(A-2)** Differential proteins in duodenal tissue. **(B)** Bar plot of GO terms of the PD and PWP groups. **(B-1)** The result were enriched in substantia nigra tissue. **(B-2)** The result were enriched in duodenal tissue. **(C)** Bar plot showing the result of KEGG pathways between the PD and PWP groups. **(C-1)** KEGG pathways in substantia nigra tissue. **(C-2)** KEGG pathways in duodenal tissues.

main therapeutic role for some diseases such as frailty, but also assisting in the treatment for others diseases such as COVID-19 (12). An US study claimed that Chinese herbal medicine was making its way into mainstream Western medicine. Patients may discover *Ping-Wei-San* in their search for a substitute for cisapride, which was partially withdrawn from the US

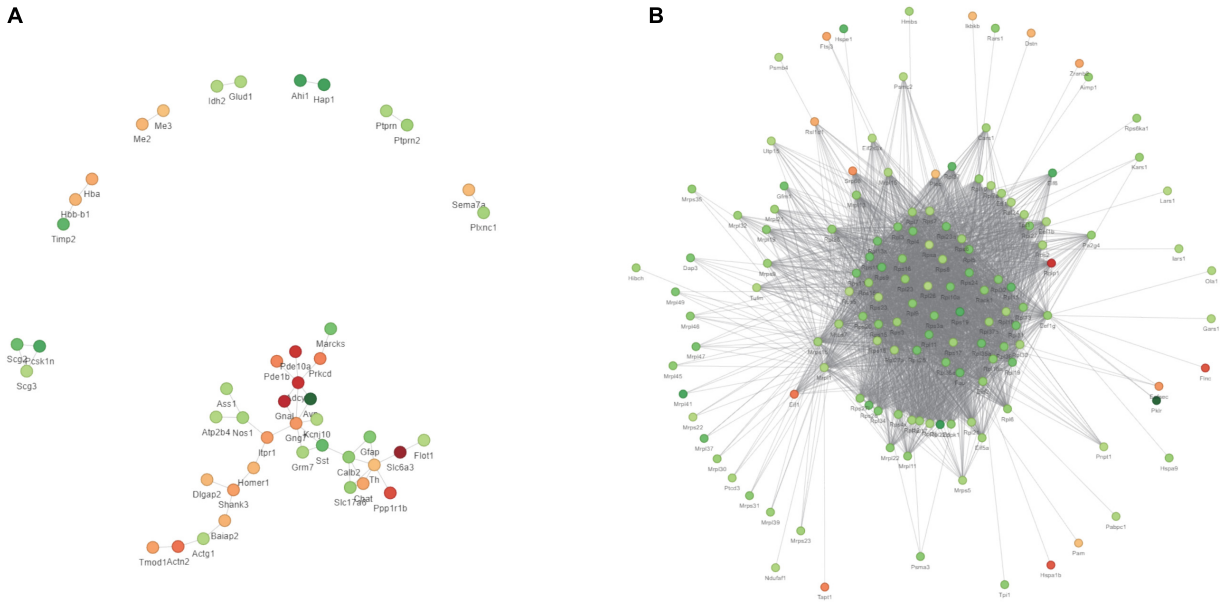

FIGURE 9
The results of TMT labeling studies. The PPI network of DEPs between the PD and PWP groups. **(A)** DEPs in substantia nigra tissues. **(B)** DEPs in duodenal tissues.

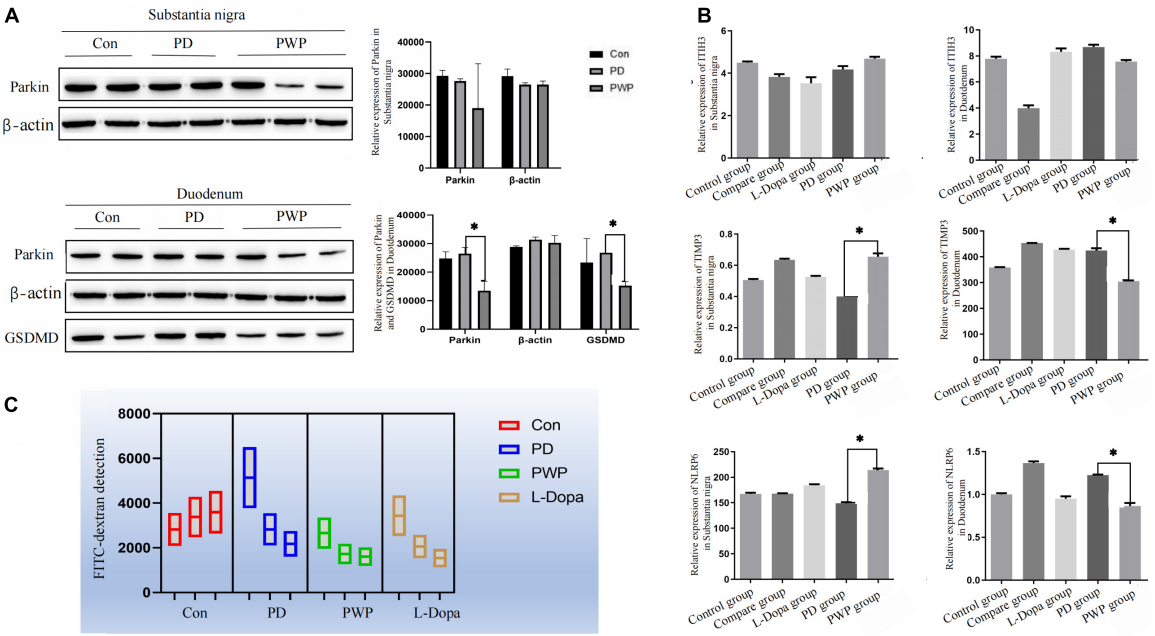

FIGURE 10
The result of protein expression in Brain and gut by WB and ELISA, as well as FITC-dextran concentrations test. **(A)** WB showing the expression of Parkin, and GSDMD in the substantia nigra and duodenum. **(B)** ELISA results showing the expression of TIMP3, NLRP6, and ITIH3 in the substantia nigra and duodenum. **(C)** FITC-dextran concentrations test in the control, PD, L-Dopa therapy, compare and PWP groups. *Means that $p < 0.1$ for significant differences.

market in July 2000 (14). One study identified patterns of TCM use including *Ping-Wei-San* (usage of 2.4%) in colon cancer patients post-surgery in Taiwan. The herbal ingredients were most commonly used for stimulate ghrelin secretion to increase food intake and had potential anti-tumor effect (15). Findings from A Matched cohort study demonstrated

that adding Chinese herbal medicines, e.g., *Ping wei san* to conventional treatment significantly reduces depression risk among patients with insomnia (16). However, the mechanism of action of TCMCs is difficult to explain precisely because of its multicomponent and multitarget properties. TCM regulates multiple systems in the human body, such as the gut microbiota, proteomics, and metabolomics, to play a therapeutic role (17–19). Therefore, this study combined gut microbiota, proteomics, and fecal metabolomics to comprehensively explore the potential mechanism of PWP in the treatment of PD from multiple perspectives and to evaluate evidence that PWP can effectively treat PD. In this study, we verified and explained the mechanism by which PWP treats and alleviates PD from multiple aspects.

First, we demonstrated that the gut bacterial community composition was different between the control, PD model, and PWP groups, belonging to multiple operational taxonomic unit (OTU) types, of which OTU735 accounted for the largest proportion. The top seven differences in gut flora between the three groups were associated with PD and the brain-gut axis (4), including *Firmicutes*, *Bacteroidetes*, *Campylobacter*, *Patescibacteriota*, *Desulfobacteriota*, *Verrucomicrobiota*, and *Deferribacterota*, which has been proved that the treatment of PWP from gut flora partly in previous studies (20–23). *Bacteroidota*, *Verrucomicrobiota*, and *Campylobacter* have been found to be an important part of the brain-gut-microbiota axis in PD. *Patescibacteriota* have been shown to play a role in the brain-gut-microbiota axis to promote the progression of brain and emotion-related diseases such as depression and brain glioma (24, 25). In addition, *Campylobacter*, *Patescibacteriota*, and *Verrucomicrobiota* were also the top three different gut flora between the PD and PWP groups, which may be the main mediators of the PWP herbal effect involving restore mitochondrial function and is neuroprotective.

Second, fecal metabolomics was used to explore the treatment mechanism of PWP acting on PD, and we found that PWP has an effect on multiple metabolites in the treatment of PD. The correlation results showed that there was a clear correlation between gut flora and fecal metabolomics. Among the differentially expressed fecal metabolites, alpha-linolenic ethanolamine has been reported to be associated with PD, a metabolite with neuroprotective effects in PD and stroke (26). 9(S)-HODE is one of the oxidation products of linoleic acid. In this experiment, under the action of PWP, both the content of 9(S)-HODE and bacteroides decreased. The former suggested that lipid peroxidation levels was low *in vivo*. Previous studies have found bacteroides trigger lipid peroxidation (27). Even though,it has been a long way to fully explore the interaction relationships between PWP, gut flora, and fecal metabolomics. In general, the holistic concept of TCM means that TCM not only treats PD from the nervous system but also exerts potential regulatory effects by regulating other systems (28); pathway classification and KEGG enrichment analysis also proved this.

Next, we used proteomics to elucidate the mechanism of action of PWP in the substantia nigra and duodenum of PD mice. As a mitochondrial electron transport chain complex I inhibitor, rotenone induces apoptosis by enhancing the production of mitochondrial reactive oxygen species. Intake of PWP herbs reduced the rotenone-induced loss of dopaminergic neurons in PD mice, as evidenced by TH immunostaining in the striatum. Although affected by the blood-brain barrier, some active ingredients still act on the substantia nigra-related proteins (29). The results of the enrichment analysis indicated the existence of multiple identical biological processes and signaling pathways, which also proved the effect of PWP on PD through the brain-gut axis. PPI also showed that centriole proteins, such as PDE10A, GNAL, SLC6A3, PPP1R1B, and ADCY5, are regulated by PWP in the substantia nigra. According to the research reports of central proteins, it is proven that PWP exerts therapeutic and regulatory effects through mechanisms such as regulating dopamine, dystonia, microglial activation and autophagy (30). The effects of rotenone in PD mice mainly involve mitogen-activated protein kinase (MAPK), which increases the levels of excitatory amino acid neurotransmitters (31, 32). Nod-like receptor 6 (NLRP6) is located at the intestinal epithelial barrier and is involved in sensing and maintaining the naturally colonized bacteria of the gut microbiome. NLRP6 plays a key role in regulating inflammation and host defense against gut microbes. Different studies have found that deubiquitination of NLRP6 prevents overproduction of interleukin 18 (IL-18) in the colonic mucosa; and when induced, NLRP6 undergoes phase separation to activate the inflammasome immune response (33). We found that NLRP6 expression was reduced in the duodenum of PWP intake mice. Gasdermin D (GSDMD) is a direct executor of inflammatory caspase-induced pyroptosis by driving mucin secretion through calcium-dependent sciderin-mediated cortical F-actin breakdown (34). TMT found that intestinal gasdermin D (GSDMD) was increased in PD mice compared with that in normal mice, and WB results showed that intestinal GSDMD expression decreased after 90 days of PWP intake, which might reveal PWP playing a role in shaping intestinal mucosal homeostasis.

Conclusion

This study combined gut microbiota, metabolomics, and proteomics to explore the mechanism of action of PWP on PD mice. The results reveal that PWP herbal treatment modulates gut microbiota abundance, alters metabolite biological pathways, and affects functional pathway protein expression in PD mice, resulting in therapeutic effects. Although

we are at the beginning of understanding the effect of PWP herbs in the microbiome that corresponds to functionally, the findings still provide new leads and testable hypotheses on the treatment of PD. This study paves the way for novel therapeutic strategies for the treatment of PD using Chinese herbal medicine.

Data availability statement

The microbiomics data presented in the study are deposited in the Sequence Read Archive repository (https://www.ncbi.nlm.nih.gov/), accession number PRJNA904900. The metabolomics data presented in the study are deposited in the MetaboLights Study repository (https://www.ebi.ac.uk/metabolights/), accession number MTBLS6642. The protein data are deposited at (https://www.iprox.cn/page/PSV023.html;?url=1671194978436ARUz) password: NGt5.

Ethics statement

The animal study was reviewed and approved by Ethics Committee of Affiliated Hospital of Qingdao University.

Author contributions

DL: formal analysis, software, and manuscript writing – original draft. H-JY: investigation and data curation. G-JH: formal analysis, data curation, and methodology. R-YY: investigation. A-MX: supervision. X-YL: conceptualization, funding acquisition, project administration, and writing review and final approval of manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgments

The 16s RNA and Fecal metabolomic data were test and analyzed on the online platform of Majorbio Cloud Platform (www.majorbio.com, Shanghai Majorbio Bio-pharm Technology Co., Ltd.). TMT analysis were performed using the online platform of PTM BIO (www.ptmbiolab.com).

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.

The reviewer KG declared a shared affiliation with the authors to the handling editor at the time of review.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.945356/full#supplementary-material

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Resveratrol regulates insulin resistance to improve the glycolytic pathway by activating SIRT2 in PCOS granulosa cells

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Scope: Insulin resistance (IR) has a close relationship with the main clinical manifestations of patients with PCOS; hence, the research and development of new drugs to treat PCOS by improving IR is a desiderate task at present. Resveratrol (RES) possesses a variety of beneficial pharmacological functions, such as antioxidation, anti-inflammatory, regulating glucose, and lipid metabolism. However, whether RES could improve IR and the underlying mechanisms remained unclear in PCOS.

Methods and results: SD rats received a high-fat diet and letrozole for 30 days to establish the PCOS model and then intervened with RES for 30 days. The results demonstrated that RES played a protective role on the IR in PCOS rats, which significantly decreased the levels of blood glucose and serum insulin, up regulated the expression of IGF1R, and down regulated the expression of IGF1. *In vitro*, KGN cells were treated with insulin, RES, and AGK2, respectively. We found that a high dose of insulin (4μg/mL) significantly inhibited KGN cell viability, decreased the level of lactic acid, and increased the level of pyruvate, while RES (25μM) attenuated the growth-inhibitory effect, as well as increased the level of lactic acid and decreased the level of pyruvate after high levels of insulin treatment. Simultaneously, RES up regulated the expression level of the crucial rate-limiting enzymes relating to glycolytic pathways, such as LDHA, HK2, and PKM2. Furthermore, AGK2 remarkably inhibited the expression level of SIRT2, which was similar to the same negative effects processed by insulin. Meanwhile, RES overtly repaired the glycolysis process by reversing the levels of lactic acid and pyruvate, together with up regulating the expression level of LDHA, HK2, and PKM2, after AGK2 treatment.

Conclusion: RES could effectively improve insulin resistance and restore the glycolysis pathway by regulating SIRT2, which may contribute to attenuating the ovarian damage of PCOS rats and provide a potential treatment for patients with PCOS.

KEYWORDS

resveratrol, PCOS, insulin resistance, SIRT2, glycolysis

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine and metabolic disorder disease in women of childbearing age and affects up to 10%, characterized by hyperandrogenism, sparse ovulation, or anovulation (1). Insulin resistance (IR) and associated compensatory hyperinsulinemia are common features of PCOS affecting 50–70% of women with the disorder (2). IR has been defined as a decreased ability of insulin to regulate the uptake and production

of glucose *in vivo*, which resulted in an increasing amount of insulin to achieve a given metabolic action (3). In the last few decades, numerous studies have identified the ovary as an important target tissue for insulin action (4). Physiological insulin levels promoted the proliferation of granulosa cells, and glucose metabolism of granulosa cells provided energy for the development of oocytes, thereby regulating follicular development (3). By contrast, hyperinsulinemia could promote the apoptosis of ovarian granulosa cells, increase the serum level of androgen, and aggravate hyperandrogenism in patients with PCOS, inhibiting the development of follicles (5–7). In addition, the literature studies have shown that IR could downregulate the expression of PI3K and inhibit PI3K/AKT pathway, reducing the function of insulin to regulate glucose metabolism, causing localized glucose metabolism disorder in the ovary (8, 9). These studies indicated that PCOS follicular dysplasia was closely related to hyperinsulinemia and IR. Meanwhile, multiple lines of evidence have reported that lactate, the main product of glycolysis, plays an important role during folliculogenesis and follicular maturation (10), and the rate of glycolysis was regulated by its rate-limiting enzymes, such as hexokinases (HKs), pyruvate kinase M (PKM), and lactic dehydrogenase (LDH) (11). Our previous research has indicated that abnormal glycolysis, in which the proteins' expression of HK2, PKM2, and LDHA were decreased, was a critical reason for follicular dysplasia in PCOS rats (12). However, the relationship between the IR and the abnormal glycolysis in the PCOS ovary remains ill-defined.

At present, vegetables and fruits rich in natural flavonoid compounds may prevent or delay the onset of diseases such as cardiovascular diseases (13). Resveratrol (3,4,5-trihydroxy-trans-stilbene, RES) is a naturally occurring compound mainly found in dietary plants including the root of polygonum cuspidate, peanut, and grape skin (14, 15). RES has several pharmacological effects including anti-inflammatory, antioxidant, and increased insulin sensitivity (14, 16). Hoseini et al. found that RES significantly decreased the level of insulin and fasting blood glucose in patients with type 2 diabetes complicated with coronary heart disease (17). Furthermore, Zou et al. have revealed that RES ameliorated metabolic disorders and IR in high-fat diet-fed mice (18). In addition, RES also has positive effects on follicular development, such as decreased number of atretic follicles, increased follicular reserve of the ovary, and prolonged ovarian lifespan (19, 20). Meanwhile, RES has a positive effect on improving the architecture of ovarian follicular (21) and suppressing the damage of the ovaries in PCOS rats by restoring glycolytic activity (12). However, the mechanism of RES on IR and the relationship between IR and glycolysis in PCOS remain unclear.

Thus, the present study aimed to examine the effect of RES on ovarian IR in PCOS rats, as well as to explore the mechanisms underlying the IR-regulated glycolysis pathway in PCOS ovarian granulosa cells, which may provide potential strategies for the treatment of PCOS.

Abbreviations: IR, insulin resistance; PCOS, polycystic ovary syndrome; PCOM, polycystic ovarian morphology; GCs, granulosa cells; RES, resveratrol; Ctrl, control; HFD, high-fat diet; CMC, carboxymethyl cellulose; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

Materials and methods

Animals and feeding

All experimental procedures were approved by the Animal Ethics Committee of the University of South China (permit number: USC2020031602). A total of 24 female Sprague-Dawley (SD) rats (5 weeks, 171 ± 11 g) were obtained from the Laboratory Animal Center of the University of South China (Hengyang, China; permit number: SYXK (Xiang) 2020-0002). The rats were raised with free access to food and water in a temperature-controlled room (23 ± 2°C) with a normal dark–light (12 h:12 h) cycle. After 7 days of acclimatization, rats were randomly divided into control (Ctrl) ($n = 8$) and PCOS ($n = 16$) groups. The control group received a normal diet, whereas the PCOS group received a high-fat diet (HFD) and letrozole to establish the PCOS model. The experimental group was then randomly divided into a PCOS group ($n = 8$) and a PCOS-RES group ($n = 8$), in which rats received oral RES (R8530; Solarbio, China).

Dosage information

In the animal modeling experiment, rats were randomly divided into two groups. Ctrl group: rats were fed a normal diet for 30 days. PCOS group: rats were fed an HFD (consisting of 61.5% ordinary feed, 12% lard, 5% sucrose, 5% milk powder, 5% peanut, 10% egg, 1% sesame oil, and 0.5% salt) and intragastric administration of letrozole [1 mg/kg/d, dissolved in 1% carboxymethyl cellulose (CMC)] for 30 days (22, 23). During the treatment experiment, rats were divided into the Ctrl group, the PCOS group, and the PCOS-RES group. The Ctrl and PCOS rats were fed as described in the modeling experiment for 30 days. The PCOS-RES rats were fed a normal diet and intragastric administration of RES (20 mg/kg/d (24, 25), dissolved in 1% CMC) for 30 days.

Determination of insulin levels

Blood was collected from the abdominal aorta of rats when killed and placed at room temperature (23 ± 2°C) for 30 min before centrifugation at 3,000 rpm for 15 min at 4°C. The level of serum insulin was detected using a radioimmunoassay (Beijing North Institute of Biological Technology, China).

Cell culture and treatment

Human ovarian granulosa-like tumor cell line KGN, which originated from a stage III invasive ovarian granulosa cell carcinoma in a 63-year-old woman, was considered a model for understanding the regulation of steroidogenesis, cell growth, and apoptosis in human granulosa cells (26). In this study, KGN cells were kindly provided by Clinical Anatomy & Reproductive Medicine Application Institute. KGN cells were cultured with Dulbecco's Modified Eagle's Medium-high glucose (DMEM, Sigma, USA) supplemented with 12% fetal bovine serum (FBS, Invitrogen Gibco, USA) and maintained in an atmosphere of 5% CO_2 at 37°C.

KGN cells were plated in 6-well plates at 10^5 per well. After starving for 24 h, the cells were treated without or with insulin (I2643;

Sigma, USA) at concentrations of 1, 2, and 4 mg/ml. Then, KGN cells were treated with insulin (4 μg/ml) and RES at concentrations of 10, 25, 50, and 100 μM for 24 h. In addition, the cells were also treated with AGK2 (10 μM, A8231; Sigma, USA) and RES (25 μM) for 24 h.

CCK-8

KGN cells were plated in 96-well plates at 10^4 per well. After starving for 24 h, the insulin group cells were treated without or with insulin at concentrations of 1, 2, and 4 μg/ml for 24 h. The KGN cells were treated with 4μg/ml of insulin, meanwhile treated with RES at concentrations of 0, 25, 50, and 100 μM for 24 h in the Ins + RES group, and the AGK2 group cells were treated with 10 μM, AGK2+RES were treated with 10 μM AGK2 and 25 μM resveratrol for 24 h. Then CCK-8 was added at 10 μl per well and incubated for 30 min in the incubator, following the manufacturer's protocol. Plates were read on a VersaMax microplate reader at 450 nm wavelength.

Enzyme-Linked Immunosorbent Assay (ELISA)

Lactic acid and pyruvate in the culture supernatant were measured using commercially available ELISA kits. Plates were read on a VersaMax microplate reader at 505 nm (pyruvate) and 530 nm (lactic acid) wavelengths.

Western blot

Ovarian tissues and cultured cells were homogenized in lysates [RIPA lysis buffer (CW2333S; CWBIO, China): PMSF (P0100; Solarbio, China) = 94:6] on ice for 30 min and subsequently centrifuged at 12,000 rpm for 20 min at 4°C. Supernatants were transferred to a clean 1.5 ml tube, and protein concentration was measured by BCA Protein Assay Kit (CW0014; CWBIO, China). Proteins were denatured by boiling at 100°C for 10 min and then separated by 10% SDS-PAGE, electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes, and blocked in PBST [phosphate-buffered saline (PBS) with 0.1% Tween-20] containing 5% skim milk powder for 2 h. After washing with PBST, the membranes were, respectively, incubated with antibodies against PKM2 (1:1,000, #4053; Cell Signaling Technology, USA), HK2 (1:1000, A0994; ABclonal, China), LDHA (1:1000, #3558; Cell Signaling Technology, USA), SIRT2 (1:1000, A12575; ABclonal, China), and GAPDH (1:1,000, #97166; Cell Signaling Technology, USA) overnight at 4°C. The membranes were further incubated with HRP-conjugated goat anti-mouse or goat anti-rabbit IgG (H+L) (1:5,000, SA00001-1/2; Protein Tech Group Inc., USA) for 2 h at room temperature. Finally, eECL (CW0049M, CWBIO, China) and the Tanon-5500 Chemiluminescence Imaging System were used to detect the chemiluminescence of protein bands.

Quantitative real-time PCR (qPCR)

Total RNA from ovary tissues or KGN cells was extracted using the TRIzol reagent (15596018; Thermo Fisher Scientific, USA). The

TABLE 1 Primer sequences used for the qRT-PCR analysis.

GENE	Sequence (5′-3′)	Annealing temperature (°C)	Product (bp)
Rat IGF1	F: GTGGTGAATGACACAGTTGG	55	164
	R: CACATTACGCATCTCTTCCA		
Rat IGF1R	F: ACATCCTGTGGCTGGACTAT	55	179
	R: TCCACTTCTGTCACCAGGTA		
	R: GCAGATGGTCGGCTTGAAC		
Rat GAPDH	F: CCTCAAGATTGTCAGCAATG	55	164
	R: CAGTCTTCTGAGTGGCAGTG		
Homo HK2	F: CGAGAGCATCCTCCTCAAGTG	55	164
	R: AGCCACAGGTCATCATAGTTCC		
Homo PKM2	F: TGGGAGAGAAGGGAAAGAACATC	55	179
	R: GCACCGTCCAATCATCATCTTC		
Homo LDHA	F: ATGAGTTGGACTGTGCCTGTTGTG	55	134
	R: GTGAAGAGCCAGGTGCCGTTG		
Homo SIRT2	F: CGCACGGCACCTTCTACACATC	55	188
	R: GGCTCTGACAGTCTTCACACTTGG		
Homo GAPDH	F: GAGTCCACTGGCGTCTTCAC	55	164
	R: GAGGCATTGCTGATGATCTTGAG		

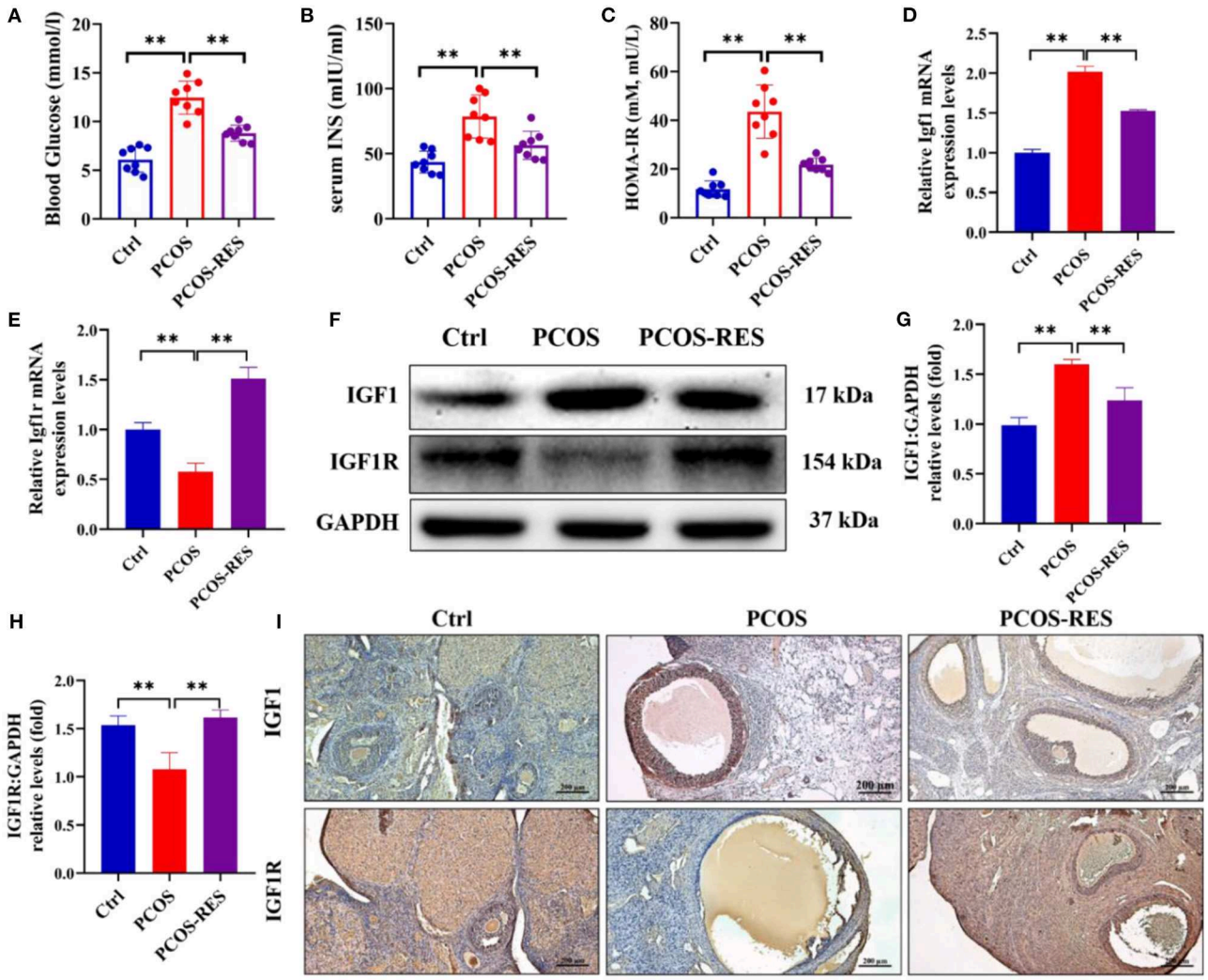

FIGURE 1
Resveratrol can enhance the ovarian insulin sensitivity in PCOS rats. The level of blood glucose **(A)**, serum insulin **(B)**, and HOMA-IR **(C)** was shown ($n = 8$). The relative mRNA expression levels of IGF1 **(D)** and IGF1R **(E)** in the ovary were determined using qPCR. The protein levels of IGF1 and IGF1R were determined by Western blot **(F)** and quantified by Image J software [IGF1 **(G)** and IGF1R **(H)**]. Immunohistochemistry analysis of the expression of IGF1 and IGF1R **(I)**. Significant differences between the groups are shown as $^{**}P < 0.01$.

synthesis of cDNA was performed with the TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (AT311-02; TransGen Biotech, China), according to the manufacturer's protocol. Real-time PCR analyses were performed with ChamQ Universal SYBR qPCR Master Mix (Q711-02; Vazyme, China) and Applied Biosystems QuantStudio 3 (Thermo Fisher Scientific, USA). GAPDH was used as the reference, and gene expression levels were calculated using the comparative Ct method (27). The primer sequences used for amplification are shown in Table 1.

Statistical analysis

Data were analyzed using GraphPad Prism 8.0 (GraphPad Software, USA) and presented as the mean ± standard deviation. Significant differences within groups were evaluated by the unpaired one-way ANOVA followed by Bonferroni's *post-hoc* test.

Results

Resveratrol enhanced the ovarian insulin sensitivity in PCOS rats

A PCOS rat model was first established to examine the effects of RES on ovarian insulin sensitivity. As shown in Figures 1A–C, HFD and letrozole treatment remarkably increased the levels of blood glucose, serum insulin, and HOMA-IR compared with that of the Ctrl group ($n = 8$ each; $p < 0.01$). The levels of blood glucose and serum insulin were obtained after rats were exposed to PCOS-inducing conditions for 30 days. RES administration significantly reduced the levels of blood glucose, serum insulin, and HOMA-IR of PCOS rats ($n = 8$ each; $p < 0.01$) (Figures 1A–C). To explore the effect of RES on ovarian insulin resistance, we detected the expressions of IGF1 and IGF1R. The results of qPCR showed the upregulation of IGF1 and downregulation of IGF1R in PCOS rats, and RES administration significantly restored the mRNA expression of IGF1 and IGF1R

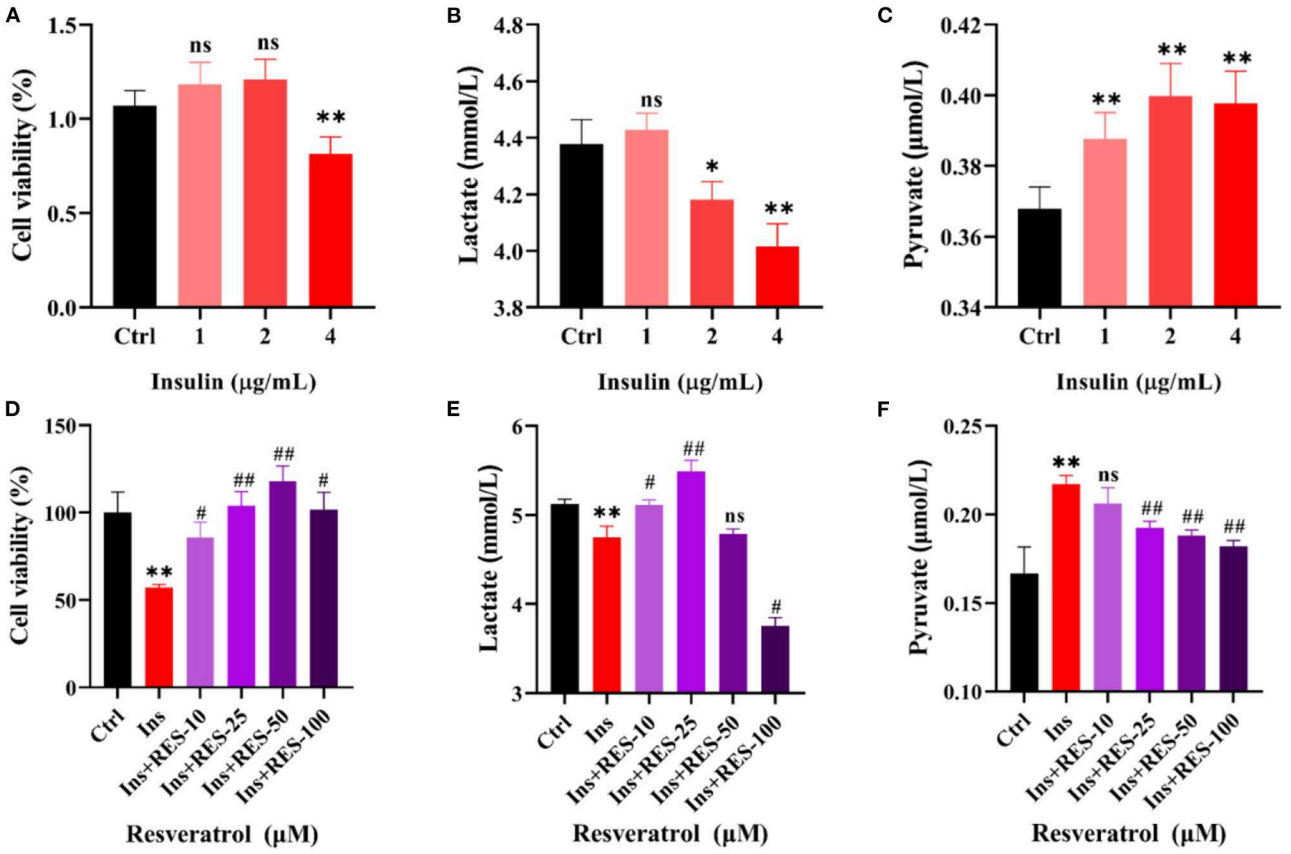

FIGURE 2
RES can improve the glycolysis process of insulin-treated KGN cells. Cell viability of KGN cells in which treatment with insulin **(A)** or insulin and RES **(D)** was determined using cck-8. The level of lactate in KGN cells, which treated with insulin **(B)** or insulin and RES **(E)** using ELISA. The level of pyruvate in KGN cells, which treated with insulin **(C)** or insulin and RES **(F)** using ELISA. *ctrl vs. insulin; #insulin vs. insulin + RES. Significant differences between the groups are shown as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$.

(Figures 1D, E). Western blot analysis confirmed the increase of IGF1 and decrease of IGF1R in the PCOS group vs. the Ctrl group, respectively, and the improvement in response to RES treatment (Figures 1F–H). Immunohistochemistry (IHC) analysis confirmed the results, showing an increase of IGF1 and a decrease of IGF1R in the PCOS group and a recovery of values in response to RES treatment (Figure 1I).

Resveratrol improved the glycolysis process of insulin-treated KNG cells

Then, we deeply explored the mechanism of RES enhancing insulin sensitivity at the cellular level. As shown in Figures 2A–C, we treated KGN cells with different concentrations of insulin and detected the changes in cell viability, and the levels of lactic acid and pyruvic acid. The results of CCK-8 showed 4 μg/ml of insulin decreased KGN cell viability compared with that of the Ctrl group, while there was no significant difference in KGN cells after being treated with 1 and 2 μg/ml of insulin. The results of ELISA showed that with the increase in the concentration of insulin, the level of lactate decreased while pyruvate increased. Thus, we choose the 4 μg/ml insulin for the next experiment. With respect to the concentration of insulin, we added different concentrations of RES to cells. The results showed that 25 and 50 μm of RES could reduce the pyruvate level and increase the lactate level of cells treated with insulin (Figures 2D–F). Lactate has long been proposed to play an important role in follicular development (10); thus, we chose 25 μm of RES, which is the most significant in increasing lactate level for the follow-up experiments.

Resveratrol improved the expression of key glycolytic enzymes of insulin-treated KNG cells

The aforementioned experimental results showed that the intervention of RES could enhance the insulin sensitivity of ovarian tissue and promote the process of glycolysis. To explore the effect of insulin resistance on glycolysis, we detected the expression of key glycolytic enzymes (HK2, PKM2, and LDHA) in KGN cells after insulin treatment and RES intervention. The results of qPCR showed the mRNA expressions of HK2, LDHA, and PKM2 were downregulated in KGN cells treated with insulin. RES administration significantly restored the

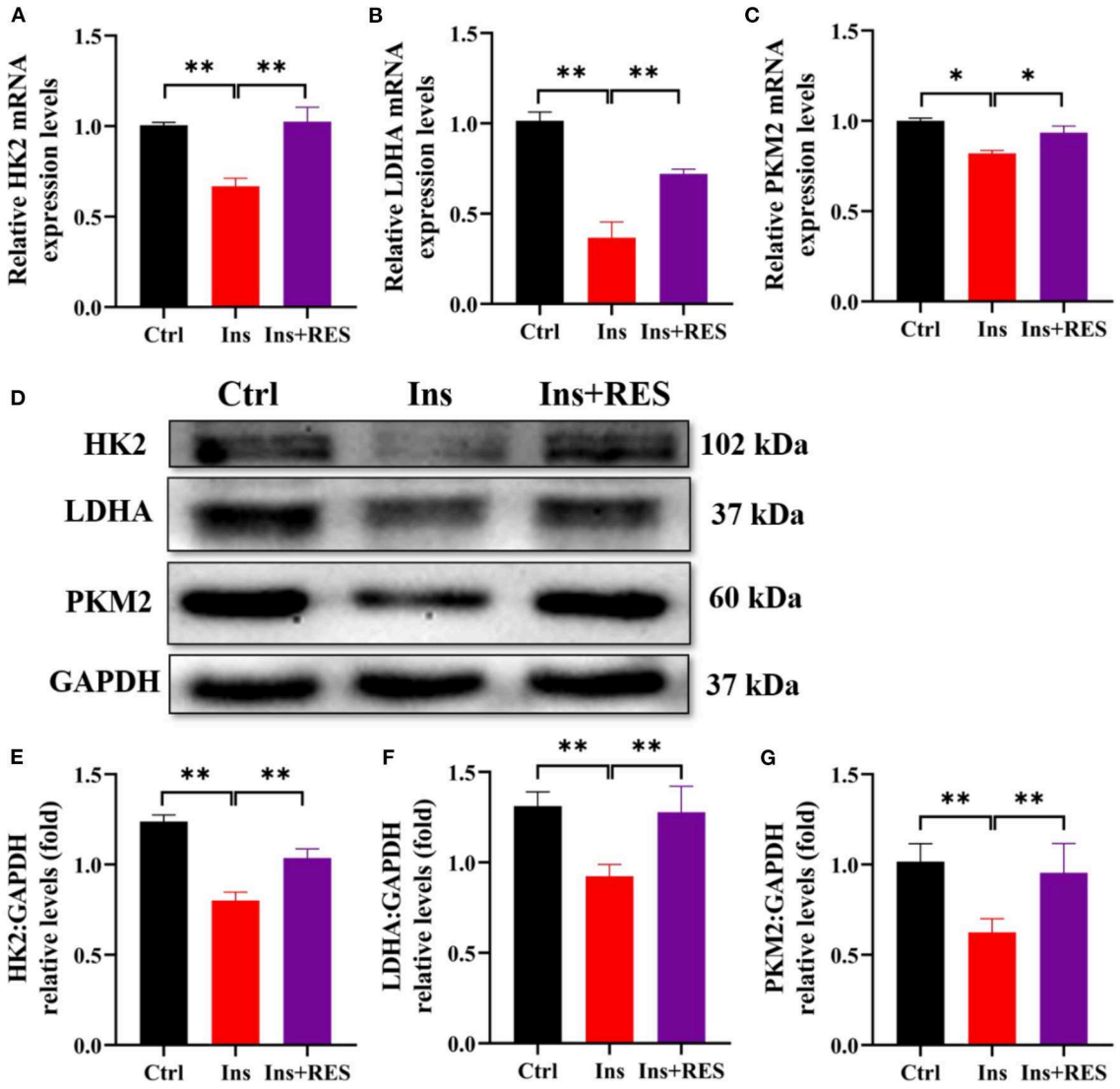

FIGURE 3
RES can improve the expression of key glycolytic enzymes of insulin-treated KGN cells. The relative mRNA expression levels of HK2 **(A)**, LDHA **(B)**, and PKM2 **(C)** in KGN cells were determined using qPCR. The protein levels of HK2, LDHA, and PKM2 were determined by Western blot **(D)** and quantified by ImageJ software **(E–G)**. Significant differences between the groups are shown as $^{*}P < 0.05$ and $^{**}P < 0.01$.

mRNA expression of HK2, LDHA, and PKM2 (Figures 3A–C). Western blot analysis confirmed that insulin decreased the protein expressions of HK2, LDHA, and PKM2 in KGN cells compared with the Ctrl group and the improvement after RES treatment (Figures 3D–G).

Resveratrol upregulated the expression of SIRT2 in KGN cells treated with insulin and AGK2

Based on the transcriptome profiling and ovary tissue results in our previous research, we found that SIRT2, an NAD^{+}-dependent deacetylase, was decreased in PCOS rats while increased in PCOS-RES rats (12). In this study, we mainly interrogated the role of SIRT2 in PCOS granulosa cells. First, we detected the mRNA level of SIRT2 in KGN cells treated with insulin and RES, and the results showed that RES could reverse the effect of insulin on SIRT2 (Figure 4A). AGK2 is a specific SIRT2 inhibitor, which is reported to confer neuroprotection (28, 29). To future confirm the effect of RES, we added AGK2 in KGN cells and found that the expression of SIRT2 was significantly downregulated after being treated with AGK2. As expected, RES reversed it (Figure 4B). Meanwhile, Western blot analysis confirmed that the protein expression of SIRT2 was decreased in KGN cells treated with insulin compared with the Ctrl group and increased after RES treatment (Figures 4C–F). To explore the correlation between insulin sensitivity and the glycolysis process in PCOS, we analyzed the

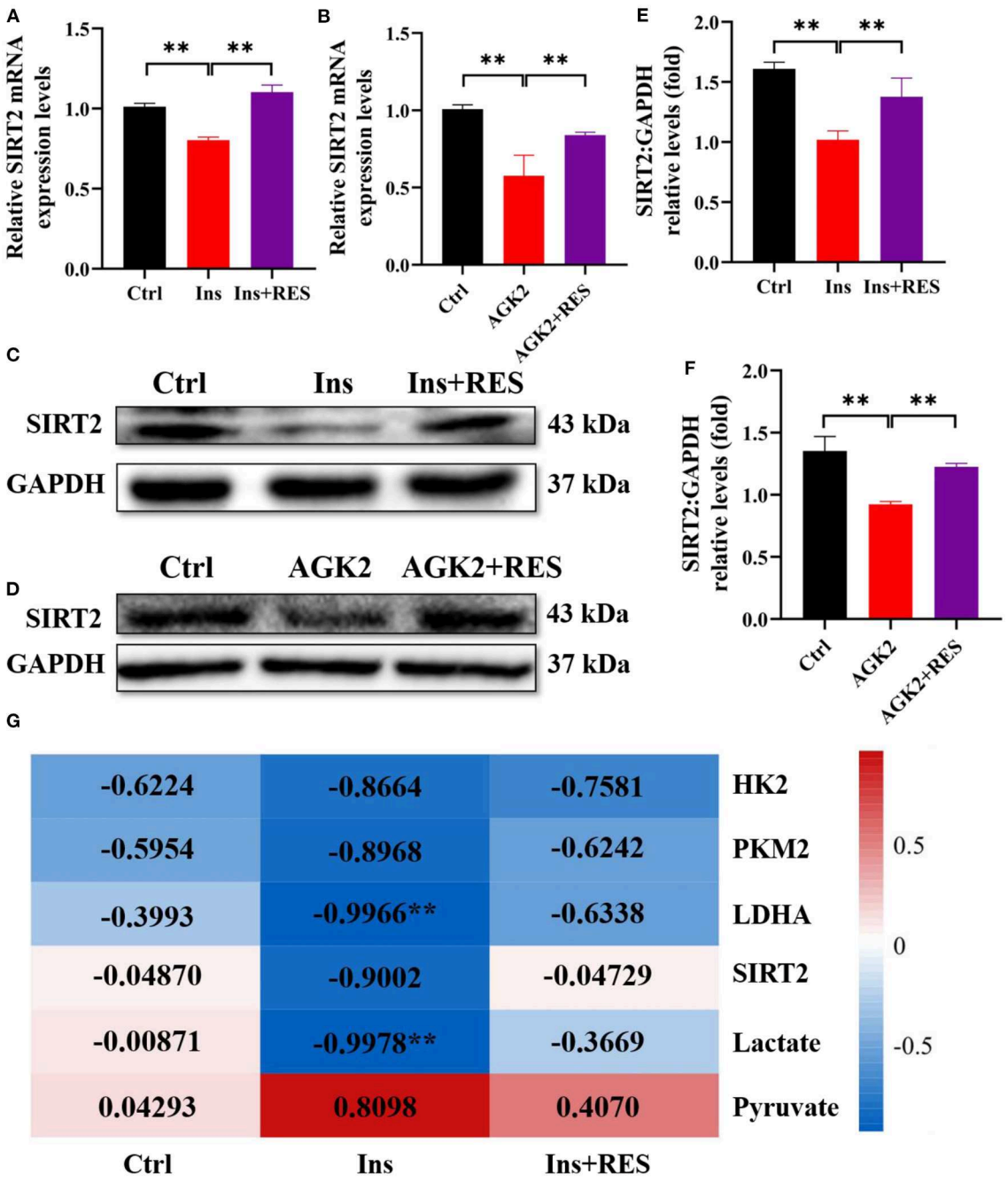

FIGURE 4
RES can upregulate the expression of SIRT2 in KGN cells treated with insulin and AGK2. The relative mRNA expression levels of SIRT2 **(A, B)** in KGN cells were determined using qPCR. The protein level of SIRT2 was determined by Western blot **(C, D)** and quantified by ImageJ software **(E, F)**. Correlation analysis between insulin sensitivity and glycolysis **(G)**. Significant differences between groups are shown as $^{**}P < 0.01$.

correlation between HOMA-IR and key rate-limiting enzymes (HK2, LDHA, and PKM2), glycolytic pathway regulatory factor (SIRT2) and glycolysis products (lactic acid and pyruvate). The results showed that HOMA-IR was negatively correlated with HK2, LDHA, PKM2, SIRT2, and lactic acid levels and had little correlation after RES intervention (Figure 4G), which indicated that RES intervention promoted glycolysis by improving ovarian insulin resistance in PCOS rats.

Resveratrol reversed the inhibitory effect of AGK2 on the process of glycolysis in KGN cells

To find out whether RES regulates glycolysis of granulosa cells through SIRT2, we used qPCR and Western blot to detect the mRNA levels and protein expressions of HK2, PKM2, and LDHA in each group. The results showed that compared with

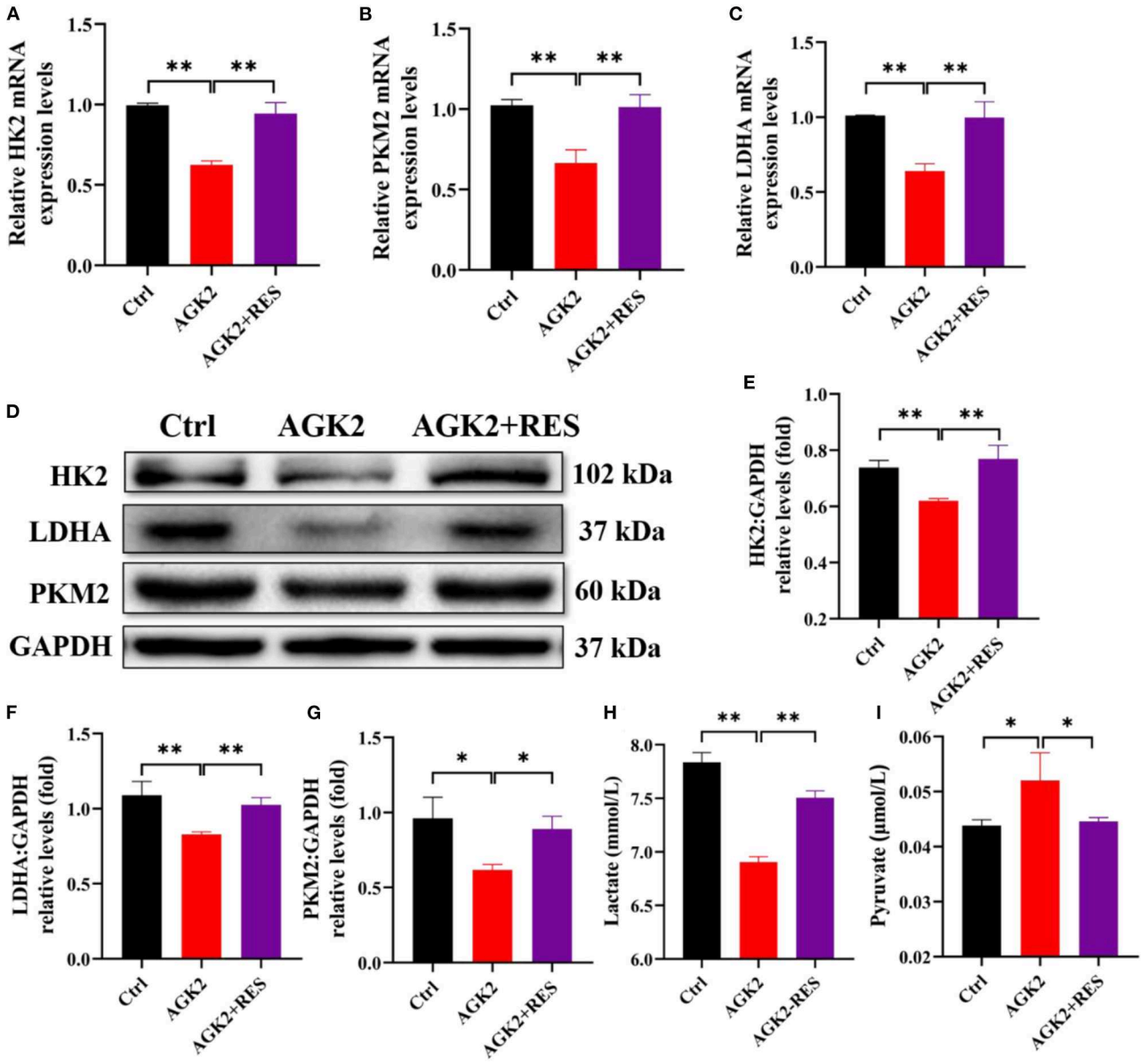

FIGURE 5
RES can upregulate the expression of SIRT2 in KGN cells treated with insulin and AGK2. The relative mRNA expression levels of HK2 **(A)**, PKM2 **(B)**, and LDHA **(C)** in KGN cells were determined using qPCR. The protein levels of HK2, LDHA, and PKM2 were determined by Western blot **(D)** and quantified by ImageJ software **(E–G)**. The levels of lactate and pyruvate in KGN cells using ELISA **(H, I)**. Significant differences between the groups are shown as $*P < 0.05$ and $**P < 0.01$.

the Ctrl group, the mRNA levels and protein expressions of HK2, PKM2, and LDHA were significantly decreased in KGN cells with AGK2 treatment while significantly increased after RES intervention (Figures 5A–C). Western blot analysis confirmed that the expression of SIRT2 in KGN cells treated with AGK2 was significantly decreased than the Ctrl group and increased after RES treatment (Figures 5D–G). Meanwhile, we detected the levels of lactic acid and pyruvic acid in each group of cells by ELISA to further verify the regulation of SIRT2 on the glycolysis activity of granulosa cells. The results showed that compared with the Ctrl group, AGK2 could significantly reduce the content of lactic acid and increase the accumulation of pyruvate, which significantly improved after RES intervention (Figures 5H, I). This is consistent with the changes in PCOS-like cells constructed by insulin and the levels of glycolysis products in PCOS rats, which further proves that SIRT2 played a key role in RES enhancing insulin sensitivity, improving the glycolysis process of granulosa cells in PCOS rats.

Discussion

Recently, the increase in the incidence of PCOS has affected the physical and mental health of women of childbearing age, even as young girls (30). RES is under consideration as a new agent for the

treatment of PCOS symptoms (21). Our previous study first explored that disorder glycolysis plays an important role in reproductive disorders of PCOS, and RES improves the PCOS rats' follicular development by regulating the glycolytic pathway (12). However, the underlying mechanism of the RES-regulated glycolytic pathway to reverse the reproductive disorders in PCOS is still unclear. Thus, in this study, we investigated the molecular mechanism by which RES improved the follicular development of PCOS. Reports showed that insulin resistance associated with compensatory hyperinsulinemia is a common feature of PCOS affecting 50–70% of women along with ovulation failure (23). Therefore, in this study, we determined the insulin resistance in PCOS rats, the results showed higher levels of glucose and insulin in PCOS rats, and HOMA-IR also increased. Those results were consistent with the study reported by Peng et al. (23), which lays a foundation for subsequent experiments.

A follicle is the basic functional unit of the mammalian ovary, which is structurally divided into oocytes, granulosa cells, and theca cells (31). Studies have shown that IGF is involved in many physiological processes such as follicular growth, steroid synthesis and secretion, follicular atresia, and oocyte maturation, and its expression changes are closely related to insulin resistance (32). It was found that the serum IGF1 level and insulin resistance index (HOMA-IR) of patients with PCOS were significantly increased, and IGF1 was positively correlated with HOMA-IR (33). Similarly, this study found that the expression of IGF1 in the ovarian tissue of PCOS rats was positively correlated with HOMA-IR. Studies have shown that lowering the level of IGF1 effectively improved the serum level of testosterone in PCOS rats and reduced the pathological damage of ovarian tissue (34). The results of this study proved that RES also reduced the expression of IGF1 in ovaries and enhanced insulin sensitivity of ovaries in PCOS rats, which provide a theoretical basis for RES to treat patients with PCOS.

Granulosa cells are the most important somatic cells in the process of follicular development, the change in their morphology and the number is the starting signal of follicular growth and development. As the oocytes lack the glycolytic rate-limiting enzymes, their ability to utilize glucose is rarely low, while granulosa cells have a strong affinity for glucose, transforming glucose into lactic acid, pyruvate, and other substrates, which are essential substances for oocyte growth and development (35, 36). Therefore, the glycolysis rate of granulosa cells is of great significance to the development of follicles and oocytes. Studies have found that the expression of key rate-limiting enzymes of glycolysis: LDHA, HK2, and PKM2 in ovaries of PCOS mice was significantly lower than that of normal mice, and lactic acid production was reduced (37, 38). However, increasing the content of lactic acid in the follicular fluid of patients with PCOS *in vitro* reduced the occurrence of atresia follicles (39). Lin et al.'s research on the follicular fluid of normal subjects and patients with PCOS showed that the proper amount of insulin could promote the production of lactic acid in the follicular fluid of normal subjects but has no significant effect on the content of lactic acid in the follicular fluid of patients with PCOS (40). *In vitro* experiments of this subject found that a high dose of insulin inhibited the activity of human ovarian granulosa cells (KGN cells) and reduced the lactic acid content in cells. Similarly, Yan et al. found that insulin resistance could reduce the expression of HK2 in neurons, resulting in the blockage of glycolysis, which confirmed that high levels of insulin could inhibit glycolysis (41). This study confirmed that the HOMA-IR of PCOS rats was negatively correlated with the rate of ovarian glycolysis, and the correlation decreased after the intervention of RES, which further clarified the mechanism of RES in improving follicular development disorder in PCOS rats by enhancing insulin sensitivity and promoting the glycolysis process of granulosa cells.

Studies have shown that RES plays an anti-inflammatory and antioxidant role by activating the expression of Sirtuins in tissues or cells (42). Sirtuins are an NAD^+-dependent deacetylase expressed in the whole life system, including SIRT1-7, and SIRT2 mainly exists in the cytoplasm and nucleus, takes key enzymes of glycolysis as targets, and participates in regulating lipid metabolism, glucose metabolism, and oxidative stress (43). Lantier et al. found that knocking out SIRT2 could reduce muscle sensitivity and promote insulin resistance in the liver (44). The results of this study showed that a high dose of insulin *in vitro* could downregulate the expression of SIRT2 in KGN cells, which indicated that the expression of SIRT2 is also affected by insulin level. In addition, SIRT2 also participated in the regulation of cell glycolysis. Knocking out SIRT2 in an islet could destroy the stability of glucokinase regulatory protein and reduce glycolysis flux (45). AGK2 is a selective SIRT2 inhibitor with an IC50 value of 3.5 μM. Previous studies showed that the addition of AGK2 inhibited SIRT2 activity and rescued the α-Synuclein-mediated toxicity of dorsomedial dopamine neurons (46). Meanwhile, in SIRT2-myc-expressing HeLa cells, AGK2 effectively inhibits the activity of SIRT2 and increases acetylated tubulin (29). *In vitro* experiments of this subject also proved that SIRT2 activity was inhibited by AGK2 and RES reversed it.

In summary, this topic elaborated the mechanism that RES could enhance the insulin sensitivity of the ovary, promote the glycolysis activity of ovarian granulosa cells, and improve the follicular development disorder of PCOS rats from the animal level and cell level. At the same time, SIRT2 may be the key factor for RES to regulate the rate of glycolysis in ovarian granulosa cells. The research results of this topic further improve the mechanism of PCOS follicular development disorder and provide a new direction and strategy for the clinical prevention and treatment of PCOS diseases.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Animal Ethics Committee of the University of South China (permit number: USC2020031602).

Author contributions

XL: conceptualization. AL, LH, JZ, DM, KL, and SL: methodology. XL and ShaL: validation. AL, LH, WZ, QW, XC, SL, and JZ: formal analysis, investigation, and data curation. AL: writing—original draft preparation. AL and XL: writing—reviewing

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.

Publisher's note

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Dried fruit intake causally protects against low back pain: A Mendelian randomization study

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Background: Low back pain is the leading cause of years lived with disability worldwide. The aim of this study was to evaluate whether dried fruit intake causally protects against low back pain using two-sample Mendelian randomization (MR).

Methods: We obtained summary-level data for dried fruit intake ($N = 421{,}764$) from the IEU Open GWAS Project. Forty-one independent genetic variants proxied dried fruit intake. The corresponding data for low back pain were derived from the FinnGen project (13,178 cases and 164,682 controls; discovery data) and the Neale lab (5,423 cases and 355,771 controls; replication data). We conducted univariable and multivariable MR analyses.

Results: In the univariable MR analysis, the inverse variance weighted estimate showed that greater dried fruit intake was associated with decreased risk of low back pain [odds ratio (OR) = 0.435, 95% confidence interval (CI): 0.287–0.659, $P = 8.657 \times 10^{-5}$]. Sensitivity analyses using the MR-Egger (OR = 0.078, 95% CI: 0.013–0.479, $P = 0.009$), maximum likelihood (OR = 0.433, 95% CI: 0.295–0.635, $P = 1.801 \times 10^{-5}$), weighted median (OR = 0.561, 95% CI: 0.325–0.967, $P = 0.038$) and Mendelian Randomization Pleiotropy RESidual Sum and Outlier (MR-PRESSO) (OR = 0.454, 95% CI: 0.302–0.683, $P = 4.535 \times 10^{-4}$) methods showed consistent results. No evidence of directional pleiotropy was identified according to the Egger intercept (intercept P-value = 0.065) or applying the MR-PRESSO method (global test P-value = 0.164). The replication analysis yielded similar results. The multivariable MR revealed that the inverse association between dried fruit intake and low back pain was consistent after adjustment for fresh fruit intake, body mass index, current tobacco smoking, alcohol intake frequency, total body bone mineral density, serum 25-hydroxyvitamin D levels, and vigorous physical activity.

Conclusion: This MR study provides evidence to support that dried fruit intake causally protects against low back pain.

KEYWORDS

Mendelian randomization, low back pain, dried fruit intake, genome-wide association studies, summary statistics

Introduction

Low back pain is a leading contributor to disability worldwide among all musculoskeletal disorders (1). For the majority of patients, low back pain is non-specific, because precise identification of the specific nociceptive source is not possible. If lacking proper diagnosis and therapy, acute low back pain cases are at risk for the development of chronic pain. This can lead to more frequent healthcare visits, increased financial costs, activity limitation, high rates of disability, and reduced quality of life. The pathogenesis of low back pain is complex and multifactorial. Numerous risk factors contribute to its pathogenesis, including

an older age, unhealthy lifestyles, physical factors, musculoskeletal tissue structural failure, and psychological factors (1). In addition, low back pain can be associated with some diseases such as tumors and infections. Epidemiological studies show that low back pain affects about 80% of people in Western countries during their lifetime (2). In Europe, the prevalence of low back pain has ranged between 6 and 11% in the general population (3). The social and economic costs related to low back pain are enormous. It is estimated that the annual economic loss caused by low back pain is about 2.8 billion pounds in the United Kingdom and 8.9 billion euros in Spain (1, 4). Low back pain has become a significant burden for society and health systems, and this burden is still rising due to an aging population.

Dried fruits are shelf-stable forms of fresh fruits, which contain low water content. They represent a small but significant proportion of human diets in modern populations. Traditional dried fruits include prunes, pears, peaches, apples, dates, raisins, mulberries, figs, and apricots. The Middle East and North Africa region has the highest per capita dried fruit consumption (>30 kg per year) (5). In contrast, dried fruit consumption is low in Europe (5). For instance, per capita consumption of dried grapes is about 1.08 kg per year in Germany.[1] Dried fruits are enriched in a variety of dietary fibers, and they are good sources of a number of micronutrients, including magnesium, potassium, iron, calcium, phosphorus, zinc, niacin, vitamin K, vitamin B6, vitamin E, and choline (6). In addition, dried fruits contain many bioactive compounds, including polyphenols, carotenoids, and flavonoids (6). Over the two past decades, experimental research and human clinical studies have reported beneficial effects of dried fruit intake on reducing inflammation, body weight, blood pressure, and glycated hemoglobin levels (7–11). In addition, dried fruits have been shown to exert anti-oxidative, anti-cancer, and anti-aging properties (5, 12–15). However, it remains unclear whether dried fruit intake has a beneficial effect on low back pain. Mendelian randomization (MR) is a statistical method using single nucleotide polymorphisms (SNPs) as instruments for inferring causality between risk factors and a disease outcome. MR can greatly reduce the risk of confounding and reverse causation, which are shortcomings of conventional epidemiological studies (16). Recently, Jin et al. (17) used inverse variance weighted MR and weighted median methods to evaluate the causality between dried fruit intake and 11 site-specific cancers, finding that dried fruit intake had protective effects against some site-specific cancers such as breast cancer and lung cancer. In the present study, we aimed to analyze the potential causal effect of genetically predicted dried fruit intake on low back pain applying the MR framework.

Methods

Ethical approval

Our study was exempt from ethical approval, because we only analyzed publicly available summary-level data of genome-wide association studies (GWAS). All GWAS summary statistics used in our MR study were obtained from the IEU Open GWAS Project[2] (18).

Genetic instruments

Summary-level data for dried fruit intake were obtained from a GWAS study ($N = 421{,}764$) of the UK Biobank, using the GWAS-ID "ukb-b-16576." The UK Biobank is a large population-based cohort of more than 500,000 participants who were recruited at ages 40 to 69 years across England, Wales, and Scotland from 2006 to 2010 (19). In the UK Biobank, dried fruit intake was available from a question "About how many pieces of dried fruit would you eat per day? (Count one prune, one dried apricot, 10 raisins as one piece; put 0 if you do not eat any)" included in the touchscreen questionnaire[3] Participants who answered >100 were rejected. Genotyping of the participants was performed using Affymetrix UK Biobank Axiom array. Extensive centralized quality control was applied for the genetic data (19). We identified SNPs robustly associated with dried fruit intake at genome-wide significance ($P < 5 \times 10^{-8}$) as instrumental variables. We restricted instrumental variables to independent SNPs without linkage disequilibrium ($R^2 < 0.001$) to minimize MR biases by using the clump_data function of the R package "TwoSampleMR" version 0.5.6[4] (18, 20). The European panel of 1,000 Genomes data was used as the reference panel (21). Palindromic SNPs with intermediate allele frequencies were not used, because they may invert the direction of causality.

Analyses were adjusted for fresh fruit intake, body mass index (BMI), current smoking status, alcohol intake frequency, total body bone mineral density, serum 25-hydroxyvitamin D levels, and vigorous physical activity applying multivariable MR. Summary-level data for these exposures were obtained from the IEU Open GWAS Project. The detailed information are shown in Table 1.

Summary-level data for low back pain

Summary-level data for low back pain in individuals of European descent were derived from the FinnGen study (13,178 cases and 164,682 controls) using the GWAS-ID "finn-b-M13_LOWBACKPAIN" (Table 1). The FinnGen study is a nationwide cohort launched in 2017. It aims to collect and evaluate genome and health data from 500,000 Finnish biobank participants (22). Low back pain was identified according to International classification of diseases (ICD) codes retrieved from nationwide registries in Finland. The effect alleles in dried fruit intake and low back pain datasets were harmonized using the harmonize_data function from the TwoSampleMR R package.

For replication analyses, summary statistics for low back pain were obtained from the Neale lab study with the GWAS-ID "ukb-d-M13_LOWBACKPAIN", including 5,423 cases and 355,771 controls (Table 1).

1 https://gfa.org.ge/wp-content/uploads/2019/05/Dried-Fruits-market-research.pdf

2 https://gwas.mrcieu.ac.uk/

3 https://biobank.ndph.ox.ac.uk/ukb/field.cgi?id=1319

4 https://github.com/MRCIEU/TwoSampleMR

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TABLE 1 Detailed information on the GWAS datasets used in this MR study.

Dataset type	Item	GWAS ID	Author	Consortium	Year	Population	Sample size	Sex
Exposure	Dried fruit intake	ukb-b-16576	Ben Elsworth	MRC-IEU	2018	European	421,764	Males and females
	Fresh fruit intake	ukb-b-3881	Ben Elsworth	MRC-IEU	2018	European	446,462	Males and females
	Body mass index	ieu-b-40	Yengo L	GIANT	2018	European	681,275	Males and females
	Current tobacco smoking	ukb-b-223	Ben Elsworth	MRC-IEU	2018	European	462,434	Males and females
	Alcohol intake frequency	ukb-b-5779	Ben Elsworth	MRC-IEU	2018	European	462,346	Males and females
	Total body bone mineral density	ebi-a-GCST005348	Medina-Gomez C	NA	2018	European	56,284	Males and females
	Serum 25-hydroxyvitamin D levels	ebi-a-GCST90000618	Revez JA	NA	2020	European	496,946	Males and females
	Vigorous physical activity	ebi-a-GCST006098	Klimentidis YC	NA	2018	European	261,055	NA
Outcome	Low back pain (discovery)	finn-b-M13_LOWBACKPAIN	NA	FinnGen	2021	European	13,178 cases and 164,682 controls	Males and females
	Low back pain (replication)	ukb-d-M13_LOWBACKPAIN	Neale lab	NA	2018	European	5,423 cases and 355,771 controls	Males and females

MRC-IEU, Medical Research Center-Integrative Epidemiology Unit; SNP, single nucleotide polymorphism; GWAS, genome wide association study; MR, mendelian randomization; GIANT, Genetic Investigation of ANthropometric Traits; NA, not applicable.

Statistical analysis

In this two-sample MR analysis, we used the inverse variance weighted method implemented in the TwoSampleMR R package as the primary MR method. This method gives reliable causal assessments and has the highest statistical power if the selected SNPs meet the instrumental variable assumptions (23). We then conducted sensitivity analyses using the MR-Egger, weighted median, weighted mode, simple mode, and maximum likelihood methods for assessing the robustness of the findings. These methods relax different MR assumptions regarding pleiotropy. For instance, the MR-Egger method can give unbiased assessments even when the exclusion restriction assumption is violated, but it has comparatively low statistical power (24). The weighted median method stipulates that at least 50% of the information is from valid instrumental variables (25). To assess the presence of horizontal pleiotropy, we used the MR-Egger regression intercept (24). If the intercept term is significantly different from zero, this is taken as evidence for horizontal pleiotropy (24). We applied the Mendelian Randomization Pleiotropy RESidual Sum and Outlier (MR-PRESSO) method to detect and correct for potentially pleiotropic outliers (26). We carried out leave-one-out sensitivity analysis to evaluate whether individual instrumental variables drive observed causal associations. A PhenoScanner[5] search was performed to identify phenotypes related to the selected instrumental variables. For evaluating the presence of heterogeneity between variant-specific estimates, we used the Cochran's Q statistical test. Besides univariable MR, we performed multivariable MR to control for potential confounders including fresh fruit intake, BMI, current smoking status, alcohol intake frequency, total body bone mineral density, serum 25-hydroxyvitamin D levels, and vigorous physical activity. Seven models for multivariable MR were taken into account: (a) model 1: adjustment for fresh fruit intake; (b) model 2: adjustment for BMI; (c) model 3: adjustment for current tobacco smoking; (d) model 4: adjustment for alcohol intake frequency; (e) model 5: adjustment for total body bone mineral density; (f) model 6: adjustment for serum 25-hydroxyvitamin D levels; and (g) model 7: adjustment for vigorous physical activity. We carried out all MR analyses using the TwoSampleMR (version 0.5.6) and MR-PRESSO (version 1.0) packages in R version 4.0.4. Statistical significance was set at $P < 0.05$.

Results

Forty-one independent SNPs were selected as instrumental variables in the assessment of dried fruit intake (Supplementary Table S1). These instrument SNPs explained 0.63% of the variance in dried fruit intake. According to the study by Jin et al. (17), the *F*-statistics of individual SNPs ranged between 17.5 and 47.9, indicating adequate instrument strength.

The inverse variance weighted MR estimate showed that greater dried fruit intake was significantly associated with decreased risk of low back pain (OR = 0.435, 95% CI: 0.287–0.659, $P = 8.657$

5 http://www.phenoscanner.medschl.cam.ac.uk/

TABLE 2 MR analysis for the association between dried fruit intake and low back pain.

Dataset for low back pain	Cases/controls	Number of instruments	MR method	OR (95% CI)	*P*-value
finn-b-M13_LOWBACKPAIN (Discovery)	13,178/164,682	41	IVW	0.435 (0.287–0.659)	8.657×10^{-5}
		41	MR-Egger	0.078 (0.013–0.479)	0.009
		41	Maximum likelihood	0.433 (0.295–0.635)	1.801×10^{-5}
		41	Weighted median	0.561 (0.325–0.967)	0.038
		41	MR-PRESSO	0.454 (0.302–0.683)	4.535×10^{-4}
ukb-d-M13_LOWBACKPAIN (Replication)	5,423/355,771	41	IVW	0.984 (0.976–0.992)	5.704×10^{-5}
		41	MR-Egger	0.961 (0.926–0.996)	0.004
		41	Maximum likelihood	0.984 (0.976–0.992)	8.193×10^{-5}
		41	Weighted median	0.985 (0.974–0.996)	0.009
		41	MR-PRESSO	0.984 (0.977–0.991)	1.240×10^{-4}

IVW, inverse variance weighted; CI, confidence interval; OR, odds ratio; MR, Mendelian randomization.

$\times 10^{-5}$) (Table 2 and Figure 1). Sensitivity analyses using the MR-Egger (OR = 0.078, 95% CI: 0.013–0.479, $P = 0.009$), maximum likelihood (OR = 0.433, 95% CI: 0.295–0.635, $P = 1.801 \times 10^{-5}$), weighted median (OR = 0.561, 95% CI: 0.325–0.967, $P = 0.038$) and MR-PRESSO (OR = 0.454, 95% CI: 0.302–0.683, $P = 4.535 \times 10^{-4}$) methods also revealed an inverse association between greater dried fruit intake and low back pain (Table 2). The Cochran's Q statistical test did not provide evidence for statistically significant heterogeneity in the causal estimate amongst instrumental variables (Q = 48.801, $P = 0.160$). No evidence of directional pleiotropy was identified according to the Egger intercept (intercept *P*-value = 0.065) or applying the MR-PRESSO method (global test *P*-value = 0.164). Results of the leave-one-out sensitivity analysis demonstrated that the observed causal associations were not driven by individual instrumental variables (Table 3). We further conducted a PhenoScanner search to identify SNPs related to other potential confounders at genome-wide significance ($P < 5 \times 10^{-8}$) (Supplementary Table S2). The result of the inverse variance weighted analysis was not significantly altered after removing these SNPs (OR = 0.567, 95% CI: 0.339–0.950, $P = 0.031$).

We replicated the protective effect of dried fruit intake on low back pain using a validation sample from the Neale lab study (GWAS ID: ukb-d-M13_LOWBACKPAIN). Consistent with the primary analysis, the replication analysis showed that greater dried fruit intake was causally associated with decreased low back pain risk (Table 2). Besides dried fruit intake, we evaluated the casual association of fresh fruit intake, BMI, current tobacco smoking, alcohol intake frequency, total body bone mineral density, serum 25-hydroxyvitamin D levels, and vigorous physical activity with low back pain using the inverse variance weighted method (Supplementary Table S3). The instrumental variables for these exposures and their association with low back pain are shown in Supplementary Tables S4–S10.

To verify whether the protective effect of dried fruit intake on low back pain was independent of fresh fruit intake, BMI, current tobacco smoking, alcohol intake frequency, total body bone mineral density, serum 25-hydroxyvitamin D levels, and vigorous physical activity, we performed multivariable MR analyses. Results of multivariable MR analyses supported that greater dried fruit intake was protective against low back pain (Table 4).

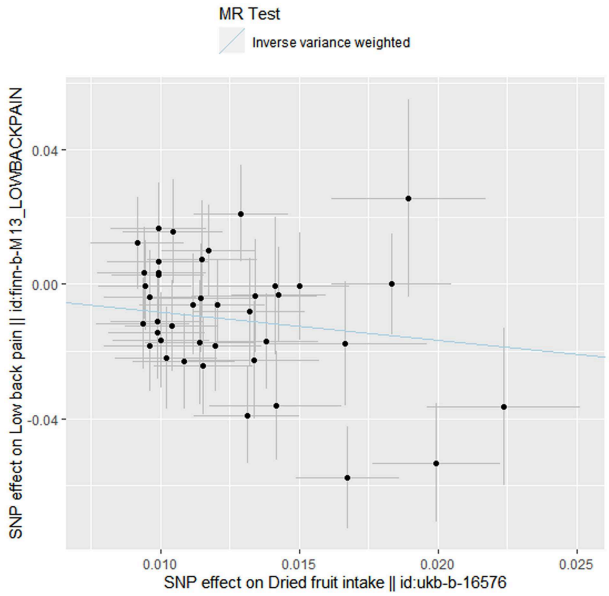

FIGURE 1
Scatter plot showing Mendelian randomization effect estimates of dried fruit intake over low back pain. Each variant-low back pain association is plotted against variant-dried fruit intake association, and the corresponding Mendelian randomization estimate for inverse variance weighted is plotted.

Discussion

To our knowledge, this is the first MR study to evaluate the casual association between dried fruit intake and low back pain. Using genetic data from individuals of European descent, our analyses showed that greater dried fruit intake was associated with decreased low back pain risk. This association was consistent after adjustment for fresh fruit intake, BMI, current tobacco smoking, alcohol intake frequency, total body bone mineral density, serum 25-hydroxyvitamin D levels, and vigorous physical activity.

TABLE 3 Leave-one-out analysis using the inverse variance weighted method.

	SNP	OR	95% lower confidence interval	95% upper confidence interval
	All	0.435	0.287	0.659
Removing	rs10026792	0.447	0.293	0.682
Removing	rs10129747	0.438	0.286	0.670
Removing	rs10740991	0.491	0.332	0.726
Removing	rs10896126	0.423	0.277	0.648
Removing	rs11152349	0.424	0.278	0.645
Removing	rs11586016	0.440	0.288	0.672
Removing	rs11632215	0.429	0.281	0.655
Removing	rs11720884	0.432	0.282	0.661
Removing	rs11772627	0.414	0.270	0.636
Removing	rs11811826	0.432	0.282	0.662
Removing	rs12137234	0.445	0.292	0.678
Removing	rs1582322	0.426	0.279	0.650
Removing	rs1622515	0.425	0.279	0.649
Removing	rs1648404	0.426	0.279	0.649
Removing	rs17175518	0.424	0.279	0.646
Removing	rs17184707	0.431	0.282	0.659
Removing	rs1797235	0.442	0.289	0.675
Removing	rs2328887	0.420	0.277	0.637
Removing	rs2533273	0.437	0.286	0.669
Removing	rs261809	0.444	0.291	0.677
Removing	rs3101339	0.425	0.277	0.651
Removing	rs34162196	0.447	0.292	0.684
Removing	rs3764002	0.466	0.309	0.702
Removing	rs4140799	0.428	0.280	0.654
Removing	rs4149513	0.414	0.273	0.629
Removing	rs4269101	0.441	0.287	0.676
Removing	rs429358	0.476	0.316	0.718
Removing	rs4800488	0.444	0.290	0.680
Removing	rs57499472	0.413	0.274	0.623
Removing	rs62084586	0.429	0.280	0.656
Removing	rs72720396	0.439	0.287	0.671
Removing	rs746868	0.403	0.269	0.604
Removing	rs75641275	0.457	0.301	0.694
Removing	rs7582086	0.431	0.282	0.659
Removing	rs7599488	0.438	0.286	0.671
Removing	rs7808471	0.448	0.294	0.684
Removing	rs7829800	0.418	0.276	0.632
Removing	rs8081370	0.438	0.286	0.671
Removing	rs862227	0.419	0.277	0.635
Removing	rs893856	0.443	0.290	0.677
Removing	rs9385269	0.430	0.281	0.660

SNP, single nucleotide polymorphism; OR, odds ratio.

MR is an effective analytic method for causal inference. It is less affected by certain fundamental shortcomings of traditional observational investigations. Recently, MR studies have been performed to assess potential risk factors for low back pain. In 2020, Elgaeva and colleagues applied inverse variance weighted meta-analysis as the main method for evaluating the causal association between BMI and back pain (27). Summary statistics for BMI were obtained from the GIANT consortium (N = 322,154), and the corresponding data for back pain and chronic back pain were derived from a large European sample (N = 453,860). They found that 1-standard deviation (4.65 kg/m^2) increase in BMI was associated with a significant increase in back pain (OR = 1.15, 95% CI: 1.06–1.25, P = 0.001) and chronic back pain (OR = 1.20, 95% CI: 1.09–1.32, P = 0.0002); the significant causal association remained in secondary analysis and sensitivity analyses. These results suggested that a higher BMI may be a risk factor for back pain. Consistent with their findings, Zhou and colleagues found a casual association between BMI and low back pain (OR = 1.28, 95% CI: 1.18–1.39, $P = 6.60 \times 10^{-9}$) using a two-sample MR design (28). An MR study by a Chinese research group recently evaluated the causal effect of plasma omega-3 levels on low back pain risk, finding that up-regulated plasma omega-3 levels were linked with reduced low back pain risk using the inverse variance weighted method ($\beta = -0.366$, OR = 0.694, P = 0.049) (29). However, this link was not supported by sensitivity analyses using the weighted mode (P = 0.281) and MR-Egger (P = 0.228) methods. In 2022, Williams et al. applied inverse weighted variance, Causal Analysis Using Summary Effect (CAUSE), and sensitivity analyses to evaluate risk factors for chronic back pain (30). Their study demonstrated that several life style factors including greater alcohol intake (inverse variance weighted OR = 1.29, 95% CI: 1.17–1.43, $P = 7.2 \times 10^{-7}$) and smoking (inverse variance weighted OR = 1.27, 95% CI: 1.19–1.35, $P = 7.0 \times 10^{-15}$) increased the risk of chronic back pain (30).

In our study, we applied a two-sample MR design to evaluate the causal association between dried fruit intake and low back pain. Consistent with previous MR studies, we used the inverse variance weighted method in the primary MR analysis. The result of the inverse variance weighted-based estimate was statistically significant; no single instrument SNPs drove the causal estimate. Multiple sensitivity analyses using various methods revealed consistent and stable results. To verify the causal association found in the discovery dataset, we used a validation sample for low back pain from another European population. The replication analysis provided similar results and suggested a causal association between dried fruit intake and low back pain. Furthermore, to adjust for potential confounding factors including fresh fruit intake, BMI, current tobacco smoking, alcohol intake frequency, total body bone mineral density, serum 25-hydroxyvitamin D levels, and vigorous physical activity, we used multivariable MR. The effect of dried fruit intake remained after adjustment for these factors. In summary, the above-mentioned efforts enhanced the robustness of the results in our study.

The mechanisms involved in the causal association between dried fruit intake and low back pain remain unclear. Dried fruits are obtained from fresh fruits by using various drying

TABLE 4 Results of multivariable MR.

Exposure	Outcome	OR (95% CI)	*P*-value	Model	Controlling for
Dried fruit intake	Low back pain	0.487 (0.292–0.810)	0.006	1	Fresh fruit intake
Dried fruit intake	Low back pain	0.417 (0.274-0.633)	3.937×10^{-5}	2	BMI
Dried fruit intake	Low back pain	0.486 (0.303–0.779)	0.003	3	Current tobacco smoking
Dried fruit intake	Low back pain	0.504 (0.323–0.788)	0.003	4	Alcohol intake frequency
Dried fruit intake	Low back pain	0.353 (0.210–0.595)	9.144×10^{-5}	5	Total body bone mineral density
Dried fruit intake	Low back pain	0.470 (0.313–0.705)	2.597×10^{-4}	6	Serum 25-Hydroxyvitamin D levels
Dried fruit intake	Low back pain	0.483 (0.303–0.771)	0.002	7	Vigorous physical activity

MR, Mendelian randomization; OR, odds ratio; CI, confidence interval; BMI, body mass index.

techniques. They are important healthful snacks and are rich sources of dietary fibers, minerals, vitamins, and a variety of bioactive compounds such as flavonoids and carotenoids (6). Dried fruits exert multiple biological effects, including anti-oxidative, anti-inflammatory, anti-atherosclerosis, and anti-cancer effects (5, 7, 10, 12, 14, 15, 31). Experimental research showed that dried fruit intake suppressed proinflammatory cytokines and promoted functions of the musculoskeletal system (32–34). In clinical studies, numerous authors found that daily intake of dried fruits had protective effects on musculoskeletal health in both men and women (35–37). The protective effect of dried fruit intake on low back pain might be related to the micronutrients and bioactive compounds dried fruits contain. For instance, vitamin E could improve musculoskeletal health by maintaining bone mineral density and reducing oxidative stress and inflammation (38, 39). In addition, some authors found that flavonoids had antioxidant and antinociceptive activities, which may be used to relieve pain (40, 41). However, since the evidence regarding the underlying mechanisms is limited, further experimental and clinical studies are required.

One limitation of this MR study is that we did not have access to individual-level data to adjust for medication usage. It has been reported that some medications such as non-steroidal anti-inflammatory drugs and opioids have beneficial effects for low back pain. Another limitation is that we only studied participants of European descent in this MR study. The association between dried fruit intake and low back pain in Asians such as Chinese and Indians remains unclear.

In summary, this MR study using genetic data from individuals of European descent provided evidence to support that greater dried fruit intake was associated with decreased risk of low back pain. The results highlighted the importance of evaluating dried fruit intake for the prevention of low back pain. Further validations using randomized controlled trials with large sample sizes are warranted.

Data availability statement

Publicly available datasets were analyzed in this study. They can be obtained at https://gwas.mrcieu.ac.uk/.

Ethics statement

Our Mendelian randomization study analyzing publicly available summary-level data was exempt from ethical approval.

Author contributions

JH contributed to study concept and design, acquisition and interpretation of data, statistical analyses, and manuscript writing and revision. Z-FX supervised the study, contributed to data interpretation, and assisted in reviewing the manuscript. Both authors read and approved the final manuscript.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1027481/full#supplementary-material

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Vitamin E intake and multiple health outcomes: an umbrella review

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Background: The benefits of vitamin E (VE) for multiple health outcomes have been well evaluated in many recent studies.

Objective: The purpose of this umbrella review was to conduct a systematic evaluation of the possible associations between VE intake and various health outcomes.

Methods: We systematically searched various databases, such as PubMed, Embase, and the Web of Science, to identify related meta-analyses of observational studies and randomized trials. We estimated the effect size of each association by using the random or fixed effects models and the 95% confidence intervals. We used standard approaches to evaluate the quality of the articles (AMSTAR) and classified the evidence into different levels of quality (GRADE).

Results: A total of 1,974 review articles were searched, and 27 articles with 28 health outcomes were yielded according to our exclusion criteria. The intake of VE was inversely associated with the risk of breast cancer, lung cancer, esophageal cancer, gastric cancer, pancreatic cancer, kidney cancer, bladder cancer, cervical neoplasms, cardiovascular disease, Parkinson's disease, depression, age-related cataracts, metabolic syndrome, and fracture. Overall, most of the quality of the evidence was low or very low. Three outcomes (stroke, age-related cataracts, obesity) were identified as having a "moderate" level of quality. The AMSTAR scores for all health outcomes ranged from 5 to 10.

Conclusion: Our study revealed that VE intake is beneficially related to multiple health outcomes. However, future studies on recommended doses and recommended populations of VE are also needed.

Systematic review registration: http://www.crd.york.ac.uk/PROSPERO/, identifier: CRD42022339571.

KEYWORDS

vitamin E, health outcomes, umbrella review, meta-analyses, intake

1. Introduction

Vitamin E (VE), a fat-soluble antioxidant, is composed of tocopherol and tocotrienol α, β, δ, γ subtypes (1). Previous studies have found a potential link between VE and many diseases (2). Since the burden of morbidity and mortality from chronic diseases and cancer is increasing, VE has been widely studied as a potential preventive measure. Oxidative stress is considered a central mechanism of carcinogenesis and is an important process in many diseases, and VE generally helps prevent multiple diseases that are caused by oxidative damage (3, 4). The effects of VE on different types of cancers (such as breast, stomach, and bladder cancer), cardiovascular diseases, and neurological disorders may all be related to it. The possible mechanisms of carcinogenesis are as follows: (1) prevention of DNA damage through scavenging lipid hydrogen peroxide radicals; (2) protection of the nerves

from free radical-mediated damage; (3) repression of the protein kinase C (PKC) pathway and enhancement of immune system function; (4) inhibition of cell cycle progression and cell proliferation via reduction of cyclin D1 and cyclin E; and (5) decrease in the expression of cyclooxygenase-2 and 8-hydroxydeoxyguanosine and type I insulin-like growth factor receptor to inhibit peroxidation and induce cell apoptosis, leading to suppression of cell proliferation (5–7). The association between VE and various health outcomes has been evaluated in a large cohort study, a case-control study, and randomized controlled trials. The results of these studies are summarized by systematic reviews and meta-analyses. However, a comprehensive review of the association between VE and multiple health outcomes (cancer and non-cancer outcomes) has been published. An umbrella review is a popular method for systematically assessing evidence from multiple sources and may be useful in assessing potential biases in the relationship between exposure and outcomes (2, 8, 9). Therefore, we conducted this study to provide a comprehensive review for investigating the relationship between VE and health outcomes reported in published systematic reviews and meta-analyses and to further assess the validity and quality of the available evidence.

2. Methods

2.1. Umbrella review methods

We systematically searched, organized, and evaluated existing evidence from numerous systematic reviews and meta-analyses on multiple health outcomes associated with VE intake (10). We included only those systematic reviews in our study that had incorporated meta-analyses. This umbrella review was registered in PROSPERO (CRD42022339571).

2.2. Literature search and eligibility criteria

We searched systematic reviews and meta-analyses of observational studies and randomized trials from PubMed, Embase, and Web of Science databases from inception to March 2022. The search strategy we used was as follows: ((((((vitamin E[Title/Abstract]) OR (Tocopherol[Title/Abstract])) OR (alpha-Tocopherol [Title/Abstract])) OR (beta-Tocopherol[Title/Abstract])) OR (gamma-Tocopherol[Title/Abstract])) OR (Tocotrienol[Title/Abstract])) AND (((systematic review[Title/Abstract]) OR (meta-analysis[Title/Abstract])) OR (systematic overview[Title/Abstract])). Meta-analyses and systematic reviews with meta-analyses of observational (cohort and case-control) and interventional (randomized and nonrandomized controlled trials) studies that evaluated VE intake and health outcomes in humans were included regardless of the race, gender, country, or region of participants. If two or more health outcomes existed in a single article, data for each outcome were extracted separately. If two or more meta-analyses revealed the same association, we chose the largest one to avoid duplicate assessments. Furthermore, articles reporting VE intake with therapeutic utilities were excluded only if nontherapeutic intake was also reported. Articles written in languages other than English and not involving humans were also excluded.

2.3. Data extraction

The following information was extracted independently by two investigators: (1) name of the first author, (2) cancer outcomes and non-cancer outcomes, (3) year of publication, (4) category of exposure (dietary and supplement VE), (5) the number of included studies, (6) the number of events and total participants in each study, (7) study design [case-control, cohort, randomized controlled trial (RCT)], (8) type of comparisons (highest v lowest dose reduction of any dietary and supplement VE), (9) the estimated summary effect (RR, relative risk; OR, odds ratio; SMD, standard mean difference) and corresponding 95% confidence intervals (CIs), (10) type of effect model (fixed or random model), and (11) *P*-value and publication bias by Egger's test. Any difference was resolved by the third investigator.

2.4. Quality of included studies and quality of evidence

The AMSTAR items (a reliable strategy for evaluating the quality of system reviews and meta-analyses) were used to evaluate the quality of the included articles (11). In our umbrella review, the Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) approach was used to assess the strength of the evidence and categorize it to grade different levels of quality ("high," "moderate," "low," and "very low") (12).

2.5. Data analysis

A summary effect size was presented with a 95% confidence interval through the fixed or random effects models reported in the meta-analysis, if available. If both the cohort and case-control studies existed in the same article, the data were extracted separately. Publication bias was assessed. I^2 statistics and Cochran's Q test were used to estimate the heterogeneity between studies (13, 14). The results of Egger's and heterogeneity tests were significantly higher than those of the control group when the $p < 0.10$. For other tests, a $p < 0.05$ was considered significant.

3. Results

3.1. Characteristics of the included meta-analysis

The flowchart of the detailed selection process is presented in Figure 1. Overall, after a systematic search, a total of 1,974 articles were identified, and 27 meta-analyses with 28 health outcomes (including 13 cancer-related and 16 non-cancer-related outcomes) were enrolled according to our exclusion criteria. The associations between VE and multiple health outcomes are shown in Figure 2,

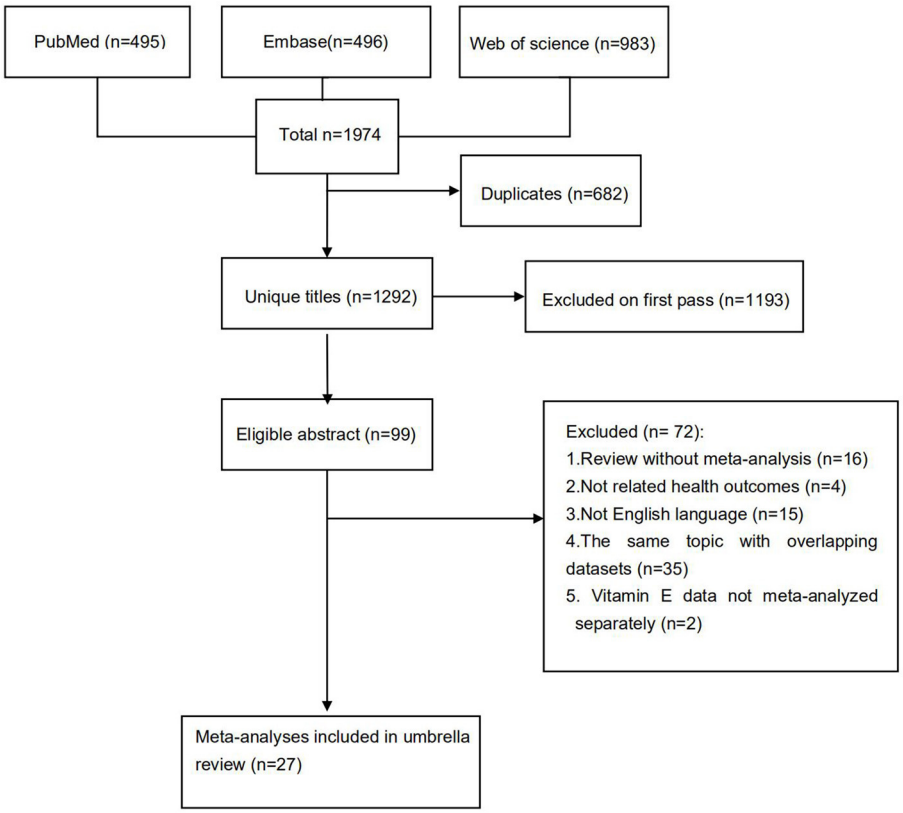

FIGURE 1
Flowchart of the systematic search and selection process.

and more details are presented in Table 1 (non-cancer outcomes) and Table 2 (cancer outcomes). The assessments of AMSTAR scores and GRADE classification are shown in Table 3.

3.2. Associations between VE intake and cancer outcomes

Comparing "the highest" with "the lowest" intake, total intake of VE and dietary intake of VE significantly reduced the risk of breast cancer by 11% (OR = 0.89, 95% CI: 0.81,0.97) and 18% (OR = 0.82, 95% CI:0.73,0.91), respectively (26). The highest dietary VE intake was significantly associated with a decreased risk of lung cancer (RR = 0.84, 95% CI = 0.76,0.93). Subgroup analysis by geographic location showed significant negative associations between dietary VE intake and the risk of lung cancer for the American and European populations (RR = 0.85, 95% CI = 0.75,0.95) but not for the Asian population. Note that there exists a linear relationship between dietary VE intake and lung cancer risk: a daily dietary intake of 2 mg of VE reduces the risk by 5% (27).

Higher dietary VE was also related to lower esophageal cancer risk (OR = 0.47, 95% CI: 0.36, 0.60), especially for esophageal squamous cell carcinoma (ESCC) (OR = 0.29, 95% CI: 0.18, 0.44). A slightly linear dose–response relationship was detected between a 3 mg/day increment of dietary VE and the risk of esophageal cancer (OR = 0.78; 95% CI: 0.57, 1.06) (28). Furthermore, a dose–response relationship was detected between 10 mg/day of dietary VE intake and gastric cancer risk (RR=0.76, 95% CI: 0.67, 0.85) (29). A significant association was found between VE intake and pancreatic cancer risk for only case-control studies (pooled OR=0.63, 95% CI 0.53, 0.75). Meanwhile, a subgroup analysis based on the geographic area found that the intake of VE was not significantly associated with pancreatic cancer risk in European countries, while the inverse association was found in other geographic areas (30).

An analysis of the highest vs. lowest VE intake revealed that the intake of VE played a protective role in bladder cancer progression (RR=0.89; 95% CI: 0.78,1.00). Moreover, a potential linear association was also detected between VE intake and bladder cancer risk (32). A significant negative association between VE intake and kidney cancer risk was found only for cohort studies (RR=0.88, 95% CI 0.72,1.08) (31). In addition, VE intake also has a significant inverse association with the risk of cervical neoplasia (OR=0.68; 95% CI: 0.49, 0.94) (33).

No significant association was observed between VE consumption and the risks of glioma (34), thyroid cancer (35), colorectal cancer (36), endometrial cancer (37), ovarian cancer (38), and non-Hodgkin lymphoma (NHL) (39). Furthermore, the association between VE and the risk of overall cancer mortality (41) or total cancer (40) is not significant. Additionally, no significant association was detected between the combined intake of VE or VE supplements and breast cancer. Additionally, when we performed

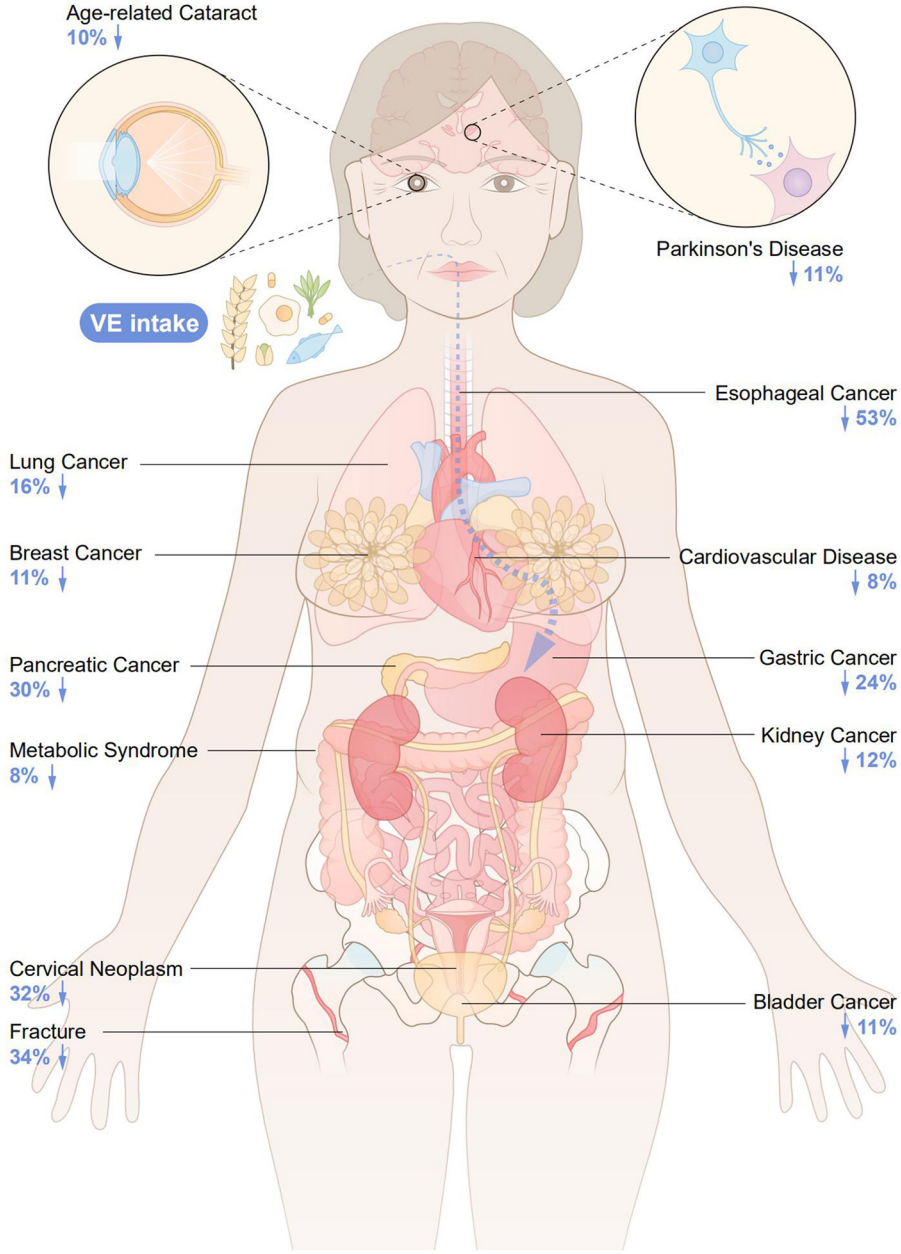

FIGURE 2
The associations between VE and multiple health outcomes.

a subgroup analysis based on the type of study, the relationship between VE intake and breast cancer or pancreatic cancer was not significant (26, 30). For kidney cancer, the association was non-significant in case-control studies (31).

3.3. Associations between VE intake and non-cancer outcomes

A higher intake of VE supplements was associated with a significant reduction in the risk of cardiovascular disease (RR=0.92; 95% CI: 0.46, 0.95) (15). Compared with the lowest category of dietary VE intake, the highest dietary VE intake was significantly associated with a 16% lower risk of Parkinson's disease in the analysis of cohort studies (RR= 0.84; 95% CI: 0.71, 0.99). A linear dose–response meta-analysis suggested that each 5 mg/day increment in VE intake was associated with a 16% lower risk of Parkinson's disease (16). In adults who are at risk of or clinically diagnosed with depression, the positive effect of VE supplements on mood outcomes was observed (SMD = −0.88; 95% CI: −1.54, −0.21) (17). A 10% reduction in the risk of age-related cataracts for individuals was found in the highest categories of VE supplements for cohort studies (RR = 0.90; 95% CI: 0.80, 1.00) (18). In addition, dietary VE intake was inversely associated with a lower risk of metabolic syndrome (MetS) for high versus low consumption

TABLE 1 Associations between Vitamin E intake and non-cancer outcomes.

Outcome	Category	References	No. of cases/ total	No. of studies	Cohort	Case-control	RCT	Meta metric	Effects model	Estimates	95%CI	I^2; Q test *P*-value	Egger's test *P*-value
Significant associations													
Cardiovascular Disease	Vitamin E supplements	Han et al. (15)	7,852/ 233,310	10	0	0	10	RR	Random	0.92	0.46,0.95	55.5%; 0.244	NA
Parkinson's Disease	Dietary vitamin E	Talebi et al. (16)	3,444/ 316,405	7	7	0	0	RR	Random	0.84	0.71,0.99	51.9%; 0.244	NA
Depression	Dietary vitamin E	Lee et al. (17)	187/ 354	7	0	0	7	SMD	Random	−0.88	−1.54,−0.21	87.0%; <0.001	NA
Age-related Cataract	Dietary vitamin E	Jiang et al. (18)	NA/ 42,147	6	6	0	0	RR	Fixed	0.90	0.80,1.00	25.4%; 0.244	0.016
Metabolic Syndrome	Dietary vitamin E	Zhang et al. (19)	NA/ 51,276	10	NA	NA	NA	RR	Random	0.92	0.85,1.00	67.1%; <0.001	NA
Fracture	Dietary vitamin E	Zhou et al. (20)	14,738/ 62,571	1	1	0	0	RR	Random	0.66	0.46,0.95	94.2%; 0.00	0.447
Non-significant associations													
Stroke	Vitamin E supplements	Loh et al. (21)	74,000/148,016	18	0	0	18	RR	Random	0.98	0.92,1.04	0.0%; 0.390	0.251
Parkinson's Disease	Dietary vitamin E	Talebi et al. (16)	1,024 / 2,604	5	0	5	0	RR	Random	0.80	0.57,1.12	23.4%; 0.262	NA
Alzheimer's Disease	Vitamin E supplements	Wang et al. (22)	1,313/ 13,311	5	5	0	0	RR	Random	0.81	0.53,1.33	69.2%; 0.012	0.659
Anxiety	Dietary vitamin E	Lee et al. (17)	153/ 306	5	0	0	5	SMD	Random	−0.86	−2.11,0.40	94.1%; <0.001	NA
Glaucoma	Dietary vitamin E	Han and Fu (23)	1,262/ 244,254	5	5	0	0	OR	Fixed	0.91	0.71,1.16	25.0%; 0.250	NA
Age-related cataract	Dietary vitamin E	Jiang et al. (18)	NA/ 92,243	6	0	0	6	RR	Fixed	0.97	0.91,1.03	0.0%; 0.937	0.016
Obesity	Vitamin E supplements	Emami (24)	NA/ 1,129	21	0	0	21	WMD	Random	0.04	−0.29,0.37	0.0%; 0.999	0.384
All-Cause Mortality	Dietary vitamin E	Jayedi et al. (25)	22,823/386,854	11	9	0	2	RR	Random	0.95	0.90,1.01	48.8%; 0.030	0.460

CI, confidence interval; RCT, randomized controlled trial; RR, relative risk; OR, odds ratio; SMD, standardized mean difference; MD, mean differences; WMD, weighted mean difference; NA, not available.

10.3389/fpubh.2023.1035674

(SMD = −0.08; 95% CI: −0.14, −0.02) (19). In terms of obesity, subgroup analysis for baseline body mass index (BMI) suggested that VE supplements had a significant effect on increasing BMI in participants with normal baseline BMI (18.5–24.9) (WMD=0.636; 95% CI: 0.01, 1.26) (24). Furthermore, the risk of fracture at all sites was significantly reduced with higher VE intake (RR = 0.66; 95% CI: 0.46,0.95), especially for men (20).

Dietary VE intake was not associated with the risk of all-cause mortality while comparing the highest group with the lowest group (RR= 0.95; 95% CI: 0.90, 1.01), even in the further subgroup analysis (25). Higher intake of VE supplements did not show a significant association with stroke (21), anxiety (17), or Alzheimer's disease (22). Dietary VE intake was also associated with a lower risk of glaucoma (23), but it was not statistically significant. In addition, the subgroup analysis did not present a significant association between dietary VE intake and the risk of Parkinson's disease in case-control studies (16), and VE supplements had no significant effect on age-related cataract risk in RCTs (18).

3.4. Heterogeneity and publication bias of included meta-analyses

Among the 28 non-overlapping meta-analyses, 13 meta-analyses reported a Q test $P < 0.10$. One meta-analysis did not report the I^2 statistic. A very high level of heterogeneity (I^2>70%) was observed in three meta-analyses, and eight meta-analyses reported moderate-to-high levels of heterogeneity (I^2 50%−70%). Sixteen meta-analyses reported low levels of heterogeneity (I^2 < 50%).

3.5. AMSTAR and GRADE evaluation of included meta-analyses

The quality of the evidence by GRADE was low (47.1%) or very low (44.1%) (Table 3). Three outcomes (stroke, age-related cataracts, and obesity) were identified as having a "moderate" level of quality. The AMSTAR scores of all health outcomes ranged from 5 to 10, with a median score of 8 (IQR 7-9) (Table 3). More details are presented in Supplementary Tables S1, S2.

4. Discussion

The associations between VE and multiple health outcomes have been reported by a large number of studies and integrated into many meta-analyses. Overall, 27 meta-analyses involving 28 unique outcomes of the correlation between VE intake and multiple health outcomes were included in this umbrella review. The results indicated that the intake of VE was related to a lower risk of subsequent cancer outcomes (breast cancer, lung cancer, esophageal cancer, gastric cancer, pancreatic cancer, kidney cancer, bladder cancer, and cervical neoplasms) and non-cancer outcomes (cardiovascular disease, Parkinson's disease, depression, age-related cataract, metabolic syndrome, and fracture). Given that most of the evidence was from observational studies, compelling evidence for VE and multiple health outcomes does not seem to exist.

There is some discrepancy in subgroup analyses based on the study type of several outcomes (breast cancer, pancreatic cancer, kidney cancer, Parkinson's disease, and age-related cataract). Some of the potential reasons are mentioned below. First, the development of some diseases, such as age-related cataracts, is a long process for healthy individuals, and it may take a decade to manifest the protective effect of VE intake (42). The lengths of follow-up in RCTs may not be long enough to observe the effects of VE supplementation on the risks of disease (43). Second, the dose of VE intake in RCTs, cohort studies, and case-control studies was different (44). Third, due to the different levels of difficulty associated with conducting different studies, there are generally more case-control studies than cohort studies and RCTs. However, for the retrospective design of the case–control studies, there was more selection and recall bias for VE intake measurement in the case-control studies (30). There were also discrepant findings in subgroup analyses of the type of VE intake. Most VE supplements were synthetic and only contained the α-tocopherol form of the eight isoforms found in natural VE. Thus, the natural form, taken in dietary form, shows a more pronounced protective effect than the synthetic form (45). This may explain the non-significant association between VE supplements or the combined intake of VE and breast cancer (26). Another possible reason may be that VE supplements are generally used by those who are more concerned about their health condition than others, which means that they are more likely to tend to adopt a healthier lifestyle (45), which may also lead to discrepancies in the results.

The results of this study suggested that higher VE intake is negatively associated with the risk of cancer outcomes (breast cancer, gastric cancer, pancreatic cancer) and non-cancer outcomes (cardiovascular disease, Parkinson's disease, depression, age-related cataract). The protective effect of higher VE intake on these outcomes may be explained by the oxidative properties of VE. A possible mechanism might be that, as an antioxidant, VE can prevent DNA from being damaged by scavenging lipid hydrogen peroxide radicals (44, 46, 47). Moreover, VE could activate apoptosis by repressing the protein kinase C (PKC) pathway, enhancing immune system function, and inhibiting cancer cell growth by decreasing the phosphoinositide 3-kinase pathway (48). Recent research found that dietary VE could inhibit the dendritic cell checkpoint SHP1 from boosting antigen presentation, strengthening antitumor T-cell immunity, and enhancing immunotherapy (49). In addition, inhibiting cell cycle progression and cell proliferation via the reduction of cyclin D1 and cyclin E could be a feasible explanation for the protective effect of VE against lung cancer and bladder cancer (50, 51). The functions of inhibiting peroxidation and inducing cell apoptosis and, in turn, leading to the suppression of cell proliferation by effectively decreasing the expression of cyclooxygenase-2 and 8-hydroxydeoxyguanosine and type I insulin-like growth factor receptors may play an important role in the significant inverse association of VE intake and kidney cancer risk (52). In previous studies, VE also displayed neuroprotective functions against free radical-mediated injury. This is exemplified by its protection of neurons in the locus coeruleus (the main site of norepinephrine

TABLE 2 Associations between Vitamin E intake and cancer outcomes.

Outcome	Category	References	No. of cases/ total	No. of studies	Cohort	Case-control	RCT	Meta metric	Effects model	Estimates	95%CI	I^2; Q test *P* value	Egger test P value
Significant associations													
Breast Cancer	total intake vitamin E	Fulan et al. (26)	NA/ NA	43	14	26	3	OR	Random	0.89	0.81,0.97	68.3%; NA	0.180
Breast Cancer	dietary vitamin E	Fulan et al. (26)	NA/ NA	29	9	20	0	OR	Random	0.82	0.73,0.91	72.1%; NA	0.150
Lung Cancer	dietary vitamin E	Zhu et al. (27)	4,164/ 435,532	9	9	0	0	RR	Fixed	0.84	0.76,0.93	41.1%; 0.075	0.246
Esophageal Cancer	dietary vitamin E	Cui et al. (28)	3,013/ 11,384	12	1	11	0	RR	Random	0.47	0.36,0.60	67.5%; <0.001	0.008
Gastric Cancer	dietary vitamin E	Kong et al. (29)	3,299/ 634,667	8	3	5	0	RR	Random	0.76	0.67,0.85	43.0%; 0.090	0.254
Pancreatic Cancer	dietary vitamin E	Chen et al. (30)	3,070/ 230,206	11	4	7	0	OR	Fixed	0.70	0.62,0.81	0.0%; 0.455	0.596
Kidney Cancer	dietary vitamin E	Shen et al. (31)	1,213/ 450,463	6	6	0	0	RR	NA	0.88	0.72,1.08	49.2%; 0.023	0.928
Bladder Cancer	dietary vitamin E	Lin et al. (32)	3,265/ 575,601	11	8	0	3	RR	Fixed	0.89	0.78,1.00	19.9%; 0.254	0.707
Cervical Neoplasm	total intake vitamin E	Hu et al. (33)	NA/ 5,301	6	0	6	0	OR	Random	0.68	0.49,0.94	70.0%; 0.005	0.530
Non-significant associations													
Glioma	dietary vitamin E	Qin et al. (34)	3180/ NA	12	2	10	0	RR	Random	0.88	0.69,1.12	64.9%; 0.001	NA
Thyroid Cancer	dietary vitamin E	Zhang et al. (35)	1,021/ 15,005	4	0	3	1	OR	NA	1.50	0.90,2.60	NA; NA	NA
Breast Cancer	vitamin E supplements	Fulan et al. (26)	NA	12	NA	NA	NA	OR	Random	0.98	0.92,1.04	0.0%; NA	0.010
Breast Cancer	combined intake vitamin E	Fulan et al. (26)	NA	14	NA	NA	NA	OR	Random	0.82	0.73,0.91	44.2%; NA	1.000
Colorectal Cancer	dietary vitamin E	Liu (36)	NA	13	NA	NA	NA	RR	Random	0.94	0.82,1.07	10.3%; NA	0.018
Kidney Cancer	dietary vitamin E	Shen et al. (31)	5,731/ 20,543	7	0	7	0	RR	NA	0.78	0.62,0.97	49.2%; 0.023	0.928

(Continued)

10.3389/fpubh.2023.1035674

TABLE 2 (Continued)

Outcome	Category	References	No. of cases/ total	No. of studies	Cohort	Case-control	RCT	Meta metric	Effects model	Estimates	95%CI	I^2; Q test *P* value	Egger test P value
Endometrial Cancer	dietary vitamin E	Bandera et al. (37)	2,800/ 3,873	6	0	6	0	OR	Random	0.91	0.84,0.99	0.0%; 0.450	NA
Ovarian Cancer	total intake vitamin E	Leng et al. (38)	4,597/ 440,532	14	5	9	0	RR	Random	0.95	0.78,1.16	53.2%; 0.019	NA
NHL	total intake vitamin E	Psaltopoulou et al. (39)	3,840/ 189,522	4	4	0	0	RR	Random	0.94	0.65,1.36	0.0%; 0.889	NA
Total Cancer	dietary vitamin E	Aune et al. (40)	5,718/ 169,236	5	NA	NA	NA	RR	Random	0.97	0.93,1.02	61.0 %; 0.030	0.070
Cancer Mortality	dietary vitamin E	Schwingshackl et al. (41)	3,605/ 279,666	3	NA	NA	NA	RR	Fixed	1.00	0.79,1.28	45.0%; NA	NA

CI, confidence interval; RCT, randomized controlled trial; RR, relative risk; OR, odds ratio; NA, not available; NHL, non-Hodgkin lymphoma.

synthesis) from death in an early model of Parkinson's disease, preventing the toxin-induced damage of striatal dopaminergic terminals, and controlling the functions of antioxidant defenses, such as glutathione and superoxide dismutase (SOD) (53–55). Furthermore, to maintain the integrity of proteins and membranes and mediate the function of the lens, VE plays a vital role in blocking the excessive activation of oxidative stress (56–58). These findings can also explain the reverse association, which is a dynamic and interactive process, between dietary VE intake and metabolic syndrome (MetS) (59, 60). Although the exact mechanism of VE supplements' effects on body mass indices (BMI) has not yet been detected, the role of VE on the activation of peroxisome proliferator-activated receptor gamma (PPARg), which could lead to the upregulation of adiponectin gene expression, could be a possible pathway. Moreover, improving insulin sensitivity and suppressing HMG-CoA reductase could also be possible mechanisms affecting body composition indicators (61–63).

For the safety of VE supplements, healthy individuals should not use more than 1,000 mg of VE per day. A daily intake of VE of up to 800 IU appears safe and beneficial (64). When the intake of VE reaches between 400 and 800 IU, healthy individuals appear to have a decreased risk of several diseases, such as CVDs (65). However, the intake of VE could promote the degradation of essential medications for conditions such as cancer, cardiovascular diseases, hypertension, or diabetes. Although a study conducted by Podszun and Frank did not find evidence for the interaction between tocopherols or tocotrienols in the body, it has been suggested that exceeding dosages of 300 mg/day may interfere with some xenobiotics, such as tamoxifen, cyclosporine A, aspirin, or warfarin (66). Under these special circumstances, it is necessary to seek the opinion of a specialist regarding the need and appropriate dosage of vitamin E supplementation. In short, to date, many studies have found potential benefits of VE for multiple disease risks, but no study suggests a specific dose or appropriate population. For healthy individuals, 1,000 mg a day is the upper limit, but more research is needed to determine whether regular supplements are recommended and how much they should be given to specific populations, such as those at high risk for disease and cancer. Prophylactic use of large doses of vitamin E supplements (>1,000 mg per day) is not recommended. When some drugs are being used, the dose of VE should be more carefully designed.

Notably, this is the first comprehensive review of available evidence on VE intake and multiple health outcomes. Standard tools were used to assess the quality of the methods in the included literature (AMSTAR) and the strength of the evidence (GRADE). To avoid possible selection bias, the study was conducted by two researchers. However, our study has several limitations. First, most of the meta-analyses included were based on retrospective studies, and the overall GRADE quality was low. Second, considering that the most common source of VE is dietary intake, it is difficult to obtain VE as the only antioxidant or key nutrient. Third, the results of the study have a large deviation, and there are more confounding factors. Micronutrient combinations have also been frequently used in studies where the interaction between multivitamins is not further elucidated, and not all meta-analyses have a subgroup analysis of these factors. Finally, the definitions of

10.3389/fpubh.2023.1035674

TABLE 3 Assessments of AMSTAR scores and GRADE classification.

Outcome	Category	Author	Year	AMSTAR	GRADE
Non-cancer outcomes					
Significant associations					
Cardiovascular disease	Vitamin E supplements	Han	2020	10	Low
Parkinson's Disease	Dietary vitamin E	Talebi	2022	8	Very low
Depression	Dietary vitamin E	Lee	2022	8	Very low
Anxiety	Dietary vitamin E	Lee	2022	8	Very low
Age-related Cataract	Dietary vitamin E	Jiang	2019	9	Very low
Metabolic Syndrome	Dietary vitamin E	Zhang	2021	9	Very low
Fracture	Dietary vitamin E	Zhou	2020	9	Low
Non-significant associations					
Stroke	Vitamin E supplements	Loh	2021	9	Moderate
Parkinson's Disease	Dietary vitamin E	Talebi	2022	8	Very low
Alzheimer's Disease	Vitamin E supplements	Wang	2021	9	Very low
Obesity	Vitamin E supplements	Mohammad	2021	10	Moderate
Glaucoma	Dietary vitamin E	Han	2022	7	Very low
Age-related Cataract	Dietary vitamin E	Jiang	2019	9	Moderate
All-Cause Mortality	Dietary vitamin E	Jayedi	2018	7	Low
Cancer outcomes					
Significant associations					
Breast Cancer	Total intake vitamin E	Fulan	2011	10	Low
Breast Cancer	Dietary vitamin E	Fulan	2011	10	Low
Lung Cancer	Dietary vitamin E	Zhu	2017	7	Low
Esophageal Cancer	Dietary vitamin E	Cui	2018	7	Very low
Gastric Cancer	Dietary vitamin E	Kong	2014	8	Low
Pancreatic Cancer	Dietary vitamin E	Chen	2016	9	Low
Kidney Cancer	Dietary vitamin E	Shen	2015	8	Low
Bladder Cancer	Dietary vitamin E	Lin	2019	8	Low
Cervical Neoplasm	Total intake vitamin E	Hu	2017	8	Very low
Non-significant associations					
Glioma	Dietary vitamin E	Qin	2014	9	Very low
Thyroid Cancer	Dietary vitamin E	Zhang	2013	6	Very low
Breast Cancer	Combined intake vitamin E	Fulan	2011	10	Low
Breast Cancer	Combined intake vitamin E	Fulan	2011	10	Low
Colorectal Cancer	Dietary vitamin E	Liu	2015	9	Very low
Kidney Cancer	Dietary vitamin E	Shen	2015	8	Low
Endometrial Cancer	Dietary vitamin E	Elisa	2008	6	Low
Ovarian Cancer	Total intake vitamin E	Leng	2019	9	Very low
NHL	Total intake vitamin E	Psaltopoulou	2018	5	Very low
Total Cancer	Dietary vitamin E	Aune	2018	7	Low
Cancer Mortality	Dietary vitamin E	Schwingshackl	2017	8	Very low

AMSTAR, a measurement tool to assess systematic reviews; GRADE, Grading of Recommendations Assessment, Development, and Evaluation; NHL, non-Hodgkin lymphoma.

maximum and minimum intakes were not clearly and uniformly quantified, making it difficult to determine the magnitude of the correlation and the impact of standardized baselines, and dose–response analyses were performed in less than half of the included meta-analyses.

5. Conclusion

In conclusion, we concluded that the intake of VE was related to a lower risk of multiple types of cancer and other diseases of diverse systems. Thus, VE intake at a safe dose is recommended to gain protect against certain diseases. However, further high-quality studies on recommended doses and recommended populations of VE are also needed. For specific populations (such as patients with high blood pressure, diabetes, and cancer) who are taking medication, additional vitamin E supplementation needs to be evaluated by a specialist before use.

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

JA and TZ conceived the project and drafted the manuscript. TZ, XY, JL, and XZ performed the screening and extraction. DL and HX performed the statistical analysis. JA revised 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.1035674/full#supplementary-material

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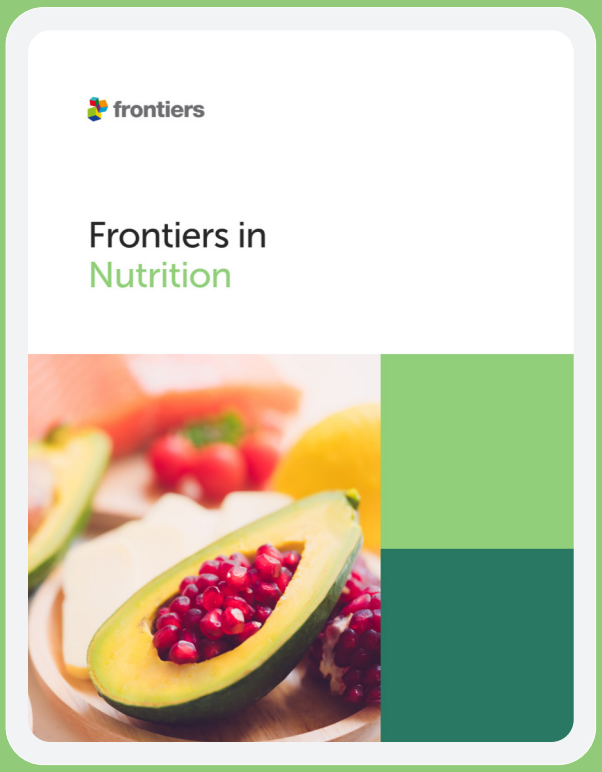

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