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Natural products and their derivatives in the treatment of inflammatory and autoimmune diseases

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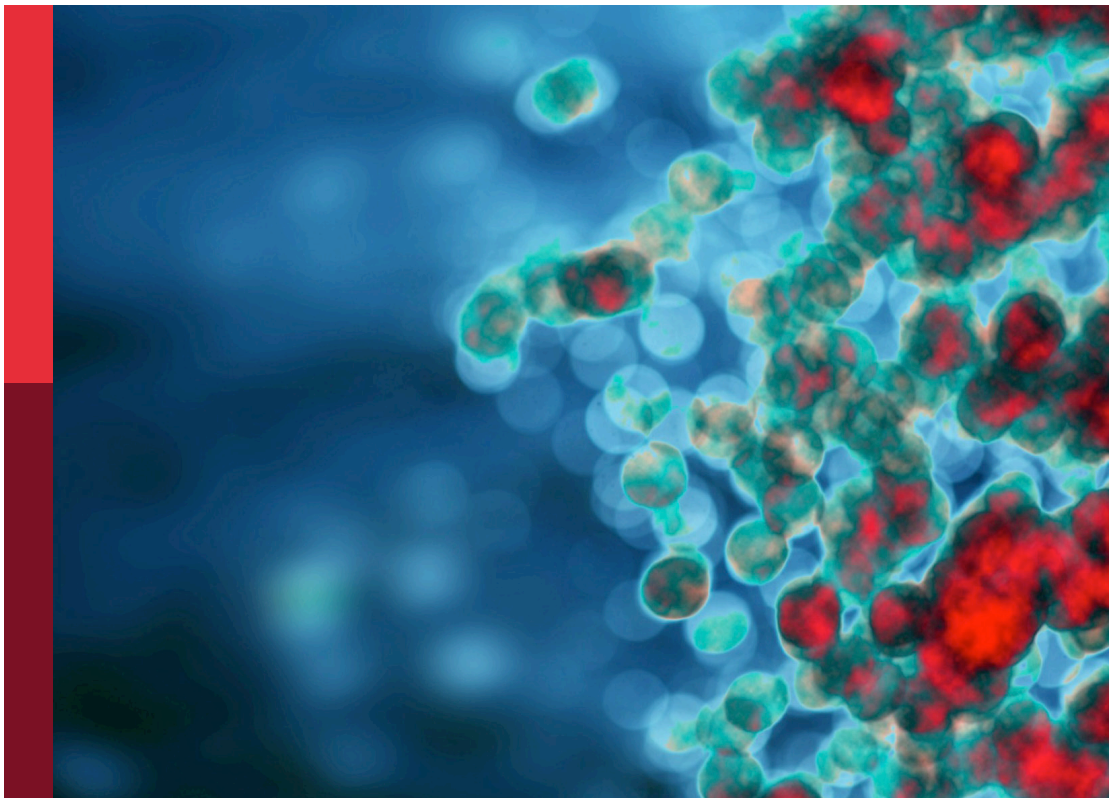

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Natural products and their derivatives in the treatment of inflammatory and autoimmune diseases

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Table of contents

04 Editorial: Natural products and their derivatives in the treatment of inflammatory and autoimmune diseases
Mohamed El-Shazly, Jiao Xiao and Jian Chen

07 Clinical and immunologic features of co-infection in COVID-19 patients, along with potential traditional Chinese medicine treatments
Guochao Zhang, Junjun Zhang, Qi Gao, Yingying Zhao and Yanjun Lai

18 Immuno-modulatory role of baicalin in atherosclerosis prevention and treatment: current scenario and future directions
Li Wang, Shenyi Huang, Xiaolun Liang, Junliang Zhou, Yifan Han, Jiangshan He and Danping Xu

25 Oral curcumin ameliorates acute murine campylobacteriosis
Markus M. Heimesaat, Soraya Mousavi, Fábia Daniela Lobo de Sá, Elisa Peh, Jörg-Dieter Schulzke, Roland Bücker, Sophie Kittler and Stefan Bereswill

39 Phenolic acids from medicinal and edible homologous plants: a potential anti-inflammatory agent for inflammatory diseases
Jingchen Xie, Suhui Xiong, Yamei Li, Bohou Xia, Minjie Li, Zhimin Zhang, Zhe Shi, Qiuxian Peng, Chun Li, Limei Lin and Duanfang Liao

65 Targeting post-stroke neuroinflammation with Salvianolic acid A: molecular mechanisms and preclinical evidence
Hongchun Yang, Muhammad Mustapha Ibrahim, Siyu Zhang, Yao Sun, Junlei Chang, Hui Qi and Shilun Yang

73 Chinese medicine PaBing-II protects human iPSC-derived dopaminergic neurons from oxidative stress
Shouhai Wu, Cuiping Rong, Ruishan Lin, Kaiyuan Ji, Tongxiang Lin, Weimin Chen, Wei Mao and Yang Xu

88 Reporting quality and risk of bias of randomized controlled trials of Chinese herbal medicine for multiple sclerosis
Jing-Ying Wu, Jiang-Li Yang, Jia-Ling Hu, Shan Xu, Xiao-Jie Zhang, Shi-Yan Qian, Min-Li Chen, Mahad Abdulkadir Ali, Juan Zhang, Zheng Zha and Guo-Qing Zheng

107 Herbal medicines for SOD1^{G93A} mice of amyotrophic lateral sclerosis: preclinical evidence and possible immunologic mechanism
Jiang-Li Yang, Jing-Ying Wu, Jing-Jing Liu and Guo-Qing Zheng

128 Advances in the study of artemisinin and its derivatives for the treatment of rheumatic skeletal disorders, autoimmune inflammatory diseases, and autoimmune disorders: a comprehensive review
Zhiyong Long, Wang Xiang, Wei Xiao, Yu Min, Fei Qu, Bolin Zhang and Liuting Zeng

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Editorial: Natural products and their derivatives in the treatment of inflammatory and autoimmune diseases

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Editorial on the Research Topic
Natural products and their derivatives in the treatment of inflammatory and autoimmune diseases

Inflammatory and autoimmune diseases such as psoriasis, inflammatory bowel disease (IBD), multiple sclerosis (MS), ankylosing spondylitis (AS), systemic lupus erythematosus (SLE), IgA nephropathy (IgAN), rheumatoid arthritis (RA), et al. represent a significant and growing burden on global health (1). Characterized by dysregulated immune responses, these conditions pose serious threats to human well-being and longevity. While decades of intensive research have illuminated the intricate molecular mechanisms underlying these pathologies, leading to the development of various therapeutic interventions, the persistent challenges of drug resistance and adverse side effects underscore the urgent need for innovative therapeutic strategies.

In this context, the realm of natural products and their derivatives emerges as a treasure trove of potential solutions. For centuries, traditional medicine systems have harnessed the power of nature to alleviate a myriad of ailments, and modern science is increasingly validating these time-honored practices. Natural products, particularly their structurally diverse monomer compounds, exhibit a remarkable array of biological activities, positioning them as invaluable sources of novel drugs and promising lead compounds for innovative drug development.

Over recent decades, researchers have achieved significant breakthroughs across chemical sciences, pharmaceutical resources, pharmacological studies, formulation technologies, and novel drug discovery. The remarkable story of Professor Tu You-You and her Nobel Prize-winning discovery of artemisinin serves as a powerful testament to the transformative potential of natural product research. Initially identified for its anti-malarial properties, artemisinin and its analogs have since demonstrated a broader spectrum of activities, including compelling anti-inflammatory and immunomodulatory effects with promising applications in the treatment of immune-related disorders (2). This success story

underscores the vast, yet largely untapped, potential residing within the natural world for addressing complex human diseases.

This Research Topic titled "*Natural products and their derivatives in the treatment of inflammatory and autoimmune diseases*" aims to highlight the exciting advancements in this critical area of research. We have gathered a Research Topic of insightful perspectives, cutting-edge original research articles, comprehensive reviews, and thought-provoking commentaries that delve into the functional implications of natural products and their derivatives in combating the intricate challenges of inflammatory and autoimmune conditions.

The contributions within this Research Topic showcase the breadth and depth of ongoing investigations. From comprehensive reviews exploring the multifaceted therapeutic potential of compounds like artemisinin in rheumatic and autoimmune disorders to preclinical studies evaluating the immunomodulatory effects of herbal medicines in neurodegenerative conditions such as amyotrophic lateral sclerosis (ALS), the articles presented here underscore the diverse applications of natural products (Long et al.). Furthermore, investigations into the quality and potential biases in clinical trials of traditional Chinese medicine for MS highlight the importance of rigorous scientific evaluation in this field (Wu et al.).

Several articles within this Research Topic delve into the specific molecular mechanisms by which natural products exert their beneficial effects. Studies targeting post-stroke neuroinflammation with Salvianolic acid A and exploring the anti-inflammatory potential of phenolic acids from medicinal plants exemplify this mechanistic focus (Yang et al.; Xie et al.). In addition, Zheng's group reported that Chinese herbal medicine represented the potential for the treatment of ALS (Yang et al.). Moreover, investigations into the effects of oral curcumin in models of campylobacteriosis and the immuno-modulatory role of baicalin in atherosclerosis further illustrate the diverse therapeutic targets and mechanisms of action of natural compounds (Heimesaat et al.; Wang et al.). Furthermore, Chinese medicine PaBing-II showed a notable protective effect on human induced pluripotent stem cell (iPSC)-derived dopaminergic neurons (Wu et al.). Beyond direct therapeutic applications, this Research Topic also emphasizes the crucial role of cutting-edge methodologies in advancing the field. The application of network pharmacology and genetic sequencing for identifying potential biomarkers and novel drug targets in inflammatory and autoimmune diseases holds immense promise for developing more precise and effective treatments.

The papers presented in this Research Topic underscores the significant progress being made in harnessing the power of natural products to address the persistent challenges of inflammatory and autoimmune diseases. We are confident that the research highlighted herein will not only contribute to a deeper understanding of the therapeutic potential of natural compounds but will also inspire further investigation and ultimately pave the way for the discovery of novel drugs with independent intellectual property rights, offering new hope for patients suffering from these debilitating conditions.

We extend our sincere gratitude to all the contributing authors for their valuable insights and rigorous research, and to the reviewers for their dedication and expertise in ensuring the high quality of this Research Topic. We hope that this Research Topic will serve as a valuable resource for researchers, clinicians, and anyone interested in the exciting and ever-evolving field of natural product-based therapeutics for inflammatory and autoimmune diseases.

Author contributions

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

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Clinical and immunologic features of co-infection in COVID-19 patients, along with potential traditional Chinese medicine treatments

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Objectives: With the increasing number of people worldwide infected with SARS-CoV-2, the likelihood of co-infection and/or comorbidities is rising. The impact of these co-infections on the patient's immune system remains unclear. This study aims to investigate the immunological characteristics of secondary infections in hospitalized COVID-19 patients, and preliminarily predict potential therapeutic effects of traditional Chinese medicine and their derivatives for the treatment of co-infections.

Methods: In this retrospective cohort study, we included 131 hospitalized patients with laboratory-confirmed COVID-19, of whom there were 64 mild and 67 severe cases. We analyzed clinical characteristics and immunologic data, including circulating immune cell numbers, levels of inflammatory factors and viral load, comparing COVID-19 patients with and without co-infection.

Results: Among 131 hospitalized COVID-19 patients, 41 (31.3%) were co-infection positive, with 33 (80.5%) having severe disease and 14 (34.1%) of them resulting in fatalities. Co-infected patients exhibited significantly higher severity and mortality rates compared to non-co-infected counterparts. Co-infected patients had significantly lower absolute counts of lymphocytes, total T lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells and B lymphocytes, while levels of hs-CRP, PCT and IL-6 were significantly elevated compared to non-co-infected patients. Additionally, the viral load of co-infected patients was significantly higher than non-co-infected patients.

Conclusion: Co-infection emerges as a dangerous factor for COVID-19 patients, elevating the risk of severe pneumonia and mortality. Co-infection suppresses the host's immune response by reducing the number of lymphocytes and increasing inflammation, thereby diminishing the antiviral and anti-infective effects of the immune system, which promotes the severity of the disease.

Therefore, it is crucial to implement infection prevention measures to minimize the spread of co-infections among COVID-19 hospitalized patients. Additionally, changes in these biomarkers provide a theoretical basis for the effective treatment of co-infections with traditional Chinese medicine.

KEYWORDS

COVID-19, SARS-CoV-2, co-infection, immune cells, inflammatory factors

Introduction

The coronavirus disease 2019 (COVID-19) is a highly infectious respiratory disease caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), which belongs to RNA beta coronavirus family. Since its emergence in 2019, COVID-19 has rapidly spread globally, posing significant threats to both human health and the global economy (1, 2). As of 27 August 2023, there have been over 770 million confirmed cases and 6.9 million deaths reported globally. The number of SARS-CoV-2 infections is increasing by more than 50,000 per day, and it is estimated that a large number of cases have not been reported (3, 4). While efforts to understand COVID-19, including its origin, pathogenesis and therapeutic strategies, are ongoing, the development of effective antiviral vaccines has been a crucial milestone in combating the epidemic. Currently, more than 200 traditional or novel vaccines are in development, with some already widely deployed in several countries (5–8). Global vaccination efforts have significantly reduced the prevalence, severity and mortality of COVID-19. However, the emergence of SARS-CoV-2 mutant strains poses a significant challenge to the effectiveness of vaccines (9, 10). As SARS-CoV-2 variants increasingly evade neutralizing antibody responses, cellular immune responses are gaining importance in the immunological context.

At present, specific antiviral agents for COVID-19 are still under study. α-Interferons, ribavirin, lopinavir/ritonavir, etc., are only approved for emergency use, and their distribution is controlled. In contrast, antibiotics are easily used to treat secondary bacterial infections. However, the empirical or preventive use of antibiotics can easily lead to the development of drug-resistant bacteria. In addition, critically ill patients, requiring respiratory support and intensive care, are particularly susceptible to secondary and opportunistic infections, including co-infections with other pathogens such as influenza viruses (11). Early studies have indicated a direct association between bacterial and/or viral co-infections and more severe outcomes during pandemics (12, 13). Bacterial infections, especially *streptococcus pneumonia, staphylococcus aureus* and fungal, are common complications of influenza-induced pneumonia, raising questions whether co-infection exacerbates the development of severe COVID-19 and how to influence the disease's progression (14). In this context, the development of new antibacterial and antiviral drugs is a global priority to meet the ongoing demand caused by COVID-19 and reduce the number of deaths associated with bacterial infections worldwide. Traditional Chinese medicine, primarily derived from plants and with a long history as sources of therapeutic agents, offers several advantages, including multiple targets, potential efficacy, and minimal toxic side effects. Research has shown that traditional Chinese medicine can lower the incidence of serious or critical events, improve clinical recovery rates, and help alleviate symptoms such as cough or fever (15). Artemisinin, one of the latest and most successful natural products, along with its derivatives, has served as a first-line antimalarial drug in dozens of countries. Compared to other antimalarial drugs, artemisinin can rapidly reduce parasitic diseases and delay drug resistance when used in combination therapy (16). Additionally, artemisinin has shown positive effects in treating malaria, cancer, viral infections, bacterial infections, and some cardiovascular diseases (17). Traditional Chinese medicine and their derivatives may also produce unexpected positive effects in the treatment of COVID-19 and the management of co-infections.

The role of the immune system in the onset and progression of co-infections in COVID-19, particularly its link to symptom severity, remains unclear. Several studies have reported immune system impairments induced by co-infections in COVID-19 (11, 13, 18–20). In co-infections in COVID-19 cases, patients often exhibit lymphopenia and elevated levels of various cytokines and chemokines, leading to a hyperinflammatory response known as a cytokine storm. To elucidate the immune response and pathogenesis of co-infections in COVID-19 patients, we conducted a study involving 131 COVID-19 patients, comparing cellular immune and inflammatory factor differences between those with and without co-infections. These findings may help us broaden our understanding of the risk factors associated with disease severity in co-infection among COVID-19 patients. Additionally, this provides a theoretical basis for the development of traditional Chinese medicine and their derivatives to effectively treat co-infections.

Materials and methods

Study design and patients

The research is a retrospective cohort study. We selected a total of 131 hospitalized patients who had been admitted to the hospital

and were confirmed with COVID-19 between December 17, 2022 to January 28, 2023, in the Ninth Hospital of Xi'an (Shaanxi, China). The diagnosis and staging of COVID-19 were determined based on the diagnosis and treatment scheme of COVID-19 (9th edition) issued by the National Health Commission of China (21). Specifically, patients were diagnosed with COVID-19 if they were tested positive for SARS-CoV-2 viral RNA and exhibited clinical symptoms such as fever and cough. Patients diagnosed with COVID-19 were categorized as asymptomatic, mild, moderate, severe and critical cases based on the severity of their symptoms. In this study, we included patients with both mild and severe symptoms. Mild patients encompassed those with moderate symptoms, while severe patients encompassed those with critical symptoms. According to whether these patients were co-infected, they were categorized into non-co-infected and co-infected groups, excluding asymptomatic patients with COVID-19. Clinical characteristics and laboratory data of the patients were obtained from medical records. The data were examined by two physicians, and a third researcher ruled any differences in interpretation between the two primary reviewers. The study was performed in accordance with Good Clinical Practice and the Declaration of Helsinki principles for ethical research. The study complied with all relevant ethical regulations and the protocol was approved by the institutional review board of the Ninth Hospital of Xi'an (NO.202311), and a waiver of informed consent was granted, due to the retrospective nature of the observational design.

Clinical classifications

According to the guidelines for the diagnosis and management of COVID-19 (9th edition, in Chinese) released by the National Health Commission of China (21), the clinical classifications of COVID-19 are as follows. Mild cases: Clinical symptoms are mild, and there are no signs of pneumonia on imaging. Moderate cases: Patients exhibit symptoms such as fever and respiratory tract symptoms, along with pneumonia manifestation visible in imaging. Severe cases: Meet any of the following criteria — respiratory distress with a respiratory rate ≥ 30 breaths/min; SpO_2 ≤ 93% at rest; and PaO_2/FIO_2 ≤ 300. Patients with over 50% lesion progression within 24 to 48 hours in pulmonary imaging should be treated as severe cases. Critical cases: Those meeting one of the following conditions — respiratory failure requiring mechanical ventilation, shock, or complications from other organ failures necessitating monitoring and treatment in the ICU.

Real time PCR for quantification of SARS-CoV-2 viral load

Pharyngeal swabs were collected and stored at -20 °C. Viral RNA was extracted following the manufacturer's instructions (Daan Genetics Co., Ltd., China). Subsequently, 20μL of RNA was mixed with a commercial real-time PCR master mix (Daan Gene Co., Ltd. Guangzhou, China) in a 20μl reaction containing primers. The detection limit of the ORF1ab/N real-time reverse transcription polymerase chain reaction (RT-PCR) assays was approximately 200 copies per milliliter. Samples with cycle threshold (Ct) values of ≤40.0 were considered positive for SARS-CoV-2 RNA. RT-PCR analyses for all assays were performed on the ABI 7500 real-time PCR system (Applied Biosystems, USA).

Lymphocyte phenotyping by flow cytometry

We collected peripheral blood from patients using EDTA anticoagulation tubes. Briefly, antibodies were added to whole blood, and samples were lysed with FACS lysing solution (Beckman Coulter, Indianapolis, IN, USA). Peripheral blood mononuclear cells (PBMC) were isolated, and subpopulations were detected by flow cytometry. All experimental procedures were conducted strictly in accordance with the instructions provided for the reagents. Antibodies were purchased from Beckman Coulter Inc. T cell were labeled using anti-CD45-FITC/anti-CD4-PE/anti-CD8-ECD/anti-CD3-PC5.5. B and NK cells were labeled using anti-CD45-FITC/anti-CD56-PE/anti-CD19-ECD/anti-CD3-PC5.5/anti-CD16-PE. Kaluza flow cytometry software version 2.1 (Beckman Coulter, Indianapolis, IN, USA) was used for data analysis.

Measurement of levels of serum hs-CRP, PCT and IL-6

All blood samples for initial high-sensitivity C-reactive protein (hs-CRP), procalcitonin (PCT) and IL-6 measurements were obtained within ≥ 48h of the clinical diagnosis of COVID-19. Whole blood was collected in serum-separating tubes. Serum biomarker levels were measured in duplicate in samples kept on ice prior to measurement. Inflammatory factors, including hs-CRP, PCT and IL-6, were tested by Siemens Immulite 1000 and Roche Cobas c411 biochemical analyzer chemiluminescence immunoassay (CLIA).

Collection of sputum samples and microbial culture

Consecutive deep sputum specimens were collected from patients with COVID-19. Subsequently, 10 μL of sputum specimen was inoculated in Columbia blood agar, Chocolate agar plus bacitracin, and chocolate agar, and incubated in air plus 5% carbon dioxide (37 °C, 48 h). Individual colonies were picked and prepared for testing. Isolates were identified by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Bruker Daltonics) and, where necessary, appropriate API kits (bioMérieux).

Statistical analyses

Statistical analyses were performed using Graphpad Prism version 9.5.0 (GraphPad, La Jolla, CA, USA). Student's t test was performed for two-group analysis. The relationship between 2 variables was examined by simple linear regression analysis. For analysis of contingency tables, Fisher's exact or χ^2 test was used. A p-value < 0.05 was considered statistically significant.

Results

Basic information of patients with COVID-19

In order to understand the immune response and clinical characteristics of COVID-19 patients, 131 COVID-19 patients were included in this retrospective cohort study. All of them were admitted to the Ninth Hospital of Xi'an and underwent CT scanning and SARS-CoV-2 viral RNA testing, with positive results confirming SARS-CoV-2 infection after admission. Among the participants, 64 patients were mild cases, and 67 patients were severe cases. The clinicopathologic characteristics of these patients were detailed in Table 1. 23 patients died during hospitalization, and 118 were discharged from the hospital. The gender distribution of mild cases is balanced, while in severe cases, the proportion of males is significantly higher than females. The average age of severe cases was significantly higher than that of mild cases. Severe cases took an average of 26.40 days from symptoms onset to discharge (deceased patients were excluded) which was significantly longer than the 17.84 days for mild cases. Most patients had underlying health conditions, with hypertension being the most common complication. Severe cases had a higher frequency of underlying diseases compared to mild cases in the context of COVID-19. It is noteworthy that Alzheimer's disease (5 cases) and Parkinson's disease (4 cases) were present among severe cases, while they were difficult to be detected in mild cases. Among severe patients, there were 23 deceased individuals, including 6 females, a significantly lower number compared to the 17 male deaths. The average age of deceased patients was over 60 years old, with 14 patients being older than 80.

TABLE 1 Demographics and clinical characteristics of patients with COVID-19.

	Mild cases (n=64)	Severe cases (n=67)	*P*
Females (%)	36 (56.3)	20 (29.9)	0.002
Males (%)	28(43.7)	47(70.01)	0.002
Age, mean, years (± SD)	71.14 (11.82)	78.57 (9.444)	0.0001
Days since symptoms onset (± SD)	17.84 (4.42)	26.40 (7.87)	<0.0001
Underlying diseases			
Chronic lung disease (%)	12 (18.8)	14 (20.9)	0.758
Chronic heart disease (%)	23 (35.9)	33 (49.3)	0.124
Chronic liver failure (%)	5 (7.8)	7 (10.4)	0.601
Chronic kidney disease (%)	1 (1.6)	5 (7.5)	0.106
Alzheimer's disease (%)	0 (0)	5 (7.5)	
Parkinson's disease (%)	0 (0)	4 (6.0)	
Type 2 diabetes (%)	11 (17.2)	19 (28.4)	0.128
Hypertension (%)	34 (53.1)	42 (62.7)	0.268
Bacterial superinfection (%)	8 (12.5)	33 (49.3)	<0.0001
Females/males who died (%)	0 (0)	6 (26)/ 17 (74)	
Deaths over 80 years old (%)	0 (0)	14 (61)	

Results are expressed as mean ± standard deviation (SD) or percentage; *P* values are from a χ^2 analysis.

During medical treatment, we found that out of the 131 patients with COVID-19, 41(31.3%) were co-infection positive and 90 were co-infection negative. Among the 90 non-co-infected patients, 56 (62.2%) were mild cases, and 34 (37.8%) were severe cases. Among the 41 co-infected patients, there were 8 (19.5%) mild cases and 33 (80.5%) severe cases. These results indicated that co-infected patients faced a significantly higher risk of developing severe symptoms. The mean duration from symptom onset to discharge was significantly longer in co-infected patients (27.07 d) compared to non-co-infected patients (19.33 d). Additionally, it was observed that 9 (10%) of the non-co-infected patients passed away, whereas 14 (34.1%) deaths occurred among co-infected patients, demonstrating a significantly higher mortality rate in this group. Among the 33 severe patients with co-infections, 18 (54.5%) had single bacterial infections, 15 (45.5%) mixed bacterial infections. In contrast, all 8 mild patients with co-infections were infected with single bacterial strains, and no mixed bacterial infections were observed. Furthermore, 18 cases with fungal infections and 14 cases with Acinetobacter baumannii were detected in severe patients with co-infections. In mild patients with co-infections, only 1 case of fungal infection was found, and Acinetobacter baumannii was difficult to detect. Notably, while isolating pathogenic bacteria from severe cases, we found one patient infected with carbapenem-resistant Pseudomonas aeruginosa (CRPA), six patients with carbapenem-resistant Acinetobacter baumannii (CRAB) and one patient with carbapenem-resistant enterobacteriaceae (CRE) infection. No resistant organisms were found in the mild cases. The basic information of patients with coinfections was presented in Table 2.

Measurement of immune cells in COVID-19 patients with co-infection

We also conducted detection of circulating immune cells in 131 patients with COVID-19, and the results are presented in Table 3. Preliminary analysis of circulating immune cells revealed that the counts of Lymphocytes, total T lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells, B cells and NK cells in mild cases were within the normal reference range and near the lower limit of reference values. In severe cases, NK cell counts were within the normal reference range,

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TABLE 2 Epidemiology of pathogen co-infections at COVID-19 admission.

	Mild cases (n=8)	Severe cases (n=33)	*P*
Single pathogen infection (%)	8 (100)	18 (54.5)	
Mixed pathogen infection (%)	0 (0)	15 (45.5)	
Fungal infection (%)	1 (12.5)	18 (54.5)	0.032
Candida albicans (%)	1 (12.5)	14 (42.4)	0.115
Aspergillus fumigatus (%)	0 (0)	4 (12.1)	
Staphylococcus aureus (%) (G^+)	2 (25)	2 (6.1)	0.105
Streptococcus pneumoniae (%) (G^+)	1 (12.5)	2 (6.1)	0.530
Enterococcus faecalis (%) (G^+)	0 (0)	1 (3.0)	
Staphylococcus hominis (%) (G^+)	0 (0)	1 (3.0)	
Pseudomonas aeruginosa (G^-)	1 (12.5)	4 (12.1)	0.977
Acinetobacter baumannii (G^-)	0 (0)	14 (42.4)	
Klebsiella pneumoniae (G^-)	2 (25)	5 (15.2)	0.507
Legionella pneumophila (G^-)	1 (12.5)	0 (0)	
Enterobacter asburiae (G^-)	0 (0)	1 (3.0)	
Stenotrophomonas maltophilia (G^-)	0 (0)	3 (9.1)	
Escherichia coli (G^-)	0 (0)	1 (3.0)	
Enterobacter cloacae (G^-)	0 (0)	2 (6.1)	
Mycoplasma pneumoniae	0 (0)	4 (12.1)	
CR-PA	0 (0)	1 (3.0)	
CR-AB	0 (0)	6 (18.2)	
CRE	0 (0)	1 (3.0)	
No. Death	0 (0)	14 (42.4)	

Results are expressed as mean ± standard deviation (SD) or percentage; *P* values are from a $\chi 2$ analysis. G+, Gram positive; G-, Gram negative; CRPA, carbapenem-resistant Pseudomonas aeruginosa; CRAB, carbapenem-resistant Acinetobacter baumannii; CRE, carbapenem-resistant Enterobacteriaceae.

while all other types of immune cell counts were below the normal reference range. In severe cases, the number of lymphocytes, total T lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells and B lymphocytes decreased significantly compared to mild cases, as shown in Figure 1A. The assay strategy was illustrated in Figure 1D. The proportion of total T lymphocytes, $CD4^+$ T cells in severe cases was significantly lower than in mild cases, whereas the proportion of B cells and NK cells was significantly higher than in mild cases. This difference may be attributed to the more significant reduction of T cells in the severe cases. There was no significant difference in the proportion of $CD8^+$ T cells between mild and severe cases.

The numbers of lymphocytes, total T lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells, and B lymphocytes were significantly lower in co-infected patients than those in non-co-infected patients, and there was no difference in the number of NK cells between the two groups, as is shown in Figure 1B. The proportion of total T lymphocytes, $CD4^+$ T cells in co-infected patients were significantly lower than those in non-co-infected patients, while the proportion of NK cells in co-infected patients was significantly higher than that in non-co-infected patients. The proportion of $CD8^+$ T cells and B cells did not differ significantly between the two groups. Additionally, we found that among mild cases, the number of lymphocytes, total T lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells and B lymphocytes in co-infected patients were slightly lower than those in non-co-infected patients. However, there was no significant difference between the groups. Among severe cases, there was no difference in circulating immune cell counts between the groups, as is shown in Figure 1C.

SARS-CoV-2 viral load in COVID-19 patients with co-infection

To assess the relationship between COVID-19 patient severity and SARS-CoV-2 viral load, we conducted tests on SARS-CoV-2 viral loads in throat swab samples from all patients. The results revealed that patients with higher SARS-CoV-2 viral loads were more prone to developing severe pneumonia. Specifically, the SARS-CoV-2 viral RNA RT-PCR CT value for severe patients was 28.65, significantly lower than that for mild patients (32.03). A smaller CT value in viral nucleic acid detection indicates a higher viral load, as demonstrated in Figure 2A. Moreover, the viral load of co-infected patients (CT value = 28.64) was significantly higher than that of non-co-infected patients (CT value = 31.06), as indicated in Figure 2B. In mild patients, the viral load of co-infected patients was significantly elevated compared to non-co-infected patients. However, among severe patients, there was no notable difference in viral load between the groups, as shown in Figure 2C. To explore the impact of SARS-CoV-2 viral load on circulating immune cells and changes in inflammatory factors, we conducted a correlation analysis, the results of which are presented in Figure 2D. With the increase in SARS-CoV-2 viral load, the counts of lymphocytes, total T lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells and B lymphocytes in patients decreased, indicating a negative correlation. Additionally, serum levels of hs-CRP, PCT and IL-6 were elevated, showing a positive correlation. Importantly, there was no significant correlation between the number of NK cells and SARS-CoV-2 viral load.

The levels of hs-CRP, PCT and IL-6 levels were measured in COVID-19 patients with co-infection

Serum inflammatory cytokines were assessed in 131 patients with COVID-19 to determine the presence of a cytokine storm. We observed that hs-CRP, PCT and IL-6 levels were higher than the upper limit of the normal reference range in nearly all mild and severe patients, as detailed in Table 3. In severe patients, hs-CRP, PCT and IL-6 levels were significantly higher than those in mild patients, as depicted in Figure 3A. Moreover, in co-infected patients, hs-CRP, PCT and IL-6 levels were significantly elevated

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TABLE 3 Immunological features of patients with COVID-19.

	reference range	Mild cases (n=64)	Severe cases (n=67)	*P*	non-co-infected cases (n=90)	co-infected cases (n=41)	*P*
Lymphocytes $\times10^6$/L (± SD)	1100 - 3200	1174 (399.3)	446 (205.5)	<0.0001	907.9 (495.2)	568.3 (357.1)	0.0001
Total T cell count, $\times10^6$/L (± SD)	723 - 2271	868.1 (323.6)	287.4 (171.1)	<0.0001	664.8 (399.7)	365.6 (265.8)	<0.0001
Total T cells, % (± SD)	61.1 - 77	73.57 (9.29)	62.14 (17.56)	<0.0001	71.06 (13.27)	60.38 (16.70)	0.0001
$CD4^+$ T cell count, $\times10^6$/L (± SD)	396 - 1309	511.8 (230.3)	159.8 (99.33)	<0.0001	387.0 (261.1)	210.5 (166.0)	0.0001
$CD4^+$ T cells, % (± SD)	25.8 - 41.6	42.92 (10.59)	35.36 (13.16)	0.0004	41.05 (11.79)	34.65 (13.08)	0.0062
$CD8^+$ T cell count, $\times10^6$/L (± SD)	224 - 1014	324.6 (155.4)	115.9 (95.03)	<0.0001	251.9 (173.8)	143.2 (114.2)	0.0004
$CD8^+$ T cells, % (± SD)	18.1 - 29.6	28.16 (10.33)	24.31 (13.31)	0.0676	27.30 (12.57)	23.76 (10.58)	0.1192
B cell count, $\times10^6$/L (± SD)	118 - 645	124.8 (78.79)	62.31 (56.05)	<0.0001	103.7 (79.67)	69.00 (56.41)	0.0132
B cells, % (± SD)	9.02 - 14.10	10.55 (6.086)	14.10 (12.78)	0.0458	11.71 (8.113)	13.81 (13.74)	0.2752
NK cell count, $\times10^6$/L (± SD)	61 - 607	171.1 (98.05)	107.7 (89.51)	0.0002	139.8 (98.11)	136.2 (101.2)	0.8466
NK cells, % (± SD)	10.04 - 19.78	14.52 (7.92)	21.72 (13.67)	0.0004	15.90 (10.09)	23.26 (13.59)	0.0007
CRP, mg/L (± SD)	0 - 3	20.80 (13.51)	121.4 (64.03)	<0.0001	49.80 (54.45)	121.6 (71.51)	<0.0001
PCT, ng/mL (± SD)	0 - 0.046	0.08 (0.11)	1.26 (1.34)	<0.0001	0.3223 (0.7042)	1.470 (1.446)	<0.0001
IL-6, pg/mL (± SD)	0 - 7	19.58 (12.63)	102.8 (77.13)	<0.0001	42.27 (46.35)	105.7 (90.11)	<0.0001

Results are expressed as mean ± standard deviation (SD) or percentage; *P* values are from a unpaired t test. CRP, C-reactive protein; PCT, procalcitonin.

compared to non-co-infected patients, as shown in Figure 3B. In mild patients, hs-CRP and IL-6 were significantly increased in co-infected patients compared to non-co-infected patients, while PCT showed no significant increase. Conversely, in severe patients, hs-CRP, PCT and IL-6 levels in co-infected patients were significantly higher than those in non-co-infected patients, as illustrated in Figure 3C.

Discussion

COVID-19 has resulted in 6.9 million deaths worldwide. A recent meta-analysis examined the impact of tuberculosis on the severity of COVID-19 and found that SARS-CoV-2 and *Mycobacterium tuberculosis* were the primary causes of death from infectious diseases. Simultaneously, the mortality rate increased among patients diagnosed with a co-infection of COVID-19 and tuberculosis (20). Another meta-analysis explored viral co-infections in COVID-19, suggesting that such co-infections may exacerbate the severity of COVID-19 and influence treatment and prevention strategies (11). However, hospitalized patients with COVID-19 are vulnerable to hospital-acquired infections, and those with co-infections face a significantly increased risk of mortality. A study revealed that 94.2% of COVID-19 patients were concurrently infected with various other microorganisms, including viruses, bacteria and fungi (22). In this study, we analyzed a total of 131 patients with COVID-19, among whom 41 were co-infected. This co-infection rate of 31.3% is significantly higher than what has been reported in previous studies (23). Compared to patients with COVID-19 alone, those with co-infections faced a 3.5 times higher risk of death. Males and older patients, especially those with underlying comorbidities such as Alzheimer's disease, Parkinson's disease and bacterial infections, were at increased risk of developing severe pneumonia. Interestingly, co-infection was rare in mild cases, whereas in severe cases, the incidence of co-infection was significantly higher. Co-infection may easily develop into severe disease. Furthermore, severe patients exhibited a significant increase in the proportion of mixed bacterial infections, fungal infections and drug-resistant bacterial infections which could be attributed to prolonged antibiotic use and nosocomial infections resulting from mechanical ventilation. These factors may play a crucial role in the deterioration and, in some cases, fatalities observed in co-infected patients.

As SARS-CoV-2 variants increasingly evade neutralizing antibody responses, cellular immune responses are gaining importance in the immunological context. During the diagnosis and treatment of COVID-19, the presence of SARS-CoV-2 alongside other respiratory pathogens may result in overlapping clinical symptoms, making it challenging for clinicians to exclude other pathogens from the infection. Co-infected patients more frequently experience respiratory distress and may develop acute respiratory distress syndrome. In terms of laboratory findings, lymphopenia is more common in co-infected cases than in non-co-infected patients. Research has found that the interaction between SARS-CoV-2 and Mycobacterium tuberculosis co-infection can modulate immune responses, particularly affecting

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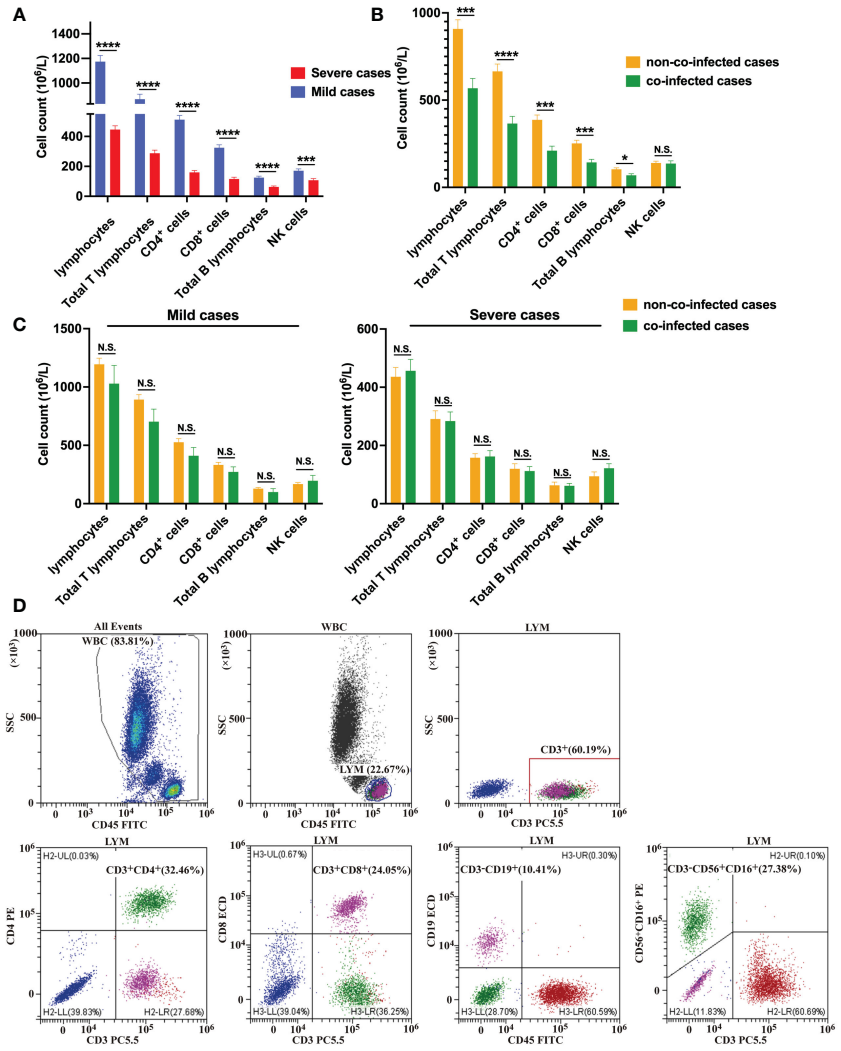

FIGURE 1
Immune cell counts in COVID-19 patients. **(A)** Comparisons of the absolute number of immune cells between severe cases (n = 67) and mild cases (n = 64). **(B)** Comparisons of the absolute number of immune cells between co-infected cases (n = 41) and non-co-infected cases (n = 90). **(C)** Comparisons of the absolute number of immune cells between co-infected and non-co-infected cases based on disease severity. **(D)** Flow cytometric analysis of lymphocytes, total B lymphocytes, NK cells, total T lymphocytes, $CD4^+$ T cells and $CD8^+$ T cells from a representative patient. All data presented as the mean ± SEM. Differences were tested using unpaired *t* test. $p<0.05$ was considered significant. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$; N.S., not significant.

monocytes, macrophages, neutrophils and T cells, resulting in weakened immunity and increased inflammation (20). Sharov et al. (19) discovered that COVID-19 led to a rapid increase in the depletion of T cells, initially caused by HIV. The numbers of $CD4^+$ and $CD8^+$ cells decreased, accompanied by a sharp rise in the number of exhausted T cell, contributing to the deterioration of COVID-19-related respiratory distress. These findings are consistent with our research. Co-infection can suppress the antiviral effect of the immune system by reducing the number of T lymphocytes. In the majority of patients with severe COVID-19 disease, decreased T-lymphocyte counts are observed, whereas only a small proportion of patients with mild disease show decreased T-lymphocyte counts. This may be attributed to the increased expression of apoptosis factors FAS and FAS-L in T cells, B cells and NK cells. Additionally, research has found that high expression of PD-1 and TIM-3 genes on $CD4^+$ and $CD8^+$ T cells leads to T cell depletion and dysregulation of inflammatory cytokine secretion (24). Specifically, $CD4^+$ T lymphocytes serve as clear indicators of impaired immune function in patients with COVID-19 and hold predictive value for the disease's severity, with their counts correlating with the seriousness of the illness (25). $CD8^+$ T lymphocytes, as effector T cells that specifically kill target cells, have the capability to inhibit and counteract viruses by releasing various cytokines to eliminate target cells. Notably, changes in $CD8^+$ T lymphocytes are not apparent in the early stage of SARS-CoV-2 infection when the condition is mild. However, in severe conditions, cellular immune function becomes significantly impaired, leading to a decrease in $CD8^+$ T lymphocytes (26). Generally, co-infection, caused by the combined action of one or more pathogens, can lead to reduced lymphocyte counts,

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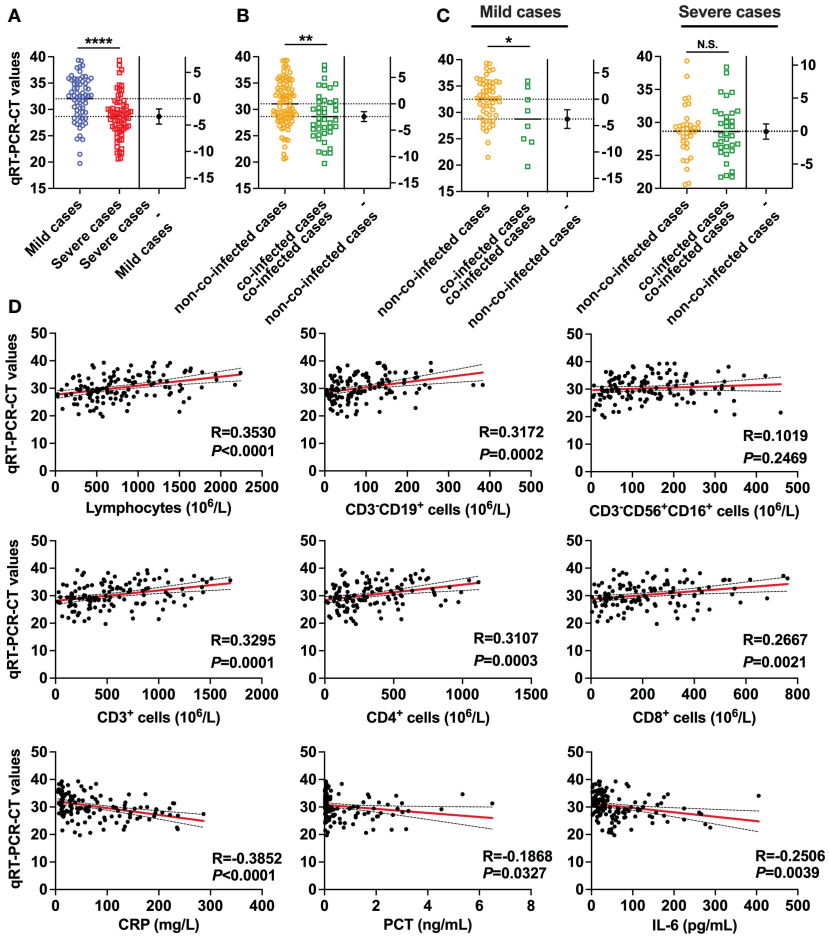

FIGURE 2
SARS-CoV-2 viral RNA RT-PCR CT values in patients with COVID-19. **(A)** Comparisons of SARS-CoV-2 viral RNA RT-PCR-CT values between severe cases (n = 67) and mild cases (n = 64). **(B)** Comparison of SARS-CoV-2 viral RNA RT-PCR-CT values between co-infected cases (n = 41) and non-co-infected cases (n = 90). **(C)** Comparison of SARS-CoV-2 viral RNA RT-PCR-CT values between co-infected cases and non-co-infected cases by disease severity. **(D)** Correlations of SARS-CoV-2 viral RNA RT-PCR-CT values with the number of Lymphocyte, Total B lymphocyte, NK cell, total T lymphocytes, $CD4^+$ T cells, and $CD8^+$ T cells, as well as the levels of hs-CRP, PCT and IL-6. All data are presented as the mean ± SEM. Differences were tested using unpaired t test. $p<0.05$ was considered significant. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$; N.S., not significant.

overexpression of inflammatory factors, immune suppression in the host, resistance to antibiotics, and overall poor prognosis (11, 18). Our findings revealed that co-infection significantly reduces the number of circulating immune cells and lymphopenia being more common in co-infected than in non-co-infected patients. In co-infected COVID-19 patients, NK cell counts were within the normal reference range, but other immune cell counts were significantly lower than those in non-co-infected patients. This decline in circulating immune cells made it challenging for adaptive immunity to exert antiviral effects, thereby facilitating mild cases progression to severe ones.

Real-Time PCR-based detection of viral nucleic acids is the gold standard for diagnosing COVID-19. This technique allows for the immediate and easy determination of viral load values, indirectly indicating the severity of the infection. Consistent with previous studies (27, 28), we observed variations in viral load among patients with different disease severities. Severe patients exhibited significantly higher viral loads compared to mild patients, suggesting that a high viral load might be a risk factor for severe disease. Older patients with more comorbidities had higher SARS-CoV-2 viral loads. In addition, in mild patients, viral loads were significantly higher in patients with co-infections. This could be attributed to the compromised immune system and diminished ability to fight the virus in older, frail individuals. Another contributing factor could be the higher levels of angiotensin-converting enzyme 2 in the alveoli of the elderly, which is considered a receptor for SARS-CoV-2 (29). These factors lead to increased viral loads and more severe disease upon admission. We also discovered an association between viral load and an elevated risk of systemic inflammation, disease progression and death. Increased viral load due to co-infection led to a decrease in the number of circulating immune cells in patients. Simultaneously, levels of inflammatory factors rose, indicating a hyperinflammatory state. Viral load negatively correlated with the count of peripheral blood lymphocytes, total T lymphocytes, $CD4^+$ T cells and $CD8^+$ T cells, while showing positive correlations with inflammatory markers such as hs-CRP, PCT and IL-6. These findings suggest that viral load, circulating immune cell count, and serum

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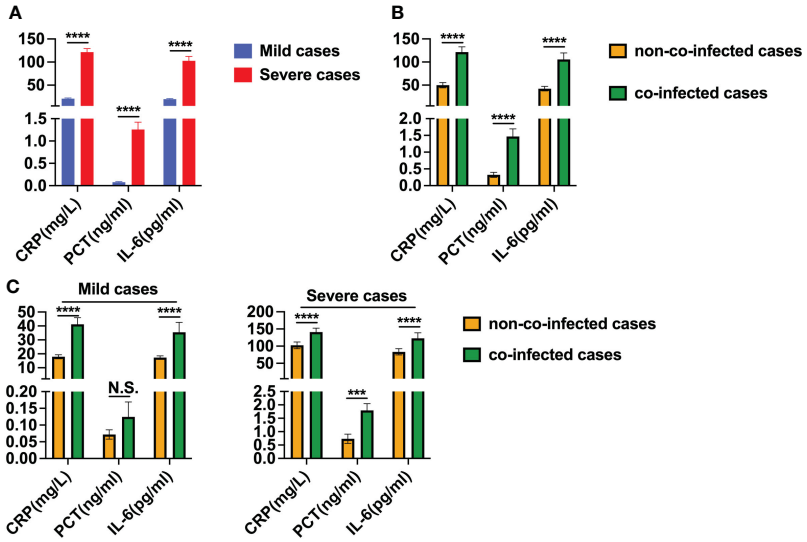

FIGURE 3
Levels of hs-CRP, PCT and IL-6 in patients with COVID-19. **(A)** Comparison of hs-CRP, PCT and IL-6 levels between severe cases (n = 67) and mild cases (n = 64). **(B)** Comparison of hs-CRP, PCT and IL-6 levels between co-infected cases (n = 41) and non-co-infected cases (n = 90). **(C)** Comparison of hs-CRP, PCT and IL-6 levels between co-infected and non-co-infected cases based on disease severity. All data are presented as the mean ± SEM. Differences were tested using unpaired t test. $p<0.05$ was considered significant. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$; N.S., not significant.

inflammatory factor concentrations play pivotal roles in the progression of COVID-19 patients and contribute to risk stratification among hospitalized COVID-19 patients. This information could aid in predicting poor prognoses.

In the COVID-19 disease process, the inflammatory response plays a pivotal role and an inflammatory cytokine storm escalates the severity of the condition (30). Infection-related biomarkers such as hs-CRP, PCT and IL-6 levels are commonly utilized laboratory indicators in diagnosing and treating infectious lung diseases. In our study, we observed elevated serum levels of hs-CRP, PCT and IL-6 in both mild and severe COVID-19 cases, with significantly higher levels in severe cases than in mild ones. These findings align with previous research emphasizing the pivotal role of inflammatory factors in the progression from mild to severe disease (31). Xia et al. (32) found that serum procalcitonin levels were significantly higher in children with COVID-19 compared to those with other common respiratory infections. This suggests that co-infection with other pathogens may contribute to the increased inflammatory response. Pink et al. (33) discovered that approximately 32% of COVID-19 patients developed secondary bacterial infections during hospitalization. Infected patients with secondary bacterial infections had significantly higher PCT and hs-CRP levels compared to their initial admission. Co-infected patients exhibited notably elevated levels of hs-CRP, PCT and IL-6. It can be inferred that hs-CRP, PCT, and IL-6 assays could assist in identifying co-infected patients, especially in severe cases. Co-infection can activate inflammatory factors, potentially exacerbating the severity of the disease. Hence, the severity of COVID-19 disease can be assessed based on the level of inflammatory factors.

During China's fight against COVID-19, traditional Chinese medicine has demonstrated significant benefits in alleviating symptoms and preventing disease deterioration. Moreover, it has proven effective in combating the spread of infectious diseases. Based on laboratory test results of co-infected patients, we speculate that traditional Chinese medicine or extracts with antiviral, antibacterial, anti-inflammatory, and immune system-improving properties have a positive effect on the treatment of co-infected patients. Honeysuckle, used in China for thousands of years, has been an effective treatment for viral infections. It can effectively inhibit virus replication *in vitro* and *in vivo*. Li M et al. (34) discovered that the Honeysuckle acids-flavonoids mixture has broad-spectrum antiviral activity and can significantly inhibit H1N1 and H3N2 influenza viruses. Additionally, honeysuckle can reduce IL-6 or TNF-α release induced by the SARS-CoV-2, inhibiting cytokine storm and virus replication by blocking the binding of the SARS-CoV-2 spike protein to the ACE2 receptor to form a syncytium (35). Research has found that honeysuckle and its extract chlorogenic acid have antibacterial and immune-enhancing effects, and can inhibit the production of multidrug-resistant bacteria by inhibiting protein synthesis (36–38). Therefore, honeysuckle and its derivatives are expected to bring good therapeutic effects to co-infected patients. Berberine, Matrine, Ginkgo Biloba Extract, Allicin, Ginsenosides, etc., are also widely used in traditional Chinese medicine for antibacterial and antimicrobial treatment (39–43). They have been validated to some extent in modern pharmacological research and show potential therapeutic effects on co-infections.

Our study has several limitations, as well. Firstly, this was a single-center observational cohort study, and the patient data were exclusively collected from northwest China. The sample quantity was not big enough, particularly for mild cases with co-infections.

Therefore, it is crucial to interpret the results cautiously, as statistical insignificance might not rule out differences between cases of COVID-19 and co-infections in mild patients. Secondly, only sputum cultures were conducted upon the patients' admission; urine and blood cultures were not performed. This limitation might have led to the underdiagnosis of some co-infections. Additionally, asymptomatic infected patients and some mild cases who declined hospitalization were not included in the study, resulting in a low hospital occupancy rate. Moreover, all the cases we analyzed were winter-onset cases, and a significant proportion of them were severe. This scenario might have contributed to higher rates of co-infections and mortality. Addressing these issues is essential to gain a deeper understanding of the complex SARS-CoV-2 pathogenesis. Finally, We have only predicted the therapeutic effects of traditional Chinese medicine and their derivatives on co-infections, and further experimental verification is necessary to understand their specific therapeutic effects.

In summary, co-infection emerges as a risk factor for COVID-19 patients, elevating the risk of severe pneumonia and mortality. Co-infection suppresses the host's immune response by reducing the number of lymphocytes and increasing inflammation, thereby diminishing the antiviral and anti-infective effects of the immune system, which promotes the severity of the disease. Implementing infection prevention measures is crucial to mitigate COVID-19 spread among hospitalized patients. Additionally, changes in these biomarkers provide a theoretical basis for the effective treatment of co-infections with traditional Chinese medicine.

Data availability statement

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

Ethics statement

The study complied with all relevant ethical regulations and the protocol was approved by the institutional review board of the Ninth Hospital of Xi'an(NO.202311). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/ next of kin because Written informed consents were waived due to the very contagious nature of COVID-19. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

ZG: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. ZJ: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft. GQ: Data curation, Formal analysis, Investigation, Writing – original draft. ZY: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. LY: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

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

Publisher's note

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Immuno-modulatory role of baicalin in atherosclerosis prevention and treatment: current scenario and future directions

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Atherosclerosis (AS) is recognized as a chronic inflammatory condition characterized by the accumulation of lipids and inflammatory cells within the damaged walls of arterial vessels. It is a significant independent risk factor for ischemic cardiovascular disease, ischemic stroke, and peripheral arterial disease. Despite the availability of current treatments such as statins, proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, and lifestyle modifications for prevention, AS remains a leading cause of morbidity and economic burden worldwide. Thus, there is a pressing need for the development of new supplementary and alternative therapies or medications. Huangqin (*Scutellaria baicalensis* Georgi. [SBG]), a traditional Chinese medicine, exerts a significant immunomodulatory effect in AS prevention and treatment, with baicalin being identified as one of the primary active ingredients of traditional Chinese medicine. Baicalin offers a broad spectrum of pharmacological activities, including the regulation of immune balance, antioxidant and anti-inflammatory effects, and improvement of lipid metabolism dysregulation. Consequently, it exerts beneficial effects in both AS onset and progression. This review provides an overview of the immunomodulatory properties and mechanisms by which baicalin aids in AS prevention and treatment, highlighting its potential as a clinical translational therapy.

KEYWORDS

baicalin, atherosclerosis, immuno-modulatory, traditional Chinese medicine, anti-inflammatory

1 Introduction

Atherosclerosis (AS) is a chronic and progressive disease characterized by inflammation within the arterial wall, accompanied by the accumulation of overactive smooth muscle cells and immune cells. This condition forms the pathophysiological basis for cardiovascular disease (CVD) and ischemic stroke, leading to significant mortality and disability worldwide (1–4). Although recent advancements in revascularization techniques and cholesterol-lowering medications (primarily statins and PCSK9 inhibitors) have notably improved AS management (5–7), the incidence of AS-induced acute events, such as acute myocardial infarction and acute ischemic stroke, continues to be alarmingly high over the past decade, contributing to substantial economic burden and mortality rates in both developed and developing countries (8). For instance, in China and many Asian countries, the number of newly diagnosed AS cases has been continuously increasing, and AS-associated cardiovascular disease or thrombosis remains a leading cause of death, which is reported to be as high as 40% (9–11). In light of these challenges, there is a consensus on the necessity for complementary and alternative treatment approaches for AS. Current guidelines strongly recommend pharmacological interventions, yet the effectiveness of traditional Chinese medicine (TCM) has garnered interest from the AS and CVD research community because of its "multiple channels and multiple targets" approach (12–14). Significantly, TCM has a higher acceptance in China and other Asian countries, owing to its strong cultural affinity. Therefore, developing TCM-based therapies for AS prevention and treatment represents a promising direction for novel strategic interventions.

Baicalin, a prominent flavonoid compound extracted from the root of Huangqin (*Scutellaria baicalensis* Georgi. [SBG]), exhibits a wide range of pharmacological effects such as anti-inflammatory, anti-apoptotic, antioxidant, and anticancer properties as well as improvement of smooth muscle cell function (15–19). In line with these properties, baicalin presents promising opportunities for AS prevention and treatment, as its therapeutic mechanisms and functional targets are likely linked to the pathophysiological processes of AS (20–22). This review draws upon the pathological basis of AS and provides a comprehensive analysis of the latest research on the anti-atherosclerotic effects of baicalin from an immunomodulatory perspective, aiming to offer insights into future directions for developing innovative treatment strategies for AS.

2 Immuno-interaction along with the processes in AS

Immune system plays a crucial role in the initiation and progression of AS. Here, we will shortly introduce the main involvements of innate and adaptive immunity during AS pathophysiological processes (Figure 1).

2.1 Innate immunity alteration in AS

In the initial stages of AS, endothelial cell damage triggered by various inflammatory responses and ischemic-hypoxia events leads

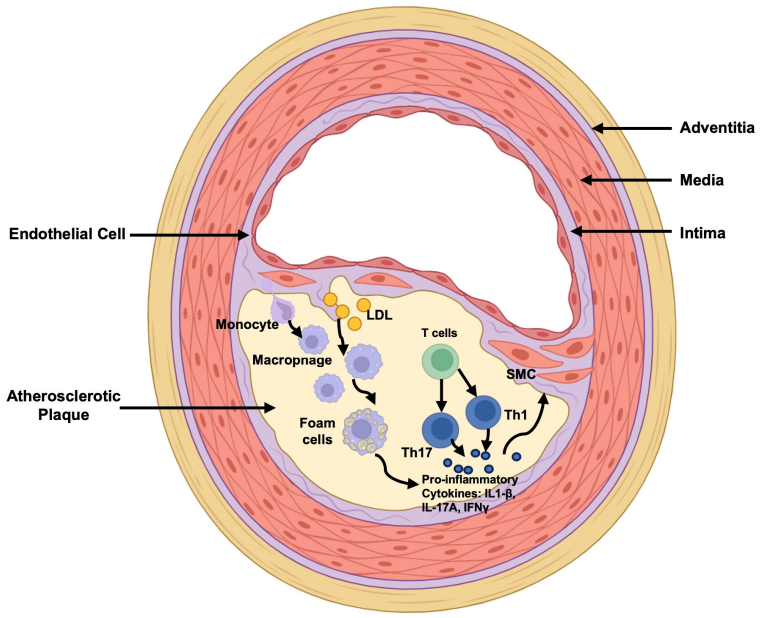

FIGURE 1
Main Immuno-alteration in Atherosclerosis. LDL retention is the marker of the initiation of atherosclerosis. Monocytes transmigrate into the subendothelial space and differentiate into macrophages, following binding to lipoproteins and forming foam cells which secret pro-inflammatory cytokines IL-1β for down-streaming smooth muscle cell activation for further pro-atherosclerotic processes. In the meantime, T cells are differentiated into Th1 and Th17 cells to secret IFNγ and IL-17A exerting pro-inflammatory effects. LDL, Low-Density Lipoprotein; Th17, T helper cells 17; Th1, T helper cells 1; IL-1β, interleukin (IL)-1β; IL-17A, interleukin (IL)-17A; IFNγ, Interferon-gamma; SMC, Smooth Muscle Cell.

to the release of adhesion and chemotactic cytokines. These cytokines attract monocytes and other leukocytes to the site of inflammation, where they mature and transition into macrophages (23–25). The intracellular accumulation of cholesterol in macrophages activates surface scavenger receptors, facilitating the binding of macrophages to lipoprotein particles and leading to foam cell formation (26, 27). Additionally, lipid accumulation prompts endothelial cells to secrete inflammatory mediators and cytokines, further accelerating AS onset. Additionally, dendritic cells (DCs) accumulating in atherosclerotic plaques play a crucial role in the disease's progression by presenting oxidized low-density lipoprotein (ox-LDL)-derived antigens, which activate both humoral and adaptive immune responses (28, 29).

2.2 Adaptive immunity alteration in AS

In the advanced stages of AS, adaptive immunity, especially cellular immunity, plays a role in T-cell infiltration (Figure 1) (30, 31). Although T cells are not as abundant as monocytes, which undergo differentiation into macrophages, they can penetrate the intima to modulate the functions of innate immune cells and smooth muscle cells. T helper cells 1 (Th1) exert a pro-inflammatory role in AS, for example, by secreting interferon-gamma (IFN-γ), which promotes monocyte infiltration, macrophage differentiation, and foam cell formation. T helper cells 2 (Th2), on the other hand, by secreting interleukin (IL)-4 to play a disease prompting role. T helper cells 17 (Th17), activated under pro-atherogenic conditions, have complex effects because of the various roles of IL-17A at different AS stages. However, undoubtedly, the proportion of Th17 cells increases in patients with acute coronary syndrome. Conversely, regulatory T (Treg) cells are present in atherosclerotic plaques at all stages of AS, although in a smaller proportion (32, 33). Animal studies have reported the exacerbating effects of depleting Tregs in AS, while reintroducing Treg cells mitigates and slows disease progression (34). Mechanistically, Treg cells secrete IL-10, playing a crucial role in preventing AS progression. Additionally, Treg cells secrete transforming growth factor-beta (TGF-β) to inhibit inflammation and the proliferation of smooth muscle cells. While the role of humoral immunity in AS development is little known to us since B cells are occasionally detected in atherosclerotic plaques. Studies on B-cell dynamics in atherosclerotic mice have yielded contradictory results: one indicates B cells mitigate AS, while another suggests B cells exacerbate it following B cell depletion (35–38). This discrepancy underscores the need for a comprehensive examination of B cell populations and their specific functions.

Collectively, these findings indicate that the pathological mechanisms of AS involve the participation of endothelial cells, monocyte-derived macrophages, smooth muscle cells, and immune cells. Consequently, understanding the role of immune interactions during AS progression could pave the way for innovative strategies targeting these immune responses. Furthermore, baicalin can modulate immune cells and their actions in AS progression, positioning it as a promising approach for AS prevention and treatment.

3 Immuno-modulatory features of baicalin in AS

Extensive research has confirmed baicalin's multifaceted pharmacological benefits, including anti-apoptotic effects, enhancement of endothelial cell functions, antioxidant properties, and modulation of host immune homeostasis. These attributes contribute to its protective role in both the initiation and progression of AS (Figure 2). This review focuses on the immunomodulatory effects of baicalin in AS prevention and treatment, underscoring its potential as a significant therapeutic agent.

3.1 Effect of baicalin on innate immunity in AS

After migrating to the intima, monocytes undergo differentiation into macrophages. These macrophages then engulf large amounts of ox-LDL through scavenger receptors, leading to the formation of foam cells (26). These foam cells play a crucial role in the progression of AS lesions, making the transition of macrophages into foam cells a critical phase in AS development and progression. Consequently, strategies aimed at promoting macrophage apoptosis and preventing of macrophage foam cell formation have emerged as key focus areas in AS prevention and treatment. Baicalin primarily functions by inhibiting macrophage foam cell formation, regulating cholesterol homeostasis, and facilitating macrophage apoptosis. Baicalin triggers macrophage apoptosis by activating three signaling pathways, namely c-Jun N-terminal kinases (JNKs), p38 mitogen activated protein kinase (p38/MAPK), and Steroid Receptor RNA Activator (SRA) (39), in response to lipopolysaccharide (LPS) and acetylated low-density lipoprotein (Ac-LDL), thus preventing foam cell formation in diseased cells. In studies utilizing an LPS-sensitized mouse macrophage model, baicalin demonstrated potential in modulating Protein kinase A (PKA) activity in macrophages, which, in turn, may suppress NLR family pyrin domain containing 3 (NLRP3) inflammasome activation and cell pyroptosis (40). Additionally, Baicalin encourages M2 macrophage polarization and enhances phagocytosis by increasing Mer Tyrosine Kinase (MERTK) receptor expression (41).

Macrophages in AS primarily display two phenotypes: M1 and M2 types. In general, M1 macrophages, also known as classically activated macrophages, are typically involved in the initial pro-inflammatory response. In contrast, M2 macrophages contribute to anti-inflammatory processes and produce anti-inflammatory cytokines. In experiments investigating the effects of baicalin on RAW 264.7 macrophages cell line, it was found that Baicalin inhibited the expression of inflammatory factors IL-1β, IL-6, Tumor Necrosis Factor-α (TNF-α), Cyclooxygenase-2 (COX-2) in macrophage-derived foam cells. This upregulation promoted the expression of immunosuppressive factors TGF-β and IL-10, leading to the polarization of macrophages from M1 to M2 type, which inhibited the inflammatory response during the AS process. Further analysis, combined with relevant literature, revealed a

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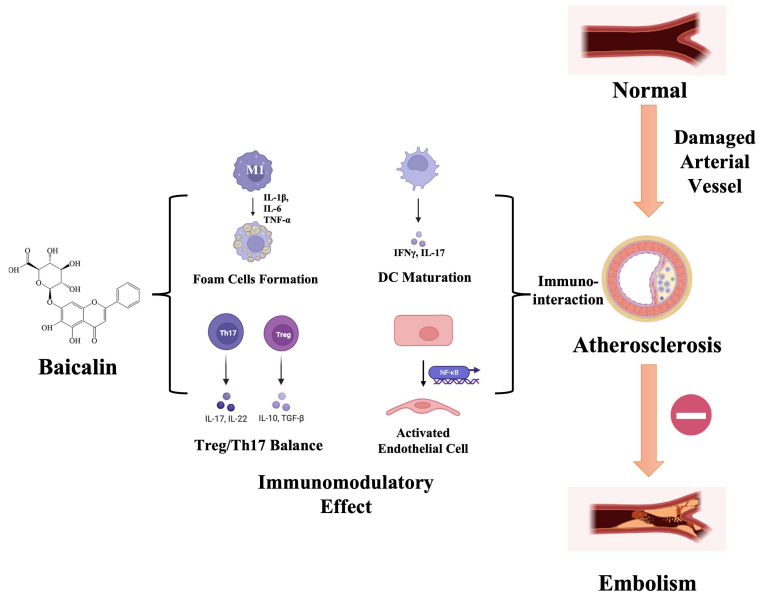

FIGURE 2
Schematic illustration of baicalin's immunomodulatory effects during atherosclerosis progression. Upon damage manifestation within arterial vessels, diverse immune cell populations undergo activation and subsequent migration toward the intima, thereby instigating the development of an inflammatory microenvironment. Baicalin, with its immunomodulatory ability, specifically targets the differentiation of macrophage-foam cells, imbalance between Treg/Th17 cells, dendritic cell (DC) maturation, and modulation of inflammatory signaling pathways. This orchestrated modulation serves a dual purpose of impeding the progression of atherosclerosis toward embolism and mitigating the risk of acute ischemic events. IL-17, interleukin (IL)-17; IFNγ, Interferon-gamma; DC, Dendritic Cell; NF-κB, Nuclear Factor-kappa B; IL-1β, interleukin (IL)-1β; IL-6, interleukin (IL)-6; TNF-α, Tumor Necrosis Factor-α; IL-22, interleukin (IL)-22; IL-10, interleukin (IL)-10; TGF-β, Transforming Growth Factor Beta; Th17, T helper cells 17; Treg, regulatory T cells.

potential association between this mechanism and Baicalin's activation of the key protein peroxisome proliferator-activated receptor gamma (PPAR-γ) (42, 43). Previous studies have shown that Baicalin exhibits anti-inflammatory activity by inhibiting the activation of the Toll-like Receptor 4 (TLR4)/Myeloid Differentiation primary response 88 (MyD88)/Nuclear Factor-kappa B (NF-κB) cell signaling pathway (44). Recent research has further confirmed that Baicalin inhibits inducible Nitric Oxide Synthase (iNOS) gene functions, providing additional insight into Baicalin's anti-inflammatory effects in AS (45). Baicalin can also inhibit macrophage proliferation, suppress excessive production of pro-inflammatory cytokines triggered by S100A8/A9, and reduce expression of the NADPH oxidase 2 (NOX2) gene, thereby inhibiting the inflammatory response (46). Despite the increasing understanding of the mechanisms by which Baicalin inhibits macrophage foam formation, further exploration is necessary to investigate its mechanisms for anti-foam cell formation and cholesterol metabolism from multiple perspectives. Additionally, compelling evidence shows that baicalin can reduce CD11c expression on DCs within atherosclerotic plaques, thus inhibiting DC maturation and the development of atherosclerotic lesions (47–49). Baicalin may also promote the transition of conventional DCs into plasmacytoid DCs and alter the expression of functional molecules by blocking the Signal Transducer and Activator of Transcription 5-Inhibitor of DNA binding 2 (STAT5-ID2) pathway, further demonstrating its protective effect against AS (50).

3.2 Effect of baicalin on cellular adaptive immunity in AS

Adaptive immunity plays a crucial role in both the onset and progression of AS (23). Activated T cells, which have encountered antigens before, are present within atherosclerotic plaques. Studies using immunodeficient apo E $^{-/-}$ animal models, which lack both T cells and B cells, have shown fewer atherosclerotic events compared with their immunocompetent counterparts (51, 52). This evidence underscores the pro-atherosclerotic influence of T cells and suggests that immunosuppressive strategies may be beneficial for treating AS. Treg cells, in particular, contribute to the protection against AS development by releasing anti-inflammatory cytokines such as IL-10 and TGF-β. Supporting this notion, baicalin can enhance Forkhead Box P3 (Foxp3) expression, thereby promoting the differentiation of Treg cells within atherosclerotic plaques, and exerting anti-arteriosclerotic effects (22, 53, 54). The underlying mechanism is believed to involve baicalin's inhibition of the Mammalian Target of Rapamycin (mTOR) pathway, leading to downregulated expression of Ras homolog gene family, member A (RhoA), Rho-Associated Coiled-Coil-Containing Protein Kinase 1 (ROCK1), phosphorylated STAT4 (p-STAT4), and p-STAT3, while simultaneously increasing phosphorylated STAT5 expression. The latter directly interacts with the Foxp3 gene, enhancing Treg cell function. Additionally, baicalin can influence the gut microbiome in an ulcerative colitis animal model, balancing Treg/Th17 ratios (55) and exhibiting anti-inflammatory

properties. This suggests potential for further research into baicalin's effects on the microbiome in AS. Baicalin also inhibits Th17 cell differentiation by downregulating IL-6 and Retinoic Acid Receptor-Related Orphan Receptor Gamma (RORctγ) expression (56). Yet, its effect on the Th1 cell population, which secretes IFN-γ and contributes to AS progression, remains less explored, warranting further investigation. Overall, the evidence supports baicalin's immunomodulatory benefits in AS, particularly regarding Treg/Th17 balance.

3.3 Effect of baicalin on humoral adaptive immunity in AS

Baicalin's effect extends beyond adaptive immune cells, also influencing humoral immunity. However, the relationship between humoral immunity and AS presents conflicting evidence, necessitating further research (23). Baicalin is reported to significantly boost B cell proliferation and IL-12 production through its interaction with TLR4 (57). Additionally, by modulating signaling pathways such as NF-κB or Phosphoinositide 3-kinase/Protein Kinase B (PI3K/Akt), which are critical for B cell survival and homeostasis, Baicalin can potentially influence the lifespan and turnover of B cells which eventually affecting the quantity and quality of antibodies produced by B cells. The interplay between innate and adaptive immunity in the onset and progression of AS highlights the potential of baicalin as a therapeutic agent, primarily through its ability to regulate immune homeostasis.

3.4 Effect of baicalin on inflammation responses in AS

Inflammation is a critical driver at all stages of AS, with chemokines, pro-inflammatory cytokines, adhesion molecules, and inflammatory signaling pathways (5) playing pivotal roles. The NF-κB signaling pathway, in particular, promotes AS by increasing the production of pro-inflammatory cytokines and chemokines such as IL-6, IL-1, Intercellular Adhesion Molecule-1 (ICAM-1), Vascular Cell Adhesion Molecule-1 (VCAM-1), and TNF-α. Therefore, inhibiting NF-κB activation is effective in reducing AS incidence. Baicalin can suppress cytokine and chemokine production by inhibiting NF-κB activation in both *in vitro* and *in vivo* studies. Baicalin pretreatment to human umbilical vein endothelial cells confers protection from damage induced by vascular inflammation, which is similar to the early stages of AS, by suppressing NF-κB activity (58, 59). Baicalin can enhance SIRT expression in models of cardiovascular AS, acting as an inhibitor of NF-κB. This suppression of NF-κB leads to a reduction in the secretion and activation of downstream inflammatory factors (60). Additionally, baicalin influences the Wnt pathway, a crucial cellular signaling network involved in numerous biological processes, playing a significant anti-atherosclerotic role. Baicalin also promotes the activation of the Wingless-related integration site/Dickkopf-1 (Wnt/DKK1) pathway, potentially mitigating AS progression (61). Baicalin can also decrease the levels of inflammatory factors by promoting the phosphorylation of glycogen synthase kinase 3β (GSK3β), enhancing β-catenin expression and nuclear translocation, which is associated with the Wnt pathway (62). In summary, these findings underscore baicalin's role in attenuating inflammatory responses, thereby decreasing the degree of AS progression.

4 Future directions of baicalin in AS

Baicalin has shown potential as a novel therapeutic agent for various diseases, but its clinical development and application (63) have been significantly hindered by its low bioavailability. To address this challenge, several innovative strategies, such as nanonization, solid dispersion, inclusion complexes, and micelles, have been designed and developed. These modifications have been proven to enhance the dissolution and solubility of baicalin. For instance, the formation of a baicalin-phospholipid complex through weak interactions under specific conditions can further improve the bioactivity of baicalin, offering enhanced anti-inflammatory effects with faster onset and prolonged duration (64). Furthermore, the advent of various baicalin-loaded liposome technologies, including folate-modified baicalin-loaded liposomes, baicalin-phospholipid nanoparticles, and baicalin-phospholipid prodrugs, has improved baicalin bioavailability in oral drug delivery systems (65). However, the exploration of baicalin's drug delivery systems in the context of AS therapies remains limited, underscoring the need for further evaluation of suitable delivery vehicles to leverage its promising anti-atherosclerotic effects.

Recent evidence has highlighted the anti-inflammatory effects of mesenchymal stem cells (MSCs) in treating multiple diseases, including AS (66, 67). Intriguingly, baicalin enhances the proliferation and survival of MSCs by modulating the hedgehog signaling pathway (68), addressing the critical challenge of MSC apoptosis in clinical applications. Moreover, baicalin pretreatment can optimize the therapeutic effects of MSC-derived exosomes by inhibiting the nuclear factor erythroid 2–related factor 2 (Nrf2) pathway (69). These findings suggest that a combined approach of baicalin and MSC therapy could offer superior therapeutic benefits over using either treatment alone. This synergy underscores the potential of developing combination strategies that integrate baicalin and MSCs, warranting further investigation.

5 Concluding remarks

Baicalin has been recognized as a promising therapeutic agent for AS prevention and treatment owing to its ability to mitigate inflammation-induced damage, regulate lipid metabolism, promote cell survival, and modulate immune responses. This discussion has focused on the immunomodulatory effects of baicalin in combating AS. Despite extensive experimental evidence supporting baicalin's immunomodulatory benefits in AS, the specific mechanisms of action, including its precise receptors and downstream signaling pathways, remain elusive. This gap in knowledge, coupled with limited clinical data, has delayed baicalin's potential clinical applications. Moreover, the physical properties of baicalin still

require optimization and technological advancements. Looking forward, initiating appropriate baicalin-based clinical trials is recommended to gather sufficient data, which could enrich our understanding of its mechanisms and enhance its therapeutic potential in AS prevention and treatment.

Author contributions

LW: Funding acquisition, Writing – original draft, Writing – review & editing. SH: Writing – original draft, Writing – review & editing. XL: Resources, Visualization, Writing – original draft. JZ: Investigation, Writing – review & editing. YH: Resources, Writing – review & editing. JH: Visualization, Writing – review & editing. DX: Supervision, Writing – review & editing.

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Conflict of interest

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

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Oral curcumin ameliorates acute murine campylobacteriosis

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Introduction: Human infections with the food-borne enteropathogen *Campylobacter jejuni* are responsible for increasing incidences of acute campylobacteriosis cases worldwide. Since antibiotic treatment is usually not indicated and the severity of the enteritis directly correlates with the risk of developing serious autoimmune disease later-on, novel antibiotics-independent intervention strategies with non-toxic compounds to ameliorate and even prevent campylobacteriosis are utmost wanted. Given its known pleiotropic health-promoting properties, curcumin constitutes such a promising candidate molecule. In our actual preclinical placebo-controlled intervention trial, we tested the anti-microbial and anti-inflammatory effects of oral curcumin pretreatment during acute experimental campylobacteriosis.

Methods: Therefore, secondary abiotic IL-10$^{-/-}$ mice were challenged with synthetic curcumin via the drinking water starting a week prior oral *C. jejuni* infection. To assess anti-pathogenic, clinical, immune-modulatory, and functional effects of curcumin prophylaxis, gastrointestinal *C. jejuni* bacteria were cultured, clinical signs and colonic histopathological changes quantitated, pro-inflammatory immune cell responses determined by *in situ* immunohistochemistry and intestinal, extra-intestinal and systemic pro-inflammatory mediator measurements, and finally, intestinal epithelial barrier function tested by electrophysiological resistance analysis of colonic *ex vivo* biopsies in the Ussing chamber.

Results and discussion: Whereas placebo counterparts were suffering from severe enterocolitis characterized by wasting symptoms and bloody diarrhea on day 6 post-infection, curcumin pretreated mice, however, were clinically far less compromised and displayed less severe microscopic inflammatory sequelae such as histopathological changes and epithelial cell apoptosis in the colon. In addition, curcumin pretreatment could mitigate pro-inflammatory innate and adaptive immune responses in the intestinal tract and importantly, rescue colonic epithelial barrier integrity upon *C. jejuni* infection. Remarkably, the disease-mitigating effects of exogenous curcumin was also observed in organs beyond the infected intestines and strikingly, even systemically given basal hepatic, renal, and serum concentrations of pro-inflammatory mediators measured in curcumin pretreated mice on day 6 post-infection. In conclusion, the anti-

Campylobacter and disease-mitigating including anti-inflammatory effects upon oral curcumin application observed here highlight the polyphenolic compound as a promising antibiotics-independent option for the prevention from severe acute campylobacteriosis and its potential post-infectious complications.

KEYWORDS

curcumin, polyphenols, preclinical placebo-controlled intervention study, *Campylobacter jejuni*, secondary abiotic IL-10 -/-mice, acute enterocolitis, campylobacteriosis model, host-pathogen interaction

1 Introduction

Human infections with the food-borne enteropathogen *Campylobacter jejuni* are responsible for increasing incidences of acute campylobacteriosis cases worldwide and are of both, tremendous medical and financial impact (1). In 2021, over 127,000 new campylobacteriosis infections were reported in Europe but the real case numbers including undiagnosed and/or non-reported illnesses are estimated to exceed the reported ones several-fold (2). Within the *Campylobacteraceae* family, the Gram-negative, microaerophilic, non-spore-forming *C. jejuni* bacteria live commensally in the intestinal tracts of warm-blooded vertebrate species, including birds, usually generating no symptoms (3, 4). However, food chain transmission via contaminated undercooked meat from poultry or other livestock, unpasteurized milk, and its byproducts, as well as surface water may all be considered as potential infectious sources upon ingestion by humans (5). After a successful gastro-duodenal passage, the highly motile *C. jejuni* bacteria invade the distal intestinal tissues, and specific bacterial cell wall components, such as the endotoxin lipo-oligosaccharide (LOS), induce the Toll-like receptor-4 (TLR-4)-dependent hyperactivation of the host immune system (6–9). In this pro-inflammatory immune cascade, both innate and adaptive immune cell subsets, including neutrophils and T cells, respectively, are recruited to the infection site, and pro-inflammatory mediators including interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and nitric oxide (NO) are released to restrict the infection, but by the expenses of exerting oxidative stress and damage to the intestinal tissues (10). This results in epithelial cell damage, induction of apoptosis, ulcerations, crypt drop-outs, and crypt abscesses, which can lead to malabsorptive dysfunctions and to the barrier-impaired "leaky gut" (11, 12). Depending on the individual immunological fitness of the human host as well as the distinct virulence genes expressed by the pathogen, infected humans may present with symptoms of different severities after an incubation period ranging from 2 to 6 days. For instance, patients may complain about general malaise, nausea, vomiting, abdominal cramps, watery or even bloody diarrhea, mucous discharge, and fever (13, 14). Typically, patients recover completely two weeks post-infection (p.i.). However, rarely, post-infectious autoimmune morbidities affecting the intestinal tract (i.e., irritable bowel syndrome (IBS), chronic inflammatory bowel disease (IBD)), the joints (i.e., reactive arthritis (RA)), and the central nervous system (i.e., Guillain Barré syndrome (GBS)) may appear weeks to months after the initial infection (14–16). It is interesting to note that the chance of developing post-infectious sequelae is strongly correlated with the severity of the previous enteritis episode, which in turn, depends on the sialylation state of the *C. jejuni*-LOS (17). Patients with campylobacteriosis are typically treated with symptomatic therapies such as analgesic, antipyretic, and spasmolytic medications, along with electrolyte replacement and rehydration. Patients with severe medical conditions, such as those with immune-suppressive disorders, may be treated with antibiotics including ciprofloxacin or erythromycin (14, 18). The increasing prevalence of infections with multi-drug resistant *C. jejuni* strains, however, can make it rather challenging to effectively treat severe campylobacteriosis cases in critically ill patients (18). Thus, it is imperative to find non-toxic, antibiotic-independent therapeutic approaches to ameliorate and even prevent acute campylobacteriosis and its post-infectious sequelae.

Our One Health approach to identifying new protective substances against campylobacteriosis focused on curcumin. The bright yellowish curcumin can be found in the roots of the turmeric plant *Curcuma longa* belonging to the ginger family and is responsible for the spicy taste of curry spices. For a long time, the polyphenolic compound has been known for its health-promoting properties and is highly appreciated in traditional medicine as remedy for many different morbidities (19). Both *in vitro* and *in vivo* studies provided evidence for potent anti-inflammatory, anti-oxidant, anti-infectious, and even anti-tumor effects of exogenous curcumin as reviewed previously (20, 21). A recent *in vitro* study revealed that curcumin was able to compromise the quorum sensing of *C. jejuni*, which is essentially involved in bacterial motility, colonization, and interaction with epithelial cells (22). Notably, oral curcumin intake could alleviate acute experimental inflammation in the small and large intestinal tract (23–25). In several randomized clinical trials, curcumin has been even shown efficient in maintaining remission in patients suffering from ulcerative colitis when administered in combination with mesalazine (26–28).

Given its known pleiotropic disease-ameliorating properties, curcumin constitutes a promising candidate molecule for combatting campylobacteriosis. This prompted us to test curcumin for its potential anti-microbial and anti-inflammatory effects during *C. jejuni*-induced enterocolitis in mice. Two prerequisites need to be taken into consideration, however. To enable *C. jejuni* to colonize stably the murine intestinal tract, mice need to be pretreated with antibiotics to deplete the commensal gut microbiota providing a protective colonization resistance against *C. jejuni* (29, 30). Furthermore, wildtype mice are known to be approximately 10,000 times more resistant to TLR-4 ligands such as lipo-polysaccharides (LPS) and LOS as compared to humans (31), whereas the *il10* gene knock-out can make mice vulnerable to *C. jejuni*-LOS, however (8). Secondary abiotic (SAB) IL-10$^{-/-}$ mice, in which the intestinal microbiota had been depleted by preceding antibiotic treatment, were shown to develop acute enterocolitis with bloody diarrhea and wasting symptoms within less than a week after oral *C. jejuni* infection. Moreover, the animals generate pro-inflammatory immune responses that did not only impact the intestinal tract but also extra-intestinal organs such as the liver, kidneys, and systemic circulation (32–34). Our previous studies underscored that the SAB IL-10$^{-/-}$ mouse model is a reliable way to assess the potential anti-microbial and disease-mitigating effects of defined compounds including phenolic molecules such as carvacrol (35, 36) and resveratrol (37, 38), for instance, during acute campylobacteriosis. In this preclinical placebo-controlled investigation, we administered synthetic curcumin orally as a preventative measure to SAB IL-10$^{-/-}$ mice beginning a week before *C. jejuni* infection. We evaluated the effects of this regimen on various parameters, including i.) fecal *C. jejuni* shedding over time p.i., ii.) gastrointestinal pathogen loads, iii.) clinical conditions, iv.) microscopic inflammatory alterations in the colonic tissues, and v.) intestinal effects as well as vi.) extra-intestinal outcomes including vii.) systemic pro-inflammatory immune responses on day 6 p.i.

2 Materials and methods

2.1 Ethics declaration

All mouse studies were approved by the commission for animal experiments led by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin, registration numbers G0104/19) and carried out in compliance with the European Guidelines for animal welfare (2010/63/EU). The clinical status of the experimental animals was evaluated daily.

2.2 Mice, gut microbiota depletion, curcumin treatment

Under specific pathogen-free (SPF) conditions, both male and female IL-10$^{-/-}$ mice on a C57BL/6j background were raised and kept within the same unit located at the Forschungseinrichtungen für Experimentelle Medizin (FEM, Charité – Universitätsmedizin Berlin). Commensal gut microbiota depletion in mice was achieved by an 8-week treatment with ampicillin plus sulbactam (2 g/L and 1 g/L, respectively; Dr. Friedrich Eberth Arzneimittel, Ursensollen, Germany) via the autoclaved drinking water (*ad libitum*) starting immediately post-weaning, as previously described (34). The day before initiation of the protective treatment, the antibiotics were stopped to ensure drug washout (i.e., day -8).

Seven days prior the *C. jejuni* infection (i.e., day -7), treatment with curcumin (from Sigma-Aldrich, München, Germany) was initiated. To improve water solubility, the synthetic compound was dissolved in 2% carboxy-methylcellulose (Sigma-Aldrich, München, Germany) which resulted in a final concentration of 0.05%. The curcumin suspensions were finally concentrated to 1.0 mg/mL resulting in daily treatment dosages of 200 mg per kilogram of body weight, equivalent to the concentration applied in acute murine ileitis previously (23). Placebo treated (and subsequently infected) mice received vehicle only (positive controls). Furthermore, naive (i.e., untreated and non-infected mice) served as negative controls.

2.3 *C. jejuni* infection and gastrointestinal colonization properties

On days 0 and 1, 10^9 colony-forming units (CFU) of the *C. jejuni* 81–176 strain were applied to sex- and age-matched littermate mice that were three months old by oral gavage. Animals were handled under rigorous aseptic circumstances and kept in a sterile environment (autoclaved food and drinking solutions). *C. jejuni* were quantitatively evaluated in feces over time p.i. and in luminal samples obtained from different regions of the gastrointestinal tract (specifically from the stomach, duodenum, ileum, and colon) at day 6 p.i. by culture, as previously reported (29, 39) allowing for the assessment of gastrointestinal colonization features. The viable pathogen detection limit was 100 CFU/g.

2.4 Clinical outcome

The clinical conditions of the mice were evaluated before and after the infection with *C. jejuni* using a standardized campylobacteriosis score (12 points maximum). This score addressed the stool consistency (0: formed feces; 2: pasty feces; 4: liquid feces), the abundance of blood in stool (0: no blood; 2: microscopic detection of blood by the Guajac method using Haemoccult, Beckman Coulter/PCD, Krefeld, Germany; 4: macroscopic blood visible), and the overall clinical aspect (0: normal; 2: ruffled fur, less locomotion; 4: isolation, severely compromised locomotion, pre-final aspect), as previously described (40).

2.5 Sampling

Mice were necrotized by CO_2 inhalation on day 6 p.i. Under sterile conditions, luminal gastrointestinal samples (i.e., from the

stomach, duodenum, ileum, and colon) and *ex vivo* biopsies were obtained from the colon, mesenteric lymph nodes (MLN), liver, and kidneys. Cardiac blood was drawn for cytokine testing in serum samples. Each mouse had its colon sample taken in parallel for studies involving microbiology, immunohistopathology, and immunology. A ruler was used to measure the large intestinal lengths (in cm).

2.6 Immunohistochemistry

Colonic *ex vivo* samples that had been instantly fixed in 5% formalin and embedded in paraffin, were used for *in situ* immunohistochemical investigations. In summary, paraffin slices of *ex vivo* biopsies originating from the colon (5 µm) were stained with primary antibodies directed against cleaved caspase 3 (Asp175, Cell Signaling, Beverly, MA, USA, 1:200), MPO7 (No. A0398, Dako, Glostrup, Denmark; 1:500), CD3 (#N1580, Dako, Glostrup, Denmark; 1:10), and B220 (No. 14–0452-81, eBioscience, San Diego, CA, USA; 1:200), respectively as reported earlier (41). Following that, positively labeled cells were analyzed in a blinded fashion using light microscopy at a 400-times magnification and the median number from 6 high-power fields (HPF, 0.287 mm^2) per mouse specimen were calculated.

2.7 Pro-inflammatory mediators

Colonic explants were cut lengthwise, cleaned in phosphate-buffered saline (PBS; Thermo Fisher Scientific, Waltham, MA, USA), and ~1 cm^2 of the tissue strips in addition to *ex vivo* biopsies derived from the liver (~1 cm^3) and kidney (half of the organ after longitudinal cut) then placed in 24-flat-bottom well culture plates (Thermo Fisher Scientific, Waltham, MA, USA) containing 500 µL of serum-free RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) plus 100 µg/mL of streptomycin and 100 IU/mL of penicillin (both from Biochrom, Berlin, Germany). Following eighteen hours at 37°C, the corresponding culture supernatants and serum samples were subjected to the Mouse Inflammation Cytometric Bead Assay (CBA; BD Biosciences, Heidelberg, Germany) using a BD FACSCanto II flow cytometer to measure IFN-γ, TNF-α, monocyte chemoattractant protein-1 (MCP-1), and IL-6. NO was measured with the Griess reaction (42).

2.8 Electrophysiological measurements

Ex vivo biopsies from the terminal large intestine were moved to Ussing chambers (remaining unstripped; 0.049 cm^2 area). An automatic clamp device (CVC6, Fiebig Hard & Software, Berlin, Germany) was applied to measure the transmural electrical resistance (R_t) for one hour at 37°C under voltage clamp conditions. The bathing solution that was equilibrated with carbogen gas (pH 7.4) had the following composition: Beta-hydroxybutyric acid (0.5 mmol/L), L-glutamine (2.5 mmol/L), D (+)-mannose (10.0 mmol/L), D(+)-glucose (10.0 mmol/L), NaCl (113.6 mmol/L), KCl (5.4 mmol/L), $CaCl_2$ (1.2 mmol/L), $MgCl_2$ (1.2 mmol/L), Na_2HPO_4 (2.4 mmol/L), NaH_2PO_4 (0.6 mmol/L), and $NaHCO_3$ (21.0 mmol/L).

2.9 Statistical analysis

GraphPad Prism (version 9; San Diego, CA, USA) was used to determine medians and significance levels following the pooling of data from three separate trials. The Anderson-Darling test was applied to evaluate the normalization of data sets. Pairwise comparisons of regularly distributed and non-normally distributed data were performed using the Student's t-test and the Mann-Whitney test, respectively. The one-way ANOVA with Tukey post-hoc test (for regularly distributed data) and the Kruskal-Wallis test with Dunn's post-hoc test (for non-normally distributed data) were used for multiple comparisons. Significant two-sided probability (p) values were defined as < 0.05.

3 Results

3.1 Gastrointestinal pathogen loads following curcumin pretreatment of *C. jejuni* infected mice

Seven days following initiation of the oral curcumin application, SAB IL-$10^{-/-}$ mice were infected with 10^9 viable *C. jejuni* bacteria on days 0 and 1 by gavage. To test the effects of exogenous curcumin on the enteropathogenic colonization of the intestinal tract, we determined the fecal *C. jejuni* loads by culture every day following infection. As early as 24 hours after the second oral *C. jejuni* challenge (i.e., on day 2 p.i.), the pathogens could be isolated from the feces of placebo controls at high median loads of 10^9 CFU per g, whereas curcumin pretreated mice exhibited approximately one order of magnitude lower median fecal *C. jejuni* bacteria numbers as compared to the latter which also held true for days 3 until 6 p.i. ($p<0.01$–0.001; Figure 1). At necropsy we further quantified luminal *C. jejuni* bacteria in distinct gastrointestinal regions. Whereas in curcumin pretreated mice approximately one order of magnitude lower median pathogen numbers could be determined in the ileal and colonic lumen if compared to placebo counterparts ($p<0.01$), even 4 log orders fewer median bacterial cells were isolated from the stomach of the former as compared to the latter on day 6 p.i. ($p<0.001$; Figure 2). Hence, curcumin pretreatment of *C. jejuni* infected mice resulted in lower gastrointestinal pathogen loads.

3.2 Inflammatory sequelae upon curcumin pretreatment of *C. jejuni* infected mice

Furthermore, we tested the impact of curcumin prophylaxis on the clinical course of *C. jejuni* infected mice with clinical campylobacteriosis scores assessing the overall clinical aspect and

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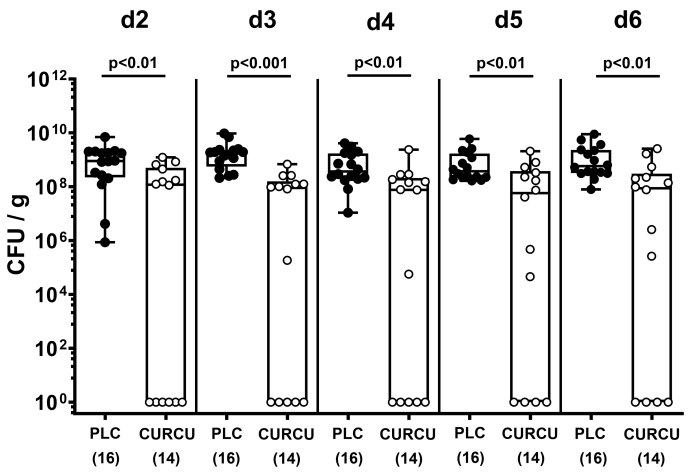

FIGURE 1
Fecal pathogen shedding over time following *C. jejuni* infection of mice with curcumin prophylaxis. Secondary abiotic IL-10$^{-/-}$ mice were pretreated with curcumin (CURCU; open circles) or placebo (PLC; closed circles) via the drinking water starting 7 days prior peroral infection with *C. jejuni* 81–176 strain on day (d) 0 and d1. Fecal *C. jejuni* numbers were determined daily post-infection by culture (in colony forming units per gram; CFU/g). Box plots (25th and 75th percentiles), whiskers (minimum and maximum values), medians (black bar in boxes), numbers of analyzed mice pooled from 3 independent experiments (in parentheses), and significance levels (p values) determined by the Mann-Whitney test are indicated.

severity of bloody diarrhea. Whereas on day 6 p.i. placebo control animals displayed highly elevated clinical scores indicative for severe acute campylobacteriosis (p<0.001 versus naive), clinical scores were lower in curcumin pretreated mice and did not even differ from basal values (not significant (n.s.) versus naive mice; Figure 3A). Remarkably, 35.7% of mice from the verum cohort did not exhibit any clinical signs of *C. jejuni* infection at all (i.e., campylobacteriosis score of 0). Since acute inflammation is known to result in enhanced shrinkage of the affected intestine (32, 43), we measured the colonic lengths in the sacrificed mice. In fact, the colonic lengths were lower in infected as compared to naive mice (p<0.05–0.001), whereas higher values could be obtained in curcumin as compared to placebo pretreated mice on day 6 p.i. (p<0.05; Figure 3B), further indicative for mitigated gross *C. jejuni*-induced disease upon oral curcumin prophylaxis.

Moreover, we analyzed potential anti-inflammatory effects of curcumin pretreatment during campylobacteriosis on the microscopic level. Therefore, we quantified the extent of histological cell damage in the infected colon and obtained increased histopathological scores in mice from the placebo and curcumin cohorts (p<0.001 and p<0.05 versus naive, respectively) on day 6 p.i., but with lower values in the latter versus the former (p<0.001; Figure 3C). Whereas the histopathological scores in the placebo control group reached mostly maximum values, those

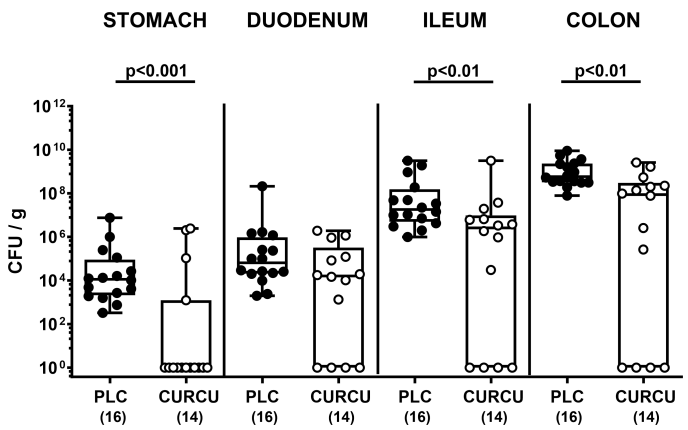

FIGURE 2
Gastrointestinal pathogen counts following *C. jejuni* infection of mice with curcumin prophylaxis. Secondary abiotic IL-10$^{-/-}$ mice were pretreated with curcumin (CURCU; open circles) or placebo (PLC; closed circles) via the drinking water starting 7 days prior peroral infection with *C. jejuni* 81–176 strain on days 0 and 1. At necropsy (i.e., day 6 post-infection), luminal *C. jejuni* bacteria were quantified in the gastrointestinal tract (as indicated) by culture (in colony forming units per gram; CFU/g). Box plots (25th and 75th percentiles), whiskers (minimum and maximum values), medians (black bar in boxes), numbers of analyzed mice pooled from 3 independent experiments (in parentheses), and significance levels (p values) determined by the Mann-Whitney test are indicated.

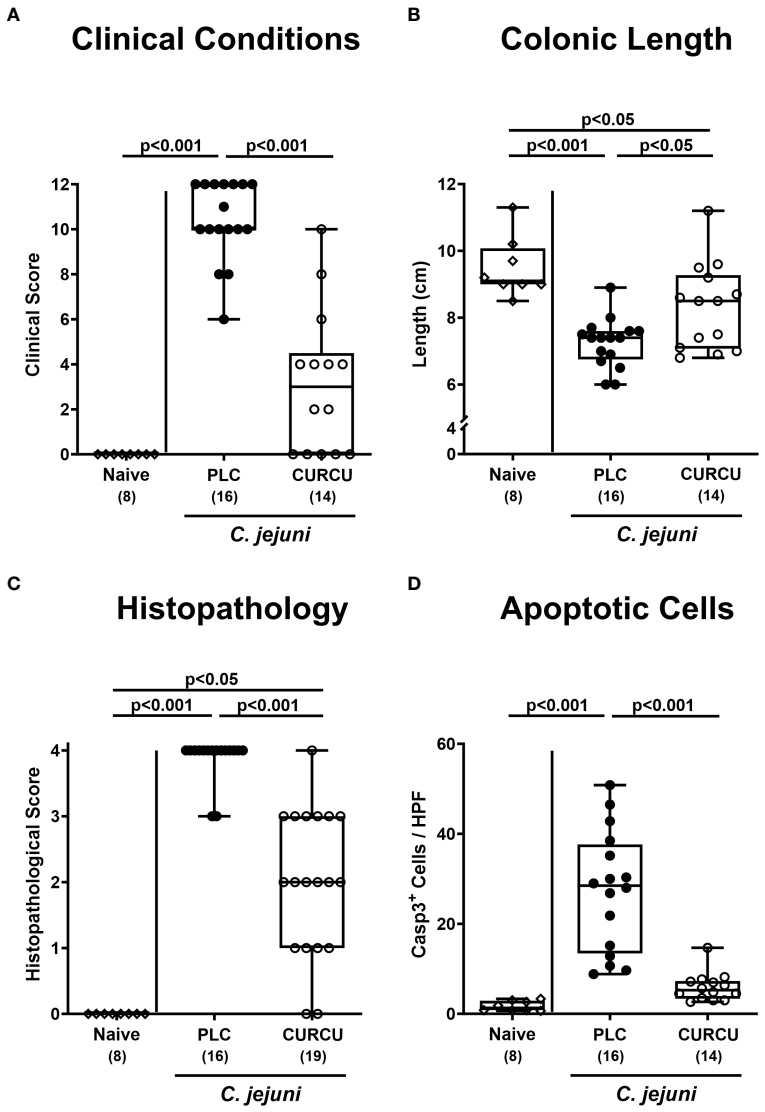

FIGURE 3
Macroscopic and microscopic inflammatory signs following curcumin pretreatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were pretreated with curcumin (CURCU; open circles) or placebo (PLC; closed circles) via the drinking water starting 7 days prior peroral infection with *C. jejuni* 81–176 strain on days 0 and 1. The macroscopic inflammatory signs including **(A)** the clinical conditions as quantified with a campylobacteriosis scoring system (see methods) and **(B)** the colonic lengths as measured with a ruler (in cm) were surveyed on day 6 post-infection. Furthermore, the microscopic inflammatory changes were quantitatively assessed in colonic paraffin sections with **(C)** histopathological scores (see methods) and **(D)** average numbers of apoptotic epithelial cells (positive for caspase3, Casp3) from 6 high power fields (HPF, 400-times magnification) per animal. Naive mice (open diamonds) served as non-infected controls without prophylaxis. Box plots (25th and 75th percentiles), whiskers (minimum and maximum values), medians (black bar in boxes), numbers of analyzed mice pooled from 3 independent experiments (in parentheses), and significance levels (p values) determined by the Kruskal-Wallis test with Dunn's post-hoc test **(A, C)** or by the one sided ANOVA test with Tukey's *post-hoc* test **(B, D)** are indicated.

obtained from mice of the curcumin cohort varied considerably with reaching the maximum score in a single animal, whereas in two mice no histopathological changes could be observed at all on day 6 p.i. (Figure 3C). To further grade the large intestinal inflammation upon enteropathogenic infection, we quantified the apoptotic epithelial cell responses in the colon applying *in situ* immunohistochemistry. Remarkably, *C. jejuni* infection resulted in increased numbers of apoptotic colonic epithelial cells in placebo ($p<0.001$ versus naive), but not in curcumin pretreated mice on day 6 p.i. (n.s. versus naive; $p<0.001$ versus placebo; Figure 3D). Hence, oral curcumin prophylaxis also mitigated *C. jejuni*-induced histopathological and apoptotic cell damage in the colon.

3.3 Colonic immune cell responses following curcumin pretreatment of *C. jejuni* infected mice

To analyze the effects of curcumin pretreatment on *C. jejuni*-induced immune cell responses, we stained colonic paraffin sections with antibodies against surface markers of defined innate and adaptive immune cell subsets. On day 6 p.i., elevated numbers of MPO7$^+$ neutrophilic granulocytes were detected in the colonic mucosa and lamina propria ($p<0.01$–0.001 versus naive), but with lower counts in curcumin as compared to placebo pretreated mice ($p<0.05$; Figure 4A). When analyzing colonic CD3$^+$ T and B220$^+$ B

lymphocyte populations, *C. jejuni*-induced increases could exclusively be observed in placebo controls ($p<0.001$ versus naive), whereas curcumin prophylaxis resulted in basal adaptive immune cell counts in the colon on day 6 p.i. (n.s. versus naive; Figures 4B, C). Hence, curcumin prophylaxis mitigated *C. jejuni*-induced innate and adaptive immune responses in the colon.

3.4 Intestinal pro-inflammatory mediator secretion following curcumin pretreatment of *C. jejuni* infected mice

Furthermore, we tested the effect of curcumin prophylaxis on *C. jejuni*-induced secretion of pro-inflammatory mediators. Our measurements revealed increased IFN-γ ($p<0.001$) and TNF-α ($p<0.01$) concentrations in colonic explants taken from placebo as opposed to curcumin pretreated mice on day 6 p.i. (Figure 5A, B), whereas colonic NO levels were comparably elevated in both infected cohorts (n.s.; Figure 5C). In the MLN draining the infected intestines, however, respective pro-inflammatory mediators were increased in infected mice from the placebo ($p<0.05$ versus naive), but not the curcumin cohort (Figures 5D–F). Hence, curcumin pretreatment mitigated *C. jejuni*-induced pro-inflammatory mediator secretion in the intestinal tract.

3.5 Colonic epithelial barrier function upon curcumin pretreatment of *C. jejuni* infected mice

In addition, we tested whether the disease-alleviating effects upon curcumin pretreatment of *C. jejuni* infected mice had an impact on large intestinal epithelial barrier function. The electrophysiological resistance measurements of colonic *ex vivo* biopsies in the Ussing chamber on day 6 p.i. revealed that *C. jejuni* infection resulted in lower transmural electrical resistances in the colon derived from placebo, but not curcumin pretreated mice when compared to naive control animals ($p<0.05$; Figure 6). Of note, the colonic transmural resistance values were even higher in curcumin pretreated mice with induced campylobacteriosis versus non-infected and untreated controls ($p<0.05$; Figure 6). Hence, curcumin pretreatment of *C. jejuni* infected mice could rescue epithelial barrier integrity.

3.6 Extra-intestinal and systemic inflammatory mediator secretion following curcumin pretreatment of *C. jejuni* infected mice

We further addressed whether the anti-inflammatory properties of curcumin pretreatment were also effective in organs beyond the infected intestines. Our measurements of pro-inflammatory mediators in liver and kidney explants revealed that hepatic as well as renal IFN-γ, TNF-α, and NO concentrations were increased in placebo ($p<0.01$–0.001 versus naive), but not in curcumin pretreated mice on day 6 p.i. (n.s. versus naive; Figure 7). Strikingly, the potent inflammation-dampening effects of curcumin could also be observed systemically given that in serum samples taken 6 days following infection of curcumin-pretreated mice, basal IFN-γ, TNF-α, MCP-1, and IL-6 concentrations were measured (n.s. versus naive), whereas systemic pro-inflammatory mediators were all elevated in placebo counterparts ($p<0.001$ versus naive; Figure 8). Hence, curcumin pretreatment could mitigate also

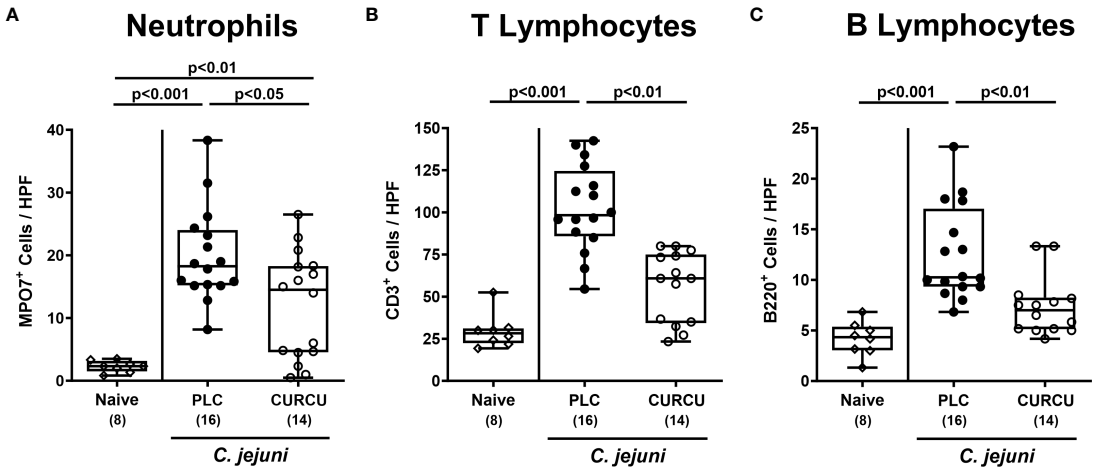

FIGURE 4
Colonic immune cell responses following curcumin pretreatment of *C. jejuni* infected mice. Secondary abiotic IL-$10^{-/-}$ mice were pretreated with curcumin (CURCU; open circles) or placebo (PLC; closed circles) via the drinking water starting 7 days prior peroral infection with *C. jejuni* 81–176 strain on days 0 and 1. On day 6 post-infection, the average numbers of **(A)** neutrophils ($MPO7^+$), **(B)** T lymphocytes ($CD3^+$), and **(C)** B lymphocytes ($B220^+$) were determined in the colonic mucosa and lamina propria from 6 high power fields (HPF, 400-times magnification) per animal in immunohistochemically stained paraffin sections. Naive mice (open diamonds) served as non-infected controls without prophylaxis. Box plots (25^{th} and 75^{th} percentiles), whiskers (minimum and maximum values), medians (black bar in boxes), numbers of analyzed mice pooled from 3 independent experiments (in parentheses), and significance levels (p values) determined by the Kruskal-Wallis test with Dunn's *post-hoc* test are indicated.

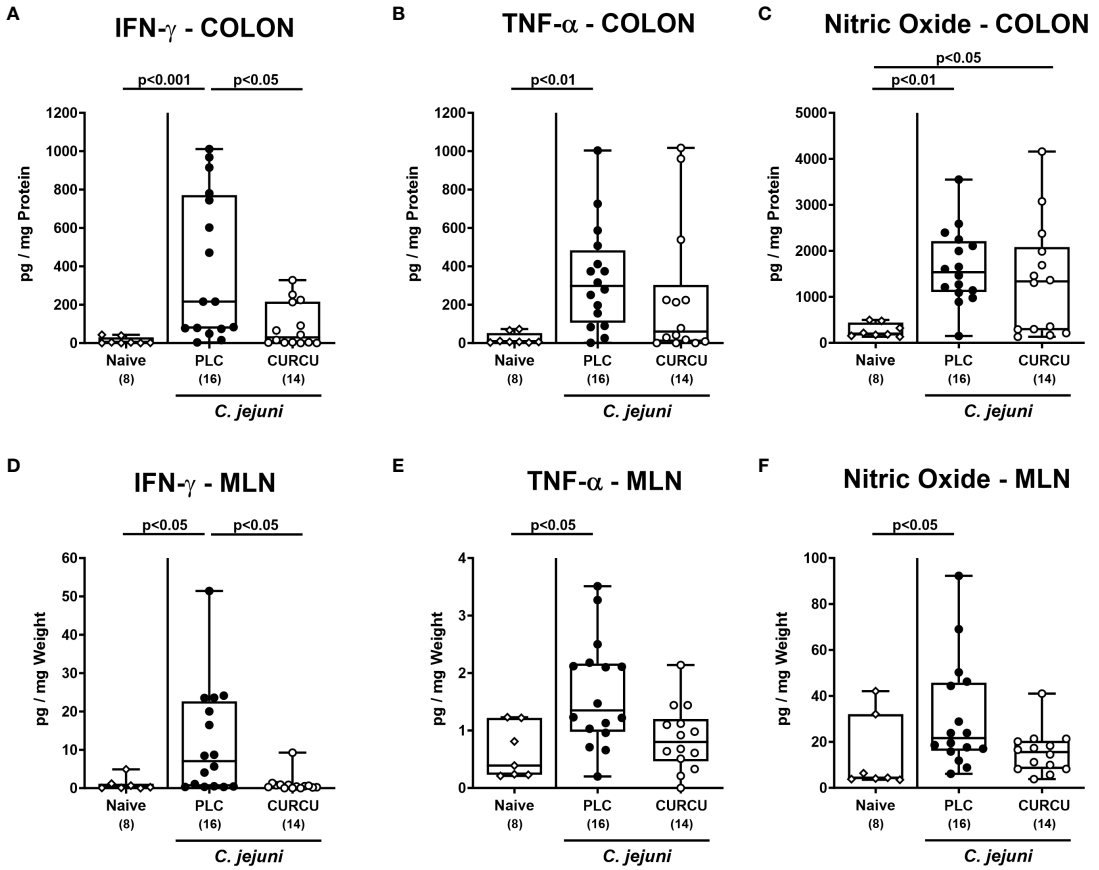

FIGURE 5
Intestinal pro-inflammatory mediators following curcumin pretreatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were pretreated with curcumin (CURCU; open circles) or placebo (PLC; closed circles) via the drinking water starting 7 days prior peroral infection with *C. jejuni* 81–176 strain on days 0 and 1. **(A, D)** IFN-γ, **(B, E)** TNF-α, and **(C, F)** nitric oxide concentrations were measured in supernatants of *ex vivo* biopsies derived from the colon **(A–C)** and mesenteric lymph nodes (MLN; **D-F**) on day 6 post-infection. Naive mice (open diamonds) served as non-infected controls without prophylaxis. Box plots (25th and 75th percentiles), whiskers (minimum and maximum values), medians (black bar in boxes), numbers of analyzed mice pooled from 3 independent experiments (in parentheses), and significance levels (p values) determined by the Kruskal-Wallis test with Dunn's post-hoc test **(A, B, D–F)** or by the one-sided ANOVA test with Tukey's *post-hoc* test **(C)** are indicated.

extra-intestinal and even systemic *C. jejuni*-induced pro-inflammatory mediator responses.

4 Discussion

In our actual preclinical placebo-controlled intervention trial, prophylactic oral curcumin application to mice starting a week before *C. jejuni* infection ameliorated the acute campylobacteriosis syndrome as evidenced by i.) an improved clinical outcome, ii.) less inflammation-induced shrinkage of the infected large intestines, iii.) less histopathological and apoptotic epithelial changes in the colon, iv.) attenuated colonic infiltration with distinct innate and adaptive immune cells, v.) diminished pro-inflammatory mediator secretion in the intestinal tract (i.e., in the colon and MLN), vi.) in extra-intestinal organs (i.e., liver and kidneys) and strikingly, vii.) even systemically if compared to placebo. In addition, viii.) the gastrointestinal *C. jejuni* numbers (i.e., in the stomach, ileum, and colon) were lower in the former versus the latter. The result of decreased gastrointestinal enteropathogen loads (Figure 2) were rather unexpected given that the applied curcumin concentration (1.0 g/L) was below the minimum inhibitory concentration (MIC) of 5.9 g/L (at pH 7.4). Nevertheless, one needs to take into consideration that the polyphenol is subjected to modification by distinct intestinal enzymes yielding metabolites with potential bacteria-toxic effects (21).

Apart from the lowered gastrointestinal *C. jejuni* loads the mitigated campylobacteriosis syndrome upon curcumin pretreatment (Figure 3) might additionally have been due to pronounced immune-modulatory effects of the polyphenol in this acute inflammatory scenario. In support, curcumin was shown to ameliorate experimental colitis of different etiologies (24, 44–52). Furthermore, our own previous work revealed that oral pretreatment of mice with the same curcumin concentration as applied in our actual trial alleviated acute *Toxoplasma gondii*-induced ileitis (23). The immune-modulatory effects of curcumin treatment were mirrored by less distinct infiltration of the infected large intestinal mucosa and lamina propria with neutrophils as well as T and B lymphocytes (Figure 4). In support, both *in vitro* and *in vivo* studies provided evidence that curcumin interacts with various

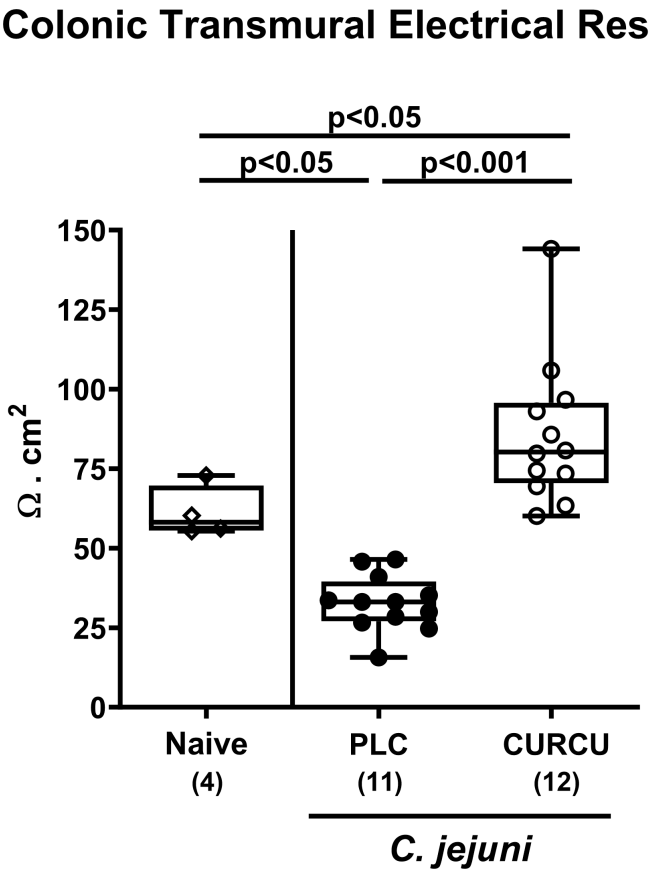

FIGURE 6
Colonic epithelial barrier function following curcumin pretreatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were pretreated with curcumin (CURCU; open circles) or placebo (PLC; closed circles) via the drinking water starting 7 days prior peroral infection with *C. jejuni* 81–176 strain on days 0 and 1. On day 6 post-infection, the transmural electrical resistance of the distal colon was measured in Ussing chambers. Naive mice (open diamonds) served as non-infected controls without prophylaxis. Box plots (25th and 75th percentiles), whiskers (minimum and maximum values), medians (black bar in boxes), numbers of analyzed mice pooled from 3 independent experiments (in parentheses), and significance levels (p values) determined by the one-sided ANOVA test with Tukey's *post-hoc* test are indicated.

immune cell subtypes of the innate and adaptive immune system including the afore-mentioned ones preventing from cellular infiltration of the target tissues (53). The attenuated immune cellular infiltration of the colonic tissue following curcumin pretreatment was accompanied by dampened intestinal secretion of IFN-γ, TNF-α, and NO as measured in the colon and in the MLN draining the infected intestinal tract on day 6 p.i. Remarkably, curcumin treated mice presented with colonic T and B cell numbers and furthermore, with intestinal pro-inflammatory mediator concentrations that did not differ from those detected in naive mice (Figure 5). Our data are well in line with previous *in vitro* and *in vivo* studies showing that curcumin was able to down-regulate the expression of respective pro-inflammatory mediators counteracting various inflammatory conditions including intestinal inflammation (53–55). In consequence of the here observed immune-modulatory effects of curcumin pretreatment, the large intestinal tissues were less distinctly exposed to cell-toxic oxidative stress resulting in less pronounced apoptotic responses in the colonic epithelial cells and in less severe histopathological damage (Figures 3C, D). Our data are supported by previous studies underscoring potent anti-apoptotic effects of exogenous curcumin as observed *in vitro* and *in vivo* (46, 52, 56–59). The alleviated *C. jejuni*-induced intestinal tissue damage upon curcumin pretreatment was accompanied by an uncompromised epithelial barrier function as indicated by transmural electrical resistance values measured in the colon of curcumin pretreated *C. jejuni* infected mice that were not only higher as compared to placebo counterparts, but also to naive controls (Figure 6), indicative for an enhanced tightening of the epithelial barrier. Our actual and previous studies further underscore potent effects of exogenous polyphenolic compounds such as curcumin and resveratrol on rescuing epithelial barrier integrity and function during acute murine campylobacteriosis (38, 60) and are supported by previous studies showing epithelial barrier-preserving capacities

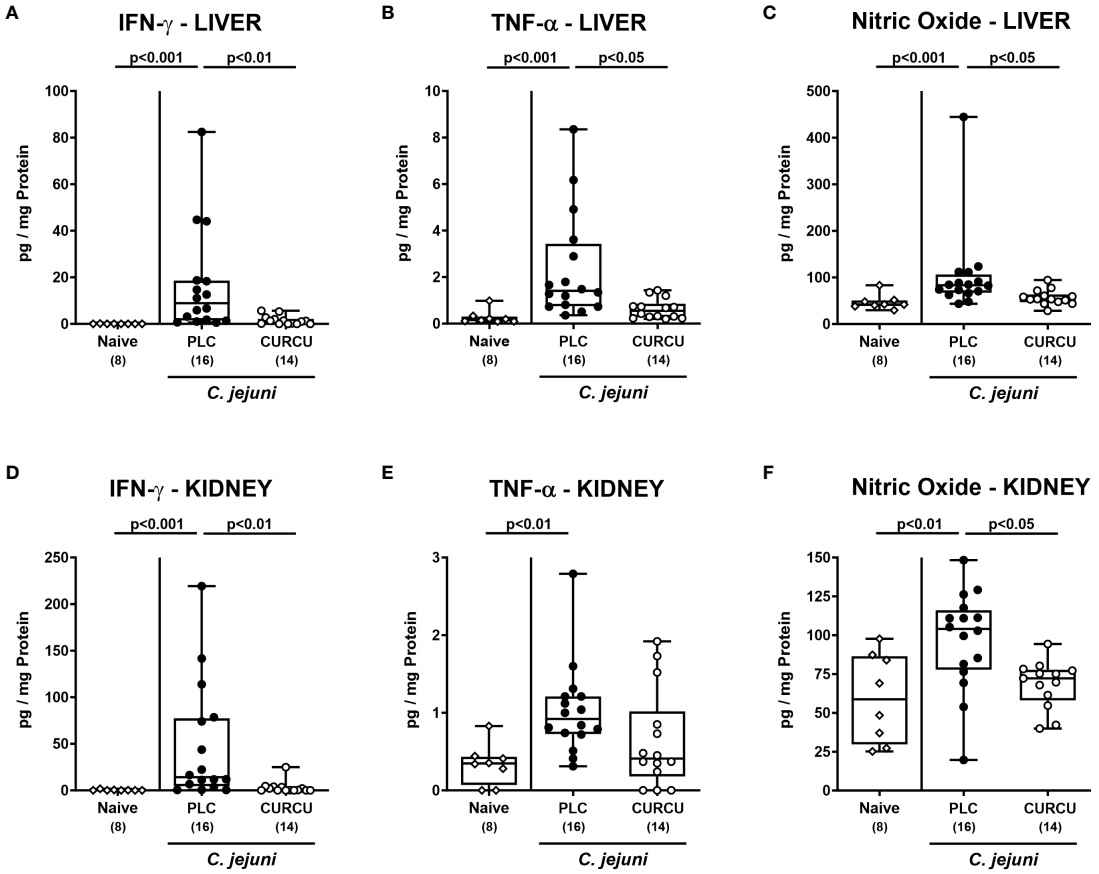

FIGURE 7
Extra-intestinal pro-inflammatory mediators following curcumin pretreatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were pretreated with curcumin (CURCU; open circles) or placebo (PLC; closed circles) via the drinking water starting 7 days prior peroral infection with *C. jejuni* 81–176 strain on days 0 and 1. **(A, D)** IFN-γ, **(B, E)** TNF-α, and **(C, F)** nitric oxide concentrations were measured in supernatants of *ex vivo* biopsies derived from the liver **(A–C)** and kidneys **(D–F)** on day 6 post-infection. Naive mice (open diamonds) served as non-infected controls without prophylaxis. Box plots (25th and 75th percentiles), whiskers (minimum and maximum values), medians (black bar in boxes), numbers of analyzed mice pooled from 3 independent experiments (in parentheses), and significance levels (p values) determined by the Kruskal-Wallis test with Dunn's *post-hoc* test **(A–C, E, F)** or by the one sided ANOVA test with Tukey's *post-hoc* test **(D)** are indicated.

of curcumin application which was accompanied by up-regulated expression of tight junction proteins such as claudin-1 and zonula occludens protein-1 (ZO-1) (61–64).

Remarkably, the disease-mitigating properties of curcumin pretreatment in *C. jejuni* infected mice could not only be assessed in the intestinal tract, but also in extra-intestinal organ such as the liver and the kidneys given hepatic as well as renal IFN-γ, TNF-α, and NO concentrations in curcumin pretreated mice on day 6 p.i. that were comparable to naive values (Figure 7). In line with our results, curcumin was shown to exert potent anti-oxidative effects in a plethora of liver diseases as shown by enhanced radical scavenging and down-regulating the inducible nitric oxidase synthase (iNOS) which resulted in decreased hepatic NO concentrations (65, 66). Furthermore, polyphenolic application led to both, decreased hepatic TNF-α expression and attenuated cytokine-induced apoptosis (65, 66). Previous studies underlined also reno-protective properties of curcumin due to down-regulated expression of TNF-α, MCP-1, and iNOS in the kidneys as reviewed previously (67), whereas polyphenolic pretreatment attenuated renal injury in LPS-induced endotoxemia (68). Strikingly, the disease-mitigating potency of curcumin pretreatment could also be observed systemically as shown by naive IFN-γ, TNF-α, MCP-1, and IL-6 concentrations measured in serum samples derived from *C. jejuni* infected mice of the verum cohort (Figure 8). In support, curcumin was shown to inhibit pro-inflammatory cytokine production in endotoxinemia (69, 70) and to prevent LPS-induced TLR-4 activation and subsequent pro-inflammatory mediator secretion by the inhibition of the TLR-4/MyD88/NF-κB signaling pathways during sepsis (71–73). The anti-TLR-4 effects of curcumin were also propagated by Lubbad and colleagues who demonstrated that curcumin treatment could ameliorate experimental colitis in a TLR-4 dependent fashion

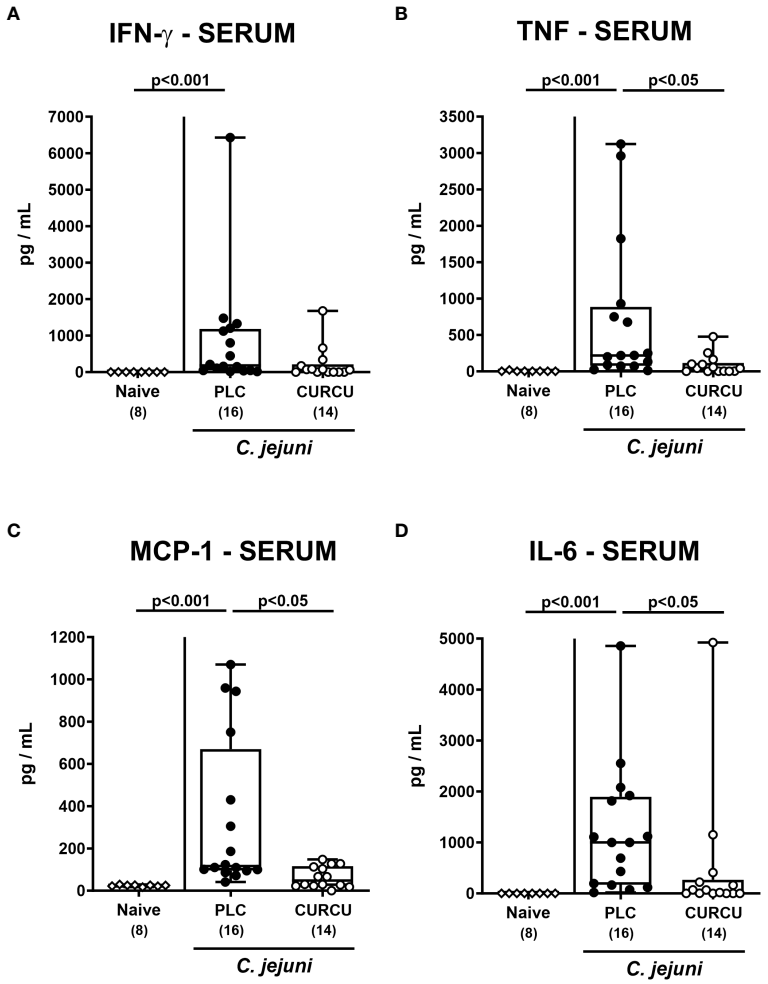

FIGURE 8
Systemic pro-inflammatory mediators following curcumin pretreatment of *C. jejuni* infected mice. Secondary abiotic IL-10$^{-/-}$ mice were pretreated with curcumin (CURCU; open circles) or placebo (PLC; closed circles) via the drinking water starting 7 days prior peroral infection with *C. jejuni* 81–176 strain on days 0 and 1. **(A)** IFN-γ, **(B)** TNF-α, **(C)** MCP-1, and **(D)** IL-6 concentrations were measured in serum samples taken on day 6 post-infection. Naive mice (open diamonds) served as non-infected controls without prophylaxis. Box plots (25th and 75th percentiles), whiskers (minimum and maximum values), medians (black bar in boxes), numbers of analyzed mice pooled from 3 independent experiments (in parentheses), and significance levels (p values) determined by the Kruskal-Wallis test with Dunn's *post-hoc* test are indicated.

(48). Moreover, in this line, in a previous study applying an experimental *C. jejuni* infection model of an immune cell – epithelial cell co-culture, we were able to show the barrier-preserving effect of curcumin which was dependent on its inhibition of NF-κB (60).

5 Conclusion

Given that the hyper-activation of the host immune system by the bacterial endotoxin LOS constitutes the main molecular mechanism underlying *C. jejuni* -induced enteritis (8) and furthermore, that the risk for developing autoimmune sequelae (such as GBS, RA, and IBD) is associated with the severity of the enteritis (17), the TLR-4 antagonist curcumin constitutes an elegant antibiotic-independent strategy to mitigate acute campylobacteriosis and furthermore, to reduce the risk for post-infectious collateral damages of *C. jejuni* infection. Curcumin may be considered as non-toxic, non-mutagenic, and overall safe given that even high doses of 6 g per day for up to 7 weeks were tolerated well in human trials (74). One should also take into consideration that the systemic concentrations of the polyphenolic compound are rather low given the poor bioavailability of curcumin after ingestion (74).

Data availability statement

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

Ethics statement

The animal study was approved by Landesamt für Gesundheit und Soziales (LaGeSo), Berlin. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MH: Conceptualization, Funding acquisition, Investigation, Supervision, Validation, Visualization, Writing – original draft. SM: Investigation, Validation, Visualization, Writing – review & editing. FL: Investigation, Visualization, Writing – review & editing. EP: Investigation, Methodology, Validation, Writing – review & editing. JS: Validation, Writing – review & editing. RB: Investigation, Validation, Visualization, Writing – review & editing. SK: Investigation, Validation, Writing – review & editing. SB: Conceptualization, Funding acquisition, Supervision, Validation, Writing – review & editing.

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Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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Phenolic acids from medicinal and edible homologous plants: a potential anti-inflammatory agent for inflammatory diseases

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Inflammation has been shown to trigger a wide range of chronic diseases, particularly inflammatory diseases. As a result, the focus of research has been on anti-inflammatory drugs and foods. In recent years, the field of medicinal and edible homology (MEH) has developed rapidly in both medical and food sciences, with 95% of MEH being associated with plants. Phenolic acids are a crucial group of natural bioactive substances found in medicinal and edible homologous plants (MEHPs). Their anti-inflammatory activity is significant as they play a vital role in treating several inflammatory diseases. These compounds possess enormous potential for developing anti-inflammatory drugs and functional foods. However, their development is far from satisfactory due to their diverse structure and intricate anti-inflammatory mechanisms. In this review, we summarize the various types, structures, and distribution of MEHP phenolic acids that have been identified as of 2023. We also analyze their anti-inflammatory activity and molecular mechanisms in inflammatory diseases through NF-κB, MAPK, NLRP3, Nrf2, TLRs, and IL-17 pathways. Additionally, we investigate their impact on regulating the composition of the gut microbiota and immune responses. This analysis lays the groundwork for further exploration of the anti-inflammatory structure-activity relationship of MEHP phenolic acids, aiming to inspire structural optimization and deepen our understanding of their mechanism, and provides valuable insights for future research and development in this field.

KEYWORDS

medicinal and edible homology, plant sources, structure and distribution, phenolic acids, anti-inflammatory, inflammatory diseases, mechanism, pathway

1 Introduction

Inflammatory diseases can trigger abnormal reactions in various body systems, leading to tissue damage and dysfunction, which seriously affects human health, and inflammation is the basis of inflammatory diseases. Inflammation is a cascade of chemical signals triggered by viral and bacterial infections, toxic compound stimulation, and tissue damage, which can activate white blood cells to produce and release inflammatory cytokines. Chronic inflammation can contribute to the development of various chronic diseases such as inflammatory diseases, autoimmune diseases, tumors, neurogenic diseases, diabetes, cardiovascular diseases, and tissue fibrosis (1, 2). Therefore, anti-inflammatory drugs and foods have always been a hot topic of research. Medicinal and edible homology (MEH) refers to natural resources offering edible and medicinal value. Being safe and healthy options with medicinal functions, MEH-based research and product development are receiving increasing attention (3, 4). In 2002, a list of items that function as both food and medicine (the catalog of "Medicinal and edible homologous" sources) was released by the former Chinese Ministry of health. As of now, there are 110 Chinese medicinal materials that have been included (3), and 102 of these are plants, accounting for nearly 95%.

Medicinal and edible homologous plants (MEHPs) are characterized by the presence of a variety of active ingredients. Phenolic acids are one of the most representative ingredients of MEHPs. Phenolic acids are a class of organic acids with directly linked phenolic groups to aromatic rings, are abundant in plants, encompassing a broad spectrum of medicinal and edible varieties, and constitute vital secondary metabolites. Currently, phenolic acids hold broad applications across various sectors, including the food industry, medicine, health supplements, and cosmetics. They are integrated into food products as natural preservatives and antioxidants, enhancing shelf life (5). Within the pharmaceutical domain, phenolic acids serve as therapeutic agents or adjuvants for combating inflammatory conditions and select cancers (6). As dietary supplements, they contribute to health promotion and disease prevention (7). Additionally, phenolic acids are leveraged in skincare for their potent antioxidant and anti-inflammatory benefits, particularly in anti-aging and protective formulations (8). The structural skeleton of phenolic acids is mainly composed of a carboxyl group and one or more hydroxyl groups bound to aromatic rings. Phenolic acids can be divided into three classes: hydroxybenzoic acids, hydroxyphenylacetic acids, and hydroxycinnamic acids, all having anti-inflammatory, anti-oxidant, anti-bacterial, and anti-viral activities (9–12). Bioactivity is closely related to the structure of MEHP phenolic acids; hence, an understanding of the varied structures of these compounds is important.

Anti-inflammatory activity is one of the main features of MEHP phenolic acids and plays an important role in the prevention and treatment of numerous inflammatory diseases (13–16). Although the pathogenesis of these diseases is different, the regulation of inflammatory signaling pathways is similar. Therefore, it is essential to elucidate the anti-inflammatory mechanisms of MEHP phenolic acids for intensive research on their anti-inflammatory diseases' activity.

We performed a comprehensive database search of PubMed, Web of Science, and Science Direct for entries up to November 2023, to systematically review the types, structures, anti-inflammatory activities, and molecular mechanisms of MEHP phenolic acids. The objective is to provide scientific basis for in-depth research and comprehensive development of the anti-inflammatory activities of MEHP phenolic acids.

2 Structure and distribution of MEHP phenolic acids

Upon conducting a thorough literature review, we discovered that 68 types of MEHP were reported to contain a comprehensive collection of 167 phenolic acids. Among these, there are 45 hydroxybenzoic acids, 113 hydroxycinnamic acids, 8 hydroxyphenylacetic acids, and 1 other phenolic acid. The 68 MEHPs belong to 35 families with 6 species from Rosaceae or Lamiaceae, 5 from Zingiberaceae, 4 from Caprifoliaceae or Compositae, and 3 from Moraceae, Rutaceae, Leguminosae, or Campanulacea.

2.1 Hydroxybenzoic acids

Hydroxybenzoic acids are based on a hydroxybenzoic acid skeleton. The hydroxybenzoic acids can be divided into simple hydroxybenzoic acids, polyhydroxybenzoic acids, hydroxybenzoates, and hydroxybenzoate glycosides. According to reports, there are 18 types of simple hydroxybenzoic acids, 6 types of polyhydroxybenzoic acids, 12 types of hydroxybenzoates, and 9 types of hydroxybenzoate glycosides. Simple hydroxybenzoic acids are the most widely distributed (including vanillic acid, gallic acid, syringic acid, salicylic acid, protocatechuic acid, p-hydroxybenzoic acid, etc.) among which vanillic acid, gallic acid, and syringic acid are distributed in 28, 25, and 24 MEHPs, respectively. Details are shown in Table 1 and the structure is shown in Figure 1.

2.2 Hydroxycinnamic acids

Hydroxycinnamic acids are the most abundant and widely distributed phenolic acids. According to structure, they can be divided into simple hydroxycinnamic acids, hydrogenated hydroxycinnamic acids, polyhydroxycinnamic acids, hydroxycinnamates, hydroxycinnamate glycosides, and hydroxycinnamate salts. Among the reported MEHP phenolic acids, there are 10 simple hydroxycinnamic acids, 7 hydrogenated hydroxycinnamic acids, 46 polyhydroxycinnamic acids, 26 hydroxycinnamates, 21 hydroxycinnamate glycosides, and 1 hydroxycinnamate salt. Among these, simple

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TABLE 1 Hydroxybenzoic acids of medicinal and edible homologous plants.

No.	Components	Molecular Formula	MEHPs
Simple hydroxybenzoic acids			
1	3-hydroxybenzoic acid	$C_7H_6O_3$	*Lycium barbarum* L (17); *Sesamum indicum* L (18); *Crocus sativus* L (19); *Amomum tsao-ko Crevost* et Lemaire (20).
2	salicylic acid	$C_7H_6O_3$	*Cichorium intybus* L (21); *Hippophae rhamnoides* L (22); *Perilla frutescens* (L.) Britt. (leaf) (23); *Sesamum indicum* L (18); *Panax ginseng* C.A.Mey (24); *Crocus sativus* L (25); *Curcuma longa* L (26); *Panax quinquefolium* L (27).
3	p-hydroxybenzoic acid	$C_7H_6O_3$	*Hippophae rhamnoides* L (28); *Hordeum vulgare* L (29); *Laminaria japonica* Aresch (30); *Houttuynia cordata* Thunb (31); *Zingiber officinale* Rosc (32); *Lycium barbarum* L (17); *Sterculia lychnophora* Hance (33); *Morus alba* L. (fruit) (34); *Nelumbo nucifera* Gaertn. (fruit) (35); *Nelumbo nucifera* Gaertn. (leaf) (36); *Cichorium intybus* L (37); *Perilla frutescens* (L.) Britt. (leaf) (38); *Sesamum indicum* L (18); *Angelica sinensis* (Oliv.) Diels (39); *Kaempferia galanga* L (40); *Crocus sativus* L (41); *Panax quinquefolium* L (27); *Gastrodia elata* B1 (42); *Piper nigrum* L (43); *Panax ginseng* C.A.Mey (44); *Coriandrum sativum* L (45).
4	anisic acid	$C_8H_8O_3$	*Kaempferia galanga* L (40).
5	pyrocatechuic acid	$C_7H_6O_4$	*Hippophae rhamnoides* L (28); *Hordeum vulgare* L (46).
6	gentisic acid	$C_7H_6O_4$	*Hippophae rhamnoides* L (28); *Nelumbo nucifera* Gaertn. (fruit) (47); *Dimocarpus longan* Lour (48); *Panax ginseng* C.A.Mey (44); *Rosa rugosa* Thunb (49); *Crocus sativus* L (25).
7	protocatechuic acid	$C_7H_6O_4$	*Hippophae rhamnoides* L (28); *Hordeum vulgare* L (46); *Ziziphus jujuba* Mill (50); *Lycium barbarum* L (17); *Gardenia jasminoides* Ellis (51); *Sterculia lychnophora* Hance (33); *Mosla chinensis* 'jiangxiangru' (52); *Morus alba* L. (fruit) (34); *Morus alba* L. (leaf) (53); *Alpinia oxyphylla* Miq (54); *Nelumbo nucifera* Gaertn. (fruit) (35); *Nelumbo nucifera* Gaertn. (leaf) (36); *Perilla frutescens* (L.) Britt. (leaf) (38); *Piper nigrum* L (43); *Panax ginseng* C.A.Mey (44); *Rosa rugosa* Thunb (49); *Prunella vulgaris* L (55); *Angelica sinensis* (Oliv.) Diels (39); *Panax quinquefolium* L (27);*Cornus officinalis* Sieb. et Zucc (56); *Eucommia ulmoides* Oliv (57).
8	isovanillic acid	$C_8H_8O_4$	*Vigna umbellata* Ohwi et Ohashi (58); *Vigna angularis* Ohwi et Ohashi (58); *Perilla frutescens* (L.) Britt. (Leaf) (38).
9	vanillic acid	$C_8H_8O_4$	*Crataegus pinnatifida* Bge (59); *Dimocarpus longan* Lour (48); *Hippophae rhamnoides* L (60); *Vigna umbellata* Ohwi et Ohashi (58); *Vigna angularis* Ohwi et Ohashi (58); *Hordeum vulgare* L (29); *Laminaria japonica* Aresch (30); *Houttuynia cordata* Thunb (31); *Hovenia dulcis* Thunb (61); *Lycium barbarum* L (17); *Morus alba* L. (fruit) (34); *Platycodon grandiflorum* (Jacq.) A.DC (62); *Nelumbo nucifera* Gaertn. (leaf) (36); *Cichorium intybus* L (21); *Perilla frutescens*(L.) Britt. (Leaf) (63); *Perilla frutescens* (L.) Britt. (fruit) (64); *Sesamum indicum* L (18); *Panax ginseng* C.A.Mey (65); *Coriandrum sativum* L (45); *Angelica sinensis* (Oliv.) Diels (39); *Kaempferia galanga* L (40); *Crocus sativus* L (41); *Curcuma Longa* L (26); *Codonopsis pilosula* (Franch.) Nannf (66); *Dendrobium officinale* Kimura et Migo (67); *Panax quinquefolium* L (27); *Gastrodia elata* B1 (68); *Dolichos lablab* L (69).
10	3,5-dihydroxybenzoic acid	$C_7H_6O_4$	*Amomum tsao-ko Crevost* et Lemaire (20).
11	veratric acid	$C_9H_{10}O_4$	*Hippophae rhamnoides* L (28).
12	gallic acid	$C_7H_6O_5$	*Portulaca oleracea* L (70); *Dolichos lablab* L (69); *Dimocarpus longan Lour* (48).; *Phyllanthus emblica* L (71); *Citrus medica* L (72); *Hippophae rhamnoides* L (60); *Vigna umbellata* Ohwi et Ohashi (58); *Vigna angularis* Ohwi et Ohashi (58); *Hordeum vulgare* L (46); *Laminaria japonica* Aresch (30); *Ziziphus jujuba* Mill (73); *Canarium album* Raeusch (72); *Houttuynia cordata* Thunb (31); *Zingiber officinale* Rosc (32); *Lycium barbarum* L (74); *Morus alba* L. (fruit) (75); *Morus alba* L. (leaf) (76); *Citrus reticulata* Blanco (77); *Nelumbo nucifera* Gaertn. (fruit) (47); *Nelumbo nucifera* Gaertn. (leaf) (36); *Perilla frutescens* (L.) Britt. (Leaf) (23); *Panax ginseng* C.A.Mey (78); *Rosa rugosa* Thunb (49); *Crocus sativus* L (41); *Curcuma Longa* L (26); *Panax quinquefolium* L (27); *Cornus officinalis* Sieb. et Zucc (79).
13	4-O-methylgallic acid	$C_8H_8O_5$	*Phyllanthus emblica* L (71); *Piper nigrum* L (43).
14	syringic acid	$C_9H_{10}O_5$	*Portulaca oleracea* L (80); *Cannabis sativa* L (81); *Dolichos lablab* L (69); *Dimocarpus longan* Lour (48); *Phyllanthus emblica* L (71); *Hordeum vulgare* L (29); *Houttuynia cordata* Thunb (31); *Zingiber officinale* Rosc (32); *Lycium barbarum* L (74); *Morus alba*

(Continued)

10.3389/fimmu.2024.1345002

TABLE 1 Continued

No.	Components	Molecular Formula	MEHPs
Simple hydroxybenzoic acids			
			L. (fruit) (82); *Citrus reticulata* Blanco (77); *Nelumbo nucifera* Gaertn. (leaf) (36); *Perilla frutescens*(L.) Britt. (Leaf) (63); *Sesamum indicum* L (18); *Piper nigrum* L (43); *Panax ginseng* C.A.Mey (44); *Coriandrum sativum* L (45); *Angelica sinensis* (Oliv.) Diels (83); *Crocus sativus* L (25); *Curcuma longa* L (26); *Dendrobium officinale* Kimura et Migo (67); *Panax quinquefolium* L (27).
15	3,4-O-dimethylgallic acid	$C_9H_{10}O_5$	*Piper nigrum* L (43).
16	5-sulfosalicyclic acid	$C_7H_6O_6S$	*Perilla frutescens* (L.) Britt. (Leaf) (63)
17	vanillic acid 4-sulfate	$C_8H_8O_7S$	*Piper nigrum* L (43).
18	ginkgolic acid	$C_{22}H_{34}O_3$	*Cistanche deserticola* Y.C.Ma (84).
Polyhydroxybenzoic acids			
19	2-O-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-phenylacetic acid	$C_{15}H_{12}O_8$	*Morus alba* L. (fruit) (85)
20	3,4-di-O-galloylquinic acid	$C_{21}H_{20}O_{14}$	*Phyllanthus emblica* L (71).
21	digallic acid	$C_{14}H_{10}O_9$	*Canarium album* Raeusch (86).
22	gallic acid O-malic acid	$C_{10}H_{10}O_9$	*Canarium album* Raeusch (86).
23	galloylquinic acid	$C_{14}H_{16}O_{10}$	*Canarium album* Raeusch (86).
24	galloylshikimic acid	$C_{14}H_{14}O_9$	*Canarium album* Raeusch (86).
Hydroxybenzoates			
25	1-O-galloyl-glycerol	$C_{10}H_{12}O_7$	*Phyllanthus emblica* L (71).
26	methylparaben	$C_8H_8O_3$	*Crocus sativus* L (41).
27	2-O-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxyphenylmethylacetate	$C_{16}H_{14}O_8$	*Morus alba* L. (fruit) (85)
28	2-O-galloylgalactaric acid	$C_{13}H_{14}O_{12}$	*Phyllanthus emblica* L (71).
29	1-methyl 2-galloylgalactarate	$C_{14}H_{15}O_{12}$	*Phyllanthus emblica* L (71).
30	3,5-dihydroxy-2-(2-methoxy-2-oxoethyl) phenyl 4-hydroxybenzoate	$C_{16}H_{14}O_7$	*Cornus officinalis* Sieb. et Zucc (56)
31	3-O-methylgallate	$C_8H_7O_5$	*Phyllanthus emblica* L (71).
32	protocatechuic acid ethyl ester	$C_9H_{10}O_4$	*Sterculia lychnophora* Hance (33); *Morus alba* L. (fruit) (85)
33	7-O-galloyl-d-sedoheptulose	$C_{14}H_{18}O_{11}$	*Cornus officinalis* Sieb. et Zucc (79)
34	protocatechuic acid methyl ester	$C_8H_8O_4$	*Kaempferia galanga* L (40); *Morus alba* L. (fruit) (85)
35	methyl gallate	$C_8H_8O_5$	*Cistanche deserticola* Y.C.Ma (84)
36	O-acetylsyringic acid	$C_{14}H_{18}O_6$	*Morus alba* L. (fruit) (82)
Hydroxybenzoate glycosides			
37	1-O,6-O-digalloyl-β-D-glucose	$C_{20}H_{20}O_{14}$	*Phyllanthus emblica* L (71).
38	β-glucogallin	$C_{13}H_{16}O_{10}$	*Phyllanthus emblica* L (71).
39	gallic acid-3,5-diglucoside	$C_{19}H_{26}O_{15}$	*Angelica sinensis* (Oliv.) Diels (83).
40	galloyl-glucoside	$C_{13}H_{16}O_{10}$	*Angelica sinensis* (Oliv.) Diels (83).

(Continued)

10.3389/fimmu.2024.1345002

TABLE 1 Continued

No.	Components	Molecular Formula	MEHPs
Hydroxybenzoate glycosides			
41	gentisic acid 5-O-D-(6'-salicyly1)-glucopyranoside	$C_{20}H_{20}O_{11}$	*Prunella vulgaris* L (87).
42	protocatechuic acid 4-O-glucoside	$C_{13}H_{16}O_9$	*Piper nigrum* L (43).
43	vanillic acid -4-O-glucoside	$C_{14}H_{18}O_9$	*Mosla chinensis* 'jiangxiangru' (52); *Sesamum indicum* L (18).
44	salicylic acid-2-O-glucoside	$C_{13}H_{16}O_8$	*Sesamum indicum* L (18).
45	1-O-4-carboxylphenyl-(6-O-4-hydroxybenzoyl)-β-D-glucopyranoside	$C_{20}H_{20}O_{10}$	*Kaempferia galanga* L (40).

hydroxycinnamic acids and polyhydroxycinnamic acids are the most diverse. The most widely distributed simple hydroxycinnamic acids include caffeic acid, ferulic acid, and p-coumaric acid, which are distributed in 39, 31, and 28 MEHPs, respectively. Most polyhydroxycinnamic acids have caffeic acid as the parent core, including caffeoylquinic acids which combine caffeic acid and quinic acid (chlorogenic acid) and rosmarinic acid which is a combination of caffeic acid and danshensu. Chlorogenic acid is the phenolic acid with the largest reported distribution in 43 MEHPs. In addition, the reported hydroxycinnamate salt (caffeic acid 3-sulfonate) was only distributed in Piper nigrum L. Detailed information is shown in Table 2 and the structure is shown in Figure 2.

2.3 Hydroxyphenylacetic acids and other acids

In contrast, hydroxyphenylacetic acids are the least abundant phenolic acids. Only 8 hydroxyphenylacetic acids have been reported in MEHPs, including 5 simple hydroxyphenylacetic acids, 2 hydroxyphenylacetates, and 1 hydroxyphenylacetate

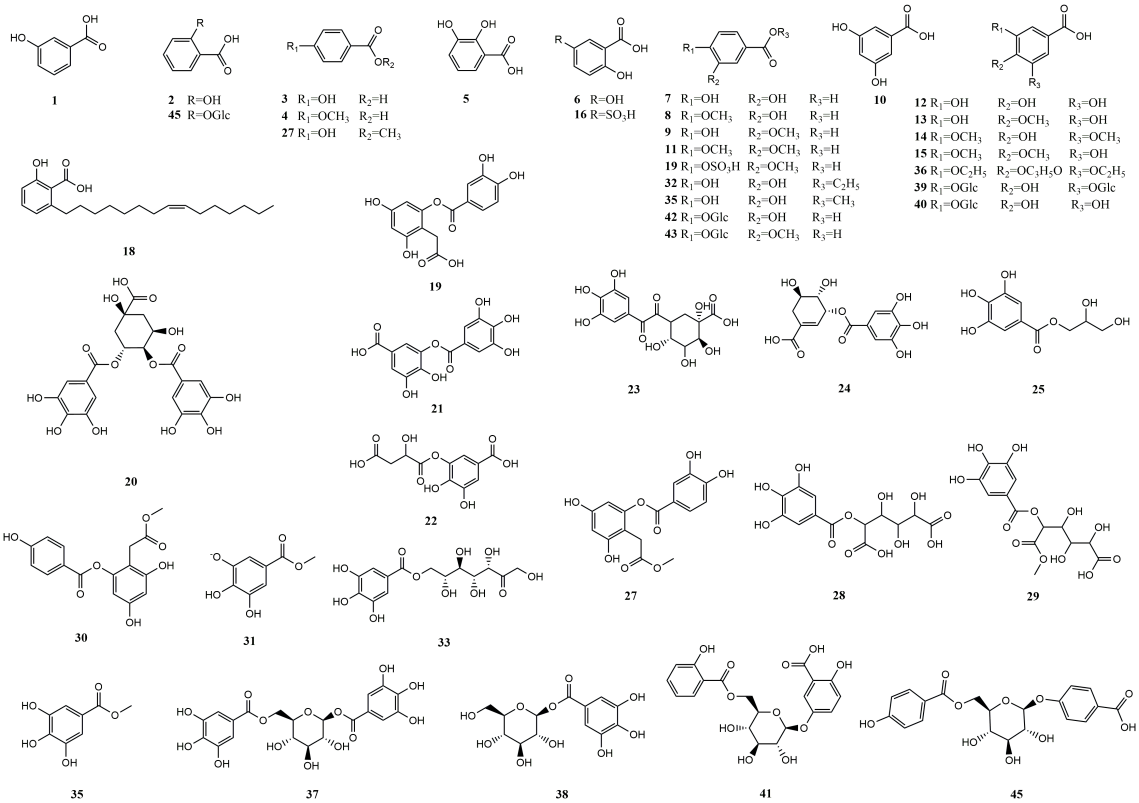

FIGURE 1
Structures of hydroxybenzoic acids from medicinal and edible homologous plants.

10.3389/fimmu.2024.1345002

TABLE 2 Hydroxycinnamic acids of medicinal and edible homologous plants.

No.	Components	Molecular Formula	MEHPs
Simple hydroxycinnamic acids			
46	m-coumaric acid	$C_9H_8O_3$	*Hippophae rhamnoides* L (28); *Piper nigrum* L (43); *Panax ginseng* C.A.Mey (44); *Morus alba* L. (fruit) (34)
47	o-coumaric acid	$C_9H_8O_3$	*Morus alba* L. (fruit) (88); *Perilla frutescens* (L.) Britt. (leaf) (63); *Panax ginseng* C.A.Mey (44); *Crocus sativus* L (19).
48	p-coumaric acid	$C_9H_8O_3$	*Cornus officinalis* Sieb.et Zucc (79); *Portulaca oleracea* L (70); *Prunella vulgaris* L (87); *Prunus mume* (Sieb.) Sieb. et Zucc (89); *Dolichos lablab* L (69); *Dendrobium officinale* Kimura et Migo (67); *Dimocarpus longan* Lour (48); *Citrus medica* L (72); *Hordeum vulgare* L (29); *Houttuynia cordata* Thunb (31); *Zingiber officinale* Rosc (32); *Lycium barbarum* L (17); *Morus alba* L. (fruit) (34); *Morus alba* L. (leaf) (90); *Alpinia oxyphylla* Miq (91); *Nelumbo nucifera* Gaertn. (fruit) (47); *Nelumbo nucifera* Gaertn. (leaf) (36); *Chrysanthemum morifolium* Ramat (92); *Cichorium intybus* L (37); *Rubus chingii* Hu (93); *Panax ginseng* C.A.Mey (44); *Coriandrum sativum* L (94); *Prunella vulgaris* L (95); *Crocus sativus* L (41); *Curcuma longa* L (26); *Panax quinquefolium* L (27); *Hippophae rhamnoides* L (96); *Kaempferia galanga* L (40).
49	trans p-methoxycinnamic acid	$C_{10}H_{10}O_3$	*Kaempferia galanga* L (40).
50	caffeic acid	$C_9H_8O_4$	*Cirsium setosum* (Willd.) MB (97); *Portulaca oleracea* L (70); *Prunus mume* (Sieb.) Sieb. et Zucc (89); *Phyllanthus emblica* L (71); *Citrus medica* L (72); *Hippophae rhamnoides* L (98); *Vigna umbellata* Ohwi et Ohashi (58); *Vigna angularis* Ohwi et Ohashi (58); *Laminaria japonica* Aresch (30); *Ziziphus jujuba* Mill (73); *Lonicera japonica* Thunb (99); *Zingiber officinale* Rosc (32); *Lycium barbarum* L (17); *Sterculia lychnophora* Hance (33); *Morus alba* L. (fruit) (34); *Morus alba* L. (leaf) (53); *Citrus reticulata* Blanco (100); *Alpinia oxyphylla* Miq (91); *Nelumbo nucifera* Gaertn. (fruit) (47); *Nelumbo nucifera* Gaertn. (leaf) (36); *Lophatherum gracile* Brongn (101); *Chrysanthemum morifolium* Ramat (102); *Perilla frutescens* (L.) Britt. (leaf) (103); *Perilla frutescens* (L.) Britt. (fruit) (64); *Sesamum indicum* L (18); *Piper nigrum* L (43); *Taraxacum mongolicum* Hand.-Mazz (104); *Mentha haplocalyx* Briq (105); *Panax ginseng* C.A.Mey (78); *Lonicera hypoglauca* Miq (106); *Lonicera macranthoides* Hand.-Mazz (107); *Coriandrum sativum* L (45); *Prunella vulgaris* L (108); *Angelica sinensis* (Oliv.) Diels (83); *Crocus sativus* L (41); *Curcuma Longa* L (26); *Codonopsis pilosula* (Franch.) Nannf (66); *Cornus officinalis* Sieb. et Zucc (79); *Eucommia ulmoides* Oliv (57).
51	Z-caffeic acid	$C_9H_8O_4$	*Sterculia lychnophora* Hance (33)
52	ferulic acid	$C_{10}H_{10}O_4$	*Prunus mume* (Sieb.) Sieb. et Zucc (89); *Cannabis sativa* L (109); *Dolichos lablab* L (69); *Dimocarpus longan* Lour (48); *Hippophae rhamnoides* L (60); *Vigna umbellata* Ohwi et Ohashi (58); *Vigna angularis* Ohwi et Ohashi (58); *Hordeum vulgare* L (29); *Laminaria japonica* Aresch (30); *Ziziphus jujuba* Mill (73); *Houttuynia cordata* Thunb (31); *Zingiber officinale* Rosc (32); *Hovenia dulcis* Thunb (61); *Lycium barbarum* L (17); *Morus alba* L. (fruit) (34); *Morus alba* L. (leaf) (76); *Citrus reticulata* Blanco (100); *Alpinia oxyphylla* Miq (91); *Nelumbo nucifera* Gaertn. (fruit) (47); *Nelumbo nucifera* Gaertn. (leaf) (36); *Perilla frutescens* (L.) Britt. (leaf) (38); *Sesamum indicum* L (18); *Panax ginseng* C.A.Mey (44); *Lonicera hypoglauca* Miq (110); *Coriandrum sativum* L (45); *Prunella vulgaris* L (55); *Angelica sinensis* (Oliv.) Diels (39); *Kaempferia galanga* L (40); *Curcuma Longa* L (26); *Dendrobium officinale* Kimura et Migo (67); *Panax quinquefolium* L (27).
53	(E)-isoferulic acid	$C_{10}H_{10}O_4$	*Panax quinquefolium* L (27)
54	3,4-dimethoxycinnamic acid	$C_{11}H_{12}O_4$	*Sesamum indicum* L (18).
55	sinapinic acid	$C_{11}H_{12}O_5$	*Portulaca oleracea* L (80); *Dimocarpus longan* Lour (48); *Hordeum vulgare* L (29); *Morus alba* L. (fruit) (82); *Alpinia oxyphylla* Miq (91); *Nelumbo nucifera* Gaertn. (leaf) (36); *Brassica juncea* (L.) Czern.et Coss (111); *Perilla frutescens* (L.) Britt. (leaf) (38); *Sesamum indicum* L (18); *Crocus sativus* L (41); *Curcuma longa* L (26); *Houttuynia cordata* Thunb (31); *Morus alba* L.(leaf) (76)
Hydrogenated hydroxycinnamic acids			
56	p-hydroxyphenylpropionic acid	$C_9H_{10}O_3$	*Mentha haplocalyx* Briq (112).

(Continued)

10.3389/fimmu.2024.1345002

TABLE 2 Continued

No.	Components	Molecular Formula	MEHPs
Hydrogenated hydroxycinnamic acids			
57	3-(2,4-dihydroxyphenyl) propionic acid	$C_9H_{10}O_4$	*Lycium barbarum* L (17).
58	dihydrocaffeic acid	$C_9H_{10}O_4$	*Eucommia ulmoides* Oliv (57); *Prunella vulgaris* L (113).
59	p-hydroxyphenyl-lactic	$C_9H_{10}O_4$	*Hippophae rhamnoides* L (28); *Angelica sinensis* (Oliv.) Diels (83); *Dendrobium officinale* Kimura et Migo (114)
60	danshensu	$C_9H_{10}O_5$	*Mentha haplocalyx* Briq (105); *Prunella vulgaris* L (113).
61	dihydroferulic acid	$C_{10}H_{12}O_4$	*Prunella vulgaris* L (113); *Panax quinquefolium* L (27).
62	(±)3-{2-[1-(3′,4′-dihydroxy-phenyl)ethyl]-4,5-dihydroxyphenyl} propanoic acid	$C_{17}H_{18}O_6$	*Eucommia ulmoides* Oliv (57).
Polyhydroxycinnamic acids			
63	1-O-caffeoylquinic acid	$C_{16}H_{18}O_9$	*Chrysanthemum morifolium* Ramat (115); *Morus alba* L. (leaf) (116)
64	2-O-caffeoylglucarate	$C_{15}H_{16}O_{11}$	*Phyllanthus emblica* L (71).
65	2-O-caffeoylhydroxycitric acid	$C_{15}H_{14}O_{11}$	*Phyllanthus emblica* L (71).
66	chlorogenic acid	$C_{16}H_{18}O_9$	*Cirsium setosum* (Willd.) MB (97); *Crataegus pinnatifida* Bge (59); *Portulaca oleracea* L (70); *Prunus mume* (Sieb.) Sieb. et Zucc (89); *Chaenomeles speciosa* (Sweet) Nakai (117); *Dimocarpus longan* Lour (48); *Cinnamomum cassia* Presl (118); *Phyllanthus emblica* L (71); *Citrus medica* L (72); *Prunus armeniaca* L (119); *Hippophae rhamnoides* L (98); *Zanthoxylum bungeanum* Maxim (120); *Vigna umbellata* Ohwi et Ohashi (58); *Vigna angularis* Ohwi et Ohashi (58); *Ziziphus jujuba* Mill (73); *Lonicera japonica* Thunb (99); *Houttuynia cordata* Thunb (121); *Lycium barbarum* L (74); *Gardenia jasminoides* Ellis (51); *Morus alba* L. (fruit) (34); *Morus alba* L. (leaf) (53); *Citrus reticulata* Blanco (77); *Alpinia oxyphylla* Miq (91); *Nelumbo nucifera* Gaertn. (fruit) (47); *Nelumbo nucifera* Gaertn. (leaf) (36); *Lophatherum gracile* Brongn (122); *Chrysanthemum morifolium* Ramat (102); *Cichorium intybus* L (123); *Perilla frutescens* (L.) Britt. (leaf) (63); *Sesamum indicum* L (18); *Piper nigrum* L (43); *Taraxacum mongolicum* Hand.-Mazz (104); *Mentha haplocalyx* Briq (105); *Panax ginseng* C.A.Mey (44); *Lonicera hypoglauca* Miq (110); *Lonicera macranthoides* Hand.-Mazz (107); *Lonicera fulvotomentosa* Hsu et S.C.Cheng; *Coriandrum sativum* L (124); *Prunella vulgaris* L (55); *Angelica sinensis* (Oliv.) Diels (83); *Crocus sativus* L (41); *Astragalus membranaceus* (Fisch.) Bge.var.mongholicus (Bge.) Hsiao (125); *Eucommia ulmoides* Oliv (57); *Codonopsis pilosula* (Franch.) Nannf (66).
67	neochlorogenic acid	$C_{16}H_{18}O_9$	*Crataegus pinnatifida* Bge (59); *Prunus mume* (Sieb.) Sieb. et Zucc (89); *Gardenia jasminoides* Ellis (51); *Mosla chinensis* 'jiangxiangru' (52); *Morus alba* L. (fruit) (34); *Morus alba* L. (leaf) (90); *Lophatherum gracile* Brongn (122); *Chrysanthemum morifolium* Ramat (102); *Cichorium intybus* L (37); *Lonicera fulvotomentosa* Hsu et S.C.Cheng (124); *Angelica sinensis* (Oliv.) Diels (83); *Lonicera japonica* Thunb (99).
68	cryptochlorogenic acid	$C_{16}H_{18}O_9$	*Lonicera hypoglauca* Miq (106); *Morus alba* L. (fruit) (126); *Lonicera japonica* Thunb (99); *Morus alba* L. (leaf) (90); *Chrysanthemum morifolium* Ramat (102); *Cichorium intybus* L (123); *Sesamum indicum* L (18); *Angelica sinensis* (Oliv.) Diels (83).
69	p-coumaroyl quinic acid	$C_{16}H_{18}O_8$	*Alpinia oxyphylla* Miq (91).
70	2-{[3-(3,4-dihydroxyphenyl) propanoyl]oxy} propanoic acid	$C_{12}H_{14}O_6$	*Eucommia ulmoides* Oliv (57).
71	3-O-feruloylquinic acid	$C_{17}H_{20}O_9$	*Lophatherum gracile* Brongn (101).
72	3-O-coumaroylquinic acid	$C_{16}H_{18}O_8$	*Lophatherum gracile* Brongn (122); *Sesamum indicum* L (18); *Alpinia oxyphylla* Miq (91).
73	3-O-sinapoylquinic acid	$C_{18}H_{22}O_{10}$	*Piper nigrum* L (43).
74	4-O-feruloylquinic acid	$C_{17}H_{20}O_9$	*Lophatherum gracile* Brongn (101); *Cichorium intybus* L (37).

(Continued)

10.3389/fimmu.2024.1345002

TABLE 2 Continued

No.	Components	Molecular Formula	MEHPs
Polyhydroxycinnamic acids			
75	4-O-coumaroylquinic acid	$C_{16}H_{18}O_8$	*Lophatherum gracile* Brongn (122); *Sesamum indicum* L (18).
76	5-O-coumaroylquinic acid	$C_{16}H_{18}O_8$	*Alpinia oxyphylla* Miq (91); *Lophatherum gracile* Brongn (122); *Sesamum indicum* L (18).
77	5-O-feruloylquinic acid	$C_{17}H_{20}O_9$	*Sesamum indicum* L (18).
78	5-O-sinapoylquinic acid	$C_{18}H_{22}O_{10}$	*Chrysanthemum morifolium* Ramat (115).
79	caffeoylmalic acid	$C_{13}H_{12}O_8$	*Phyllanthus emblica* L (71).
80	caftaric acid	$C_{13}H_{12}O_9$	*Phyllanthus emblica* L (71); *Cichorium intybus* L (123); *Taraxacum mongolicum* Hand.-Mazz (104).
81	feruloyl tartaric acid	$C_{14}H_{14}O_8$	*Piper nigrum* L (43).
82	p-coumaroyl glycolic acid	$C_{11}H_{10}O_5$	*Piper nigrum* L (43).
83	p-coumaroyl malic acid	$C_{13}H_{12}O_7$	*Piper nigrum* L (43); *Alpinia oxyphylla* Miq (91).
84	P-coumaroyl tartaric acid	$C_{13}H_{12}O_8$	*Perilla frutescens* (L.) Britt. (leaf) (127); *Piper nigrum* L (43).
85	piscidic acid	$C_{11}H_{12}O_7$	*Angelica sinensis* (Oliv.) Diels (83).
86	rosmarinic acid	$C_{18}H_{16}O_8$	*Vigna umbellata* Ohwi et Ohashi (58); *Mosla chinensis* 'jiangxiangru' (52); *Morus alba* L. (leaf) (76); *Nelumbo nucifera* Gaertn. (fruit) (47); *Perilla frutescens* (L.) Britt. (leaf) (103); *Perilla frutescens* (L.) Britt. (fruit) (64); *Piper nigrum* L (43); *Pogostemon cablin* (Blanco) Benth (128); *Coriandrum sativum* L (45); *Prunella vulgaris* L (108).
87	p-coumaroylcaffeoyltartaric acid	$C_{22}H_{18}O_{11}$	*Sesamum indicum* L (18).
88	cichoric acid	$C_{22}H_{18}O_{12}$	*Cichorium intybus* L (123); *Taraxacum mongolicum* Hand. Mazz (104).
89	avenanthramide 2f	$C_{17}H_{15}NO_6$	*Piper nigrum* L (43).
90	4-O-caffeoyl-5-O-feruloylquinic acid	$C_{26}H_{26}O_{12}$	*Chrysanthemum morifolium* Ramat (115).
91	4,5-di-O-p-coumaroylquinic acid	$C_{25}H_{24}O_{10}$	*Lonicera hypoglauca* Miq (106).
92	4,5-di-O-caffeoylquinic acid	$C_{25}H_{24}O_{12}$	*Chrysanthemum morifolium* Ramat (102); *Cichorium intybus* L (129).
93	3-O-methylrosmarinic acid	$C_{19}H_{18}O_8$	*Piper nigrum* L (43).
94	3-O-methoxyoxaloyl-1,5-di-O-caffeoylquinic acid	$C_{28}H_{26}O_{15}$	*Chrysanthemum morifolium* Ramat (115).
95	3′-dehydroxylation rosmarinic acid	$C_{18}H_{16}O_7$	*Perilla frutescens* (L.) Britt. (leaf) (130)
96	3,5-di-O-p-coumaroylquinic acid	$C_{24}H_{23}O_{10}$	*Lonicera hypoglauca* Miq (106).
97	3,5-di-O-caffeoylquinic acid	$C_{25}H_{24}O_{12}$	*Lonicera japonica* Thunb (99); *Morus alba* L. (fruit) (126); *Chrysanthemum morifolium* Ramat (102); *Cichorium intybus* L (129); *Taraxacum mongolicum* Hand.-Mazz (104); *Lonicera fulvotomentosa* Hsu et S.C.Cheng (131); *Morus alba* L. (leaf) (53)
98	3,4-di-O-caffeoylquinic acid	$C_{25}H_{24}O_{12}$	*Lonicera japonica* Thunb (99); *Chrysanthemum morifolium* Ramat (102); *Lonicera fulvotomentosa* Hsu et S.C.Cheng (131); *Gardenia jasminoides* Ellis (132)
99	1,5-di-O-caffeoylquinic acid	$C_{25}H_{24}O_{12}$	*Morus alba* L. (fruit) (82); *Morus alba* L. (leaf) (53)
100	1,4-di-O-caffeoylquinic acid	$C_{25}H_{24}O_{12}$	*Lonicera japonica* Thunb (133).

(Continued)

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TABLE 2 Continued

No.	Components	Molecular Formula	MEHPs
Polyhydroxycinnamic acids			
101	caffeoyl-ferulic acid	$C_{19}H_{16}O_7$	*Morus alba* L. (fruit) (82)
102	rosmarinic acid decarboxylation	$C_{18}H_{16}O_7$	*Prunella vulgaris* L (113).
103	1,3,5-tricaffeoylquinic acid	$C_{34}H_{30}O_{15}$	*Morus alba* L. (leaf) (134)
104	3,4,5-tricaffeoylquinic acid	$C_{34}H_{30}O_{15}$	*Morus alba* L. (leaf) (134); *Chrysanthemum morifolium* Ramat (115).
105	salvianolic acid A	$C_{26}H_{22}O_{10}$	*Angelica sinensis* (Oliv.) Diels (83).
106	salvianolic acid B	$C_{36}H_{30}O_{16}$	*Angelica sinensis* (Oliv.) Diels (83); *Mentha haplocalyx* Briq (105).
107	salvianolic acid C	$C_{26}H_{20}O_{10}$	*Angelica sinensis* (Oliv.) Diels (83).
108	salvianolic acid L	$C_{36}H_{30}O_{16}$	*Angelica sinensis* (Oliv.) Diels (83); *Mentha haplocalyx* Briq (105).
Hydroxycinnamates			
109	methyl caffeate	$C_{10}H_{10}O_4$	*Prunella vulgaris* L (113).
110	methyl (2R,3S)-2,3-dihydroxy-3-(4-methoxyphenyl) propanoate	$C_{11}H_{14}O_6$	*Kaempferia galanga* L (40).
111	ethyl (2R,3S)-2,3-dihydroxy-3-(4-methoxyphenyl) propanoate	$C_{12}H_{16}O_6$	*Kaempferia galanga* L (40).
112	trans ethyl p-methoxycinnamate	$C_{11}H_{12}O_3$	*Kaempferia galanga* L (40).
113	sinapine	$C_{16}H_{24}NO_5$	*Brassica juncea* (L.) Czern.et Coss (111)
114	methyl rosmarinate	$C_{19}H_{18}O_8$	*Perilla frutescens* (L.) Britt. (fruit) (64); *Perilla frutescens* (L.) Britt. (leaf) (103); *Prunella vulgaris* L (113).
115	p-hydroxyphenethyl trans-ferulate	$C_{18}H_{18}O_5$	*Angelica sinensis* (Oliv.) Diels (135).
116	p-coumaric acid methyl este	$C_{10}H_{10}O_3$	*Sesamum indicum* L (18); *Cannabis sativa* L (109).
117	p-coumaric acid ethyl ester	$C_{11}H_{12}O_3$	*Sesamum indicum* L (18).
118	methyl coumaroyl quinic acid	$C_{17}H_{20}O_8$	*Morus alba* L. (fruit) (82)
119	methyl 3,5-di-O-caffeoylquinate	$C_{26}H_{26}O_{12}$	*Lonicera fulvotomentosa* Hsu et S.C.Cheng (131); *Lonicera japonica* Thunb (133).
120	methyl 3,4-di-O-caffeoylquinate	$C_{26}H_{26}O_{12}$	*Lonicera fulvotomentosa* Hsu et S.C.Cheng (131);
121	methyl 3-(3,4-dihydroxyphenyl)-propanoate	$C_{10}H_{12}O_4$	*Eucommia ulmoides* Oliv (57).
122	methyl (2R,3S)-2,3-dihydroxy-3-(4-methoxyphenyl) propanoate	$C_{11}H_{14}O_5$	*Kaempferia galanga* L (136).
123	ethyl(2R,3S)-2,3-dihydroxy-3-(4-	$C_{12}H_{16}O_5$	*Kaempferia galanga* L (136).

(Continued)

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TABLE 2 Continued

No.	Components	Molecular Formula	MEHPs
Hydroxycinnamates			
	methoxyphenyl) propanoate		
124	ethyl rosmarinate	$C_{20}H_{20}O_8$	*Prunella vulgaris* L (87).
125	ethyl caffeate	$C_{11}H_{12}O_4$	*Lonicera fulvotomentosa* Hsu et S.C.Cheng (137); *Prunella vulgaris* L (113).
126	dihydroconiferyldihydro-p-coumarate	$C_{19}H_{22}O_5$	*Dendrobium officinale* Kimura et Migo (138);
127	caftaric acid monomethyl ester	$C_{14}H_{14}O_9$	*Cornus officinalis* Sieb. et Zucc (56)
128	cis ethyl p-methoxycinnamate	$C_{12}H_{14}O_3$	*Kaempferia galanga* L (40).
129	angeliferulate	$C_{21}H_{24}O_8$	*Angelica sinensis* (Oliv.) Diels (135).
130	butyl rosmarinate	$C_{22}H_{24}O_8$	*Prunella vulgaris* L (87).
131	3,4,α-trihydroxy-methyl phenylpropionate	$C_{10}H_{12}O_5$	*Prunella vulgaris* L (87).
132	2-{[3-(3,4-dihydroxyphenyl) propanoyl]oxy} propanoic acid methyl	$C_{13}H_{16}O_6$	*Eucommia ulmoides* Oliv (57).
133	3,4,α-trihydroxy-butyl phenylpropionate	$C_{13}H_{18}O_5$	*Prunella vulgaris* L (87).
134	(±)3-{2-[1-(3′,4′-dihydroxy-phenyl)ethyl]-4,5-dihydroxyphenyl} propanoic acid methyl	$C_{18}H_{20}O_6$	*Eucommia ulmoides* Oliv (57).
135	(Z)-methyl p-hydroxycinnamate	$C_{10}H_{10}O_3$	*Cannabis sativa* L (109).
136	caffeoyltartaric acid dimethyl ester	$C_{15}H_{16}O_9$	*Cornus officinalis* Sieb. et Zucc (79)
Hydroxycinnate glycosides			
137	caffeoylglucose	$C_{15}H_{18}O_9$	*Sterculia lychnophora* Hance (33); *Morus alba* L. (fruit) (82)
138	1-O-[(E)-p-Coumaroyl]-D-glucose	$C_{15}H_{18}O_8$	*Alpinia oxyphylla* Miq (91).
139	sinapic acid glucoside	$C_{17}H_{22}O_{10}$	Alpinia oxyphylla Miq (91); *Nelumbo nucifera* Gaertn. (fruit) (139)
140	3'-dehydroxyl-rosmarinic acid-3-o-β-D-glucoside	$C_{23}H_{24}O_{12}$	*Perilla frutescens* (L.) Britt. (fruit) (64)
141	6-O-feruloyl-D-glucose	$C_{16}H_{20}O_9$	*Alpinia oxyphylla* Miq (91).
142	caffeic acid 4-O-glucoside	$C_{15}H_{18}O_9$	*Chrysanthemum morifolium* Ramat (115).
143	caffeic acid dihexoside	$C_{21}H_{28}O_{14}$	*Codonopsis pilosula* (Franch.) Nannf (66).
144	caffeic acid trihexoside	$C_{27}H_{38}O_{19}$	*Codonopsis pilosula* (Franch.) Nannf (66).
145	caffeic acid-3-O-glucoside	$C_{15}H_{18}O_9$	*Phyllanthus emblica* L (71); *Piper nigrum* L (43); *Perilla frutescens* (L.) Britt. (fruit) (64)
146	codonosides A	$C_{38}H_{48}O_{20}$	*Codonopsis tangshen* Oliv (140).
147	codonosides B	$C_{38}H_{48}O_{20}$	Codonopsis tangshen Oliv (140).
148	coumaroylglucose	$C_{15}H_{18}O_8$	*Perilla frutescens* (L.) Britt. (leaf) (130)

(Continued)

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TABLE 2 Continued

No.	Components	Molecular Formula	MEHPs
Hydroxycinnate glycosides			
149	dihydroferulic acid hexoside	$C_{16}H_{22}O_9$	*Codonopsis pilosula* (Franch.) Nannf (66).
150	ferulic acid 4-O-glucoside	$C_{16}H_{20}O_9$	*Morus alba* L. (fruit) (82); *Sesamum indicum* L (18); *Piper nigrum* L (43).
151	regaloside B	$C_{20}H_{26}O_{11}$	*Lilium lancifolium* Thunb (141).
152	regaloside C	$C_{18}H_{24}O_{11}$	*Lilium lancifolium* Thunb (141).
153	regaloside E	$C_{18}H_{24}O_{10}$	*Lilium lancifolium* Thunb (141).
154	rosmarinic acid glucuronide	$C_{24}H_{26}O_{13}$	*Prunella vulgaris* L (113).
155	salviaflaside	$C_{24}H_{26}O_{13}$	*Perilla frutescens* (L.) Britt. (leaf) (103); *Perilla frutescens* (L.) Britt. (fruit) (64); *Prunella vulgaris* L (142).
156	sinapate 4-O-*β*-D-glucopyranoside	$C_{17}H_{22}O_{10}$	*Nelumbo nucifera* Gaertn. (fruit) (35)
157	dihydroferulic glucuronide	$C_{16}H_{20}O_{10}$	*Prunella vulgaris* L (113).
Hydroxycinnate salts			
158	caffeic acid 3-sulfate	$C_9H_8O_7S$	*Piper nigrum* L (43).

glycoside. There are only 10 MEHPs reported. In addition, another type of phenolic acid was found in Piper nigrum L.: 5-(3',4'-dihydroxyphenyl)-valeric acid. Details are shown in Table 3 and the structure is shown in Figure 3.

We found that 5 MEHPs contain more than 20 phenolic acids: *Morus alba* L. (fruit) (26), *Piper nigrum* L (23), *Prunella vulgaris* L (23), *Sesamum indicum* L (22), *Perilla frutescens* (L.) Britt. (leaf) (20). Among these, *Prunella vulgaris* L. and *Perilla frutescens* (L.) Britt (leaf) belong to Lamiaceae, indicating that phenolic acids may be the main active compounds in Lamiaceae plants. Among the 167 identified MEHP phenolic acids, hydroxycinnamic acids were the most numerous and widely distributed, with chlorogenic acid present in 43 MEHPs, highlighting its accessibility and potential for development.

3 Anti-inflammatory activity and mechanism of MEHP phenolic acids

Recognizing the pivotal role of inflammatory response in inflammatory diseases, anti-inflammatory drugs occupy a central position in their management and treatment. The intricate relationship between the anti-inflammatory mechanism and inflammatory diseases underscores their interconnectedness. Presently, the anti-inflammatory drugs available in the market primarily function through various pathways, including nuclear factor- kappa B (NF-κB), mitogen activated protein kinase (MAPK), NOD-like receptor protein 3(NLRP3), nuclear factor E2-related factor 2 (Nrf2), toll-like receptors (TLRs), and interleukin-17 (IL-17). Additionally, the regulation of gut microbiota and immune response mechanisms contribute significantly to their effectiveness. Notably, MEHPs phenolic acids exhibit remarkable anti-inflammatory activity, as evidenced in numerous studies on inflammatory diseases. Their diverse anti-inflammatory mechanisms of action offer promising potential for further development.

3.1 NF-κB pathway

Nuclear factor- kappa B (NF-κB) is an important nuclear transcription factor in cells, formed by dimerization of Rel proteins (p50, p52, p65, c-Ral, and RalB). NF-κB pathway consists of canonical and non-canonical pathways. (1) Canonical: NF-κB (p65/p50) and inhibitor of NF-κB (IκBα) are bound in the cytoplasm with an inactive dimer. When subjected to reactive-oxygen species (ROS), toll-like receptors (TLRs), interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α), inhibitor of κB kinase (IKKβ) is activated, then IκB is degraded, and p65/p50 dimer is dissociated. Subsequently, p65 is phosphorylated and translocated to the nucleus to activate the target genes, inducing the transcription of TNF-α, IL-1β, and interleukin 6 (IL-6) (143). (2) Noncanonical: RalB binds to p100 as inactive dimer in the cytoplasm. Lymphotoxin β(LTβ), B cell activating factor (BAFF), and tumor necrosis factor receptor superfamily member 5(CD40) stimulate the accumulation of NF-κB-inducing kinase (NIK) and activate IKKα. Then, p100 is degraded to p52, and the RalB/p52 dimer is translocated to the nucleus, to induce the transcription of related genes (144). MEHP phenolic acids can inhibit the NF-κB pathway by inhibiting canonical and non-canonical pathways (Figure 4).

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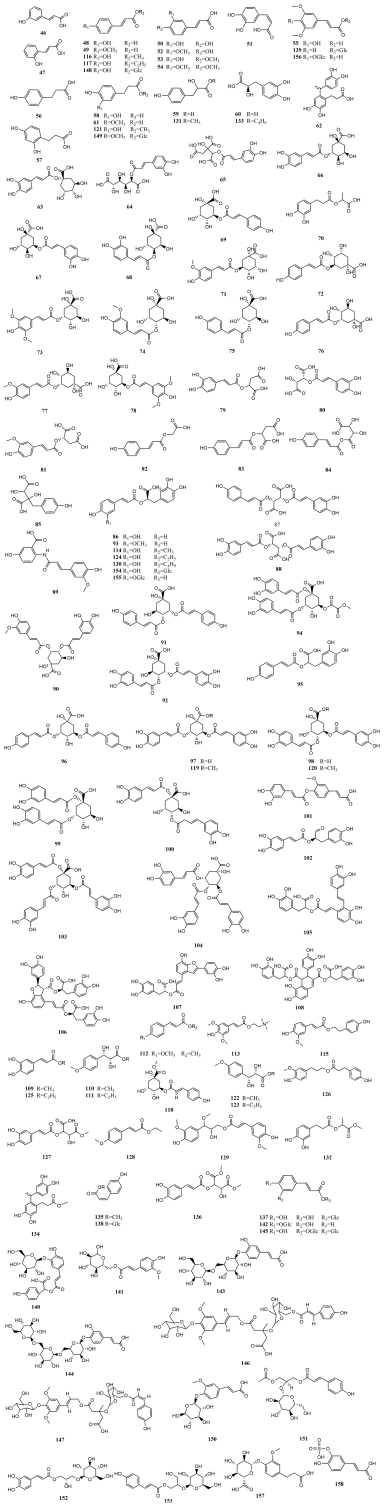

FIGURE 2
Structures of hydroxycinnamic acids from medicinal and edible homologous plants.

3.1.1 Inhibiting the canonical pathway

In canonical pathway, MEHP phenolic acid can inhibit the NF-kB pathway by suppressing the activation and nuclear translocation of NF-kB and blocking the binding of NF-kB to target genes.

TABLE 3 Hydroxyphenylacetic acids in medicinal and edible homologous plants.

No.	Components	Molecular Formula	MEHPs
Simple hydroxyphenylacetic acids			
159	m-hydroxymandelic acid	$C_8H_8O_4$	*Panax quinquefolium* L (27).
160	O-hydroxybenzene acetic acid	$C_8H_8O_3$	*Hippophae rhamnoides* L (98).
161	3,4-dihydroxyphenylacetic acid	$C_8H_8O_4$	*Sesamum indicum* L (18); *Piper nigrum* L (43).
162	homogentisic acid	$C_8H_8O_4$	*Perilla frutescens* (L.) Britt. (leaf) (63)
163	homovanillic acid	$C_9H_{10}O_4$	*Lycium barbarum* L (17); *Mentha haplocalyx* Briq (105).
Hydroxyphenylacetates			
164	4-hydroxyphenylacetic acid methyl ester	$C_9H_{10}O_3$	*Morus alba* L. (fruit) (85)
165	ethyl 3,4-dihydroxy-phenyl lactate	$C_{11}H_{14}O_5$	*Prunella vulgaris* L (87).
Hydroxyphenylacetate glycosides			
166	5,7-dihydroxy-4-((2R)-2-methylbutan-1-onyl)-phenylacetic acid 7-O-b-D-apiofuranosyl (1–3)-β-D-glucopyranoside	$C_{25}H_{33}O_{14}$	*Pogostemon cablin* (Blanco) Benth (128).
Others			
167	5-(3',4'-dihydroxyphenyl)-valeric acid	$C_{11}H_{14}O_4$	*Piper nigrum* L (43).

Various hydroxycinnamic acids with caffeic acid as the parent nucleus, such as caffeic acid, chlorogenic acid, salvianolic acid B, and rosmarinic acid, have inhibitory effects on multiple links of canonical NF-kB pathway. Caffeic acid inhibits phosphorylation of IKKα/β and IκBα to inhibit activation of NF-κB, playing a key role in anti-rheumatoid arthritis (145), it can also inhibit nuclear translocation of p-p65, and alleviating inflammation to protect ischemia/reperfution(I/R)-injury in rats (25). Chlorogenic acid inhibits phosphorylation of IκBα and the p65 protein levels to interfere with NF-κB pathway, showing anti-arthritis (146) and anti-mastitis (147) effects. Meanwhile, it inhibits nuclear translocation of p-p65 to block NF-κB signaling pathway alleviating LPS-induced inflammation in Caco-2 (148), RAW 264.7 (149), and rat hepatic stellate cells (150). salvianolic acid B inhibits activation of NF-κB pathway by inhibiting the phosphorylation of p65 (151), alleviating inflammation of arthritis mouse, it can also reduce the release of TNF-α, IL-1β, and IL-6 by inhibiting nuclear translocation of p65 producing anti-

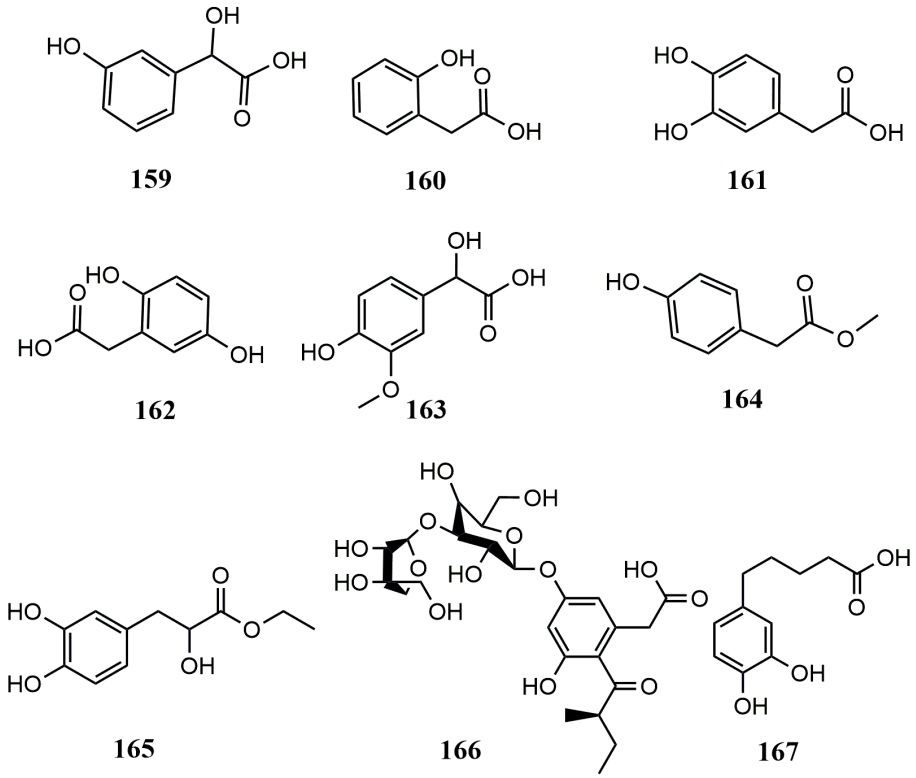

FIGURE 3
Structures of hydroxyphenylacetic acids from medicinal and edible homologous plants.

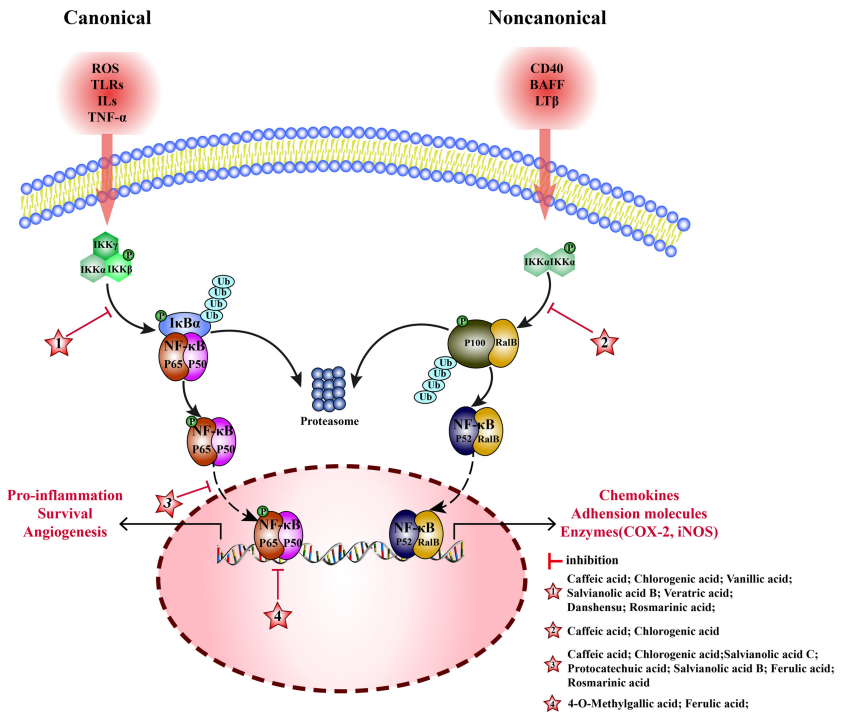

FIGURE 4
Molecular mechanism of the anti-inflammatory activity of phenolic acids from medicinal and edible homologous plants against the NF-κB pathway.

atherosclerotic effect (152). In an inflammatory model of human skin fibroblasts (HSF) induced by TNF-α, rosmarinic acid has been shown to inhibit the phosphorylation and degradation of IκBα and the activation of NF-κB (153). Additionally, rosmarinic acid can alleviate acute pancreatitis induced by sodium taurocholate by inhibiting the nuclear translocation of p65 (154). In LPS-induced acute kidney injury in mice, ferulic acid can inhibit inflammation by inhibiting nuclear translocation of p65 (155). Moreover, ferulic acid cuts off the combination between p-NF-κB and the transcription factor cAMP-response element binding protein(CREB), inhibiting NF-κB binding to DNA, helping repair acute liver injury induced by cecal ligation perforation (CLP) in mice (156).

Furthermore, two additional hydroxycinnamic acids have also been reported to have inhibitory effects on the canonical NF-kB pathway. Danshensu reduces expression of p-IKKα/β, p-IκBα, and p-p65, and upregulates expression of IκBα to alleviate chronic kidney disease in mice (157). Salvianolic acid C blocks NF-κB signaling pathway by inhibiting nuclear translocation of p-p65 and suppresses inflammation in BV2 cells induced by LPS (158).

Four hydroxybenzoic acids also possess inhibitory effects on the canonical NF-kB pathway. Vanillic acid inhibits phosphorylation IκBα, alleviating inflammation of chondrocytes in patients with arthritis (159). In acute lung injury mice, veratric acid inhibits the phosphorylation of IκB and p65, regulating the NF-κB signaling pathway to alleviate inflammatory damage induced by LPS (160). Protocatechuic acid inhibits nuclear translocation of p65, protein and mRNA expression of TNF- α, IL-1 β, and IL-6 in SH-SY5Y cells, and promotes repair after cerebral hemorrhage in mice (161). 4-O-methylgallic acid can modify the DNA binding domain of NF-κB to directly block NF-κB binding with DNA in the nucleus, thereby inhibiting leukocyte adhesion to endothelial cells and preventing vascular inflammation (162).

3.1.2 Inhibiting the noncanonical pathway

In noncanonical pathways, an accumulation of NIK promotes phosphorylation of IKKα, activating NF-κB (RalB/p52); therefore, NIK is a key kinase. Two hydroxycinnamic acids belonging to the caffeic acid category have been found to exert inhibitory effects on the noncanonical NF-kB pathway. Caffeic acid inhibits phosphorylation of NIK and IKK and the activation of noncanonical NF-κB pathway, alleviating inflammation in endothelial cells (163). Chlorogenic acid inhibits the expression of RalB and p52 to exert anti- liver cancer effects (164).

In comparison to non-canonical pathways, MEHP phenolic acids exert a more pronounced inhibitory effect on canonical pathways. Seven hydroxycinnamic acids and four hydroxybenzoic acids possess inhibitory effects on the non-pharmacological pathway, demonstrated across various models and conditions. Notably, four caffeic acid-like hydroxycinnamic acids are capable of simultaneously targeting diverse stages of the canonical pathway to alleviate inflammatory conditions, including NF-kB activation, nuclear translocation, and binding to target genes. Furthermore, two types of hydroxycinnamic acid, both belonging to the caffeic acid category, can inhibit the non-pharmacological pathway.

3.2 MAPK pathway

Mitogen activated protein kinase (MAPK) is a serine threonine protein kinase. The MAPK pathway is composed of a tertiary kinase pattern, including mitogen-activated protein kinase kinase kinase (MKKKs), mitogen-activated protein kinase kinases (MKKs), and MAPKs. MAPKs comprises four subfamilies: extracellular regulated protein kinases (ERK), mitogen-activated protein kinase p38 (p38), c-Jun N-terminal kinase (JNK), and extracellular regulated protein kinases 5 (ERK5), and these pathways are named accordingly. Among them, ERK, p38, and JNK are the three canonical MAPK pathways, which are closely associated with inflammation. MEHP phenolic acids can inhibit the MAPK pathway and exert anti-inflammatory activity by inhibiting the activation of kinases (Figure 5).

3.2.1 Inhibiting MKKKs

The MKKKs family of Serine/threonine-protein kinase (Raf), encompassing A-Raf, B-Raf, and Raf1, plays a pivotal role in the activation of the ERK pathway. Concurrently, MKKKs such as serine/threonine-protein kinase RIM15 (TAK1), mitogen-activated protein kinase kinase kinase (MEKK), and mitogen-activated protein kinase kinase kinase 5 (ASK1) are instrumental in triggering the p38 and JNK pathways. Gentisic acid inhibits the expression of Raf in ankle and knee tissues and regulates Raf/ERK signaling, thus alleviating rheumatoid arthritis in rats (165). Caffeic acid exerts anti-gastritis effects by inhibiting interleukin-1 receptor-associated kinase 1 (IRAK1), interleukin-1 receptor-associated kinase 4 (IRAK4), and TAK1 by interfering with the JNK/MAPK pathway (166). Caffeic acid can inhibit the phosphorylation of c-Raf and the activation of ERK1/2, reduce the release of inflammatory factors, and exert a detoxifying effect on liver toxicity induced by acetaminophen(APAP) in mice (167). Ferulic acid alleviates LPS-induced inflammation of RAW 264.7 cells by inhibiting the phosphorylation of TAK1, interfering with the p38/MAPK pathway to inhibit the activation of NF-κB (168).

3.2.2 Inhibiting MKKs

The three canonical MAPK pathways correspond to distinct MKKs, the ERK pathway is associated with MEK, the p38 pathway aligns with MKK3/6, and the JNK pathway is linked to MKK4/7. Two hydroxycinnamic acids, with caffeic acid serving as their central component, exert a significant inhibitory influence on MKKs. Caffeic acid can exert anti-gastritis effects by inhibiting MKK4/7 to inhibit the JNK/MAPK pathway (166). Salvianolic acid A can effectively mitigate the inflammatory response in the lungs of patients suffering from acute lung injury by suppressing LPS-induced phosphorylation of MEK, and ERK within the lung tissue (169).

3.2.3 Inhibiting MAPKs

In mice with colitis, chlorogenic acid reduces the expression ERK1/2, p-ERK, p38, p-p38, JNK, p-JNK, p-IκB, and p-p65 in tissues, blocks the ERK/JNK pathway, and reduces symptoms of

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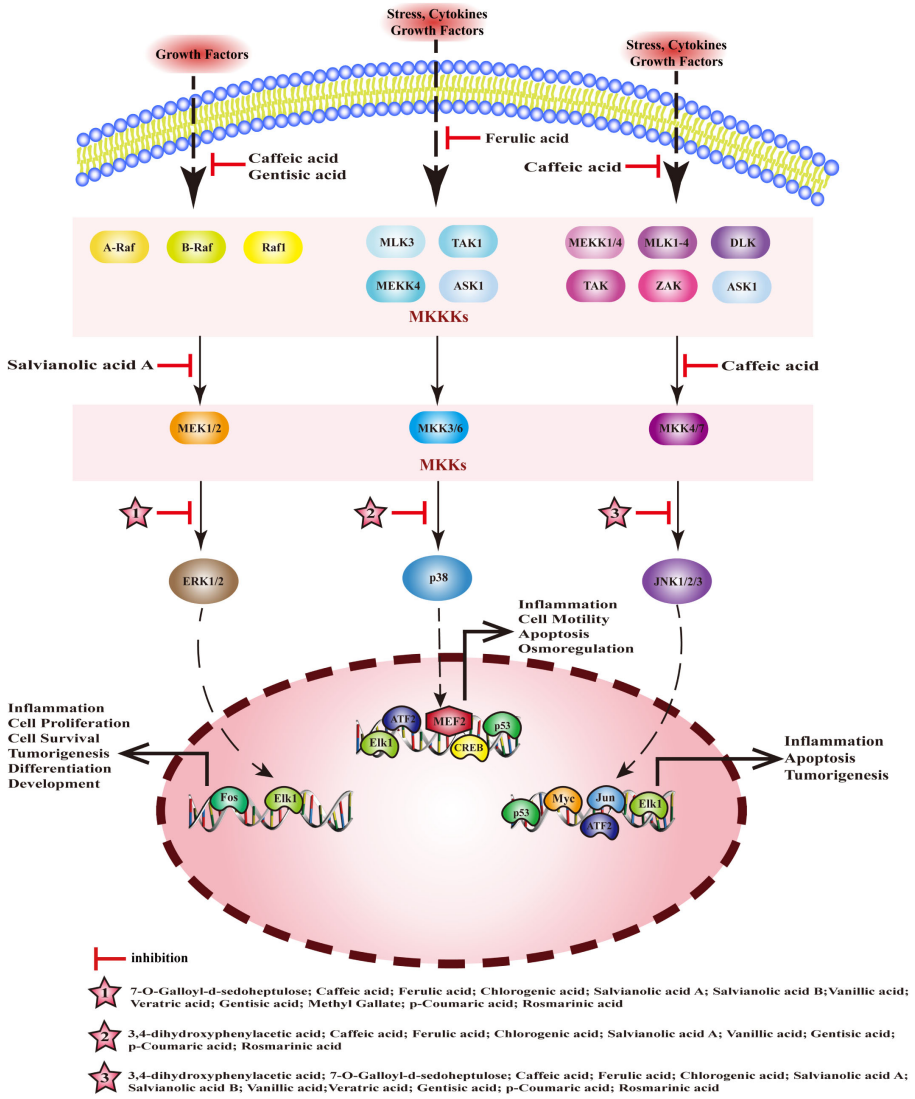

FIGURE 5
Molecular mechanism of the anti-inflammatory activity of phenolic acids from medicinal and edible homologous plants against the MAPK pathway.

colitis (170). In rats with arthritis, p-coumaric acid promotes the inactivation of MAPK pathway, inhibits inflammation, cartilage degeneration, and osteoclast formation by downregulating the expression of JNK, p-JNK, and ERK1/2[187]; it can also inhibit the expression of p-p38/pJNK/pERK and p-IKKβ/p-IκB/NF-κB, block caspase-1/MAPK/NF-κB signaling cascade to inhibit the inflammation of activated mast cell and splenocyte[188].Ferulic acid can inhibit NF-κB pathway by reducing the phosphorylation of p38 and JNK, thereby preventing endometritis (171). Salvianolic acid A inhibits the activation of p38, JNK, and ERK, blocking the activation of MAPK pathways, and exerts anti-inflammatory effects in mice with arthritis (172). 3,4-dihydroxyphenylacetic acid inhibits inflammation and repairs the intestinal barrier dysfunction in mice with type 2 diabetes by inhibiting the activation of JNK and p38 (173). 7-O-galloyl-d-sedoheptulose can inhibit the activation of NF-κB and AP-1 and plays a key role in liver protection in type 2 diabetes by inhibiting phosphorylation of ERK1/2 and JNK (174). Caffeic acid significantly inhibits the expression of p-p38, regulates inflammation and apoptosis through p53 and p38/MAPK signaling pathways, and prevents atherosclerosis (175). In addition, it can inhibit the phosphorylation of JNK, p38, and c-Jun in a dose-dependent manner, and block phosphorylation of ERK1/2 to alleviate LPS-induced inflammation in bovine mammary epithelial cells (bMECs) (176). Salvianolic acid B can downregulate the expression of p-ERK and p-JNK, inhibit the transcription of inflammatory factors, and produce anti-pneumonia effects (177). Vanillic acid significantly reduces the levels of pERK, pJNK, and p-p38, regulates the NF-κB/MAPKs signaling pathway to alleviate the allergic inflammation of HMC-1 (178). Methyl gatellate can inhibit LPS-induced inflammation in mouse macrophages by inhibiting the activation of ERK (179). Rosmarinic acid can inhibit the activation of ERK, JNK, and p38, block MAPK/NF-κB signaling pathway to improve LPS-induced inflammation in vascular smooth muscle cells (180).

When it comes to inhibiting the MAPK pathway, MEHP phenolic acids demonstrate the most profound inhibitory effect on MAPKs. Among the compounds tested, seven hydroxycinnamic acids, three hydroxybenzoic acids, and one hydroxyphenylacetic acid all exhibit inhibitory effects on MAPKs, with most of them capable of suppressing multiple types of MAPKs. In terms of inhibiting MKKKs, one hydroxybenzoic acid and two hydroxycinnamic acids are effective, while two caffeic acid-like hydroxycinnamic acids specifically demonstrate an inhibitory effect on MKKs.

3.3 NLRP3 pathway

NOD-like receptor protein 3(NLRP3) is an inflammasome sensor protein, and the activation of NLRP3 can generate an oligomer complex "Inflammamasone", which includes apoptosis-associated speck-like protein containing CARD(ASC) and caspase-1. The activation of the typical NLRP3 inflammasome pathway requires two stages: Signal 1 (priming): upregulation of the protein expressions related to inflammasomes (including inflammasome sensor proteins, IL-1β, and IL-18) by upregulating the transcriptional activity of NF-κB. Signal 2 (activation): NLRP3 interacts with pro-caspase-1 after assembly with ASC, then produces a large amount of caspase-1, which catalyzes the dissociation of pro-IL-1β and pro-IL-18 and initiates inflammatory response. MEHP phenolic acids can exert anti-inflammatory activity by inhibiting the NLRP3 pathway; the mechanism is shown in Figure 6.

In acute gouty arthritis, ferulic acid can exert anti-inflammatory effects by inhibiting the activation of NLRP3 inflammasomes (181). Caffeic acid downregulates mRNA expression of IL-1β and IL-18 to reduce inflammatory reaction of human umbilical vein endothelial cell (HUVEC) induced by advanced glycation end products (AGEs) (182). Rosmarinic acid exerts anti-inflammatory effects by inhibiting the activation and assembly of NLRP3 inflammasomes in psoriasis (183), liver injury (184), and neuroinflammation (185). Vanillic acid can inhibit the activation of NLRP3 inflammasomes and the expression of IL-18 and IL-1β to alleviate arthritis in rats by downregulating the expression of caspase-1, ASC, and NLRP3 (186). Chlorogenic acid improves pneumonia induced by Klebsiella pneumoniae (187), and inhibits periodontal disease (188) by inhibiting activation of NLRP3 inflammasome. Methyl gallate can inhibit the assembly of NLRP3 inflammasome by blocking oligomerization of NLRP3 to alleviate the inflammatory response in mice with hyperuricemic nephropathy (189). Salvianolic acid B attenuates cell death mediated by endoplasmic reticulum stress, by inhibiting NLRP3 inflammasome and reducing the secretion of caspase-1, IL-1β, and IL-18 (190). Cichoric acid decreases the levels of NLRP3, IL-1β, caspase-1, ASC oligomer, and ASC monomer and the release of IL-1β and TNF-α, inhibiting the inflammation in THP-1-derived macrophages (THP-Ms) induced by monosodium urate (MSU) (191).

Reports indicate that two hydroxybenzoic acids and six hydroxycinnamic acids possess the ability to suppress the NLRP3 pathway. Notably, five of these hydroxycinnamic acids share caffeic acid as their common backbone, suggesting that caffeic acid-derived phenolic acids exert the most pronounced inhibitory effect on the NLRP3 pathway.

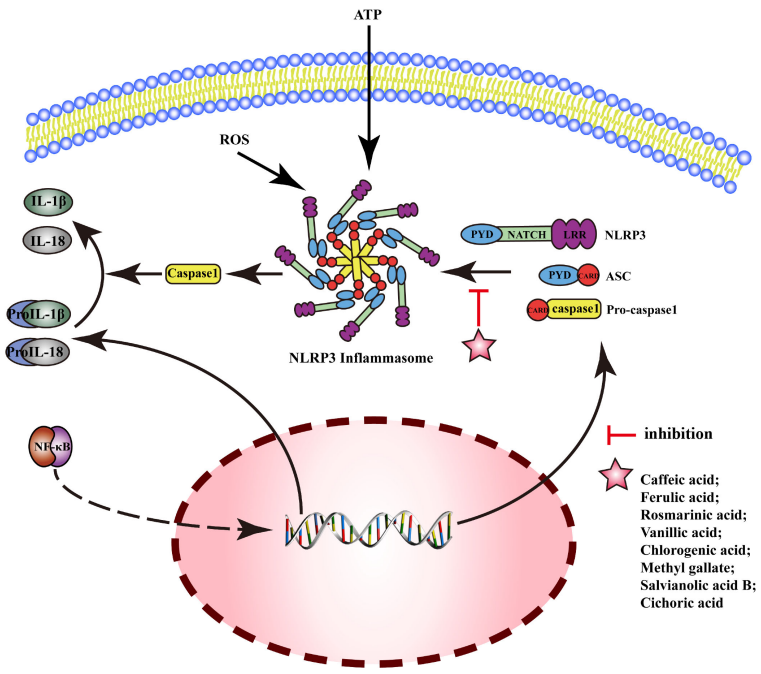

FIGURE 6
Molecular mechanism of the anti-inflammatory activity of phenolic acids from medicinal and edible homologous plants against the NLRP3 pathway.

3.4 Nrf2 pathway

Nuclear factor E2-related factor 2 (Nrf2) is a key transcription factor, that normally, binds to kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm, rapidly degrading under the action of ubiquitin proteasome pathway. When cells are stimulated by ROS or other nucleophilic agents, Nrf2 uncouples with Keap1 and is activated by phosphorylation. It is then transported into the nucleus where it competes with p65/p50 to activate the transcription factor CBP, inhibits the binding of p65/p50 to target genes and reduce the transcription of TNF-α, IL-1β, and IL-6 to inhibit the inflammatory response. Therefore, activation of Nrf2 and nuclear translocation of Nrf2 are key links in regulating Nrf2 pathway. The mechanism of MEHP phenolic acids exerting anti-inflammatory activity through Nrf2 pathway is shown in Figure 7.

3.4.1 Promoting the activation of Nrf2

Caffeic acid exerts an anti-hepatitis effect by upregulating the expression and phosphorylation of P62 (an autophagy substrate), promoting its binding and degradation with Keap1, inducing an increase in Nrf2 expression (192). Chlorogenic acid activates Nrf2/HO-1 pathway to alleviate oxidative stress and inflammatory response, repairs intestinal barrier, and effectively improves DSS-induced colitis (193). Gallic acid inhibits NF-κB pathway by binding to Keap1 and mediating Nrf2 activation, thus exerting anti-pneumonia effect (194). Rosmarinic acid can bind to Keap1, blocking the association between Keap1 and Nrf2 and activating Nrf2, thereby relieving bacterial pneumonia (195). Salvianolic acid A can directly bind to Keap1, promote the activation of Nrf2, and alleviate the inflammatory response in Schwann cells induced by high glucose (196).

3.4.2 Promoting nuclear translocation of Nrf2

3,4-dihydroxyphenylacetic acid can inhibit ethanol-induced hepatotoxicity by increasing Nrf2 protein expression and nuclear translocation (197). Ferulic acid increases the nuclear translocation of Nrf2 to inhibit LPS-induced inflammation in bMECs (198). Chlorogenic acid can improve ischemic brain injury (199), relieve endometritis (200), and regulate blood sugar (201) by increasing nuclear translocation of Nrf2 and inhibiting NF-κB pathway.

Among all the MEHP phenolic acids, hydroxycinnamic acid stands out for its remarkable promoting effect on the Nrf2 pathway. Specifically, five hydroxycinnamic acids, all belonging to the caffeic acid family, can enhance the activation of Nrf2. Furthermore, gallic acid, a hydroxybenzoic acid, also demonstrates a similar effect. Additionally, two hydroxycinnamic acids and one hydroxyphenylacetic acid contribute to the nuclear translocation of Nrf2. Notably, chlorogenic acid is unique in its ability to concurrently promote both the activation and nuclear translocation of Nrf2, thereby exerting significant anti-inflammatory effects.

3.5 TLRs pathway

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) that recognize microorganisms when they invade the body and activate immune responses. In general, TLRs mainly transduce signals through myeloid differentiation factor 88 (MyD88) or TIR-domain containing adaptor inducing interferon-β(TRIF) pathways. MyD88 signals induce the production of inflammatory factors (such as TNF, IL-6, IL-1β) and chemokines (such as C-C motif ligand 4, CCL4). MyD88 binds to TLRs and recruits IRAK4 and IRAK1/2 to Myddosome, which activates (TNF receptor-associated factor 6) TRAF6, induces the activation of NF-κB and MAPK pathways and the expression of proinflammatory cytokines (202). MEHP phenolic

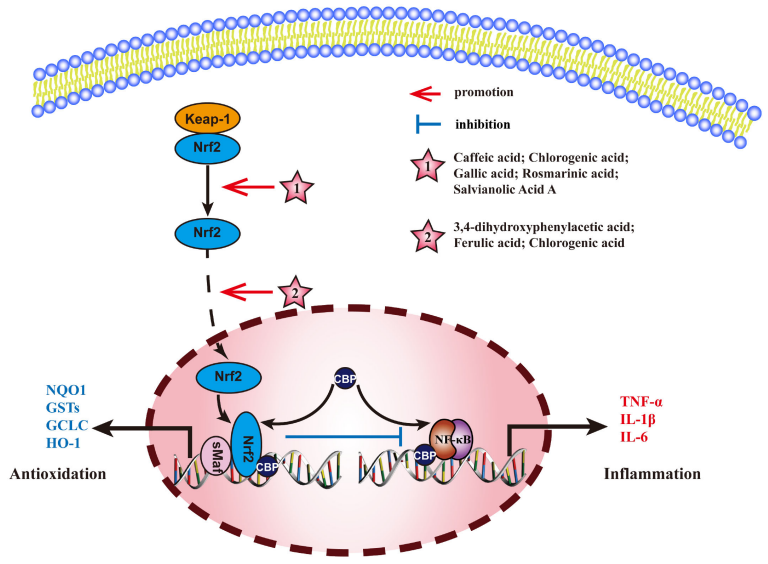

FIGURE 7
Molecular mechanism of the anti-inflammatory activity of phenolic acids from medicinal and edible homologous plants against the Nrf2 pathway.

acids exert anti-inflammatory effects mainly by interfering with the TLRs/MyD88 pathway (Figure 8).

For influenza A in mice (203), acute pancreatitis in rats (204), protocatechuic acid alleviates inflammatory response by reducing the activation of TLR4 and inhibiting NF-κB pathway. Rosmarinic acid can improve neuroinflammation after spinal cord injury (205), relieve hyperlipidemia (206), and inhibit mastitis (207) by inhibiting TLR4/MyD88-NF-κB pathway. Salvianolic acid B attenuates PM 2.5-induced tracheitis in mice by inhibiting TLR4, MyD88, and TRAF6, interfering MAPK pathway and blocking NRLP3 activation (208). Chlorogenic acid inhibits expression of TLR4 and MyD88, interferes with their downstream pathways to improve the intestinal barrier damage in weaned piglets (209), alleviates hepatitis in mice (210) and alcoholic hepatitis in rats (211), and reduces inflammation in mouse glial cells (BV2) (212) and human gingival fibroblasts (HGFs) (213) induced by LPS, and Escherichia coli-induced inflammation in sheep endometrial epithelial cells (SEECs) (214). Chlorogenic acid may also exert anti-inflammatory effects by interfering with other TLRs such as, by inhibiting TLR2/TLR9-Myd88 signaling pathway to attenuate the inflammatory response in herpes encephalitis (215), down-regulating expression of TLR2/4 to decrease activity of NF-κB signaling pathway in epidermal cells, and inhibiting skin inflammation in mice (216). Methyl gallate can inhibit the activation of TLR2 to inhibit NF-κB and MAPK pathway and alleviate toe swelling in mice (217).

Researchers have identified the anti-inflammatory potential of MEHP phenolic acids, primarily by modulating the TLR/MyD88 pathway, showcasing their efficacy in various inflammatory models. Two hydroxybenzoic acids and three caffeic acid based hydroxycinnamic acids exhibit inhibitory effects on the TLRs pathway. Specifically, protocatechuic acid, rosmarinic acid, and salvianolic acid B can suppress the TLR4 pathway, whereas methyl gallate demonstrates inhibitory action towards the TLR2 pathway. Remarkably, chlorogenic acid possesses the ability to simultaneously inhibit the TLR2, TLR4, and TLR9 pathways, thereby exerting anti-inflammatory effects in a diverse range of diseases. These findings signified a wide spectrum of potential MEHP phenolic acid-mediated therapeutic interventions targeting the TLR-mediated inflammatory pathways.

3.6 IL-17 pathway

Interleukin-17 (IL-17) is a potent pro-inflammatory cytokine, which binds to its receptor IL-17R and activates TRAF6 through Act1, leading to the triggering of NF-kB and MAPK pathways.

There are few reported MEHP phenolic acids that can regulate the IL-17 pathway, only three of which are hydroxycinnamic acid. Caffeic acid inhibits expression of IL-17 mRNA in intestinal tissue and alleviates DSS-induced colitis in mice (218). Ferulic acid inhibits secretion of IL-17 and blocks the combination of IL-17A and IL-17RA, thus improving skin inflammation in psoriatic mice (219). Rosmarinic acid can alleviate psoriasis-like dermatitis in mice by decreasing the differentiation of Th17 cells and inhibiting the expression of IL-17A (220) (Figure 9).

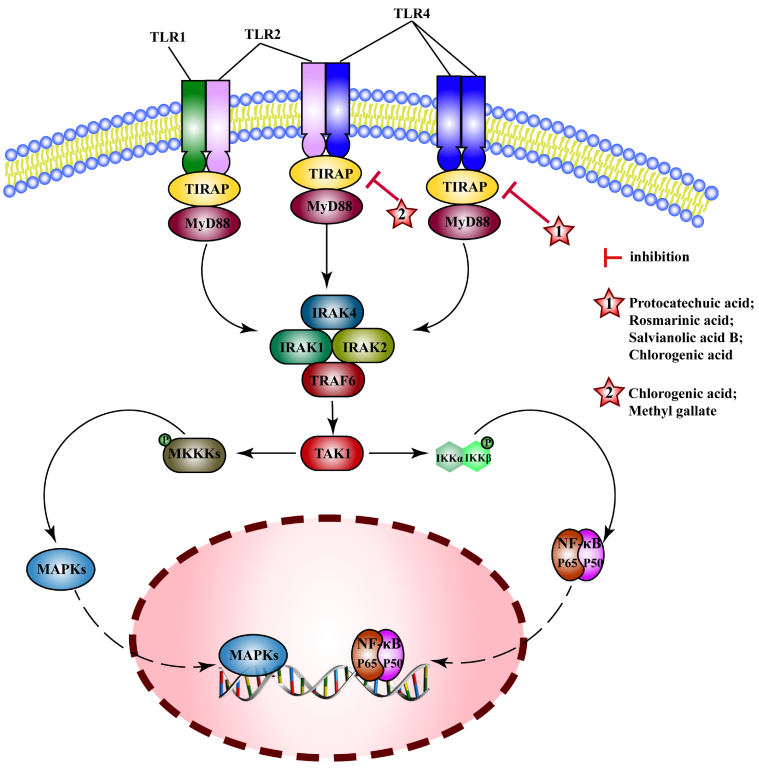

FIGURE 8
Molecular mechanism of the anti-inflammatory activity of phenolic acids from medicinal and edible homologous plants against the TLRs pathway.

3.7 Regulating intestinal microflora

Numerous studies show that intestinal microbial species are closely related to anti-inflammatory effects and the by-products of bacterial metabolism, including some short-chain fatty acids (SCFA), can play a role in inhibiting inflammation. Ferulic acid significantly increases intestinal SCFA producing bacteria, as *Olsenella, Eisenbergiella, Dubosiella, Clostridiales_unclassified*, and *Faecalibaculum*, reduces endotoxin-producing and obesity-related bacteria, and inhibits the intestinal barrier functional damage induced by a high-fat diet in mice (221). Chlorogenic acid increases the abundance of SCFA-producing bacteria, such as *Dubosiella, Romboutsia, Mucispirillum*, and *Faecalibaculum*, as well as *Akkermansia*, enhanced the integrity of the intestinal barrier, while successfully preventing glucose metabolic disorders and endotoxemia (222).

Gut microbiota abundance and richness are closely associated with inflammation. The increase of Firmicutes and the decrease of Bacteroidetes could inhibit the development of inflammation (223). Protocatechuic acid can enhance the diversity of cecal microbiota, decrease the occurrence of Bacteroidota, Proteobacteria, and *Escherichia Shigella*, while promoting the abundance of Firmicutes and *Lactobacillus*, and mitigating *Salmonella Typhimurium*-induced intestinal barrier damage and inflammatory response in yellow chickens (224). Syringic acid enriches the abundance of *Alistipes* and *norank_f_norank_o_Gastranaerophilales* in mice, improving intestinal inflammation (225). Caffeic acid modulates the composition of the gut microbiome by reducing the relative abundance of *Bacteroides* and *Turicibacter*, while simultaneously increasing the relative abundance of *Alistipes* and *Dubosiella*, enhancing the abundance of *Dubosiella* and *Akkermansia*, effectively alleviating DSS-induced colitis in mice (226). Vanillic acid improves LPS-induced intestinal inflammation in weaned piglets by increasing the proportion of Firmicutes/Bacteroidetes, reducing the abundance of Prevotellaceae, and increasing the abundance of *Lachoiraceaea*, *Lachnospira*, *Eubacterium eligens*, and *Eubacterium* (227). Chlorogenic acid can alleviate colitis induced by a high fat diet in obese rats by reducing the abundance of *Blautia*, *Sutterella*, and *Akkermansia* bacteria and increasing the abundance of *Ruminococcus* (228).

The gut microbiota boasts a rich and diverse composition, and MEHP phenolic acids can exert anti-inflammatory effects by enhancing its diversity and modulating its richness. Notably, two hydroxybenzoic acids and four hydroxycinnamic acids possess significant effects, with ferulic acid and chlorogenic acid can increase the abundance of bacteria responsible for producing short-chain fatty acids (SCFAs), thereby promoting their production and exerting anti-inflammatory benefits.

3.8 Regulating immune responses

Immune response is a self-protective function of the body, where the appropriate immune response can clear pathogens, but excessive immune response can cause harm to the body; inflammation is a result of a severe immune response. Five distinct types of caffeic acid based hydroxycinnamic acid exhibit

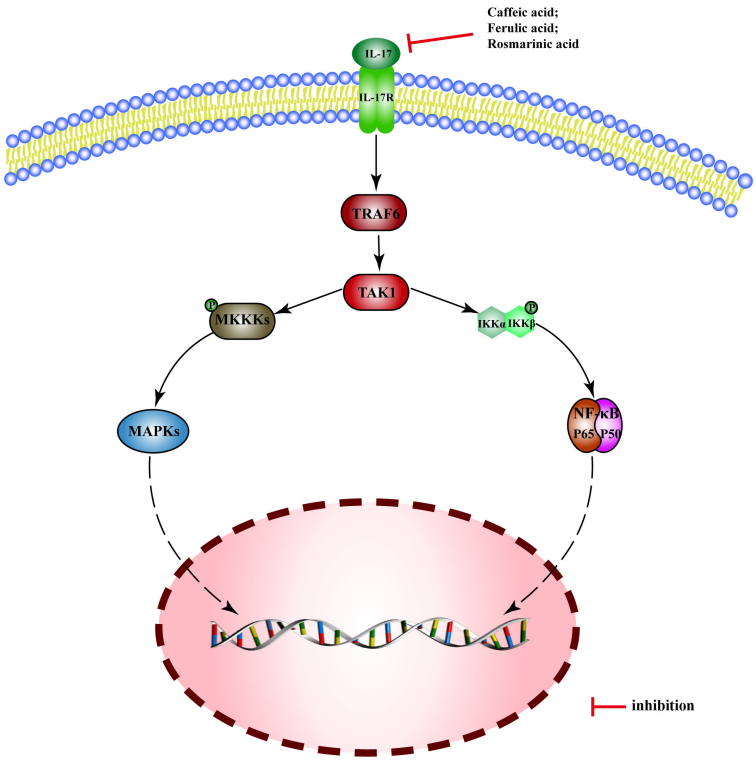

FIGURE 9
Molecular mechanism of the anti-inflammatory activity of phenolic acids from medicinal and edible homologous plants against the IL-17 pathway.

outstanding immune responses regulatory effects. Ethyl caffeate can alleviate collagen-induced arthritis in mice by inhibiting Th1 immune response and IFNγ-related signaling pathways (229). Salvianolic acid A regulates the immune response of dermis and inhibits the immune response of Th2/Th17/Th1 to alleviate atopic dermatitis in mice (230). Salvianolic acid B increases the percentage of $CD3^+CD4^+/CD3^+CD8^+$, restores balance of Th1 and Th2 type cytokines to inhibit the inflammatory response induced by a high fat diet (231). Rosmarinic acid inhibits production of IFN-γ and IL-4 from activated $CD4^+$ cells, reduces the infiltration of $CD4^+$, $CD8^+$, and mast cells, slowing down the development of mouse atopic dermatitis (232). Chlorogenic acid can inhibit microglial polarization toward the M1 phenotype and improve neuroinflammation (233).

Collectively, these findings underscore the potent immune-regulatory capabilities of caffeic acid-based hydroxycinnamic acids in various inflammatory and autoimmune conditions, highlighting their potential for therapeutic applications in immune-mediated diseases.

4 Conclusion and future prospects

MEHP phenolic acids exhibit strong and varied anti-inflammatory mechanisms, highlighting their potential for therapeutic innovations. Their action in crucial pathways like NF-κB, MAPK, NLRP, Nrf2, TLRs, and IL-17, along with the regulation of gut microbiota and immune responses, amplifies their effectiveness.

Overall, hydroxycinnamic acid displays the most potent anti-inflammatory activity among MEHP phenolic acids, likely due to its carboxyl group's adjacent double bond. The number, position, and types of substituents on hydroxyl groups significantly affect the anti-inflammatory effects. Compounds like protocatechuic acid, 4-O-methylgallic acid, 3,4-dihydroxyphenylacetic acid, gentic acid, gallic acid, danshensu, caffeic acid, etc with two or more hydroxyl groups, mainly have hydroxyl substitutions in the para position. Phenolic acid molecules with alkoxy (e.g., methoxy) or alkyl (e.g., methyl) substituents might enhance their compatibility with biomolecules (like enzymes or receptors) by increasing their lipid solubility or by stabilizing hydroxyl radicals, thus amplifying their anti-inflammatory potential. This is observed in compounds such as vanillic acid, ferulic acid, etc. Specifically, phenolic acids with catechol-like configurations, exhibiting two adjacent hydroxyl groups, are characterized by their robust antioxidant capabilities, enabling them to effectively neutralize free radicals and display pronounced anti-inflammatory properties. This is exemplified by Protocatechuic acid among all coffee acid derivatives. Caffeic acids, including caffeic acid, rosmarinic acid, chlorogenic acid, etc stand out due to their structural benefits, playing pivotal roles across varied anti-inflammatory pathways. This highlights a promising strategy for the structural refinement and enhancement of phenolic acids to bolster their therapeutic outcomes.

While advancements have been noted in the research of MEHP phenolic acids, several hurdles remain for their clinical utilization. A significant challenge is pinpointing the dosage that is both efficacious and safe, given the potential for toxicity at elevated levels. Moreover, the interplay between phenolic acids and other medications could potentially influence their therapeutic efficacy. Furthermore, variability across batches of MEHP phenolic acids demands stringent standardization and quality control measures. Additionally, the long-term safety and any adverse effects of phenolic acids are subjects that warrant further investigation. Lastly, there is an evident need for more clinical trials to substantiate the therapeutic efficacy and safety of phenolic acids in managing inflammatory conditions.

To meaningfully tackle these challenges, considerable research on MEHP phenolic acids remains to be conducted. Firstly, delving into the correlation between the molecular structure of phenolic acids and their biological activity is essential, enabling the design and development of more potent phenolic acid derivatives. Secondly, the innovation of drug delivery systems should be prioritized to enhance the bioavailability and stability of these compounds. Personalization of phenolic acid therapy, tailored to an individual's genetic and metabolic profile, presents a promising avenue for exploration. Moreover, investigating the synergistic use of phenolic acids with other pharmaceuticals or therapeutic approaches could potentially amplify their therapeutic impact. Notably, unraveling the intricate molecular mechanisms of phenolic acids, particularly their influence on cellular signaling pathways, is also a critical area for further research. Lastly, an increased number of clinical trials are imperative to yield conclusive evidence regarding the efficacy and safety of phenolic acids in combating inflammatory diseases.

In essence, MEHP phenolic acids possess significant commercial potential as both "anti-inflammatory drugs" and " anti-inflammatory functional foods," thereby fostering a healthier future for all.

Author contributions

JX: Writing – original draft. SX: Writing – original draft. YL: Writing – review & editing. BX: Writing – review & editing. ML: Writing – review & editing. ZZ: Writing – review & editing. ZS: Writing – review & editing. QP: Writing – review & editing. CL: Writing – review & editing. DL: Conceptualization, Project administration, Writing – review & editing. LL: Conceptualization, Project administration, Writing – review & editing.

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Conflict of interest

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

Publisher's note

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Targeting post-stroke neuroinflammation with Salvianolic acid A: molecular mechanisms and preclinical evidence

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Salvianolic acid A (SalA), a bioactive compound extracted from Salvia miltiorrhiza, has garnered considerable interest for its potential in ameliorating the post-stroke neuroinflammation. This review delineates the possible molecular underpinnings of anti-inflammatory and neuroprotective roles of SalA, offering a comprehensive analysis of its therapeutic efficacy in preclinical studies of ischemic stroke. We explore the intricate interplay between post-stroke neuroinflammation and the modulatory effects of SalA on pro-inflammatory cytokines, inflammatory signaling pathways, the peripheral immune cell infiltration through blood-brain barrier disruption, and endothelial cell function. The pharmacokinetic profiles of SalA in the context of stroke, characterized by enhanced cerebral penetration post-ischemia, makes it particularly suitable as a therapeutic agent. Preliminary clinical findings have demonstrated that salvianolic acids (SA) has a positive impact on cerebral perfusion and neurological deficits in stroke patients, warranting further investigation. This review emphasizes SalA as a potential anti-inflammatory agent for the advancement of innovative therapeutic approaches in the treatment of ischemic stroke.

KEYWORDS

Salvianolic acid A, ischemic stroke, anti-inflammation, neuroprotection, natural medicine

1 Introduction

Ischemic stroke is the primary cause of mortality and long-term disability of adult, posing a major global public health challenge (1). Ischemic stroke can result in devastating consequences, including paralysis, speech impairment, cognitive decline, and emotional disturbances, greatly impacting the quality of life for individuals and their families. The prognosis of stroke is influenced by various factors, including the severity of the initial ischemic insult, the extent of the resulting brain injury, and the body's inflammatory response (2–4). Neuroinflammation plays a pivotal role in the pathobiology of ischemic stroke, exacerbating the damage to brain tissue and impeding the recovery process (5, 6). Despite advancements in acute stroke treatment due to mechanical thrombectomy and thrombolytic drugs, the available pharmacological interventions have shown limited efficacy in mitigating the long-term consequences of ischemic brain injury (7, 8). Neuroprotective agents have been a central area of investigation for nearly half a century (9), with the goal of safeguarding the brain from damage and fostering recovery. Despite numerous efforts, the success rate of these drugs in clinical trials has been limited. Of the more than 114 global clinical trials conducted, only a select few have managed to substantiate the efficacy of neuroprotective drugs (10), highlighting the profound scientific and clinical challenges in the field. The complexity of the underlying pathological processes, including the intricate interplay between ischemia and inflammation, has posed a significant challenge in the development of effective stroke therapies. In the face of these limitations, the exploration of natural products as potential therapeutic candidates has gained attention in the field of stroke research (11–13). Certain natural compounds, such as those derived from medicinal plants, have demonstrated promising neuroprotective and anti-inflammatory properties, offering potential avenues for improving stroke outcomes (14–17). These natural products may provide a complementary or alternative approach to conventional pharmacological interventions, potentially targeting multiple pathways involved in post-stroke neuroinflammation and facilitating recovery.

Salvia miltiorrhiza, also known as red sage or Danshen in Chinese, is recognized in traditional Chinese medicinal herb for its properties that enhance blood circulation, alleviate blood stasis, and improve microcirculation (18, 19). Among its bioactive constituents with a well-defined chemical structure, Salvianolic acid A (SalA), (2R)-3-(3,4-dihydroxyphenyl)-2-[(E)-3-[2-[(E)-2-(3,4-dihydroxyphenyl)ethenyl]-3,4-dihydroxyphenyl]prop-2-enoyl]oxypropanoic acid, has been identified as a particularly effective agent that possesses anti-inflammatory and antioxidant properties, as well as the ability to modulate the integrity and functionality of the blood-brain barrier (BBB) (20, 21). In line with these properties, SalA holds significant promise for the treatment of ischemic stroke, with its therapeutic mechanisms and functional targets potentially converging with the cascade of post-stroke neuroinflammation (22, 23). This review focuses on the pathophysiology of post-stroke neuroinflammation and summarizes the multifaceted pharmacological of SalA, particularly focusing on its immunomodulatory role in the treatment of ischemic stroke.

1.1 The inflammatory cascade in ischemic stroke

Ischemic stroke leads to interruption or reduction of cerebral blood flow, leading to a depletion of energy in the ischemic central region and subsequent irreversible neuronal necrosis (24). Disruptions in glucose and energy metabolism within the ischemic penumbra result in diminished Na^+/K^+-ATPase activity, subsequently perturbing ion homeostasis (25). Cell membrane depolarization causes Ca^{2+} influx and thus increases intracellular calcium concentrations, which induces an excessive release of the neurotransmitter glutamate (26, 27). The binding of glutamate to the receptors increases entry of Ca^{2+}, thus causing mitochondrial dysfunction and inducing necrosis, which promotes cytotoxic oedema and inflammation in the surrounding tissue (28). Following a cerebral infarction, activated microglia release vasoactive mediators and pro-inflammatory cytokines such as interleukins (ILs) and tumor necrosis factor-α (TNF-α), which promote significant leukocyte infiltration and initiate neuroinflammation. These inflammatory cells also stimulate the generation of reactive oxygen species (ROS), leading to oxidative stress and further exacerbating the inflammatory reaction (29). Additionally, oxidative stress triggers the production and activation of matrix metalloproteinases (MMPs), leading to the degradation of tight junction proteins (TJPs) in endothelial cells and compromising the integrity of the BBB (30). This disruption allows toxic substances from the blood and peripheral immune cells to enter the affected brain regions, exacerbating brain edema and neuroinflammation.

Collectively, ischemic stroke triggers a cascade of pathological mechanisms that lead to neuronal damage, with inflammation playing a central role. SalA emerges as a potential modulator of the post-stroke neuroinflammation. This intervention has the potential to induce positive outcomes through the reduction of ROS and pro-inflammatory cytokines, ultimately leading to potential improvements in BBB integrity and mitigation of post-stroke neuroinflammation.

1.2 Pharmacokinetic characteristics of SalA in ischemic stroke

In order to preliminarily analyze the druggability of SalA, toxicological assessment and pharmacokinetic studies were conducted in non-diseased animals following oral or intravenous administration (31–33). The acute toxicity studies reported an LD50 of 1161.2 mg/kg in mice and identified a lethal dose range for Beagle dogs between 455–682 mg/kg through single intravenous SalA injections (31). In the subchronic toxicity study, intravenous SalA injections at 20, 80, and 300 mg/kg over four weeks in dogs revealed the no observed adverse effect level of 20 mg/kg, with higher doses associated with transient hepatic and renal effects and reversible thymus weight reduction (31). Notably, SAA showed no genotoxic effects in both the Ames test and the *in vivo* bone marrow micronucleus assay. These findings emphasize the importance of

liver and kidney function monitoring during SAA administration, while its non-genotoxic nature supports its potential use in clinical settings and as a functional food ingredient.

Oral dosing in rats has illustrated a linear correlation between doses and the maximum plasma concentration (Cmax), which were measured as 31.53 μg/L for a 5 mg/kg dose, 57.39 μg/L for a 10 mg/kg dose, and 111.91 μg/L for a 20 mg/kg dose. However, despite its linear characteristics, the oral bioavailability of SalA was determined to be suboptimal, with a range of 0.39% to 0.52%. The limited permeability of SalA across the heterogeneous human epithelial colorectal adenocarcinoma cell (Caco-2) monolayer may account for this observed constraint in bioavailability, as indicated by an apparent permeability coefficient of less than 10^{-6} cm/s (32). In a distinct investigation utilizing beagle dogs, SalA exhibited prompt absorption subsequent to oral delivery, achieving maximum plasma levels within a two-hour timeframe. The absolute bioavailability was determined with a range of 1.47% to 1.84%, indicating quantifiable absorption via the oral route albeit at a diminished efficacy (33) Intravenous administration in rats delineated a mean residence time (MRT) of 2.91 hours and a half-life (t1/2) of 1.96 hours at a 5 mg/kg dose, indicating a relatively rapid systemic clearance (32).

Contrastingly, within the context of ischemic stroke, the pharmacokinetic profile of SalA is markedly altered due to the pathophysiological changes in the brain. A comparative pharmacokinetic analysis of SalA revealed that systemic circulation exposure to SalA was similar between sham controls and rats undergoing ischemia/reperfusion (I/R). However, there was a significant increase in brain exposure to SalA in the I/R group compared to the sham controls, with a fold change of 9.17, particularly evident at the early time point of 0.5 hours post-treatment (22). The increased permeability of the blood-brain barrier following I/R injury indicates that SalA may penetrate the compromised barrier and potentially provide neuroprotective benefits directly to brain tissue (22). Furthermore, metabolomic analysis demonstrated that SalA administration effectively mitigated metabolic disruptions associated with I/R injury, identifying 47 relevant metabolites in contrast to minimal metabolic changes observed in serum samples (22). Taken together, these findings suggest that SalA may serve as a promising therapeutic agent for treating ischemic stroke, given its pharmacokinetic characteristics that align well with the therapeutic requirements of the pathological condition of ischemic stroke.

2 Anti-inflammatory features of SalA in ischemic stroke

2.1 Inhibition of pro-inflammatory cytokines

The depletion of cellular energy and subsequent necrosis of cells within the ischemic penumbra initiates a cascade of signaling events that stimulate inflammatory pathways, resulting in the secretion of inflammatory cytokines including IL-1β, IL-6, and TNF-α. These cytokines are pivotal in the development of ischemic stroke and contribute to the advancement of neuronal injury. Specifically, IL-1β and IL-6 play a crucial role in amplifying the inflammatory response, potentially leading to the activation of other immune cells such as microglia and astrocytes, which are intrinsic immune cells within the central nervous system (CNS). TNF-α has the ability to intensify inflammation by increasing the expression of adhesion molecules on endothelial cells, thereby promoting the migration of leukocytes into the ischemic area. SalA has shown significant efficacy in decreasing levels of IL-1β, IL-6, and TNF-α by inhibiting toll-like receptor (TLR) 2 and 4 signaling in microglia in both *in vivo* and *in vitro* models of ischemic stroke (34). Importantly, SalA downregulated TLR2/4 expression at both the mRNA and protein levels in the affected ipsilateral hemispheres, while no significant changes were observed in the non-ischemic contralateral hemispheres. Additionally, deficiency of TLR2/4 significantly decrease the anti-inflammatory efficacy of SalA, which revealed the TLR2/4-dependent inflammatory inhibition of SalA in microglia. The anti-inflammatory efficacy of SalA by inhibiting the TLR2/4 was also confirmed in hepatic ischemia-reperfusion (35) and heart failure (36). These findings suggest that TLR2/4 may represent prime targets for the anti-inflammatory therapeutics of SalA (left parts of Figure 1).

SalA has also been recognized to modulate key signaling pathways involved in the response to stroke-induced inflammation and apoptosis. SalA has been demonstrated to regulate the GSK3β/Nrf2/HO-1 pathway, which plays a crucial role in cellular defense mechanisms against oxidative stress. The protective function of heme oxygenase-1 (HO-1) is supported by its ability to inhibit the NF-κB signaling pathway, resulting in decreased TNF-α expression and subsequently mitigating inflammatory and apoptotic pathways (37). Furthermore, SalA has been demonstrated to have a direct action on the regulation of the NF-κB (38), a transcription factor crucial in immune response regulation and responsible for promoting the production of pro-inflammatory cytokines. Additionally, research has demonstrated that SalA exerts an influence on the PKA/CREB/c-Fos pathway (39), a signaling cascade that is integral in regulating various cellular processes, including cell viability and immune response. Through its multifaceted interactions, SalA potentially attenuates pro-inflammatory pathway and production of pro-inflammatory cytokines within the infarcted region, suggesting an anti-inflammatory and neuroprotective properties against ischemic injury and therapeutic potential in the context of stroke.

2.2 Regulation of immune cell infiltration

The BBB serves as a crucial interface connecting the CNS with the peripheral circulation, consisting of endothelial cells, tight junction proteins (TJPs), and regulated by a complex interplay of astrocytes, pericytes, and other CNS cells (40). Under physiological conditions, the BBB enforces a stringent control over the paracellular and transcellular transport pathways, as well as a minimal level of transcytosis, thereby preventing the infiltration of potentially neurotoxic substances from the bloodstream into the

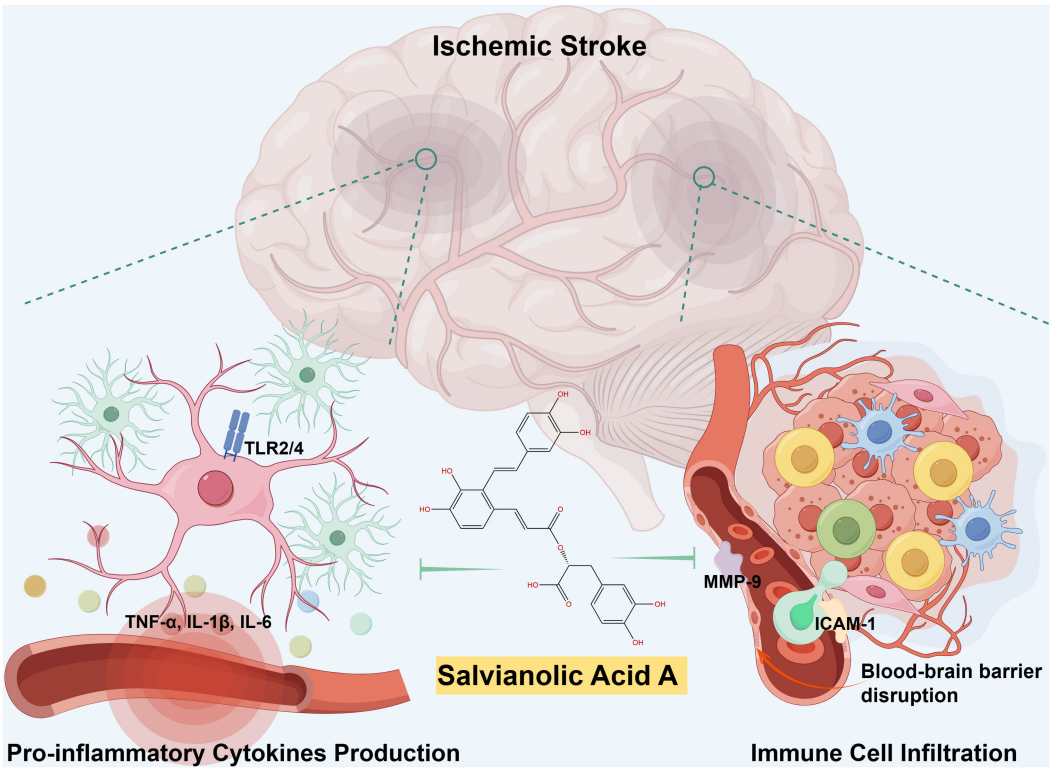

FIGURE 1
Schematic illustration of SalA's anti-inflammatory effects during ischemic stroke. Following a cerebral infarction, activated microglia release vasoactive mediators and pro-inflammatory cytokines, thereby promoting significant leukocyte infiltration and initiate neuroinflammation. SalA, with its anti-inflammatory effects, specifically targets TLR2/4-mediated inflammatory pathways, the release of pro-inflammatory cytokines, MMP-9-mediated BBB leakage, and peripheral immune cell infiltrations. The synergistic effects of targeting multiple factors contributed to the improvement of post-stroke neuroinflammation recovery.

CNS and maintaining its homeostasis (41). Serious BBB leakage induced by ischemic stroke causes peripheral immune cell infiltration, which exacerbates neuroinflammation and induces brain dysfunction and even death. Consequently, BBB protection is a standing topic in anti-inflammatory therapy of ischemic stroke.

Previous research indicated that SalA may protect against peripheral immune cell infiltration after ischemic stroke by inhibition of the CD11b/CD18 complex, intercellular adhesion molecule-1 (ICAM-1), soluble epoxide hydrolase (sEH), and granulocyte adherence (42–47). The modulation of these targets by SalA is instrumental in attenuating adhesion of peripheral immune cells to endothelial cells, a critical step in immune cells migration and subsequent inflammatory cascade. By inhibiting cell adhesion, SalA diminishes the inflammatory response associated with ischemic stroke. Moreover, SalA's suppression of sEH enhances the protective effects of lipid mediators, which are vital for endothelial integrity and contribute to the mitigation of vascular injury and inflammation.

Matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9, are implicated in the breakdown of TJPs like ZO-1, claudin-5, and occludin, which are critical for BBB integrity (48). The degradation of these TJPs by MMPs can lead to disruptions in BBB function (49). It was observed that I/R led to a significant decrease in the levels of TJPs, while treatment with SalA effectively prevented this decrease by suppressing the I/R-induced increase in MMP-9 (50). This protective mechanism of SalA is further supported by the significant reduction in Evans Blue extravasation, indicating a restoration of BBB permeability. The contributions of SalA on the BBB are further corroborated by its ability to reduce matrix metalloproteinase-9 (MMP-9) levels and increase tissue inhibitor of metalloproteinases-1 (TIMP-1) levels, both of which are essential for maintaining the structural and functional integrity of the BBB (50). Furthermore, the protective role of SalA toward the myocardial ischemia has also been attributed to its suppression of MMP-9 activity (51). Together, SalA decreases the migration of peripheral immune cells following ischemic stroke by reducing the expression of vascular adhesion molecules and inhibiting the breakdown of TJPs, resulting in vascular protection and ultimately reducing post-stroke neuroinflammation (right parts of Figure 1).

2.3 Limitations of current research on SalA

While the collective research efforts have shed light on the multifaceted anti-inflammatory actions of SalA in targeting various inflammatory pathways and mediators in ischemic stroke, there is a notable gap in our understanding of its precise molecular targets.

Despite the promising findings that SalA modulates the activity of inflammatory signaling pathways and reduces the expression of inflammatory mediators, potentially through a TLR2/4 dependent mechanism (34), the precise molecular targets of SalA remain elusive. The anti-inflammatory effects attributed to SalA, although significant, are based on observations of downstream effects rather than direct interactions. It is postulated that SalA's actions may be mediated through its binding to other proteins, thereby influencing downstream signaling pathways or secondary effects. However, the lack of direct evidence identifying the specific molecular targets of SalA limits our comprehensive understanding of its mechanism of action.

To elucidate the direct molecular targets of SalA, advanced techniques such as Activity-based Probes (ABPs) (52), which can provide insights into the enzymatic activity and binding preferences of the compound, are necessary. Furthermore, the application of Drug Affinity Responsive Target Stability (DARTS) analysis (53) could offer a means to assess the stability of the target protein in the presence of SalA, thereby indicating the compound's binding affinity and specificity.

Additionally, the Stability of Proteins from Rates of Oxidation (SPROX) analysis (53) could reveal the impact of SalA on the oxidative stability of potential target proteins, which is particularly relevant given the oxidative stress associated with ischemic stroke. Cellular Thermal Shift Assay (CETSA) (54) and Thermal Proteome Profiling (TPP) (55) are other valuable methodologies that could be employed to assess the thermal stability of proteins upon SalA binding, offering further evidence of direct interactions.

The absence of such biophysical and biochemical analyses in the current research corpus means that while we can infer the anti-inflammatory and neuroprotective properties of SalA, we cannot conclusively demonstrate its direct molecular targets. This gap in knowledge is a significant limitation, as it impedes the full realization of SalA's therapeutic potential and the development of more targeted and effective treatments for ischemic stroke.

3 Clinical efficacy and characteristics of salvianolic acid (SA) in acute stroke treatment

3.1 Clinical studies of SA in acute stroke treatment

Although there have been limited clinical studies specifically on SalA for ischemic stroke treatment, numerous studies have examined the therapeutic properties of salvianolic acids (SA) extract, a group of hydrophilic phenolic compounds sourced from Salvia miltiorrhiza, which contain defined SalA (56, 57). A recent clinical study was designed to evaluate the impact of salvianolic acid (SA) on improving blood flow to the brain in patients with ischemic stroke (57). The inclusion criteria mandated that patients be admitted within 72 hours of the onset of acute ischemic stroke symptoms, diagnosed with ischemic stroke confirmed by DWI, and have a Glasgow Coma Scale score above 5. Conversely, patients with a history of brain hemorrhage, very low consciousness levels, or allergies to contrast agents used in MRI were excluded to avoid complications. This study included a total of 159 patients, 85 in the SA group and 74 in the control group, with a mean age of approximately 60 years and an approximately equal gender distribution. The study was a randomized controlled trial, where patients were allocated to either the SA group or the control group. Notably, the study does not specify whether it was double-blinded, which is a methodological detail that could affect the results' interpretation. The SA group received a daily dose of 130 mg SA for 14 days, administered intravenously dissolved in 250 ml of normal saline, in addition to standard therapy including aspirin and atorvastin. The control group received standard therapy and an equivalent volume of normal saline intravenously. The results indicated that patients treated with SA showed significant improvements in NIHSS and mRS scores at the 90-day follow-up, suggesting a potential neuroprotective effect of SA. Notably, the relative cerebral blood volume in patients with hypoperfusion improved markedly following SA treatment, as evidenced by perfusion-weighted magnetic resonance imaging (PWI) images. This suggests that SA may enhance perfusion in hypoperfused brain tissues, thereby improving neurological outcomes in acute stroke patients.

3.2 Expanding the clinical understanding of SalA: recommendations for future research and methodological considerations

In contrast to the well-defined pharmacological agents commonly used in Western medicine, the active constituents of natural medicines, such as SA and the individual components like SalA, are often not clearly identified. The underlying mechanisms by which these natural compounds induce neuroprotection remain largely elusive. the mixtures of multiple ingredients often overshadow lesser-known drug interactions, and pharmacokinetic interactions have been identified within the constituents of the Salvia miltiorrhiza extract (58, 59). A previous study demonstrated that Sal A increased the area under measured plasma concentration-time curve of denshensu ((R)-3-(3,4-Dihydroxyphenyl)-2-hydroxypropanoic acid) and salvianolic acid B and substantially decreased their clearances, possibly via the plasma protein binding replacement (59). In light of the identified pharmacokinetic interactions within the constituents of the Salvia miltiorrhiza extract, it is crucial for future studies to delve into the mechanisms of these interactions. Understanding the interplay between SA and other medications will be paramount in ensuring patient safety and optimizing the efficacy of SA treatment protocols. Moreover, in recognition of the complexity of SA which comprises a suite of compounds including SalA, future clinical investigations should specifically focus on the use of pure SalA in monotherapy settings. This targeted approach is designed to yield a more precise elucidation of SalA's intrinsic therapeutic effects, unencumbered by the potential interactions or influences of other SA components. This

approach will provide a comprehensive understanding of SalA's therapeutic potential as an adjunct therapy, enhancing our knowledge of its role in the overall treatment strategy for ischemic stroke without compromising the standard of care.

Furthermore, it is noteworthy that the existing randomized controlled trials involving SA have predominantly enrolled Chinese patients (56). This demographic limitation raises questions regarding the generalizability of our findings to other ethnicities and populations. To address this, it is essential that forthcoming research endeavors to include diverse patient cohorts from various geographical regions. Such inclusivity will not only validate the universal applicability of SA (or SalA) but also uncover any potential variations in therapeutic responses across different populations.

In summary, while SA has demonstrated promise in the treatment of ischemic stroke, a more expansive and rigorous clinical research agenda is warranted. This includes broadening the scope to encompass larger and more diverse patient populations, employing multicenter study designs, implementing double-blind methodologies, and incorporating assessments of inflammatory biomarkers. Such an approach will not only bolster our comprehension of SA (or SalA)'s therapeutic potential but also pave the way for more efficacious treatment strategies for individuals afflicted with ischemic stroke and its sequelae.

4 Conclusion

In summary, Salvianolic acid A (SalA) has been recognized as a multifaceted therapeutic agent showing considerable promise for the treatment of ischemic stroke. The anti-inflammatory properties of SalA, which involve the suppression of pro-inflammatory cytokines, regulation of crucial signaling pathways, and direct effects on endothelial cells, suggest a potential neuroprotective role that could yield significant clinical advantages for individuals suffering from stroke. The pharmacokinetic profile of SalA, particularly its enhanced brain exposure following I/R injury, indicates a good fit for the therapeutic demands of stroke treatment. Clinical studies, albeit limited, have demonstrated promising findings on the effectiveness of SalA in enhancing neurological outcomes and cerebral perfusion in individuals with acute stroke. Further targeted clinical trials are required to fully understand the therapeutic capabilities of SalA and to establish the most effective treatment protocols. The incorporation of SalA into existing strategies for treating ischemic stroke may provide a supplementary method to traditional pharmacological interventions, potentially resulting in enhanced patient outcomes and quality of life.

Author contributions

HY: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Writing – original draft, Writing – review & editing. MI: Methodology, Writing – review & editing, Project administration, Data curation, Formal analysis, Investigation, Resources, Writing – original draft. SZ: Methodology, Resources, Software, Writing – review & editing. YS: Data curation, Investigation, Writing – original draft, Software, Visualization. JC: Writing – review & editing, Project administration, Supervision. HQ: Investigation, Project administration, Supervision, Writing – review & editing, Conceptualization, Formal analysis, Funding acquisition, Methodology. SY: Data curation, Visualization, Writing – original draft, Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – review & editing, Resources, Validation.

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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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Chinese medicine PaBing-II protects human iPSC-derived dopaminergic neurons from oxidative stress

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Background: PaBing-II Formula (PB-II) is a traditional Chinese medicine for treating Parkinson's disease (PD). However, owing to the complexity of PB-II and the difficulty in obtaining human dopaminergic neurons (DAn), the mechanism of action of PB-II in PD treatment remains unclear. The aim of this study was to investigate the mechanisms underlying the therapeutic benefits of PB-II in patients with PD.

Methods: hiPSCs derived DAn were treated with H_2O_2 to construct the DAn oxidative damage model. SwissTargetPrediction was employed to predict the potential targets of the main compounds in serum after PB-II treatment. Metascape was used to analyze the pathways. Sprague-Dawley rats were used to construct the 6-hydroxydopamine (6-OHDA)-induced PD model, and the duration of administration was four weeks. RNA sequencing was used for Transcriptome analysis to find the signal pathways related to neuronal damage. The associated inflammatory factors were detected by enzyme-linked immunosorbent assay (ELISA). We identified PB-II as an Nrf2 activator using antioxidant-responsive element luciferase assay in MDA-MB-231 cells.

Results: *In vitro* experiments showed that the treatment of PB-II-treated serum increased the percentage of TH^+ cells, decreased inflammation and the apoptosis, reduced cellular reactive oxygen species, and upregulated the expression of Nrf2 and its downstream genes. Pathway analysis of the RNA-seq data of samples before and after the treatment with PB-II-treated serum identified neuron-associated pathways. *In vivo* experiments demonstrated that PB-II treatment of PD rat model could activate the Nrf2 signaling pathway, protect the midbrain DAn, and improve the symptoms in PD rats.

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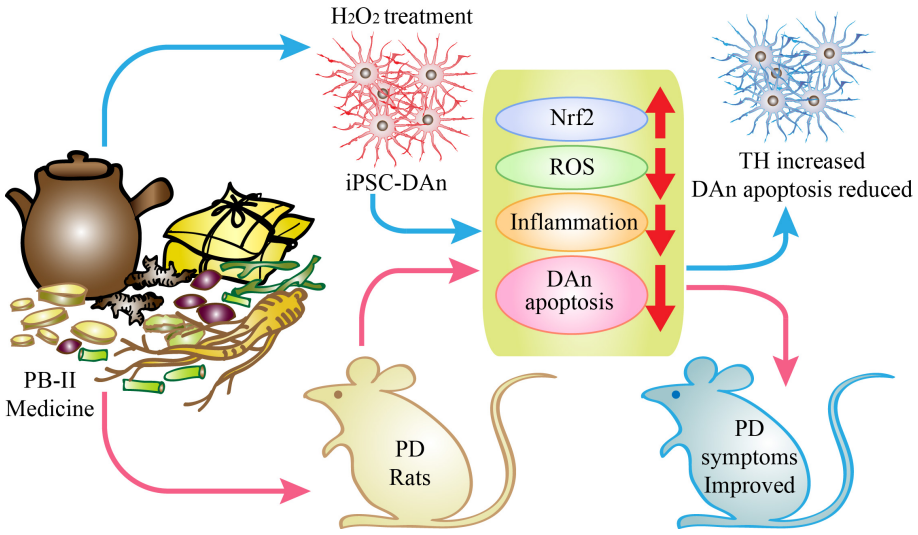
Conclusion: PB-II significantly protects DAn from inflammation and oxidative stress via Nrf2 pathway activation. These findings elucidate the roles of PB-II in PD treatment and demonstrate the application of hiPSC-derived DAn in research of Chinese medicine.

KEYWORDS

Parkinson's disease, hiPSCs, DAn, Nrf2/ARE, ROS, inflammation



GRAPHICAL ABSTRACT
PaBing-II Formula (PB-II) is a traditional Chinese medicine for treating Parkinson's disease (PD). To investigate the mechanisms underlying the therapeutic benefits of PB-II in patients with PD, we developed human dopaminergic neurons (DAn) oxidative damage models by treating human induced pluripotent stem cells (hiPSCs) derived DAn with H_2O_2 as well as the 6-hydroxydopamine (6-OHDA)-induced PD rat model. Through *in vitro* and *in vivo* studies, we demonstrate that PB-II significantly protects DAn from oxidative stress and inflammation via Nrf2 pathway activation, and is manifested as a treatment for Parkinson's disease.

Introduction

Parkinson's disease (PD) is a common progressive neurodegenerative disease characterized by tremors and bradykinesia (1). As patients age, their symptoms worsen (2). The hallmarks of PD are the degeneration of dopaminergic neurons (DAn) in the substantia nigra (SN) and the presence of Lewy bodies (2, 3). Although the mechanisms underlying PD pathology remain unclear, extensive evidence from postmortem brain tissue suggests that oxidative stress and deficiency of complex I activity and inflammation are related to PD pathogenesis (4–6). There is currently no cure for PD. The primary treatment for PD is dopamine supplementation or promotion of endogenous dopamine release. Levodopa, commonly used in PD treatment, can significantly improve the motor symptoms of PD; however, it cannot prevent disease progression and eventually leading to the death of DAn. Long-term use of Levodopa can also cause serious complications such as movement disorders, insomnia, and anxiety (7).

The PaBing-II Formula (PB-II), a Traditional Chinese Medicine, was developed and has been used to treat PD at the Second Affiliated Hospital of Guangzhou University of Chinese Medicine for over 20 years. The composition of PB-II is listed in Table 1. PB-II has significant therapeutic effects on patients with PD, particularly during the early stages (8, 9). PB-II can relieve the motor and non-motor symptoms of patients with PD, improve the therapeutic effects of dopaminergic drugs, reduce drug side effects, and improve their quality of life (10, 11). Previous studies have reported that gavaging rats with PD induced by 6-hydroxydopamine (6-OHDA) with PB-II improved rotational behavior (12), and protected DAn from apoptosis (13, 14). 6-OHDA can damage DAn in the SN of the midbrain, leading to PD symptoms in mice. The unilaterally 6-OHDA-lesioned rat model of PD has been invaluable in advancing our understanding

TABLE 1 Composition of PB-II.

Latin name	Family	The part used	Proportion
Prunus mume (Siebold) Siebold & Zucc	*Rosaceae*	Fruit	20 g
Coptis chinensis Franch.	*Ranunculaceae*	Rhizome	3 g
Paeonia lactiflora Pall.	*Paeoniaceae*	Root	20 g
Angelica sinensis (Oliv.) Diels	*Apiaceae*	Root	10 g
Aconitum carmichaelii Debeaux	*Ranunculaceae*	Root	10 g
Rehmannia glutinosa (Gaertn.) DC.	*Plantaginaceae*	Root	10 g
Reynoutria multiflora (Thunb.) Moldenke	*Polygonaceae*	Root	20 g
Ligusticum striatum DC.	*Apiaceae*	Rhizome	10 g
Pueraria lobata (Willd.) Ohwi	*Leguminosae*	Root	20 g
Panax ginseng C.A.Mey.	*Araliaceae*	Root and Rhizome	10 g
Acorus tatarinowii Schott	*Acoraceae*	Rhizome	5 g
Gastrodia elata Blume	*Orchidaceae*	Rhizome	10 g
Chinemys reevesii (Gray)		Carapax and plastron	10 g
Glycyrrhiza uralensis Fisch.	*Leguminosae*	Root and Rhizome	3 g

The names correspond to the latest version in The Plant List (http://www.theplantlist.org) or the Chinese Pharmacopoeia 2020 edition.

of the mechanisms underlying parkinsonian symptoms and is widely used in PD research (15). However, the effects of PB-II on human DAn and the mechanisms underlying its therapeutic benefits remain unclear.

Because of the ability of induced pluripotent stem cells (iPSCs) to differentiate into all cell types in the body, iPSC technology is an essential tool for studying human diseases in dishes (16). In the present study, DAn were derived from hiPSCs and treated with H_2O_2 to establish DAn oxidation model. Using this model, the mechanisms underlying the therapeutic benefits of PB-II in PD patients were investigated.

Materials and methods

PB-II treated serum preparation

Refer to a previous report (12) regarding the original PB-II recipe. We prepared slices of Chinese crude drugs from the pharmacy of Guangdong Province Hospital of Chinese medicine, which were decocted twice with 10 and 8 times of water, filtered, and concentrated in a water bath at 80°C to 1.6 kg/L (measured by crude drug weight/volume). The rats were administered PB-II twice daily at 32 g/kg (crude drug weight/body weight). After gavaging for 2 weeks, the rats under 30 mg/kg pentobarbital sodium anesthesia were bled from the arterial blood and separated into medicated serum according to a previously reported method (17). The treated and control sera were inactivated at 56°C for 30 min and stored at -80°C before use.

Liquid chromatography and mass spectrometry

LC-MS was conducted using a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) and a Q Exactive Orbitrap mass spectrometer. After screening, the analysis was performed using Waters (Milford, MA, USA) UPLCTM HSS T3 C18 (2.1 × 100 mm, 1.7 µm). Chromatographic conditions: Gradient elution was performed using acetonitrile (A) and -0.1% formic acid in water (B). The elution procedure was: 0 min, 10% A; 5 min, 20% A; 20 min, 60% A; 25 min, 90% A; 28 min 90% A; 29–33 min 10% A. The flow rate was 0.2 mL/min. Mass spectrometry conditions: Samples were ionized using an ESI ion source and analyzed using a Q Exactive Orbitrap high-resolution mass spectrometer. The main parameters of the ESI ion source were as follows: spray voltage 3500 V (Anion voltage, -3500 V); capillary temperature, 350°C; sheath gas, 40; auxiliary gas, 15. All other parameters were set to default values. A liquid eluent with a retention time of 0.5–29 min was selected for mass spectrometry analysis using an automatic switching valve.

Network pharmacology analysis of PB-II treated serum

The targets were predicted using network pharmacology after identifying the main compounds in PB-II-treated serum. Probable targets of the identified compounds were predicted using SwissTargetPrediction (http://www.swisstargetprediction.ch/) (18). Targets with a probability score >0 were used to analyze the Gene Ontology (GO) functional annotations and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using Metascape (https://metascape.org/gp/index.html#/main/step1) (19).

Derivation of human DAn from iPSCs

In our previous studies, we established multiple iPS cell lines from human fibroblasts (20). This study selected an iPS cell line preserved in our laboratory for research. Based on our previous reports (21), DAn were differentiated from iPSC using the necessary media and related cytokines. iPSCs were plated at a density of 4 × 10^4 cells/cm^2 on Matrigel (BD)-coated tissue culture dishes for differentiation. Differentiation was performed in KSR medium. On day 3, the cells were nearly confluent (>80% confluency). The culture medium was gradually changed to N2 medium, supplemented on days 0–5 with SB431542 (5 µM) + LDN-193189 (100 nM) + Iwp2 (1 µM) to obtain retinal progenitors, and then split in a 1:3 ratio for the next six passages using Accutase. The cells

were cultured using neural induction media supplemented with 3 μM CHIR99021 and 2 μM on X-ray-inactivated MEF feeders or Matrigel-coated plates. On days 6–10, induction factors were withdrawn, and PD173074 (0.2 μM) + DAPT (10 μM) was used for retinal ganglion cell induction. On days 10–15, the medium was replaced with N2, B27, 300 μg/mL cAMP (Sigma-Aldrich, St. Louis, MO, USA), 100 ng/mL SHH (C24 II), and 100 ng/mL FGF8b. Next, 10 ng/mL BDNF, 10 ng/mL GDNF, 10 ng/mL IGF-1, 1 ng/mL TGF-β, and 0.5 mM db-cAMP were added and the cells were cultured for 30 days. The culture medium for this stage was named DAn-modified Eagle's medium (DAn-MEM), which was used for subsequent DAn cultures. The cells were collected and dopaminergic neuronal markers such as tyrosine hydroxylase (TH) and β3-Tubulin (TUJ1) were detected using immunofluorescence staining and RT-qPCR. TH antibody (CST-2791), TUJ1 (TU-20) antibody (CST-4466), and DAPI (Sigma-D9542) were used for immunofluorescence detection. The primers used for DAn-specific genes are listed in Table 2. As described in our previous report (21), we utilized electrophysiological analysis to confirm the identity of human DAn.

Immunofluorescence

The cells were fixed using 4% (v/v) paraformaldehyde (Alfa Aesar, Haverhill, MA, USA), washed three times with phosphate-buffer saline (PBS) containing 0.2% v/v Tween (PBST; Thermo Fisher Scientific), and permeabilized using 0.15% v/v TritionX-100 (Sigma-Aldrich) in PBS for 1 h at 25°C. After gentle removal of PBST, the cells were incubated with primary antibodies (Nrf2, TH and TUJ1) in PBST overnight at 4°C. Subsequently, the cells were washed three times with PBST and stained with the secondary antibody for 1 h at 37°C. The cells were thrice washed in PBST, stained with DAPI, and viewed under a laser scanning confocal microscope (Carl Zeiss-710). To observe the ratio of DAn, the proportion of $TH^+/TUJ1^+$ double-positive cells among all cells was statistically analyzed. The quantitative statistics of fluorescence intensity and localization of the related proteins were performed as we previously reported (21).

The establishment of a DAn oxidation model

Cultured DAn were treated with 100 μM H_2O_2 for 12 h according to a previously reported protocol (22). After treatment, the apoptosis and reactive oxygen species (ROS) levels in the cells were examined with flow cytometry. Immunofluorescence (IF) data showed that the percentage of TH/TUJ1 double-positive cells in H_2O_2-treated cells was decreased significantly, which is considered a model of oxidative damage of DAn.

Experimental grouping

DAn culture were randomly divided into four groups: control (Ctrl), oxidative damage model (Model), model treated with blank serum (Blank Serum), and model treated with PB-II-treated serum group (PB-II Serum). Control cells were cultured under normal conditions (DAn-MEM), whereas the other cultures were treated

TABLE 2 Primers applied in the RT-qPCR.

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)
Homo-*GAPDH*	CGGAGTCAACGGATTTGGTC	GACAAGCTTCCCGTTCTCAG
Homo-*TUBB3*	GCCTCTTCTCACAAGTACGTGCCTCG	GGGCGAAGCCGGGCATGAACAAGTGCA
Homo-*TH*	GCCCTACCAAGACCAGACGTA	CGTGAGGCATAGCTCCTGAG
Homo-*FOXA2*	GGGAGCGGTGAAGATGGA	TCATGTTGCTCACGGAGGAGTA
Homo-*PITX3*	GTGCGGGTGTGGTTCAAGAA	AGCTGCCTTTGCATAGCTCG
Homo-*HO-1*	AAGACTGCGTTCCTGCTCAAC	AAAGCCCTACAGCAACTGTCG
Homo-*NQO1*	GAAGAGCACTGATCGTACTGGC	GGATACTGAAAGTTCGCAGGG
Homo-*MRP2*	AGTGAATGACATCTTCACGTTTG	CTTGCAAAGGAGATCAGCAA
Homo-*GPX2*	CTGGTGGTCCTTGGCTTC	TGTTCAGGATCTCCTCATTCTG
Homo-*Bcl2*	GCGACTCCTGATTCATTGGG	ACTTCCTCTGTGATGTTGTATTT
Homo-*Bax*	CATGGGCTGGACATTGGACT	GAGAGGAGGCCGTCCCAA
Rat-*GAPDH*	CCTCGTCTCATAGACAAGAT	GGGTAGAGTCATACTGGAA
Rat-*HO-1*	TGCACATCCGTGCAGAGAAT	CTGGGTTCTGCTTGTTTCGC
Rat-*NQO1*	AGGATGGGAGGTACTCGAATC	TGCTAGAGATGACTCGGAAGG
Rat-*MRP2*	GCCCCTCAAGCACTCTGAC	GCTTTGTGTCCCAGATGGACT
Rat-*GPX2*	GAGCTGCAATGTCGCTTTCC	TGGGTAAGACTAAAGGTGGGC

separately. We added 10% rat serum to the Blank Serum group and 10% PB-II-treated medicated serum to the PB-II Serum group for 24 h. The following day, the three treatment groups (Blank Serum, PB-II Serum, and Model) were treated with 100 μM H_2O_2 for another 12 h. Finally, all cell samples were examined for apoptosis, DAn neuronal activity, ROS, and Nrf2 signaling pathway and associated gene expression.

Flow cytometry analysis

Flow cytometry was used to analyze TH-positive cells, apoptosis, and ROS levels. Intracellular staining was performed to detect THs. The cells were digested, fixed with 4% paraformaldehyde, blocked with BSA, and incubated with anti-TH antibody (ab75875, 1/100 dilution) for 30 min at 22°C. The secondary antibody used was DyLight- 488 goat anti-rabbit IgG (H+L) (ab96899) at a 1/500 dilution for 30 min at 22°C. A total of >5,000 events were acquired. For apoptosis detection, the KEYGEN apoptosis kit (#KGA108–1) was used according to the manufacturer's instructions. Annexin V-FITC/PI staining was performed using flow cytometry software (BD Biosciences, Franklin Lakes, NJ, USA). ROS were detected using the Reactive Oxygen Species kit (#KGT010–1) according to the manufacturer's instructions. ROS detection was based on the fluorescent probe DCFH-DA. Intracellular ROS can oxidize non-colored DCFH to fluorescent DCF. Thus, flow cytometry can be used to detect ROS fluorescence intensity.

Establishment of a reporter cell line and luciferase reporter gene assay

Based on prior research in our lab, anti-oxidant responsive element (ARE)-luciferase plasmid was constructed by inserting a 39-bp ARE-containing sequence from the promoter region of the human NAD(P)H quinone oxidoreductase 1 (*NQO1*) gene into the cloning site of the pGL4.22[luc2CP/Puro] plasmid. We transfected ARE-luciferase plasmid into MDA-MB-231 cells and used 1.5μg/mL puromycin to selected the stable reporter cell lines. For the dual luciferase reporter gene assay, MDA-MB-231 cells were co-transfected with ARE-luciferase plasmid and Renilla luciferase plasmid. The transfected cells were treated with H_2O_2 or PB-II for 24 h. Luciferase activity was monitored with Dual-Luciferase Reporter Assay (Promega, J3082, USA) according to manufacturer's instructions.

Animal experiment

Sprague-Dawley rats (male, 220–250 g) were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). All animal experiments were approved by the Animal Review Board of Guangdong Provincial Hospital of Chinese Medicine (approval number: 2018009). Rats were housed under constant temperature (20–22°C) and a 12 h light-dark cycle, with free access to food and water. After one week of acclimation to the environment, the APO-induced rotation test (see section "APO-induced rotation test") was performed before being used for modeling (23). Rats without rotation were used for stereotaxic injections.

Rats were separated into four groups (n = 5): control, sham, model, and PB-II. The rats in the control group were intragastrically administered with distilled water. The sham group was injected with vehicle and distilled water. The model group was injected with 6-OHDA (see the injection procedure) and distilled water. The PB-II group was injected with 6-OHDA and then administered with PB-II (32 g/kg). All intragastric treatments were continued for four weeks after modeling.

The stereotaxic injection procedure was as following: After being anesthesized with 3% pentobarbital sodium (50 mg/kg, i.p.), the rats were placed in a stereotaxic apparatus. 6-OHDA (3 μL, 5 mg/mL; 0.02% of ascorbic acid in normal saline) was injected unilaterally into each injection site of the left striatum (coordinates from bregma: site 1: antero-posterior: 1.0 mm, medio-lateral: 4.4 mm, dorso-ventral: -4.5 to -6.5 mm; site 2: antero-posterior: -1.2 mm, medio-lateral: 2.2 mm, dorso-ventral: -4.0 to -6.0 mm). The injection rate was 1 μL/min. Rats in the sham group were injected with 0.02% ascorbic acid in normal saline.

APO-induced rotation test

An APO-induced rotation test was conducted to evaluate motor function at weeks two, four, six, and eight post-6-OHDA injection. The APO was dissolved in normal saline containing 0.02% ascorbic acid. Rats were subcutaneously injected with 0.5 mg/kg APO (24). After 5 min, the number of contralateral rotations was recorded for 30 min.

Immunohistochemistry

The rats were anesthetized with 3% pentobarbital sodium (50 mg/kg, i.p.) and perfused with normal saline and 4% paraformaldehyde. Brains were isolated and fixed in 4% paraformaldehyde for 24 h. Brain tissues were dehydrated and embedded in paraffin. The coronal brain sections were prepared using a microtome. Sections were dewaxed, hydrated, and heated in citric acid buffer (pH 6.0) for 20 min. After incubation with 3% hydrogen peroxide for 15 min, the sections were washed with PBS and incubated with blocking solution for 30 min at 37°C. The primary antibody (TH 1:1000) was added to the sections and incubated overnight at 4°C. The sections were then washed with PBS and incubated with HRP-conjugated secondary antibody for 30 min at 37°C. Another three-wash step involved a DAB reaction for 5 min. Finally, sections were washed with water and dehydrated using an ethanol gradient. The images were captured using a BX61 microscope (Olympus, Tokyo, Japan).

ELISA

The following human DAn ELISA kits were used: Monocyte Chemoattractant Protein-1 (MCP-1) (EHC113.96, Neobioscience, Shenzhen, China), tumor necrosis factor-α (TNF-α) (CSB-E09315h, Cusabio, Wuhan, China), interleukin 6 (IL-6) (CSB-E04638h, Cusabio, Wuhan, China), and interleukin 10 (IL-10) (CSB-E04593h, Cusabio, Wuhan, China). The following rat nigrostriatal region tissue ELISA kits were used: MCP-1 (CSB-E07429r, Cusabio, Wuhan, China), TNF-α (ERC102a.96, Neobioscience, Shenzhen, China), IL-6 (ERC003.96, Neobioscience, Shenzhen, China), and IL-10 (CSB-E04595r, Cusabio, Wuhan, China). Analyses were performed according to the manufacturer's instructions.

Transcriptome analysis

Total RNA from rat midbrains was extracted with TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and then enriched and purified using Oligo(dT) magnetic beads (Thermo Fisher Scientific) through direct targeted hybridization. mRNA-seq libraries were prepared and constructed using the VAHTS Stranded mRNA-seq Library Prep Kit (Vazyme Biotech, Jiangsu, China), following the manufacturer's instructions. The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) at the National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA008871) and are publicly accessible at https://ngdc.cncb.ac.cn/gsa. Qualified libraries were sequenced using an Illumina NovaSeq 6000 system in a paired-end format. Reads were mapped to the reference genome (Rnor_6.0.104, ENSEMBL) using Hisat2 and feature counts were used to count the reads. Fragments per kilobase million were calculated after normalization to the trimmed mean of M values. The edgeR package was used to analyze significantly differentially expressed genes. Gene set enrichment analysis (GSEA) was performed using the pre-ranked method in GSEA Java implementation (25). The WIKI pathway database (MsigDB, http://software.broadinstitute.org/gsea/) was used for gene annotation, and a *P*-value <0.05 was considered statistically significant.

Nrf2-antioxidant response element signal detection

Western blotting was performed to detect the Nrf2 protein in cells and the midbrains of rats after various treatments. ImageJ software was used to analyze the protein gray values. RT-qPCR was used to detect the mRNAs of Nrf2 downstream genes, such as heme oxygenase-1 (*HO-1*), *NQO1*, multidrug resistance-associated Protein 2 (*MRP2*), and glutathione peroxidase 2 (*GPX2*). The primer sequences are shown in Table 2.

Quantification and statistical analysis

Data are represented as the mean ± SEM unless otherwise indicated, and Student's *t*-test was used to compare two groups. One-way ANOVA was used to compare multiple groups. GraphPad Prism 5 software was used for statistical analyses. Differences between two groups were considered significant when the *P*-value was <0.05.

Results

Quality control test of medicated serum

LC-MS was used to analyze the active ingredients in the medicated serum and the six main compounds in the recipe, citric acid, hypaconitine, stilbene glucoside, glycyrrhizin, paeoniflorin, and Ginsenoside Rg1, were analyzed (Figure 1). Identification and simultaneous detection were performed to provide a reference for the quality control of PB-II. A detailed map is shown in Figure 1.

Generation of hiPSC and their differentiation into DAn

An iPSC line was reprogrammed from healthy human skin fibroblasts as previously described (20). iPS cells were cultured in ESC medium containing DMEM/F12 (11330; Gibco, Billings, MT, USA), KnockOut™ Serum Replacement (Gibco), MEM non-essential amino acids (Gibco), L-glutamine (Gibco), 55 mM β-mercaptoethanol (Gibco), and 20 ng bFGF (Gibco) on feeders made from CF1 mouse embryos and subjected to radioactive irradiation at a dose of 30 Gy. Colonies with the hESC morphology were observed between days 25 and 45. These cells were selected and expanded under hESC culture conditions. The pluripotency of the hiPSC line was confirmed by colony morphology, expression of the pluripotency markers OCT4 and TRA-1–81, and the formation of teratomas in NSG mice (Figures 2A–C). These data confirm the pluripotency of hiPSCs.

Using a previously described protocol (26), hiPSCs were differentiated into DAn. The presence of DAn in differentiating cultures was confirmed using DAn-specific markers. DAn-specific genes such as *TH*, *TUBB3* (*TUJ1*), *FOXA2*, and Engrailed 1 (*EN1*) were significantly upregulated in iPSC-derived DAn (Figure 2D). In addition, cell morphology confirmed the morphological characteristics of DAn (Figure 2E). IF analysis demonstrated that approximately 60% of iPSC-derived cells expressed the neuron-specific marker TUJ1 and approximately 40% of iPSC-derived

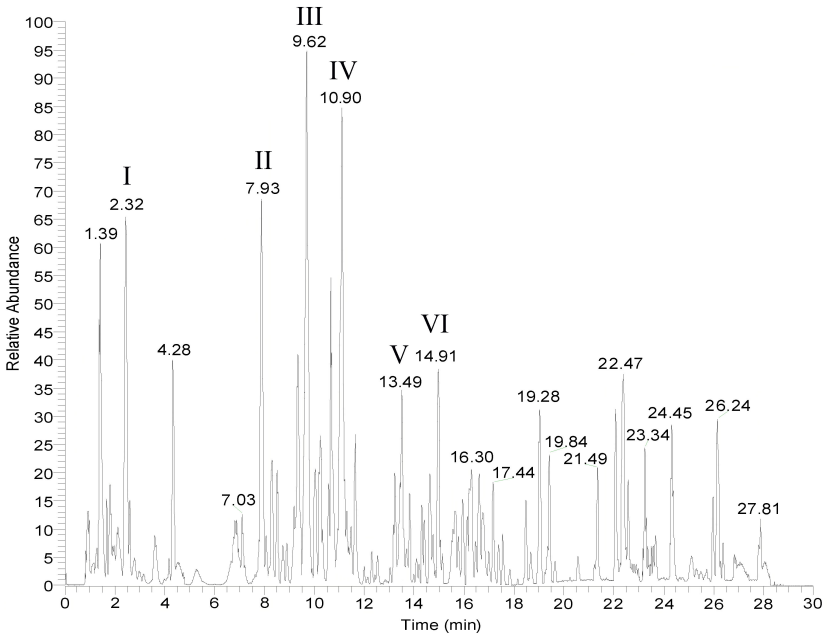


FIGURE 1
LC-MS spectrum for PB-II. The mass spectrum of PB-II was obtained using LC-MS in drug-containing serum. (I) Citric acid; (II) Hypaconitine; (III) Stibene glucoside; (IV) Liquiritin; (V) Paeoniflorin; (VI) Ginsenoside Rg1.

neurons were $TH^+/TUJ1^+$, confirming the presence of hiPSC-derived DAn (Figure 2F). Electrophysiological assays showed that the DAn possessed electrophysiological phenomena specific to human dopaminergic neurons (Figures 2G–I).

PB-II protects DAn from oxidative damage induced by H_2O_2

Immunofluorescence data showed more $TUJ1^+/TH^+$ neurons in the PB-II Serum samples than in the Model samples prepared using H_2O_2 damage and Blank Serum samples (Figure 3A). While $TUJ1^+/TH^+$ neurons decreased in the Model group after treatment with H_2O_2, PB-II Serum significantly increased the number of $TUJ1^+/TH^+$ neurons, indicating that PB-II Serum protected $TUJ1^+/TH^+$ neurons from oxidative stress (Figure 3A). In support of this conclusion, flow cytometric analysis indicated that the percentage of TH^+ cells in the PB-II Serum group was significantly higher than that in the Model or Blank Serum groups (Figures 3B, C). The percentage of apoptotic cells was analyzed in each experimental group, indicating that PB-II Serum protected DAn from apoptosis after H_2O_2 treatment (Figures 3D, E). Furthermore, when compared to that in the Ctrl sample, the expression of *Bcl-2* mRNA and protein in Model was significantly decreased, while the expression of *Bax* mRNA and protein was significantly increased (Figures 3F-J), indicating that apoptosis of DAn was significantly increased after oxidative stress. PB-II could significantly reduce the apoptosis of DAn after oxidative stress (Figures 3D–J).

PB-II activates the Nrf2/ARE signaling pathway and reduces cellular ROS

To explore the mechanism by which PB-II protects DAn from oxidative stress and inflammation, the ROS levels and inflammatory cytokines in hiPSC-derived neuronal cultures were examined after various treatments. Although ROS levels were similar between the Model and Blank Serum treatment groups, PB-II Serum significantly decreased cellular ROS levels in DAn after oxidative stress, supporting the role of PB-II in reducing oxidative stress in DAn (Figures 4A, B; Supplementary Figures S1E, F). Collectively, these findings support the hypothesis that PB-II protects DAn from oxidative stress by reducing cellular ROS levels.

As previously reported, ROS trigger the redox system by activating Nrf2 partly by promoting its nuclear translocation, leading to the increased expression of its downstream genes, such as *HO-1*, *NQO1*, *MRP2*, and *GPX2*. After treatment with H_2O_2 (100 μM) for 12 h, Nrf2 protein levels and downstream gene expression were similar to those of the Ctrl group (Figures 4C–F; Supplementary Figures S1A–D). However, PB-II Serum treatment significantly increased the expression of Nrf2 protein and its downstream genes, such as *NQO1* (Figures 4C–F; Supplementary Figures S1A–D). PB-II Serum significantly induced Nrf2 nuclear translocation in oxidative stress DAn by H_2O_2 intervention with effects comparable to artemisitene (ATT), a known Nrf2 activator (Supplementary Figures S2A–D). Employing the MDA-MB-231 cells stably transfected with an ARE-luciferase reporter as a screening platform (27), we identified that PB-II could induce the expression of the ARE-dependent luciferase gene (Figure 4G).

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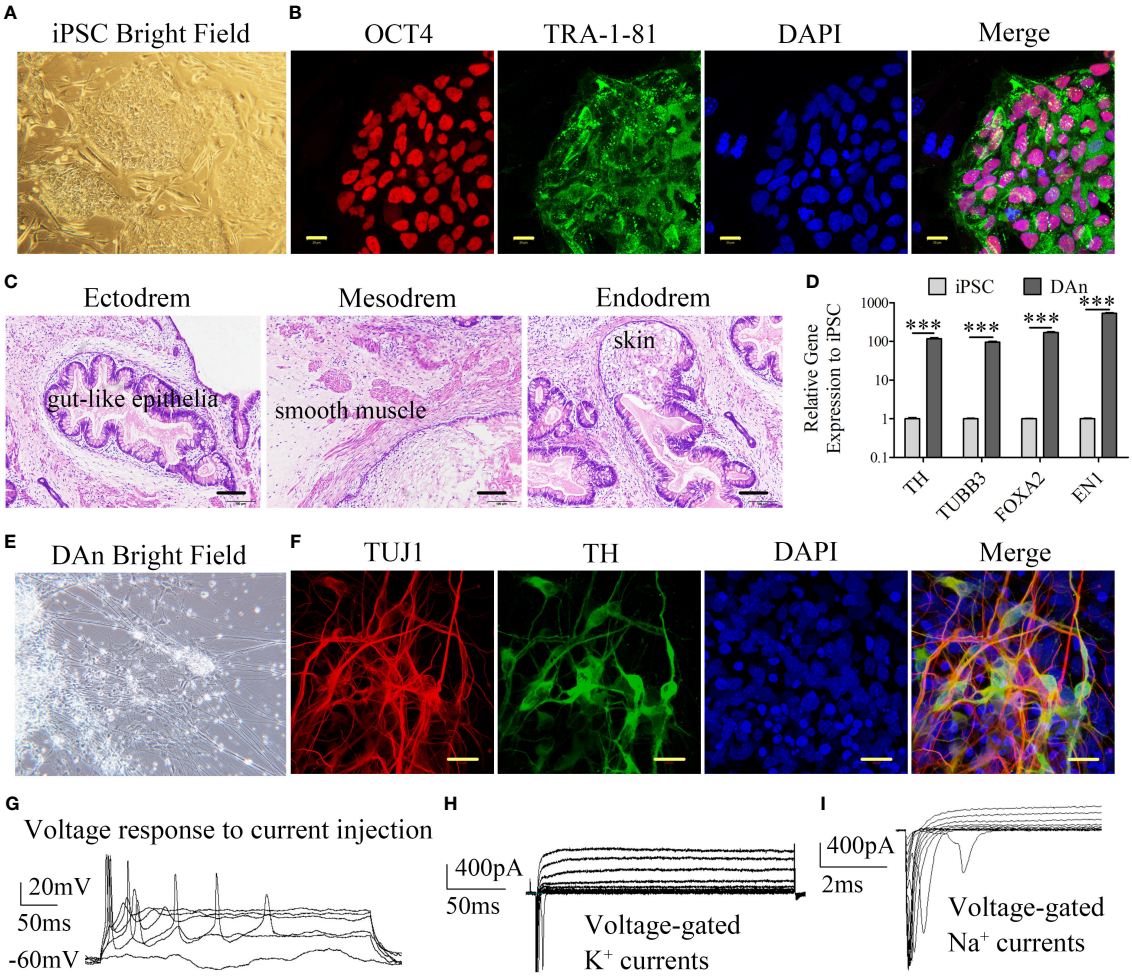

FIGURE 2
Generation of iPSC and differentiation into the dopaminergic neuron. **(A, B)** Representative colonies of passage-20 iPSC stained positive for the pluripotency-associated markers OCT4 and TRA-1–81 (scale bars, 20 μm). **(C)** Teratomas derived from iPSC have the pluripotency of three germ layers. The sections with hematoxylin and eosin staining showed the germ layers of ectoderm, mesoderm, and endoderm differentiation (scale bars, 100 μm). **(D)** RT-qPCR showed that the iPSC-DAn expression to the iPSC lines is approximately 100 times that of neuron relative genes such *TH*, *TUBB3*, *FOXA2*, and *EN1* (n=3, ***P<0.001). **(E)** Differentiated cells have the morphology of DAn. **(F)** Immunofluorescence staining of neuronal cultures derived from iPSC for neuron-specific TUJ1 (red), the DA marker TH (green), and nuclear DAPI (blue) (scale bars, 20 μm). **(G)** DAn fired evoked action potential and had voltage-gated K^+ and Na^+ currents **(H, I)** shown by cell patch clamp electrophysiology.

ELISA detection showed that H_2O_2 treatment significantly increased the expression of pro-inflammatory factors in DAn cells, such as MCP-1, TNF-α and IL-6, while inhibiting the expression of anti-inflammatory factors, IL-10. The treatment of PB-II could significantly reduce the inflammatory response of the Model group (Figures 4H–K; Supplementary Figures S1G-J). Therefore, PB-II activates the Nrf2/ARE signaling pathway to protect DAn from oxidative stress and inflammation.

PB-II improves the symptoms of PD rats by activating the Nrf2/ARE signaling pathway

Network pharmacology analysis of the main identified compounds of PB-II-treated serum showed that several neuronal signalings were involved in the GO functions, such as "regulation of neurotransmitter levels" (Figure 5A), "oxidoreductase activity" (Figure 5B), "postsynapse", and "neuronal cell body" (Figure 5C). The top 20 KEGG pathways of PB-II-treated serum also included neuron-associated pathways, such as "serotonergic synapse", "alcoholism", and "pathways of neurodegeneration-multiple diseases" (Figure 5D). Dopaminergic neuronal signaling has been implicated in neurotransmission, alcoholism, and neurodegenerative diseases. Transcriptome analysis was performed to better understand the mechanism of action of PB-II in PD. GSEA analysis showed that leading-edge genes associated with "dopaminergic neurogenesis" and "Parkinson's disease" pathways were highly expressed, following PB-II treatment as compared to the model group (Figures 5E, F), suggesting that PB-II regulates dopaminergic neuronal signaling and the PD pathway.

To further validate that PB-II can activate the Nrf2/ARE signaling pathway to protect DAn from oxidative stress and inflammation, the effects of PB-II in PD rat models were tested

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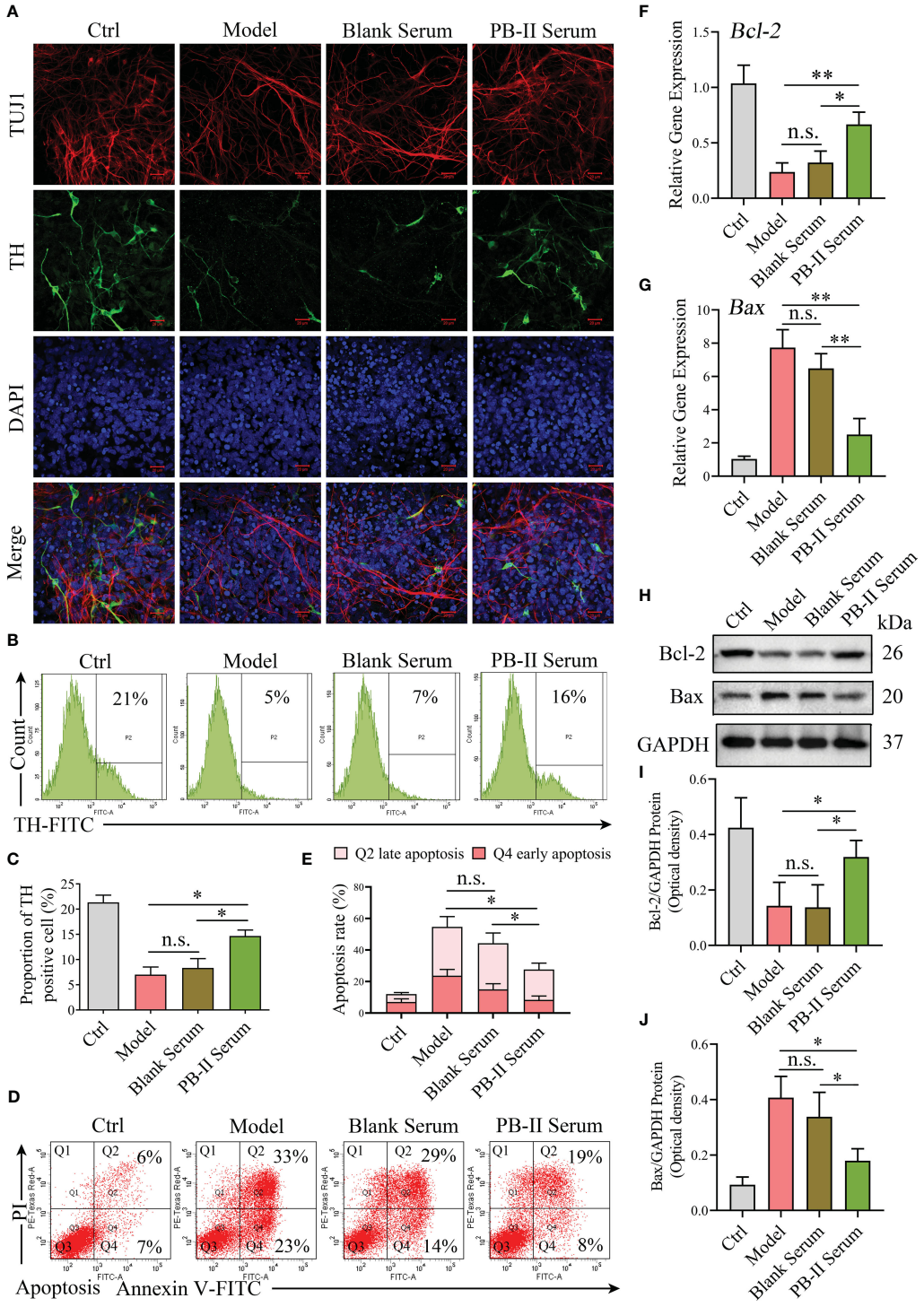

FIGURE 3
PB-II protects the neuronal cell with activated neuronal expressions. **(A)** Immunofluorescence data showed more TUJ1 and TH neuronal staining in PB-II Serum sample than that in the Model and Blank Serum samples (scale bars, 20 μm). **(B, C)** Flow cytometry analysis of the proportion of TH^+ cells in each experimental group, compared with that of the Model and Blank Serum groups, the PB-II Serum group had a higher percentage of TH^+ cells, with statistically significant differences (n = 3, *P<0.05; *n.s.*, no significance). **(D, E)** Flow cytometry detected the proportion of apoptosis in each experimental group. Compared with that of the Model and Blank Serum groups, the apoptosis in the PB-II Serum group decreased significantly (n = 3, *P<0.05; *n.s.*, no significance). **(F, G)** RT-qPCR data of *Bcl-2* and *Bax* mRNA expression in DAn of each experimental group (n = 3, *P<0.05; **P<0.01; n.s., no significance). **(H–J)** Immunoblotting data of Bcl-2 and Bax protein expression in DAn of each experimental group (n = 3, *P<0.05; n.s., no significance).

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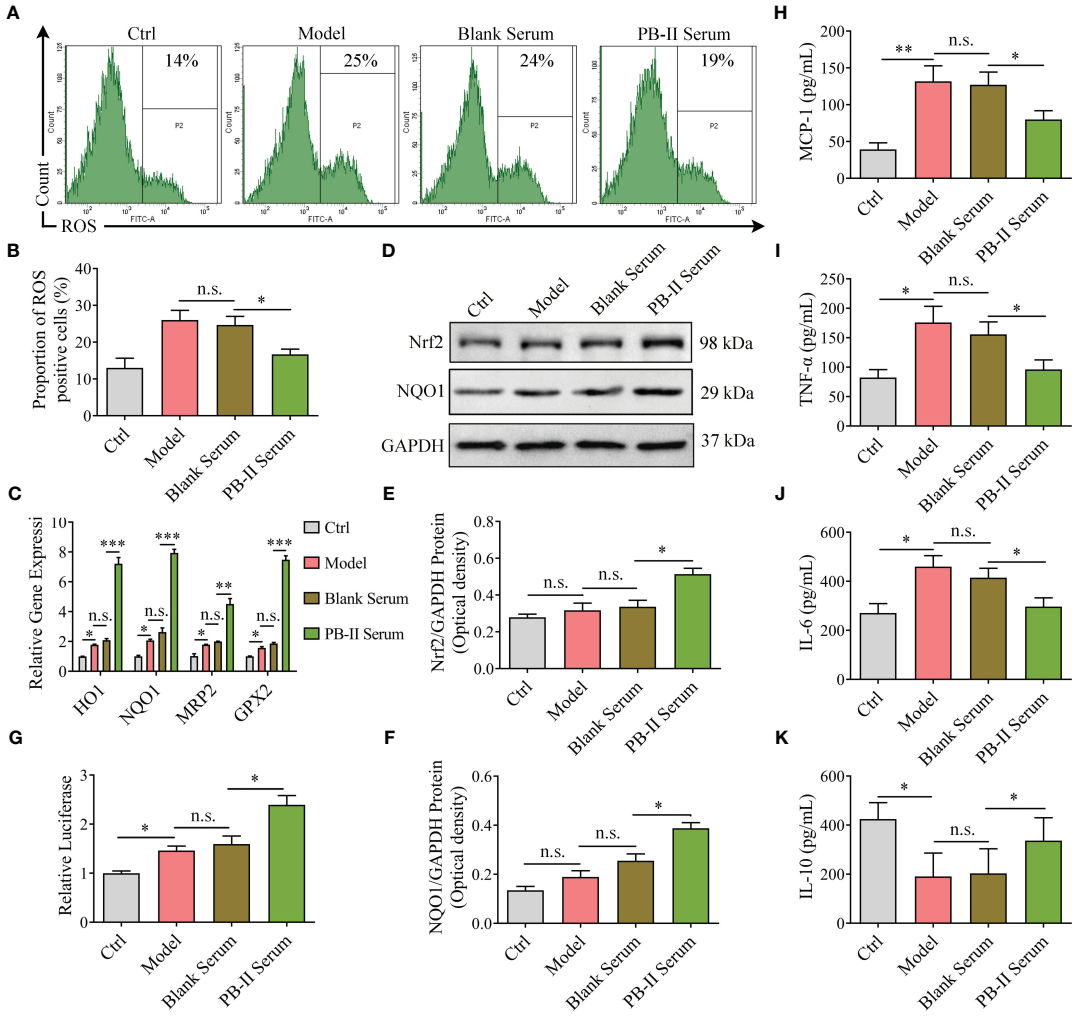

FIGURE 4
Nrf2 pathway genes are activated in medicated cells and reduce ROS and inflammation. **(A, B)** The higher ROS level activated under H_2O_2 stress was inhibited by PB-II Serum (n = 3, *P<0.05; *n.s.*, no significance). **(C)** The Nrf2 signaling pathway downstream genes, *HO-1*, *NQO1*, *MRP2*, and *GPX2*, were found in higher levels in PB-II Serum samples than in Model or Blank Serum samples (n = 3, *P<0.05, **P<0.01, ***P<0.001; *n.s.*, no significance). **(D–F)** Western blotting showed that the Nrf2 and NQO1 proteins in PB-II Serum samples are significantly more increased than those in the other samples (n = 3, *P<0.05; *n.s.*, no significance). **(G)** Luciferase activity was tested with corresponding Kit (n = 3, *P<0.05; *n.s.*, no significance). **(H–K)** ELISA data showed that the pro-inflammatory factors, MCP-1, TNF-α, and IL-6 in the model group increased, while the anti-inflammatory factor IL-10 decreased. The intervention of PB-II could reduce pro-inflammatory factors and increase anti-inflammatory factors (n = 3, *P<0.05, **P<0.01; *n.s.*, no significance).

by injecting 6-OHDA into the striatum of rats to induce midbrain DAn death (model group). The sham operation group was injected with an equal volume of normal saline (sham group). The PB-II group was administered 32 g/kg PB-II via gavage. During the four-week treatment course, the behavioral symptoms of the PD rats were measured weekly. Spinal behavior in PD rats was induced by subcutaneous injection of APO into the back of the neck, and the number of rotations was recorded within 30 min. During the initial stage of treatment (week 0), the rats in the control and sham groups showed no symptoms of *in situ* circles. However, the model and PB-II groups showed severe rotationary behavior, with more than 210 rotations in 30 min, and there was no significant difference between the two groups. During the third week of treatment, the number of rotations was significantly reduced in the PB-II group (Figure 6B). After four weeks of treatment, the rats were euthanized and tissues of the nigrostriatal region were obtained. The number of TH^+ neurons in the substantia nigra of rats in the PB-II group was significantly higher than those in the sham and model groups (Figure 6A). In addition, transcriptome analysis showed that overall gene expression in the PB-II group was similar to that in the sham group (Figure 6C). GSEA revealed that the target genes associated with the Nrf2 pathway were highly expressed after PB-II treatment compared to those in the model group (Figure 6D). Furthermore, the expression of *HO-1*, *NQO1*, *MRP2*, and *GPX2* in the midbrain of the PB-II group was also significantly increased, indicating the activation of the Nrf2/ARE signaling pathway by PB-II (Figure 6E). The Nrf2 and NQO1 protein levels in the midbrain DAn of rats in the PB-II group were significantly higher than those

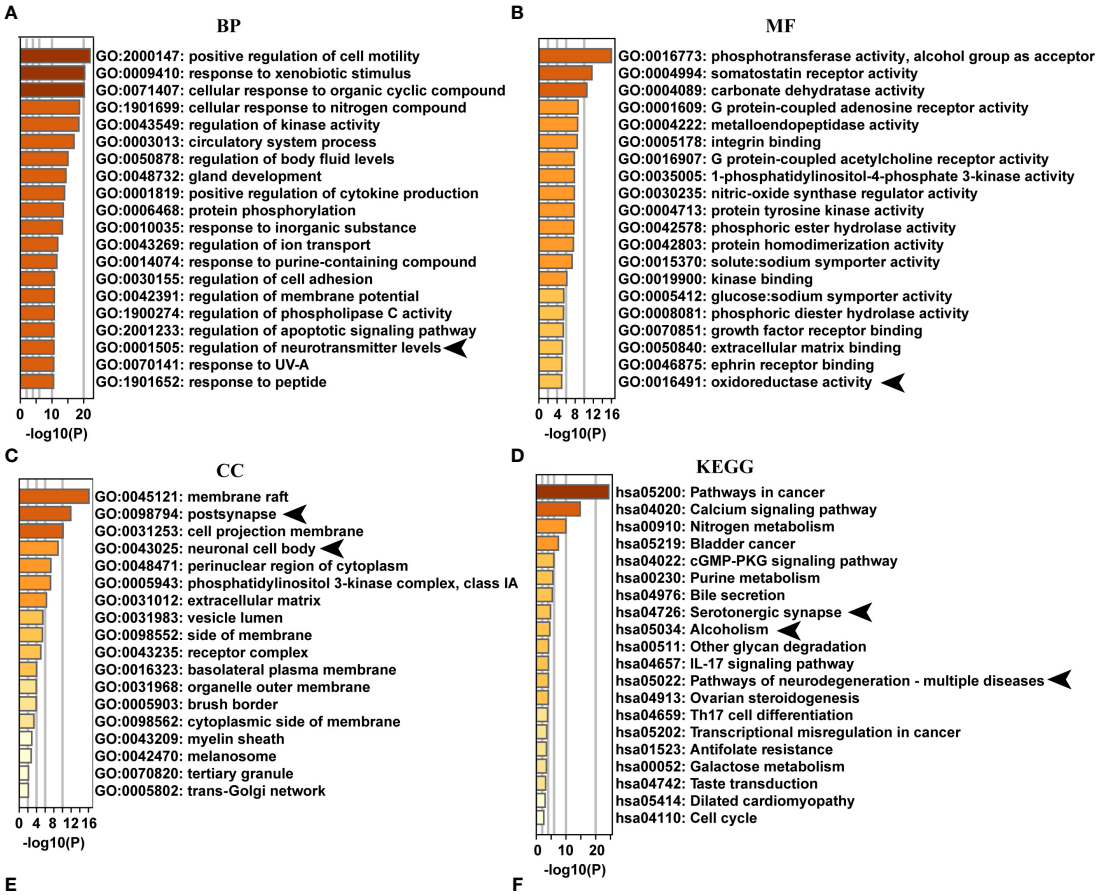

FIGURE 5
PB-II targets PD pathway. **(A)** Top 20 clusters of GO biological process enrichment. **(B)** Top 20 clusters of GO molecular functions enrichment. **(C)** Top 20 clusters of GO cellular components enrichment. **(D)** Top 20 clusters of KEGG enrichment. **(E)** GSEA analysis of dopaminergic neurogenesis pathway between PB-II and model groups (NES = 2.07, $P<0.01$). **(F)** GSEA analysis of Parkinson's disease pathway between PB-II and model groups (NES = 1.78, $P < 0.01$). CC, cellular components; BP, biological processes; MF, molecular functions. Arrows indicate the neuronal signaling associated with GO enrichment items and KEGG pathways.

in the sham and model groups (Figures 6F–H). Meanwhile, the inflammatory response in the model group was intensified, while PB-II treatment significantly reduced the inflammation (Figures 6I–L). These data confirm that PB-II activates the Nrf2/ARE signaling pathway in the midbrain of PD rats by activating Nrf2 to reduce oxidative stress and inflammation in the DAn, thus protecting the DAn from oxidative stress and inflammation-induced apoptosis.

Discussion

Oxidative stress is believed to cause the death of DAn (28–30), and the activation of the endogenous antioxidant system may protect cells from oxidative damage (30). Multiple studies in various organs have confirmed that the Nrf2-ARE pathway acts as an endogenous antioxidant pathway that antagonizes oxidative stress injury (31). In cells of the central nervous system, such as DAn, astrocytes, and

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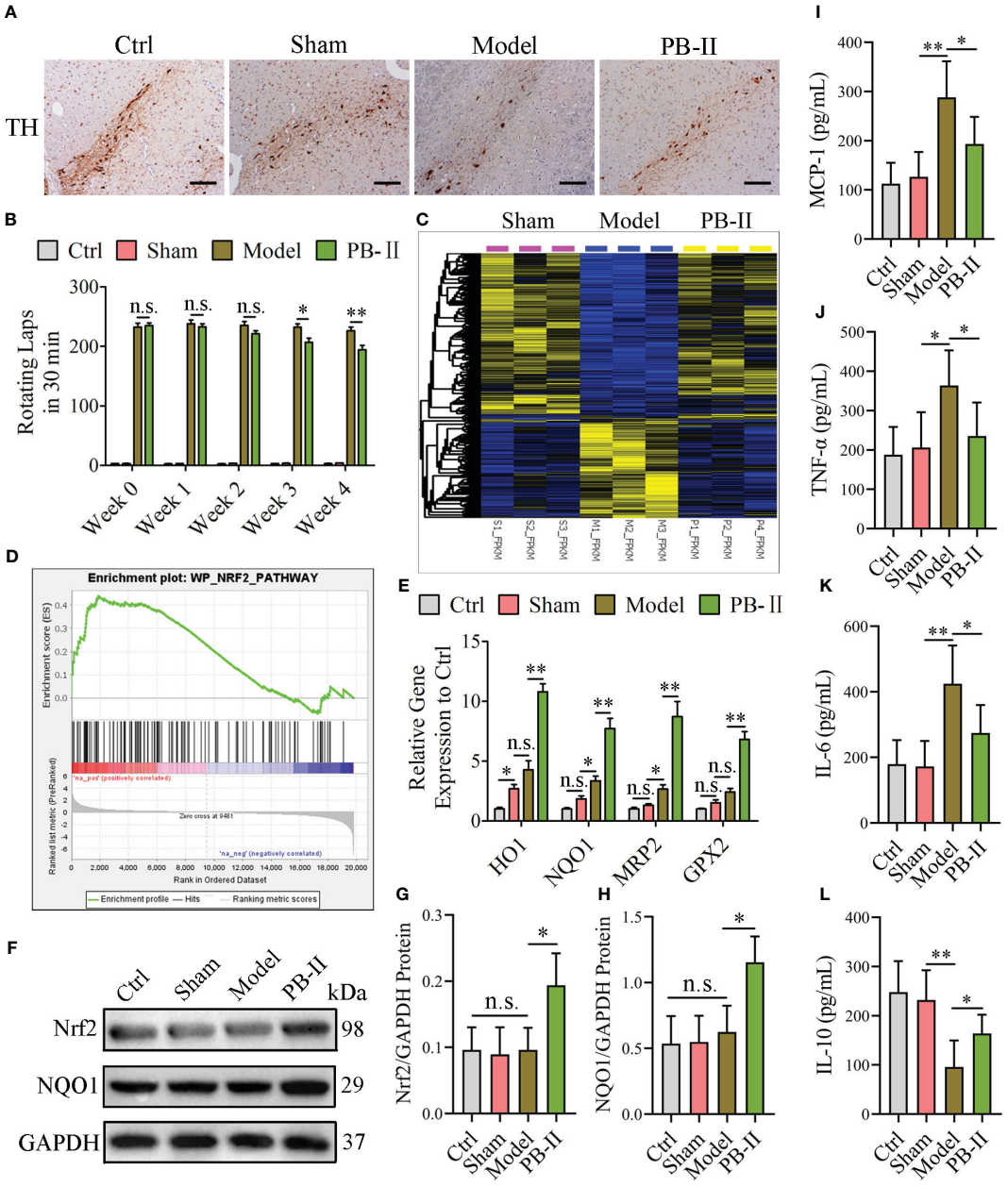

FIGURE 6
PB-II protects PD rat DAn by activating the Nrf2/ARE signaling pathway. **(A)** Immunohistochemical results showed that the TH-positive neurons in the substantia nigra of the PB-II group were significantly increased compared with the sham and model groups (scale bars, 120 μm). **(B)** The number of rotations of the rats in each experimental group within 30 min was calculated. From the third week, the number of rotations of the PD-rats in the PB-II group decreased compared with the model group (n = 5, *P<0.05; n.s., no significance) and reached a very significant difference in the fourth week (n = 5, **P<0.01; n.s., no significance). **(C)** Heat map of differentially expressed genes among the sham (pink), model (blue), and PB-II groups (yellow) obtained from the transcriptome analysis (n = 3). **(D)** GSEA analysis of the NRF2 pathway between the PB-II and model groups showed that the NRF2 pathway was highly expressed in the PB-II group (NES = 1.53, P < 0.01). **(E)** RT-qPCR results showed that the expression of Nrf2 downstream genes in the substantia nigra tissues of rats in the PB-II group was significantly increased, suggesting the activation of the Nrf2 signaling pathway of PB-II on DAn cells under 6-OHDA toxicity (n = 3, *P<0.05, **P<0.01; n.s., no significance). **(F–H)** Western blotting detection results showed that Nrf2 and NQO1 protein expression in cells of the substantia nigra region of the midbrain in the PB-II group of rats increased significantly (n = 3, *P<0.05; n.s., no significance). **(I–L)** ELISA assay results showed that the MCP-1, TNF-α, and IL-6 increased in the model group, while the IL-10 decreased. PB-II treatment could reduce pro-inflammatory factors and increase IL-10 (n = 3, *P<0.05, **P<0.01; n.s., no significance).

microglia, Nrf2 maintains redox balance through the upregulation of antioxidant genes (32). Activation of the Nrf2 signaling pathway can also reduce inflammatory responses (33). Previous research has verified that Nrf2 is mostly translocated to the nucleus of DAn in the substantia nigra of patients with PD, whereas it is present in the cytoplasm of a matched normal control group of the same age (17, 34, 35). Studies have also demonstrated that Nrf2 overexpression can reduce the damage caused by 6-OHDA in DAn (28, 36). Under physiological conditions, Nrf2 protein expression is low in cells and mainly in the cytoplasm where it interacts with Kelch-like ECH-

associated protein-1 (Keap1) (37, 38). In response to oxidative stress, Nrf2 is dissociated from KEAP1 and translocated into nucleus to regulate ARE, inducing the expression of downstream target genes such as *HO-1* and *NQO1*, and thus enhancing the detoxification and antioxidant ability of cells (39–41). *In vitro* experiments have also shown that the upregulation of HO-1 and NQO1 can protect cells against oxidative damage caused by glutamic acid, hydrogen peroxide, and amyloid-beta proteins (42–44).

Activation of the Nrf2 signaling pathway reduces the inflammation via multiple pathways, such as the inhibition of the nuclear factor kappa-B (NF-κB) (45) or PI3K/Akt (46) signaling pathways. In addition, a reduction in ROS would significantly reduce inflammation. In this context, ROS could activate NF-κB signal pathway (47), promoting inflammatory response and inducing α-synuclein aggregation in PD. In this context, ROS activates microglia to secrete several pro-inflammatory cytokines, such as TNF-α, IL-6, and MCP-1 (48). The inflammatory factors can also induce the expression of major histocompatibility complex (MHC) class II, which are associated with neuronal damage in PD patients.

Because traditional Chinese medicine (TCM) with herb components is delivered through the digestive system instead of intravenous injection, it is known that the digested and metabolized materials absorbed into the blood after digestive processes are the functional elements of TCM. Therefore, it is routine to use TCM-treated serum instead of TCM itself in functional studies of TCM. Our experiments show that PB-II-treated serum effectively reduced ROS levels and inflammation in an oxidative model of DAn, protecting DAn from apoptotic cell death. These results suggest that the Nrf2/ARE pathway-mediated antioxidant and anti-inflammatory mechanism may play a role in the effective treatment of neurodegenerative diseases. In support of this notion, our results show that PB-II plays a protective role against oxidative stress and inflammation in neurons by inducing the nuclear translocation and phosphorylation of Nrf2 as well as the expression of Nrf2 target genes.

PB-II contains 14 traditional berbs and its therapeutic efficacies for treating PD has been confirmed by many years of clinical practice (8–11). Furthermore, several studies have reported its protective effects on midbrain DAn against 6-OHDA toxicity in the substantia nigra of rat PD models (12–14). These studies further show the effects of PB-II in reducing apoptosis of DAn in the PD rat model, promoting cell regeneration, and improving PD symptoms in rats. However, the mechanisms underlying the protective roles of PB-II has been unclear. Our study provides a underlying mechanism by reducing the oxidative stress and inflammation through activation of Nrf2 pathway. Importantly, this study further confirms the powerful application of hiPSC derived neural cells in mechanistic studies of the complex TCM.

Conclusions

PB-II activates the Nrf2 signaling pathway in DAn after oxidative stress, increasing the expression of antioxidant target genes of Nrf2, thereby improving the antioxidant capacity and survival of neurons by reducing ROS and inflammation. These findings explain the therapeutic efficacy of PB-II in the treatment of PD.

Data availability statement

The raw transcriptome sequence data presented in the study are deposited in the Genome Sequence Archive (https://ngdc.cncb.ac.cn/gsa), accession number CRA008871.

Ethics statement

The studies involving humans were approved by the Institutional Review Board and TCM Review Board for Ethics of Guangdong Provincial Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by the Animal Review Board of Guangdong Provincial Hospital of Chinese Medicine. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SW: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing, Software, Supervision. CR: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Writing – review & editing, Resources, Visualization. RL: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – review & editing, Conceptualization. KJ: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – review & editing. TL: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Writing – review & editing. WC: Data curation, Investigation, Methodology, Software, Supervision, Writing – review & editing, Visualization. WM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Visualization, Writing – review & editing. YX: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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Supplementary material

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Reporting quality and risk of bias of randomized controlled trials of Chinese herbal medicine for multiple sclerosis

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Background: Multiple sclerosis (MS) is the most common non-traumatic disabling disease affecting young adults. A definitive curative treatment is currently unavailable. Many randomized controlled trials (RCTs) have reported the efficacy of Chinese herbal medicine (CHM) on MS. Because of the uncertain quality of these RCTs, the recommendations for routine use of CHM for MS remain inconclusive. The comprehensive evaluation of the quality of RCTs of CHM for MS is urgent.

Methods: Nine databases, namely, PubMed, Embase, Web of Science, Cochrane Library, EBSCO, Sinomed, Wanfang Database, China National Knowledge Infrastructure, and VIP Database, were searched from inception to September 2023. RCTs comparing CHM with placebo or pharmacological interventions for MS were considered eligible. The Consolidated Standards of Reporting Trials (CONSORT) and its extension for CHM formulas (CONSORT-CHM Formulas) checklists were used to evaluate the reporting quality of RCTs. The risk of bias was assessed using the Cochrane Risk of Bias tool. The selection criteria of high-frequency herbs for MS were those with cumulative frequency over 50% among the top-ranked herbs.

Results: A total of 25 RCTs were included. In the included RCTs, 33% of the CONSORT items and 21% of the CONSORT-CHM Formulas items were reported. Eligibility title, sample size calculation, allocation concealment, randomized implementation, and blinded description in CONSORT core items were reported by less than 5% of trials. For the CONSORT-CHM Formulas, the source and authentication method of each CHM ingredient was particularly poorly reported. Most studies classified the risk of bias as "unclear" due to insufficient information. The top five most frequently used herbs were, in

Abbreviations: MS, multiple sclerosis; CNS, central nervous system; DMTs, disease-modifying therapies; CHM, Chinese herbal medicine; EAE, experimental autoimmune encephalomyelitis; ASI, astragaloside IV; RCTs, randomized controlled trials; EDSS, Expanded Disability Status Score; CONSORT, Consolidated Standards of Reporting Trials; CONSORT-CHM, CONSORT extension criteria on reporting herbal interventions; WM, Western medicine; CIs, confidence intervals; CS, Cordyceps sinensis.

order, *Radix Rehmanniae Preparata*, *Radix Rehmanniae Recens*, *Herba Epimedii*, *Scorpio*, and *Poria*. No serious adverse effect had been reported.

Conclusions: The low reporting of CONSORT items and the unclear risk of bias indicate the inadequate quality of RCTs in terms of reporting completeness and result validity. The CONSORT-CHM Formulas appropriately consider the unique characteristics of CHM, including principles, formulas, and Chinese medicinal substances. To improve the quality of RCTs on CHM for MS, researchers should adhere more closely to CONSORT-CHM Formulas guidelines and ensure comprehensive disclosure of all study design elements.

KEYWORDS

autoimmune diseases, natural products, traditional Chinese medicine, herbal, quality of reporting, ROB

1 Introduction

Multiple sclerosis (MS) is an autoimmune-mediated degenerative disease of the central nervous system (CNS), characterized by inflammatory demyelination (1). MS is the most common non-traumatic disabling disease to affect young adults (2). MS can lead to muscle weakness, sensory deficits, cognitive impairment, and fatigue (3), which ultimately affect quality of life. Despite extensive research, the underlying pathophysiology of MS remains poorly elucidated, and a definitive curative treatment is currently unavailable (4). The current treatment of MS mainly includes hormone therapies in the acute phase, disease-modifying therapies (DMTs), and symptomatic therapies in the remission phase (2). DMTs have been shown to decrease the frequencies of relapse and the accumulation of disability (5). However, the exacerbation and even new occurrence of several autoimmune diseases associated with MS have been reported as a result of DMTs (6). A network meta-analysis of the Cochrane database found that drugs used for immunotherapy may increase withdrawals (7). The American Academy of Neurology practice guideline on the efficacy and safety of DMTs in MS mentions the following (8): "Immunosuppressive medications may increase the risk of opportunistic infection and malignancy, especially with prolonged use". Cryptococcal infections with fingolimod use and herpes family virus infections with natalizumab use have been reported (9, 10). DMTs may also cause other adverse events, including heart blocks, bradycardia, macular edema, and secondary autoimmune adverse effects (11).

Many patients with MS resorted to modalities of complementary and alternative medicine, which is used by 57%–81% of patients with MS in developed countries (12). More and more research focused on the efficacy of Chinese herbal medicine (CHM) on MS. For example, astragaloside IV (ASI) is an active monomer isolated from the Chinese medicine *Astragalus membranaceus*. In mice with experimental autoimmune encephalomyelitis (EAE), an ideal animal model for MS, early administration of ASI can delay onset and reduce disease severity (13). Bushen Yisui Formula contains 10 kinds of herbs, such as *Radix Rehmanniae Preparata, Radix Rehmanniae Recens, Scorpio*, and *Polygonum Multiflorum*, and exhibits neuroprotective effect against EAE by promoting oligodendrocyte progenitor cells' proliferation and differentiation, thus facilitating remyelination (14). Two meta-analyses (15, 16) have proved the effectiveness of CHM in the treatment of MS. There are also a large number of randomized controlled trials (RCTs) demonstrating the effectiveness of CHM for MS. High-quality RCTs, particularly double-blind placebo-controlled trials, are generally considered to be the highest level of evidence for judging the therapeutic efficacy and safety of interventions. The credibility of the evidence supporting treatment depends on the quality of RCTs. However, an overwhelming body of evidence suggests that the quality of RCT reports remains sub-optimal (17). The reporting of methodology and bias in herbal RCTs is particularly inadequate (18). One review concluded that less than 10% of herbal medicine trials used an appropriate randomization method (19). It is noteworthy that no research has evaluated the quality of RCTs of CHM on MS currently.

The Consolidated Standards of Reporting Trials (CONSORT) checklist is commonly used to evaluate the reporting quality of RCT. Introduced in 1996 (20), it was further revised in the current 2010 version. Compliance with CONSORT has been studied in many medical fields with the general conclusion that reporting quality needs to be improved (21–23). Different CHM formulas exhibit discrepancies in composition, dosage, and duration of interventions, which may translate into high variability and low reproducibility in the outcomes assessed. Therefore, we need an adequately robust design of RCT about CHM formulas. The CONSORT extension for CHM Formulas was created in 2017 (24).

Reporting checklists just evaluate whether a study is reported in detail or not, or if important information is provided to allow reproducibility (25). They do not assess whether the procedure reported was, in fact, the correct one to use. Therefore, reporting checklists do not have adequate content validity to assess whether a study is of good/bad quality or whether a study has a high or low

risk of bias (26). The Cochrane Risk of Bias tool was developed to assess the degree to which the results of a study "should be believed" (27). Therefore, we used the CONSORT, CONSORT-CHM Formulas checklists, and the Cochrane Risk of Bias tool simultaneously for assessing the quality of the RCTs of CHM for MS in terms of completeness of reporting and validity of results, to provide a more comprehensive update on RCTs that investigated the efficacy and safety of CHM in the treatment of MS.

2 Methods

2.1 Data sources and search

Nine databases, namely, PubMed, Embase, Web of Science, Cochrane Library, EBSCO, Sinomed, Wanfang Database, China National Knowledge Infrastructure, and VIP Database, were searched from the establishment of the database to September 2023. MS and its synonyms in combination with the terms of CHM or their proprietary names were used as search terms, all as MeSH and as free-text words. Chinese databases were also searched using the above search terms in Chinese.

2.2 Eligibility criteria

The study design was RCTs that evaluate CHM in the treatment of MS, regardless of language or publication status. Patients were diagnosed with MS according to recognized criteria internationally, such as Poser (28) and McDonald (29, 30), regardless of sex, age, race/ethnicity, geographical residence, or course of disease. The experimental group used CHM as monotherapy or adjuvant therapy, not including acupuncture and needle punching. There is no restriction on the frequency and dosage of CHM. The duration of the treatment course was at least 3 weeks. The intervention for the control group was placebo plus Western medicine (WM), or WM alone or placebo alone.

2.3 Exclusion criteria

Exclusion criteria included animal experiments, case reports, reviews, retrospective studies, repeated publications, and historical controlled studies.

2.4 Data extraction

Two independent researchers (JW and JY) extracted the following data from the included studies using a standard table, including author, year, the criteria for diagnosing MS, intervention, dosage, control, number of participants, age, sex, treatment duration, follow-up period, outcome measures, and intergroup differences. Any disagreements on data extraction were resolved by a third reviewer (SX).

2.5 Reporting quality

We used the CONSORT 2010 and CONSORT-CHM Formulas 2017 checklists as assessment tool for reporting quality. Two researchers (JW and JH) independently extracted information according to two checklists, who were blinded to each other's ratings. "1" or "0" was scored to represent whether the RCT had reported the relevant item/subitem or not. "0" indicates no description of the corresponding item/subitem, and "1" indicates that the author had mentioned the description of the item/subitem in the report. Discrepancies were resolved by a third reviewer (MC). Furthermore, we summarized the CONSORT checklist into five sections: Title/Abstract and Introduction, Methods, Results, Discussion, and Other Information. We also grouped and compared manuscripts revised before CONSORT (before 2010) and after it has been revised. The CONSORT-CHM Formulas checklist was published in 2017, and we compared manuscripts published before with those published after 2017.

2.6 Risk of bias

The risk of bias was evaluated by using the RCT risk of bias assessment tool recommended by the Cochrane Handbook (31) and was performed independently by two researchers (JW and JH). If there was disagreement, they were discussed with and resolved by a third reviewer (SQ).

2.7 Description of the CHMs

The selection criteria of high-frequency herbs in the treatment of MS were those with cumulative frequencies over 50% among the top-ranked herbs. We also summarized the mechanisms of Chinese medicine monomers for MS that have been reported in previous studies.

2.8 Data analysis

We used Microsoft Excel 2016 for descriptive statistical analysis and counted the total number of RCTs corresponding to each CONSORT project. The subsequent results shown as percentages and 95% confidence intervals (CIs) were calculated for each overall ratio. SPSS (version 25.0) was used for statistical calculation. The significance level was presumed as $p < 0.05$.

3 Results

3.1 Study selection

The flowchart of the research selection process is listed in Figure 1. A total of 4,029 potentially relevant articles were identified, and 1,249 duplicates were excluded. Another 2,736

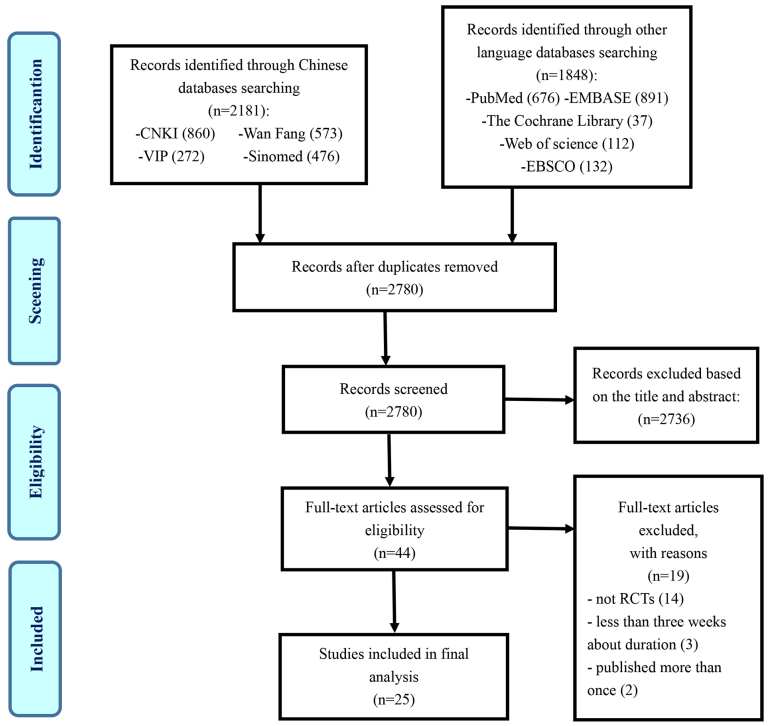

FIGURE 1
Flowchart of research selection process.

articles were excluded through screening titles and abstracts, leaving 44 articles for further evaluation. Nineteen studies were excluded for the following reasons: 14 articles were not RCTs, 3 articles were less than 3 weeks in the duration of treatment course, and 2 articles were suspected of being published more than once. Ultimately, 25 eligible studies were selected in this study (32–56).

3.2 Study characteristics

Table 1 shows partial information regarding the study characteristics. More details about the studies are shown in Additional file 1. A total of 1,450 participants were included in the 25 studies from 2004 to 2020, of whom 742 were in the treatment group, and 708 were in the control group. The ages ranged from 20 to 75 years old. The participants were 830 women and 607 men. Two studies (44, 45) did not report the sex of the participants. All the studies did not report sex disparity in response to treatment. All of the studies were conducted in China. One study was published in English (46), and the other 24 studies were published in Chinese. Eight studies (32, 33, 35–38, 53, 55) were diagnosed according to the Poser criteria; 17 studies (34, 39–52, 54, 56) were diagnosed according to the McDonald criteria. A total of 24 RCTs used CHM in combination with WM as the treatment group, and only 1 RCT used CHM alone as the treatment group. The duration of the studies lasted from 3 weeks to 135 days. As for outcome measure, 15 studies (32, 34–37, 40, 41, 43, 46, 48–50, 52, 54, 56) used the Expanded Disability Status Score (EDSS), 7 studies (32, 36, 37, 44, 46, 48, 49) used annual relapse frequency, 2 studies (46, 55) used annual relapse rate, and 2 studies (42, 44) used annual relapse interval. The total clinical efficacy rate was observed in 16 studies (32–35, 38–41, 43, 45, 51–56). Two studies used (50, 53) the Barthel index, and 2 studies (53, 56) used the SF-36 score.

Adverse effects were reported in 6 studies (32, 33, 38, 43, 45, 52), while the remaining 19 studies did not mention them. Four (32, 38, 43, 52) out of the six studies reported that no adverse effects happened in the CHM group. Wang et al. (33) reported two cases of adverse events in the CHM group and three cases in the WM group. Obesity and acne (45) were reported in the CHM group. Obesity, acne, liver dysfunction, and neuropsychiatric symptoms were reported in the WM group (43, 45). However, all studies did not mention life-threatening adverse effects.

3.3 Reporting quality

The 25 included studies consisted of 24 CHM formula studies and 1 CHM monomer study (51). The CONSORT-CHM Formulas checklist is used to evaluate the reporting quality of CHM formula RCTs. We therefore performed CONSORT scores on all included studies and CONSORT-CHM scores on 24 studies except for the monomer study. The distribution of the number of CONSORT and CONSORT-CHM Formulas items satisfied by the included studies is shown in Figures 2, 3. The CONSORT and CONSORT-CHM Formulas checklist sections are summarized in Figure 4 and individual items are described in Table 2.

TABLE 1 The characteristics of the included trials.

Study	Trial	Trial (male/female; age; duration)	Control (male/female; age; duration)	Treatment during	Outcome measure	Intergroup differences
Wu et al., 2020 (56)	Yangganyishen formula plus–minus 1 dose/d (200 mL) + CT	22 (M: 9, F: 13) Mean age: 36.80 y Mean disease duration: 3.00 ± 1.50 y	23 (M: 10, F: 13) Mean age: 35.40 y Mean disease duration: 4.30 ± 0.90 y	1 m	1. EDSS 2. Total clinical efficacy rate 3. SF-36 score	1. $p < 0.05$ 2. $p < 0.05$ 3. $p < 0.05$
Shi 2020 (55)	Improved pingfu decoction 2–3 doses/w (150–200 mL) + CT	35 (M: 18, F: 17) Mean age: 31.49 ± 2.06 y Mean disease duration: 4.01 ± 1.20 y	35 (M: 15, F: 20) Mean age: 32.22 ± 2.15 y Mean disease duration: 3.58 ± 1.21 y	1 m	1. Total clinical efficacy rate 2. Annual relapse rate	1. $p < 0.01$ 2. $p < 0.05$
Qian and Wang 2020 (54)	Ziyinguben granule 6 g/tid + CT	30 (M: 12, F: 18) Mean age: 33.47 ± 11.15 y Mean disease duration: 19.38 ± 7.12 m	30 (M: 10, F: 20) Mean age: 34.07 ± 10.92 y Mean disease duration: 19.41 ± 6.11 m	27 d	1. EDSS 2. Total clinical efficacy rate 3. Neurological deficit scale	1. $p < 0.05$ 2. $p < 0.05$ 3. $p < 0.05$
Huang et al., 2018 (53)	Bushentianjing formula 1 dose/d (400 mL) + CT	21 (M: 9, F: 12) Mean age: 43.4 ± 10.5 y Mean disease duration: 17.3 ± 11.8 m	21 (M: 10, F: 11) Mean age: 41.9 ± 12.3 y Mean disease duration: 16.8 ± 12.1 m	1 m	1. Total clinical efficacy rate 2. Neurological deficit scale 3. Barthel index 4. SF-36 score	1. $p < 0.05$ 2. $p < 0.05$ 3. $p < 0.05$ 4. $p < 0.05$
Fan et al., 2018 (52)	Bushenyisui capsule 6#tid + CT	24 (M: 4, F: 20) Mean age: 31.63 ± 10.05 y Mean disease duration: 65.63 ± 69.85 m	26 (M: 6, F: 20) Mean age: 32.73 ± 9.84 y Mean disease duration: 43.85 ± 51.37 m	3 m	1. EDSS 2. Total clinical efficacy rate 3. Adverse events	1. $p > 0.05$ 2. $p < 0.01$
Li 2017 (51)	*Tripterygium wilfordii* polyglycosides tablet 1 mg/(kg·d) bid + CT	45 (M: 15, F: 30) Mean age: 34.24 ± 5.21 y Mean disease duration: 1.24 ± 0.21 y	45 (M: 14, F: 31) Mean age: 34.21 ± 5.24 y Mean disease duration: 1.21 ± 0.25 y	3 m	1. Total clinical efficacy rate 2. Neurological Symptom Score (NSS)	1. $p < 0.05$ 2. $p < 0.05$
Wu et al., 2016 (50)	Yangganyishen formula plus–minus 1 dose/d (200 mL) + CT	21 (M: 6, F: 15) Mean age: 36.8 ± 3.6 y Mean disease duration: 3 ± 1.5 y	19 (M: 7, F: 12) Mean age: 35.4 ± 4.8 y Mean disease duration: 4.3 ± 0.9 y	1 m	1. EDSS 2. Barthel index	1. $p < 0.05$ 2. $p < 0.05$
Lu 2016 (49)	Self-made jieduyimian decoction 1 dose/d + CT	36 (M: 14, F: 22) Mean age: 41.2 ± 7.6 y Mean disease duration: 11.8 ± 4.6 y	36 (M: 15, F: 21) Mean age: 42.7 ± 7.2 y Mean disease duration: 12.1 ± 4.7 y	135 d	1. EDSS 2. Annual relapse frequency	1. $p < 0.05$ 2. $p < 0.01$
Chen and Wang 2016 (48)	Dihuangheji 6#tid + CT	54 (M: 30, F: 24) Mean age: 66.3 ± 5.63 y Mean disease duration: N.R.	54 (M: 30, F: 22) Mean age: 68.1 ± 4.86 y Mean disease duration: N.R.	135 d	1. EDSS 2. Annual relapse frequency	1. $p < 0.01$ 2. $p < 0.05$
Chen and Fan 2016 (47)	Bushenhuatan formula plus–minus 1 dose/d + CT	30 (M: 4, F: 26) Mean age: 39.2 ± 10.8 y Mean disease duration: 3.64 ± 4.17 y	30 (M: 6, F: 24) Mean age: 37.46 ± 11.09 y Mean disease duration: 4.43 ± 3.2 y	3 m	1. Multiple Sclerosis Impact Scale, MSIS - 29 2. Modified Fatigue Impact Scale, MFIS	1. $p < 0.05$ 2. $p < 0.05$

(Continued)

TABLE 1 Continued

Study	Trial	Trial (male/female; age; duration)	Control (male/female; age; duration)	Treatment during	Outcome measure	Intergroup differences
Zhou and Fan 2015 (46)	Erhuang formula 1 dose/d (200 mL) + CT	43 (M: 11, F: 32) Mean age: 30.77 ± 9.82 y Mean disease duration: 2.81 ± 2.05 y	24 (M: 9, F: 15) Mean age: 36.54 ± 11.64 y Mean disease duration: 2.71 ± 1.6 y	N.R.	1. EDSS 2. Annual relapse rate 3. Annual relapse frequency	1. $p > 0.05$ 2. $p < 0.01$ 3. $p < 0.01$
Li et al., 2015 (45)	CHM 1 dose/d + CT	24 (N.R.) Mean age: N.R. Mean disease duration: N.R.	24 (N.R.) Mean age: N.R. Mean disease duration: N.R.	1 m	1. Total clinical efficacy rate 2. Treatment onset time 3. Average length of stay 4. Adverse events	1. $p < 0.05$ 2. $p < 0.05$ 3. $p < 0.05$
Zhou et al., 2013 (44)	Shuganjianpigusui formula1 dose/d (400 mL) + CT	14 (NR) Mean age: 48.86 ± 10.54y Mean disease duration: 43.36 ± 39.7 m	21 (NR) Mean age: 46 ± 10.25 y Mean disease duration: 48.38 ± 40.52 m	3 w	1. Annual relapse frequency 2. Annual relapse interval	1. $p < 0.05$ 2. $p < 0.05$
Zhao 2013 (43)	Bushengusui tablet 6#tid + CT	18 (M: 9, F: 9) Mean age: 40.2 y Mean disease duration: 25 m	18 (M: 10, F: 8) Mean age: 40.5 y Mean disease duration: 22 m	3 m	1. Total clinical efficacy rate 2. EDSS 3. Average length of stay 4. Adverse events	1. $p < 0.05$ 2. $p < 0.01$ 3. $p < 0.05$
Wei 2012 (42)	CHM 1 dose/d + CT	25 (M: 4, F: 21) Mean age: N.R Mean disease duration: N.R	20 (M: 5, F: 15) Mean age: N.R Mean disease duration: N.R	3 m	1. Annual relapse interval	1. $p < 0.05$
Pu 2012 (41)	CHM 1 dose/d (200 mL) + CT	22 (M: 80, F: 14) Mean age: 34.5 ± 12.69 y Mean disease duration: 16.3 m	21 (M: 7, F: 14) Mean age: 38.95 ± 14.09 y Mean disease duration: 17.1 m	1 m	1. EDSS 2. Total clinical efficacy rate	1. $p < 0.05$ 2. $p < 0.05$
Li and Zhao 2012 (40)	CHM decoction 1 dose/d + CT	30 (M: 12, F: 18) Mean age: 40.24 ± 2.53 y Disease duration: 3.31 ± 1.25 y	30 (M: 13, F: 17) Mean age: 42.31 ± 1.24 y Disease duration: 3.26 ± 1.08 y	3 m	1. EDSS 2. Total clinical efficacy rate	1. $p < 0.01$ 2. $p < 0.05$
Yang et al., 2009 (39)	Simiaoyongan decoction and marrow storing pill plus–minus 1 dose/d	30 (M: 11, F: 19) Mean age: 35.67 ± 12.23 y Mean disease duration: N.R	15 (M: 5, F: 10) Mean age: 35.17 ± 12.64 y Mean disease duration: N.R	3 m	1. Total clinical efficacy rate	1. $p < 0.05$
Zeng et al., 2009 (38)	Buyanghuanwu decoction plus–minus 1 dose/d + CT	35 (M: 15, F: 20) Mean age: 42 y Mean disease duration: N.R	30 (M: 11, F: 19) Mean age: 40 y Mean disease duration: N.R	1 m	1. Total clinical efficacy rate 2. Adverse events	1. $p < 0.05$
Gao et al., 2008 (37)	Dihuanheji capsule 4#tid + CT	38 (M: 21, F: 17) Mean age: 37.10 ± 7.56 y Mean disease duration: N.R	40 (M: 24, F: 16) Mean age: 36.35 ± 7.67 y Mean disease duration: N.R	3 w	1. EDSS 2. Annual relapse frequency	1. $p < 0.001$ 2. $p < 0.05$
Fan et al., 2007 (36)	Erhuang formula + CT	30 (M: 9, F: 21) Mean age: 38.1 ± 12.48 y Mean disease duration: N.R	35 (M: 8, F: 27) Mean age: 36.46 ± 14.13 y Mean disease duration: N.R	N.R.	1. EDSS 2. Annual relapse frequency	1. $p < 0.05$ 2. $p > 0.05$

(Continued)

TABLE 1 Continued

Study	Trial	Trial (male/female; age; duration)	Control (male/female; age; duration)	Treatment during	Outcome measure	Intergroup differences
Zuo and Jia 2006 (35)	Yishengujintongluo formula 1 dose/d + CT	30 (M: 13, F: 17) Mean age: 33.4 ± 10.5 y Mean disease duration: 17.3 ± 11.8 m	30 (M: 12, F: 18) Mean age: 31.9 ± 12.3 y Mean disease duration: 16.8 ± 12.1 m	2 m	1. Total clinical efficacy rate 2. EDSS	1. $p > 0.05$ 2. $p < 0.01$
Zhang and Zhang 2006 (34)	Gusuitongluo decoction 1 dose/d + CT	30 (M: 13, F: 17) Mean age: 33.41 ± 10.52 y Mean disease duration: 17.32 ± 11.82 m	30 (M: 12, F: 18) Mean age: 31.93 ± 12.31 y Mean disease duration: 16.82 ± 12.14 m	2 m	1. EDSS 2. Total clinical efficacy rate	1. $p < 0.05$ 2. $p < 0.05$
Wang et al., 2006 (33)	Jiweiling decoction 1 dose/d + CT	36 (M: 20, F: 16) Mean age: 26.25 ± 6.70 y Mean disease duration: 2.58 ± 0.34 m	32 (M: 14, F: 18) Mean age: 27.65 ± 5.8 y Mean disease duration: 2.64 ± 0.41 m	2 m	1. Total clinical efficacy rate 2. Adverse events	1. $p < 0.01$
Shi and Wang 2004 (32)	Jiannaogusui decoction 1 dose/d + CT	19 (M: 7, F: 12) Mean age: 32.5 ± 11.6 y Mean disease duration: 40.5 ± 37.6 m	19 (M: 8, F: 11) Mean age: 33.1 ± 10.2 y Mean disease duration: 41.3 ± 31.53 m	3 m	1. Total clinical efficacy rate 2. EDSS 3. Annual relapse frequency 4. Adverse events	1. $p < 0.05$ 2. $p < 0.01$ 3. $p < 0.01$

CHM, Chinese herbal medicine; MPPT, methylprednisolone; N.R., no reported; EDSS, Expanded Disability Status Scale; CT, control therapy; d, day(s); w, week(s); m, month(s); y, year(s); tid, ter in die; bid, bis in die; ivgtt, intravenously guttae; po, per os; M, male; F, female.

3.3.1 CONSORT

Most of the items were satisfied by a few studies. Analysis of the included studies showed that, on average, 33% of the recommended items were reported. Fan et al. (52) reported the highest percentage of recommended items, at 47%, and Chen and Wang (48) and Yang et al. (39) reported the lowest percentage of recommended items, at 24%.

Only one (4%) trial could be identified as RCT after reading the title. Abstracts were structured appropriately in 92% of included studies and introductions were structured appropriately in 82%.

Seventeen items related to the methods section and were included in just 35% of included studies. Eight CONSORT items were not described in any of the articles (0%), and they were description of significant changes in the experimental method (item 3b), whether there are changes in the trial outcomes after the commencing of the experiment (item 6b), how sample size was determined (item 7a) and the explanation of any interim analysis and stopping guidelines (item 7b), the type of randomization (item 8b), the mechanism used to implement the random allocation sequence (item 9), the similarity of interventions (item 11b), and methods for additional analyses (item 12b).

The results sections included 11 items and were underreported. Overall, just 22% of included studies reported these items. None of the articles (0%) described the treatment progress with a diagram (item 13a), the reasons why the trial ended or was stopped (item 14b), the estimated effect size (item 17a), absolute or relative effect sizes (item 17b), and results of any other analyses performed (item 18).

Three items related to the discussion section, outlined study limitations, generalizability, and interpretation of results, and were reported in 44% of included studies. Information about the trial registration number, availability of the full protocol, and funding sources was reported in 0%, 0%, and 4% of included studies, respectively.

Studies published after the CONSORT checklist was revised in 2010 reported more items, although not significantly. Until 2010, 12 ± 2, or 32% of the items were reported in studies, and from 2010 onwards, 13 ± 2, or 34% were reported ($p = 0.657$, $t = 0.449$).

3.3.2 CONSORT-CHM Formulas

The CONSORT-CHM Formulas checklist items were not satisfactorily reported. Of the 39 items, an average of just 21% was reported in the included studies. Fan et al. (52) reported the highest percentage of recommended items, at 41%, and Shi (55), Chen and Wang (48), and Yang et al. (39) reported the lowest percentage of recommended items, at 13%.

Abstracts need to illustrate the name and form of the formula used and the TCM pattern applied. This was reported in only two (8%) of the included studies. None of the articles determined appropriate keywords, including “Chinese herbal medicine formula” and “RCT”. Introductions satisfied the criteria in 25% of the studies.

The methods section was poorly described, with only 21% of items reported on average. Four (17%) trials stated whether participants with a specific TCM pattern were recruited. Three (13%) articles reported the outcome measures related to TCM syndrome in detail. However, none of the articles described the

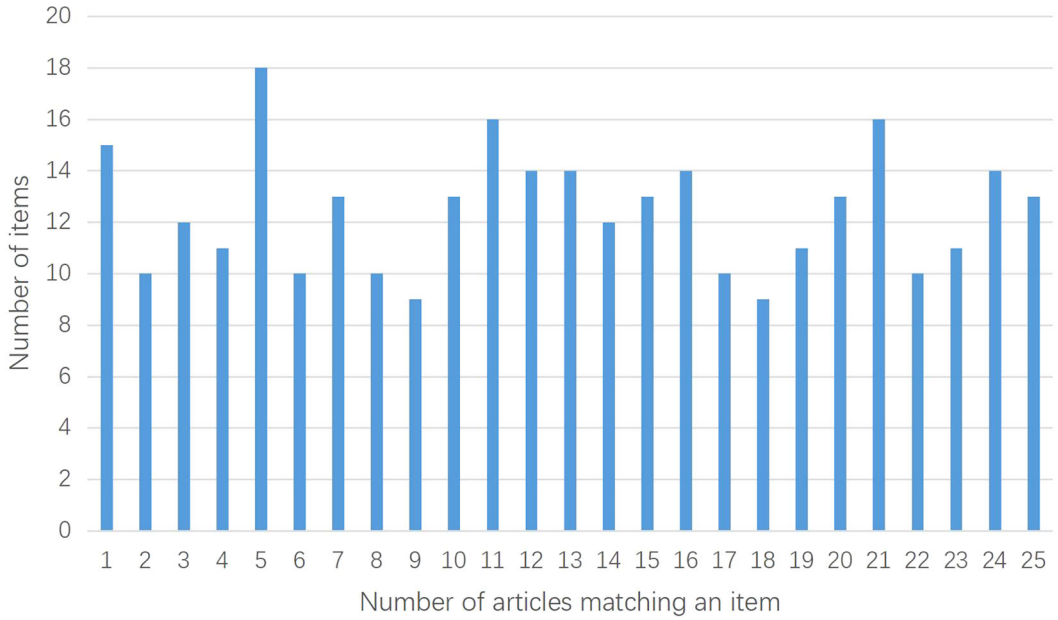

FIGURE 2
Distribution of the number of CONSORT items satisfied by the included studies.

CHM formula in detail, especially the source and authentication method of each CHM ingredient.

Items for the results section were the same as CONSORT. Discussion sections satisfied the criteria in 46% of the studies, but only three (13%) articles provided the discussion of how the formula works on different TCM patterns on disease.

After the CONSORT-CHM Formulas was published in 2017, RCT quality did not improve significantly. Until 2017, 8 ± 2, or 21% of the items were reported in studies, and from 2017 onwards, 8 ± 4, or 21% were reported (p = 0.894, t = 0.135).

3.4 Risk of bias

The summary and graph of the risk of bias are shown in Figures 5, 6. Although all the included trials claimed

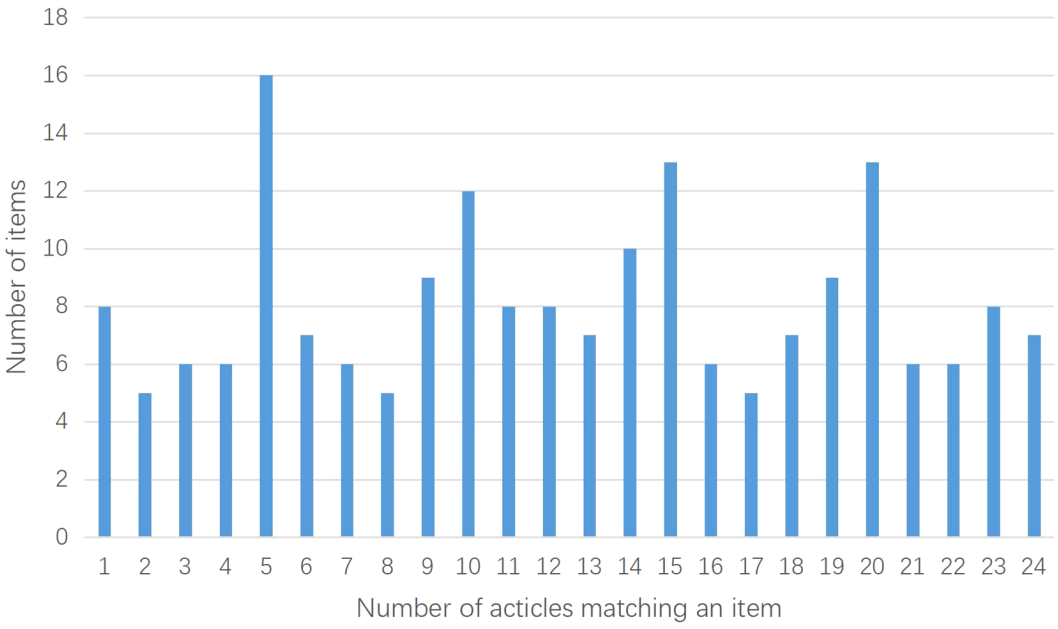

FIGURE 3
Distribution of the number of CONSORT-CHM Formulas items satisfied by the included studies.

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Section	Number of Items (before publication)	Percentage of Items (%)	Number of Items (after publication)	Percentage of Items (%)
CONSORT	Before 2010		After 2010	
Title/abstract-Introduction (4)	3	75%	3	75%
Methods (17)	6	35%	6	35%
Results (11)	3	27%	2	18%
Discussion (3)	1	33%	2	67%
Other information (3)	0	0%	0	0%
Total (38)	12	32%	13	34%
CONSORT-CHM	Before 2017		After 2017	
Title/abstract-Introduction (5)	1	20%	0	0%
Methods (17)	3	18%	4	24%
Results (11)	2	18%	2	18%
Discussion (3)	1	33%	1	33%
Other information (3)	0	0%	0	0%
Total (39)	8	21%	8	21%

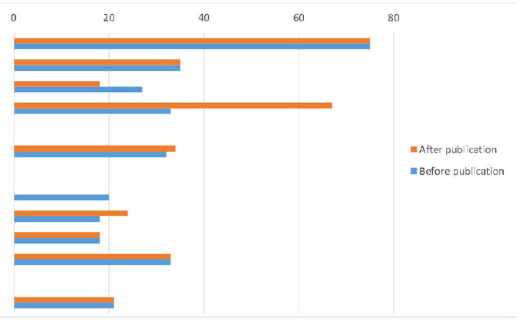

FIGURE 4
Numbers and percentages of CONSORT and CONSORT-CHM Formulas checklist sections reported by time period.

TABLE 2 Number and percentage of CONSORT and CONSRT-CHM Formulas checklist items reported in the included studies.

Section/ Topic	Item no.	Checklist item	*n*	CONSORT% (*n*/25) CONSORT% CHM (*n*/24)	95% CI
Title and abstract	1a	Identification as a randomized trial in the title	1	4%	[0 to 20]
	1a*	Statement of whether the trial targets a TCM pattern, a Western medicine-defined disease, or a Western medicine-defined disease with a specific TCM pattern	1	4%	[0 to 21]
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance, see CONSORT for abstracts)	23	92%	[74 to 99]
	1b*	Illustration of the name and form of the formula used, and the TCM pattern applied, if applicable	2	8%	[1 to 27]
	1c*	Determination of appropriate keywords, including "Chinese herbal medicine formula" and "RCT"	0	0%	[0 to 0]
Introduction					
Background and objectives	2a	Scientific background and explanation of rationale	19	76%	[55 to 91]
	2a*	Statement with biomedical science approaches and/or TCM approaches	6	25%	[10 to 47]
	2b	Specific objectives or hypotheses	22	88%	[69 to 98]
	2b*	Statement of whether the formula targets a Western medicine-defined disease, a TCM pattern, or a Western medicine-defined disease with a specific TCM pattern	6	25%	[10 to 47]
Methods					
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	24	96%	[80 to 100]
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	0	0%	[0 to 0]
Participants	4a	Eligibility criteria for participants	15	60%	[39 to 79]
	4a*	Statement of whether participants with a specific TCM pattern were recruited	4	17%	[5 to 37]
	4b	Settings and locations where the data were collected	24	96%	[80 to 100]

(Continued)

10.3389/fimmu.2024.1429895

TABLE 2 Continued

Section/ Topic	Item no.	Checklist item	*n*	CONSORT% (*n*/25) CONSORT% CHM (*n*/24)	95% CI
Methods					
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	25	100%	[100 to 100]
	5*	Description(s) for different types of formulas should include specific contents	0	0%	[0 to 0]
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	25	100%	[100 to 100]
	6a*	Illustration of outcome measures with pattern in detail	3	13%	[3 to 32]
	6b	Any changes to trial outcomes after the trial commenced, with reasons	0	0%	[0 to 0]
Sample size	7a	How sample size was determined	0	0%	[0 to 0]
	7b	When applicable, explanation of any interim analyses and stopping guidelines	0	0%	[0 to 0]
Randomization					
Sequence generation	8a	Method used to generate the random allocation sequence	8	32%	[15 to 54]
	8b	Type of randomization; details of any restriction (such as blocking and block size)	0	0%	[0 to 0]
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	0	0%	[0 to 0]
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	1	4%	[0 to 20]
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	1	4%	[0 to 20]
	11b	If relevant, description of the similarity of interventions	0	0%	[0 to 0]
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	22	88%	[69 to 98]
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	0	0%	[0 to 0]
Results					
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analyzed for the primary outcome	0	0%	[0 to 0]
	13b	For each group, losses and exclusions after randomization, together with reasons	2	8%	[1 to 26]
Recruitment	14a	Dates defining the periods of recruitment and follow-up	23	92%	[74 to 99]
	14b	Why the trial ended or was stopped	0	0%	[0 to 0]
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	7	28%	[12 to 49]

(Continued)

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TABLE 2 Continued

Section/ Topic	Item no.	Checklist item	*n*	CONSORT% (*n*/25) CONSORT% CHM (*n*/24)	95% CI
Results					
Numbers analyzed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	21	84%	[64 to 96]
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	0	0%	[0 to 0]
	17 b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	0	0%	[0 to 0]
Ancillary analyses	18	Results of any other analyses performed, including and analyses, distinguishing pre-specified from exploratory	0	0%	[0 to 0]
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	7	28%	[12 to 49]
Discussion					
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	6	24%	[9 to 45]
Generalizability	21	Generalizability (external validity, applicability) of the trial findings	8	32%	[15 to 54]
	21*	Discussion of how the formula works on different TCM patterns or diseases	3	13%	[3 to 32]
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	25	100%	[100 to 100]
	22*	Interpretation with TCM theory	24	100%	[100 to 100]
Other information					
Registration	23	Registration number and name of trial registry	0	0%	[0 to 0]
Protocol	24	Where the full trial protocol can be accessed, if available	0	0%	[0 to 0]
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	1	4%	[0 to 20]
Total mean score of CONSORT$_a$			12.4 ± 2.4		
Total mean score of CONSORT-CHM$_a$			8 ± 2.8		

CONSORT-CHM Formulas, CONSORT extension for Chinese herbal medicine formulas.
Items with * indicate that this item is CONSORT-CHM Formulas.
$_a$Mean ± SD.

randomization, only eight trials (33, 36, 37, 40, 41, 47, 52, 56) reported the method of random sequence generation. Only one study (52) mentioned allocation concealment, the blinding of participants, and investigator and outcome assessment. All studies reported complete outcome data. All of the studies were free of selective reporting except one study (42). All studies had low risk of other bias, which included incomparable baseline characteristics between the groups. The average item of low risk of bias for the 25 trials was 3.4, accounting for 49% of the total items.

3.5 Description of the CHMs

A total of 92 herbs were included in the 25 studies. The top 29 most frequently used herbs were ordinally Prepared Rehmannia Root (*Radix Rehmanniae Preparata*), Fresh Rehmannia Root (*Radix Rehmanniae Recens*), Epimrdii (*Herba Epimedii*), Scorpion (*Scorpio*), Indian Bread (*Poria*), Asiatic Cornelian Cherry Fruit (*Fructus Corni*), Stiff Silkworm (*Bombyx Batryticatus*), Root of Membranous Milkvetch (*Radix Astragali*), Root of Medicil

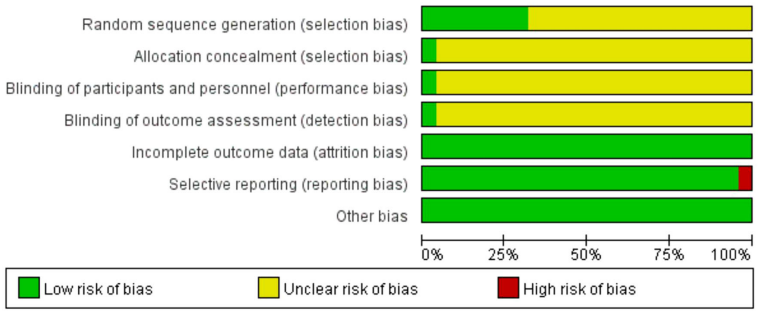

FIGURE 5
Risk of bias graph.

cyathula (*Radix Cyathulae*), Root of Chinese Angelica (*Radix Angelicae Sinensis*), Root of Ural Licorice (*Radix Glycyrrhizae*), Red Peony Root (*Radix Paeoniae Rubra*), Root Pilose Asiabell (*Radix Codonopsis pilosulae*), Dodder Seed (*Semen Cuscuta*), Tuber Fleeceflower (*Polygonum Multiflorum*), Seed of Jobstears (*Semen Coicis*), White Peony Root (*Radix Paeoniae Alba*), Desertliving Cistanche (*Herba Cistanches*), Phellodendron Bark (*Cortex Phellodendri*), Earthworm (*Pheretima*), Tall Gastrodia (*Gastrodia Elata*), Common Yam Rhizome (*Rhizoma Dioscoreae*), Eucommia Bark (*Cortex Eucommiae*), Fruit of Chinese Wolfberry (*Fructus Lycii*), Dried Tangerine Peel (*Pericarpium Citri Reticulatae*), Bulb of Fritillary (*Bulbus Fritillariae*), Leech (*Hirudo*), Szechuan Lovage Root (*Rhizoma Chuanxiong*), and Tuber of Pinellia (*Rhizoma Pinelliae*), which were used more than four times (Table 3). The cumulative percentage of these herbs was 62%.

The summary of the mechanisms of Chinese medicine monomers in the treatment of MS is shown in Table 4. Most Chinese medicine monomers can relieve inflammatory injury by inhibiting PI3K/Akt, NF-κB, and TLR4 signaling pathways and reducing the release of inflammatory factors TNF-α, IL-17A, IL-6, IL-23, IL-1β, and IFN-γ (58, 65, 68). Ginsenoside-Rg3 can regulate NOX2/4 oxidative stress-related pathways, reduce the expression of NOX, exert the effect of anti-oxidative stress, and improve the blood–brain barrier integrity (64). Ginsenoside Rd, Tanshinon IIA, Cordyceps sinensis extract, Rhodiola rosea, Baicalein, and Periplocoside A regulate the imbalance of Th17/Th1/Treg subsets and modulate immunity (63, 67, 70, 71, 73). Emodin and Matrine can upregulate the expression of the neurotransmitter-nutrient factor BDNF, improve the microenvironment of nerve survival, promote myelin repair and regeneration, and exert neuroprotective effects (65, 69).

4 Discussion

4.1 Principal findings

This article revealed that less than 50% of CONSORT and CONSORT-CHM Formulas items were reported in RCTs of CHM for MS. Moreover, revision of the CONSORT checklists and publication of CONSORT-CHM Formulas checklists had not significantly improved the quality of reporting in these studies. Consistently, the risk of bias in most RCTs was classified as "unclear." The low proportion of CONSORT items reported and the unclear risk of bias indicated that the quality of RCTs of CHM for MS was inadequate in terms of completeness of reporting and validity of results.

4.1.1 Reporting quality

RCTs of CHM in the treatment of MS had poor compliance with CONSORT and CONSORT-CHM Formulas statements. The average CONSORT and CONSORT-CHM Formulas proportions of all included studies were only 33% and 21%, respectively. To sum up, these studies had the following shortcomings:

From the perspective of CONSORT (1): The title did not indicate that the corresponding article is an RCT. Only one article in this study could be seen as an RCT based on the title (2). No article explained how to calculate the sample size. Relevant studies have found that if the pre-test sample size is not estimated, there is a lack of statistical ability to ensure the proper estimation of the treatment effect (74) (3). Most articles lacked the description of the randomization process, allocation concealment, and the blind method. However, randomization is necessary to avoid selection bias and the blinding procedure is an essential method for preventing research outcomes from being influenced by either the placebo effect or observer bias (4). No article showed a diagram of participant flow. Dropouts were only reported in one trial. These might reduce the credibility of the results (5). The estimated effect size or absolute and relative effect size can help readers better understand the benefits of drugs, but no article in this study provided the corresponding content (6). In the discussion, only a few articles explained the limitations and generalizability (7). Among other things, none of the trials in this study were registered and provided trial protocol. It was not conducive to improve transparency and accountability (75). Only one article explained the source of funds and conflict of interest.

From the perspective of CONSORT-CHM Formulas (1): No article added "Chinese herbal medicine formula" and "RCT" to the keywords (2). None of the articles reported in detail the source and authentication method of each CHM ingredient of CHM formula (3). All articles described the outcome indicators in detail, but only

FIGURE 6
Risk of bias summary.

13% of them reported the outcome indicators related to traditional Chinese medicine (TCM) syndromes.

The poor reporting quality of RCTs related to CHMs may not only affect the judgment of commentators and readers on their efficacy and safety (76) and reduce the value of CHMs, but also finally hinder the application and development of CHMs in the treatment of MS. Using the CONSORT guideline to ensure complete reporting is not difficult, and even brief additions during manuscript writing can resolve important omissions. For example, merely adding the words "random" or "randomized" to the title increases the likelihood that readers can readily identify RCTs (77). Although blinding itself may not always be feasible, reporting the extent to which blinding was addressed is crucial to enable readers to judge whether the presence or absence of blinding may have influenced results. Registration of study protocols allows readers to contrast planned and reported study methods, assists researchers in identifying ongoing work, and reduces unnecessary duplication (78). Furthermore, descriptions of detailed information about the herb would help readers to generalize and replicate the study (79). If reporting guidelines such as CONSORT-CHM Formulas are used at the outset of study design, the impact on reporting quality may be the greatest.

4.1.2 Association and comparison between the risk of bias and reporting quality

In this article, we found that the study with the highest percentage of CONSORT and CONSORT-CHM Formulas items had the lowest risk of bias. Studies in other medical fields showed similar findings to our study, in that better reporting quality was associated with lower risk of bias (80–82). However, the study with the lowest percentage of CONSORT and CONSORT-CHM Formulas items did not have the worst performance in the risk of bias. This indicated that although studies with poor reporting quality were incomplete and had low repeatability, the risk of bias might not be high and the results were valid to some extent. In this article, the study with the highest risk of bias did not have the lowest percentage of CONSORT and CONSORT-CHM Formulas items. It showed that the study with a high risk of bias lacked description of random sequence generation, allocation concealment, and outcome assessment blinding, but this did not mean the authors of the study used inappropriate research methods. Therefore, reporting quality and risk of bias are completely different concepts and still appear to create confusion in how they are being applied in the biomedical literature (26).

The risk of bias assesses the internal validity of RCTs, in other words, an evaluation of the true effect estimate. The reporting quality assesses the completeness of reporting. The risk of bias tool is most useful for assessing the authenticity of the result and bias retrospectively, but the CONSORT checklists are more appropriately applied in the guidance of prospective clinical studies (79). If researchers expect to obtain more complete and transparent RCTs in retrospective studies, the CONSORT checklists can also be a good choice.

It is worth noting that the herbal compounds are often in their natural form and quality control including authentication of constituents can be variable (83). Furthermore, effective application of traditional medicine theory to ensure data are valid and can be properly interpreted is difficult (84). The CONSORT-CHM Formulas adequately take into account the unique characteristics of TCM—theory, principles, formulas, and Chinese medicinal substances (24), which offer tailored guidance for assessing the methodological rigor and transparency of RCTs in CHM formulas. However, despite the availability of the CONSORT-CHM Formulas, introduced in 2017, its

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TABLE 3 Analysis of high-frequency herbs in the treatment of MS.

Chinese name	English name	Latin name	Frequency	The total frequency%	Cumulative percentiles%
Shudihuang	Prepared Rehmannia root	*Radix Rehmanniae Preparata*	11	4%	4%
Shengdihuang	Fresh Rehmannia root	*Radix Rehmanniae Recens*	9	4%	4%
Yinyanghuo	Epimrdii	*Herba Epimedii*	8	3%	3%
Quanxie	Scorpion	*Scorpio*	8	7%	7%
Fuling	Indian bread	*Poria*	8	3%	3%
Shanzhuyu	Asiatic cornelian cherry fruit	*Fructus Corni*	8	10%	10%
Jiangcan	Stiff silkworm	*Bombyx Batryticatus*	7	3%	3%
Huangqi	Root of membranous milkvetch	*Radix Astragali*	7	13%	13%
Chuanniuxi	Root of Medicil cyathula	*Radix Cyathulae*	7	3%	3%
Danggui	Root of Chinese Angelica	*Radix Angelicae Sinensis*	7	16%	16%
Gancao	Root of ural licorice	*Radix Glycyrrhizae*	6	3%	3%
Chishao	Red peony root	*Radix Paeoniae Rubra*	6	29%	29%
Dangshen	Root of pilose asiabell	*Radix Codonopsis pilosulae*	6	3%	3%
Tusizi	Dodder seed	*Semen Cuscuta*	6	18%	18%
Heshouwu	Tuber fleeceflower	*Polygonum Multiflorum*	6	3%	3%
Yiyiren	Seed of jobstears	*Semen Coicis*	6	21%	21%
Baishao	White peony root	*Radix Paeoniae Alba*	5	2%	44%
Roucongrong	Desertliving cistanche	*Herba Cistanches*	5	2%	46%
Huangbo	Phellodendron bark	*Cortex Phellodendri*	5	2%	47%
Dilong	Earthworm	*Pheretima*	4	1%	49%
Tianma	Tall gastrodia	*Gastrodia elata*	4	1%	50%
Shanyao	Common yam rhizome	*Rhizoma Dioscoreae*	4	1%	52%
Duzhong	Eucommia bark	*Cortex Eucommiae*	4	1%	53%
Gouqizi	Fruit of Chinese wolfberry	*Fructus Lycii*	4	1%	55%
Chenpi	Dried tangerine peel	*Pericarpium Citri Reticulatae*	4	1%	56%
Beimu	Bulb of fritillary	*Bulbus Fritillariae*	4	1%	58%
Shuizhi	Leech	*Hirudo*	4	1%	59%
Chuanxiong	Szechuan lovage root	*Rhizoma Chuanxiong*	4	1%	61%
Banxia	Tuber of pinellia	*Rhizoma Pinelliae*	4	1%	62%

utilization appears to be less widespread compared to other CONSORT extensions (85). CONSORT extensions for nonpharmacological treatments have proven valuable in evaluating the reporting quality of relevant RCTs in corresponding domains (86). In this article, we recognized the potential of CONSORT-CHM Formulas in improving the reporting quality of RCTs in CHM formulas. Therefore, to develop better-quality RCTs of CHM for MS, a rigorous design that integrates safety, efficacy, and patient-centered endpoints in accordance with the CONSORT-CHM Formulas guidelines is needed.

4.2 CHM in the treatment of MS

In the included studies, there were seven (33, 36, 37, 41, 47, 52, 55) studies with a low bias score of 4 and above, and CONSORT and CONSORT-CHM scores of 8 and above, which were of higher quality in terms of result validity and reporting completeness. A total of 33 herbs were used in these seven studies. Prepared Rehmannia Root (*Radix Rehmanniae Preparata*) and Fresh Rehmannia Root (*Radix Rehmanniae Recens*) were used five and four times, respectively, and were also the top two herbs in our count of high-frequency herbs. The

TABLE 4 The mechanisms of Chinese medicine monomers in the treatment of MS.

English name of Chinese medicine	Latin name	Monomer	Dosage	Treatment duration	Mechanism of action
Rehmannia glutinosa Libosch	*Radix Rehmanniae Preparata*	Catalpol	10 mg/kg	Injected intraperitoneally 40 d	Increase tyrosine hydroxylase expression and noradrenaline levels, neuroprotective effect (57)
Epimrdii	*Herba Epimedii*	Icariin	25 mg/kg	Administered intragastrically 42 d	Reduce AKT, iNOS, TNF-α, TGF-β1, and NF-κB, inhibition of oxidative stress and inflammatory response (58)
Asiatic cornelian cherry fruit	*Fructus Corni*	Cornuside	150 mg/kg/d	Administered intragastrically 28 d	Reduce IL-17A, IL-6, and IL-23, anti-inflammatory and immunosuppressive effects by inhibiting Th17 cells (59)
Root of membranous milkvetch	*Radix Astragali*	Total flavonoids of Astragalus	25 and 50 mg/kg/d	Administered intragastrically 21 d	Reduce NO, TNF-α, IL-6, and IL-1β, inhibition of microglia-mediated inflammation (60)
		Astragaloside IV	20 mg/kg	Injected intraperitoneally 10 d	Suppress the maturation and function of dendritic cells (13)
Root of ural licorice	*Radix Glycyrrhizae*	Glycyrrhizin	10, 25, and 50 mg/kg/d	Injected intraperitoneally 23 d	Reduce TNF-α, IFN-γ, IL-17A, and IL-6, and increase IL-4, inhibition of inflammatory response (61)
White peony root	*Radix Paeoniae Alba*	Total glucosides of paeony	0.2 g/kg, 0.4 g/kg	Administered intragastrically 14 d	Reduce mTOR and HIF-1α, anti-inflammatory and immune regulation (62)
Ginseng	*Radix Ginseng*	Ginsenoside Rd	20, 40, and 80 mg/kg	Administered intragastrically 21 d	Reduce IFN-γ, IL-6, and IL-10, and regulate the imbalance of Th17/Th1/Treg, anti-inflammatory, and immune regulation (63)
Red ginseng	*Radix Ginseng Rubra*	Ginsenoside-Rg3	500 mg/kg	Administered intragastrically 33 d	Reduce NOX2 and NOX4 expression to improve the blood–brain barrier integrity, neuroprotective, anti-inflammatory, and anti-oxidative effects (64)
Root and rhizome of sorrel rhubarb	*Radix et Rhizoma Rhei*	Emodin	30 and 60 mg/kg/d	Administered intragastrically 21 d	Increase MBP and BDNF. Reduce p-Akt, p-PI3K, and NF-κB, inhibition of microglial activation, anti-inflammatory, and neuroprotective effects (65)
Tarragon	*Artemisia dracunculus*	Artemisia dracunculus extracts	500 mg/kg/d	Administered intragastrically 33 d	Reduce IL-17 and IL-23, inhibition of inflammatory effect (66)
Root of ligulilobe sage	*Radix Salviae Liguliobae*	Tanshinon IIA	25 μmol/L	Injected intraperitoneally 11d	Reduce the demyelination and the number of inflammatory cells, and increase regulatory T cells (67)
Common threewingnut root	*Radix Tripterygii Wilfordii*	Tripterygium glycosides	9 mg/kg	Administered intragastrically 5 d	Reduce TLR4, TLR9, TNF-α, IL-1β, and IL-6, anti-inflammatory and immune regulation (68)
Root of lightyellow sophora	*Radix Sophorae Flavescentis*	Matrine	200 mg/kg/d	Injected intraperitoneally 23 d	Increase MBP and PLP, reduce p-PI3K, p-Akt, p-mTOR, and promote oligodendrocyte differentiation and myelination (69)
Chinese caterpillar fungus	*Cordyceps*	Cordyceps sinensis extract	1 g/kg, 5 g/kg	Administered intragastrically 30 d	Reduce the number of Th1 cells, inflammatory infiltration, and demyelination (70)
		Cordycepin (3'-deoxyadenosine)	50 mg/kg	Injected intraperitoneally 21 d	Reduce IFN-γ, IL-6, TNF-α, and IL-17, inhibit leukocyte infiltration and dendritic cell activation and migration, and reduce neuroinflammation
Grass of rhodiola	*Radix et Rhizoma Rhodiolae*	Rhodiola rosea	50, 100, and 200 mg/kg	Administered intragastrically 28 d	Regulate Th17/Th1 and Th17/Treg ratios, immune regulation (71)

(Continued)

TABLE 4 Continued

English name of Chinese medicine	Latin name	Monomer	Dosage	Treatment duration	Mechanism of action
Scutellarin baicalensis	*Radix Scutellariae*	Baicalein	25 mg/kg	Administered intragastrically 21 d	Suppress pathogenetic CXCR6 CD4 cells $^{+}$, reduce IL-17A production, and inhibit Th17 differentiation (72)
Periploca sepium Bge	*Periploca nigrescens*	Periplocoside A	25 and 50 mg/kg	Administered intragastrically 25 d	Suppress IL-17 production and the differentiation of Th17 cells, immunosuppressive and anti-inflammatory effects (73)

remaining seven herbs that were used three times were as follows: Scorpion (*Scorpio*), Tuber Fleeceflower (*Polygonum Multiflorum*), Bulb of Fritillary (*Bulbus Fritillariae*), Leech (*Hirudo*), Motherwort (*Herba Leonuri*), Tall Gastrodia (*Gastrodia Elata*), and Weeping Forsythia Capsule (*Fructus Forsythiae*), all of which were also high-frequency herbs, except for Motherwort and Weeping Forsythia Capsule. The seven studies reported four outcome measures. Four (36, 37, 41, 52) studies used EDSS, and three (36, 37, 41) studies showed a significant effect of CHM in reducing EDSS compared to the WM group. Four (33, 41, 52, 55) studies reported that CHM significantly improved the total clinical efficacy rate. One (33) out of two (33, 36) studies reported that CHM significantly reduced the annual relapse frequency. One (55) study reported that CHM significantly reduced the annual relapse rate. The selected high-frequency herbs from the present study mostly tonified the liver and kidney. In TCM, it is widely believed that the pathogenesis of MS is related to deficiencies in the kidney, liver, and spleen. These high-frequency herbs are promising candidates for future clinical applications and MS trials.

The major challenge in the treatment of MS remains understanding and targeting the continuous neurodegeneration in people with MS at present (87). The mechanisms that lead to neurodegeneration in MS involve a complex interplay between neuroinflammation, oxidative stress and mitochondrial dysfunction, and iron toxicity (88). Chinese medicine monomers have significant biological activity proved by many experiments. They can act on multiple aspects of the pathogenesis of MS, such as inflammatory response, immunity, apoptosis, and nerve injury (89). Our summary of previous research mechanisms reveal the following: The Chinese medicine monomers can reduce the release of inflammatory factors and relieve inflammatory injury by inhibiting PI3K/Akt, NF-κB, and TLR4 signaling pathways (58, 65, 68). They can also reduce NOX2 and NOX4 expression to exert the effect of anti-oxidative stress (64). The Chinese medicine monomers regulate the imbalance of Th17/Th1/Treg subsets and modulate immunity (63, 67, 70, 71, 73). The monomers can upregulate the expression of the neurotransmitter-nutrient factor BDNF and promote myelin repair and regeneration (65, 69). However, the current research mechanisms of Chinese medicine monomers are mostly limited to the study of inflammatory factors, chemokines, and common inflammatory metabolic pathways. There are few studies that discuss the potential molecular mechanisms of promoting myelin regeneration. The research on the internal relationship between various factors and the crosstalk mechanism between multiple signaling pathways is relatively scarce. Furthermore, the latest progress of MS reported in *The Lancet Neurology* journal states that "oxidative stress and mitochondrial dysfunction contributing to glial and neuronal injury, axonal energy failure, and loss of neuronal network function may be key molecular mechanisms driving disease progression. Excessive iron deposition in CNS parenchyma has been hypothesized to be a source of oxidative stress in MS (90)." Chinese medicine monomers are less studied in this area and need to be further explored.

The safety of CHM therapy for MS remains inconclusive. In this study, 24% (6/25) of RCTs mentioned the safety of interventions or investigated adverse effects. In four out of the six studies, it was reported that no adverse effects occurred in the CHM group. In the remaining studies, the adverse effects observed in the CHM group were fewer compared to the WM group. Studies have reported that herbal medicines are generally well tolerated, with adverse effects being limited to mild to moderate (91). Some herbal supplementations did not report risks of drug interactions with conventional MS drugs (4). There is still a need for powerful real-world evidence regarding the safety of CHM and more attention should be given to both recording and reporting the adverse effects of CHM therapy.

4.3 Strengths and limitations

To our knowledge, this is the first time the CONSORT, CONSORT-CHM Formulas checklists, and risk of bias have been simultaneously used to assess the quality of RCTs of CHM for MS.

MS remains one of the most common causes of neurological disability in the young adult population (87). A definitive curative treatment of MS is currently unavailable. The prolonged therapy of drugs constantly results in resistance and side effects. CHM formulas may provide a completely new area for the management of MS. Evaluating the quality of RCTs in CHM formulas helps bridge the gap between MS treatments using CHM formulas and modern WM. Our research may help provide a more rigorous understanding of CHM's efficacy and safety by evaluating the quality of RCTs in CHM formulas for MS. Furthermore, in the modern era of evidence-based practice, our findings will help

improve the quality of RCT in CHM formulas for MS, aiding clinicians in making informed decisions of CHM formulas based on the best available evidence. Finally, we screened high-frequency herbs as promising candidates for future clinical applications and MS trials. Mechanistic studies of Chinese medicine monomers in the field of oxidative stress, mitochondrial dysfunction, iron toxicity, and remyelination are rare and worthy of further research.

This study had some limitations. First, the number of RCTs included in this study was not enough, and there might be a risk of bias. Second, for many elements of reporting checklists and risk for bias, inconsistent judgment may have arisen from raters' different understanding or difficulties in discerning information from the published reports. Third, the 25 included studies reported different CHMs with variation in terms of composition, dosage, and duration of interventions. Different concentration ratios of the components of CHMs produced different effects, which could influence the bias of the research. This makes it difficult to recommend specific CHMs for clinics. Fourth, exact diagnostic criteria for patients with MS were not considered, as multiple diagnostic criteria were reported in the included RCTs. Different inclusion criteria of the participants may contribute to the heterogeneity of the included studies.

5 Conclusions

The low reporting of CONSORT items and unclear risk of bias indicate inadequate quality of RCTs in terms of reporting completeness and result validity. The CONSORT-CHM Formulas appropriately consider the unique characteristics of CHM, including principles, formulas, and Chinese medicinal substances. To improve the quality of RCTs on CHM for MS, researchers should adhere more closely to CONSORT-CHM Formulas guidelines and ensure comprehensive disclosure of all study design elements. High-frequency herbs are promising candidates for future clinical applications and MS trials.

Data availability statement

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

Ethics statement

This article is a literature study of previously published data and therefore does not require ethical approval or consent procedures. The manuscript does not contain any personal data in any form (including personal details, pictures or videos).

Author contributions

J-YW: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. J-LY: Data curation, Writing – original draft. J-LH: Data curation, Writing – original draft. SX: Formal analysis, Writing – review & editing. X-JZ: Formal analysis, Writing – review & editing. S-YQ: Formal analysis, Writing – review & editing. M-LC: Formal analysis, Writing – review & editing. MA: Formal analysis, Writing – review & editing. JZ: Formal analysis, Writing – review & editing. ZZ: Formal analysis, Writing – review & editing. G-QZ: Formal analysis, Funding acquisition, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1429895/full#supplementary-material

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Herbal medicines for SOD1^{G93A} mice of amyotrophic lateral sclerosis: preclinical evidence and possible immunologic mechanism

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Currently, there is no cure or effective treatment for Amyotrophic Lateral Sclerosis (ALS). The mechanisms underlying ALS remain unclear, with immunological factors potentially playing a significant role. Adhering to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA), a systematic review of preclinical studies was conducted, searching seven databases including PubMed, covering literature from the inception of the databases to April 10, 2024. Methodological quality of the included literature was assessed using CAMARADES, while the risk of bias in the included studies was evaluated using SYRCLE's ROB tool. Review Manager 5.4.1 statistical software was used for meta-analysis of the outcomes. The scoping review followed the Joanna Briggs Institute Methodological Guidelines and reporting of this review followed the PRISMA-extension for Scoping Reviews (PRISMA -ScR) checklist to explore the immunological mechanisms of Herbal Medicine (HM) in treating ALS. This systematic review and meta-analysis involved 18 studies with a total of 443 animals. The studies scored between 4 to 8 for methodological quality and 3 to 7 for risk of bias, both summing up to 10.A remarkable effects of HM in ALS mice, including onset time(Standardized Mean Difference(SMD): 1.75, 95% Confidence Interval(CI) (1.14 ~ 2.36), Z = 5.60, $P < 0.01$), survival time(SMD = 1.42, 95% CI (0.79 ~ 2.04), Z = 4.44, P < 0.01), stride length(SMD=1.90, 95% CI (1.21 to 2.59), Z = 5.39, P < 0.01) and duration time (Mean Difference(MD)=6.79, 95% CI [-0.28, 13.87], Z=1.88, P =0.06), showing HM's certain efficiency in treating ALS mice. The scoping review ultimately included 35 articles for review. HMs may treat ALS through mechanisms such as combating oxidative stress, excitatory amino acid toxicity, and calcium cytotoxicity, understanding and exploring the mechanisms will bring hope to patients. Individual herbs and their formulations within HM address ALS through a variety of immune pathways, including safeguarding the blood-brain barrier, countering neuroinflammation, impeding complement system activation, mitigating natural killer cell toxicity, and regulating T cell-

mediated immune pathways. The preclinical evidence supports the utilization of HM as a conventional treatment for ALS mice. Growing evidence indicates that HM may potentially delay neurological degeneration in ALS by activating diverse signaling pathways, especially immune pathways.

KEYWORDS

amyotrophic lateral sclerosis, herbal medicine, meta-analysis, scoping review, mechanism of immunology

1 Introduction

Amyotrophic Lateral Sclerosis(ALS)is characterized by dysfunction of upper motor neurons and lower motor neurons, affecting the medulla, cervical, thoracic, and/or lumbar segments. This results in progressive weakening of voluntary skeletal muscles, leading to symptoms such as limb movement impairment, swallowing difficulty, speech problems (dysarthria), and respiratory dysfunction (1). The median survival time of ALS is reported as 20 to 48 months after the onset of symptoms, among which 90% to 95% are sporadic ALS, and 5% to 10% of patients are familial ALS (2). As ALS remains incurable, treatment is focused on using disease modifying therapies and maximizing quality of life (1). Some countries have approved riluzole and edaravone as medications for slowing the progression of ALS. Riluzole, an anti-glutamate agent, prolongs survival in ALS patients in clinical trials and post-marketing analyses, but whether this occurs in all stages of ALS or only in advanced disease remains controversial (3). Some studies have reported that people with ALS who meet certain criteria may benefit from the use of edaravone, which has antioxidant properties (4–6). However, possibly because the study design lacked general applicability to the wider population of ALS patients, post-marketing analyses have raised questions about the safety and benefits of edaravone. As a result, the use of edaravone is still controversial and does not yet have regulatory approval around the world (1).

An increasing number of people with ALS resort to HMs because of the modest benefits of current therapies. In China and several other Asian countries, HM is widely used alongside Western Medicine (WM), with both systems cooperating to provide healthcare services for the population. However, the diverse responses to HM continue to be a subject of ongoing debate and challenge (7). In recent decades, numerous studies have investigated the effectiveness of HMs in treating ALS. Previous systematic reviews have indicated that short-term adjunctive use of HM may improve ALS Functional Rating Scale (ALSFRS) scores and clinical outcomes, with a favorable safety profile compared to placebo or riluzole alone. However, further research is required to evaluate the long-term efficacy of patient-oriented outcomes (8). Additionally, there is very low to low-quality evidence indicating that HMs may produce superior treatment responses for ALS without an increased risk of adverse events (9). Nevertheless, with their widespread use, HMs have attracted both praise and criticism. A single-center cohort study found that certain HMs were associated with a poorer prognosis in ALS patients (10).

ALS is a fatal central nervous system neurodegenerative disease. At present, the etiology and pathogenesis of ALS are still unclear. Evidence from clinical studies suggests that dysregulated immune responses contribute to heterogeneity in the clinical presentation of ALS (11). Immune inflammation caused by abnormal immune disorders, such as microglial activation, astrocyte proliferation and T cell infiltration, can be observed at the site of motor neuron degeneration (12), and immune cell infiltration can accelerate disease progression (13), All these suggest that abnormal immune disorders play an important role in the occurrence and development of ALS, and this treatment direction will be an effective treatment strategy. Exactly, in animal models of ALS and *in vitro*, the effects of HMs have been consistently praised. This phenomenon creates confusion among researchers and patients regarding the efficacy of HM in ALS studies, leading to questions about its utility and study design implications (10). The efficacy and mechanisms of HMs for experimental ALS have not been systematically evaluated yet. In addition, preclinical systematic review of animal data can provide preclinical evidence for the potential translational value from animal models to human disease. Thus, the present study aims to evaluate the efficacy and immunologic mechanisms of HMs through experimental ALS animal models.

Abbreviations: ALS, Amyotrophic Lateral Sclerosis; HM, Herbal medicine; WM, Western Medicine; ALSFRS, ALS Functional Rating Scale; CNS, Central nervous system; ROS, Reactive oxygen species; RNS, Reactive nitrogen species; GSH, Glutathione peroxidase; CAT, Catalase; ATP, Adenosine triphosphate; Nrf2, Nuclear factor erythroid 2-related factor 2; OS, Oxidative stress; PD, Parkinson's disease; MS, Multiple sclerosis; GEN, Geniposide; PGSF, Polyhemigosaponin F; TLR4, Toll-like receptor 4; iNOS, Nitric oxide synthase; COX-2, Circular cell enzymase-2.

2 Methods

2.1 Systematic analysis

2.1.1 Approach

We followed the guidelines outlined in the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) (14). There was no need for ethical approval because this was a literature research.

2.1.2 Data sources and search strategy

Two experienced researchers (YJL and WJY) independently carried out extensive searches for studies on HM for ALS. We searched the following electronic databases from their inception until April 10, 2024: PubMed, EMBASE, Web of Science, Cochrane Library, Wan fang database, Vasoactive Intestinal Polypeptide (VIP), China National Knowledge Infrastructure (CNKI), and Sinomed. The following keywords were used for the preclinical evidence: ("Chinese herbal medicine" OR "herbal medicine" OR "Traditional Chinese medicine" OR "Chinese Drug" OR "Korean Medicine" OR "East Asia Medicine") AND ("Amyotrophic Lateral Sclerosis" OR "motor neuron disease" OR "Gehrig's Disease" OR "Motor System Diseases"). Moreover, reference lists of potential articles were searched for relevant studies. All the studies included were limited on animals (Supplementary Data Sheet 1).

2.1.3 Inclusion and exclusion criteria

Literature screening was conducted collaboratively by a minimum of two members of our research team. In the initial screening phase, a comprehensive plan for full-text screening was meticulously devised. Each researcher independently assessed the abstracts and methodologies of the literature, initially selecting relevant articles. Subsequently, selected literature underwent thorough full-text review, with articles meeting the criteria being ultimately chosen. The screening results were then integrated and consolidated by the research team members, leading to the creation of corresponding flowcharts. In cases of differing opinions among team members during the screening process, careful negotiation and discussion were undertaken to reach a unanimous final decision.

The studies meeting the inclusion criteria were included in the meta-analysis: (1) Studies of HMs for ALS; (2) Inclusion of studies with Riluzole, Edaravone, normal saline and distilled water as control groups; (3) HM as monotherapy was used in the intervention group; (4) Identified $SOD1^{G93A}$ transgene mouse; (5) The primary outcome measures were onset time or survival time, while the secondary outcome measure was stride length and duration time. When the mice on the transfer bar movement for the longest time less than 5 min record the day as the onset time (15). After mice will lie down if it cannot turn to normal within 30 seconds gesture, determine its death, the death date of record this day for mice (16).

Exclusion criteria for the studies were as follows: (1) Lack of a control group; (2) Case reports, clinical experiments, reviews; (3) Cell experiments; (4) Repeated publications or studies with missing data.

2.1.4 Data extraction

Two separate authors independently extracted the following details from the included studies: (1) The primary author's name and the year of publication; (2) The specific details of animals for each study, including species, quantity, gender; (3) Accepted ALS mouse model; (4) The specifics of the treatment group, including the dosage, administration method of the therapeutic drug, treatment duration, and corresponding details for the control group; and (5) Efforts were made to contact authors for supplementary information when certain publications contained only graphical data. In instances of no response, numerical data were extracted from graphs using digital ruler software.

2.1.5 Study quality and risk of bias

In the methodology section of this study, we will employ two evaluation tools, CAMARADES (Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies) (17) and SYRCLE's ROB tool (Systematic Review Centre for Laboratory animal Experimentation's Risk of Bias) (18), to assess the quality and risk of bias in the included studies. CAMARADES will be used to evaluate the experimental design and methodological quality of the studies, such as sample size, randomization, and blinding. SYRCLE's ROB will be used to assess the risk of bias in each study during the experimental process, including selective reporting and sample size calculation. By utilizing these two evaluation tools in conjunction, we aim to comprehensively assess the quality and risk of bias in animal experimental studies, providing stronger support for the interpretation and generalization of research results.

CAMARADES primarily focused on ten aspects of the literature: (1) Publication in a peer-reviewed journal; (2) statement of temperature control; (3) randomization to treatment group; (4) allocation concealment; (5) blinded assessment of outcome; (6) avoidance of anesthetics with known notable intrinsic neuroprotective properties; (7) Use appropriate ALS animal models; (8) sample size calculation; (9) compliance with animal welfare regulations; (10) declared any potential conflict of interest. The scoring method of this scale involves assigning scores on a scale ranging from 0 to 10.

In contrast, SYRCLE's ROB primarily focused on ten aspects of the literature: (1) Randomization (selection bias); (2) Random sequence generation (selection bias); (3) Baseline characteristics (selection bias); (4) Allocation concealment; (5) Random housing (performance bias); (6) Blinding of personnel (performance bias); (7) Random outcome assessment (detection bias); (8) Blinding of outcome assessment (detection bias); (9) Incomplete outcome data (attrition bias); (10) Selective reporting (reporting bias). According to the scoring method of this scale, scores are assigned on a scale from 0 to 10.

2.1.6 Statistical analysis

Review Manager 5.4.1 statistical software was used for meta-analysis of the included literature. Stata 15 software is used to conduct sensitivity analysis to assess the robustness of data analysis. The outcome measures selected in our study include: onset time, survival time, stride length, and disease duration. All outcome measures are continuous. For these variables, we applied Weighted Mean Difference (WMD) or Standardized Mean Difference (SMD) as aggregate statistics. Each effect size included a 95% confidence interval (95% CI), and a combined p-value ≤ 0.05 was deemed statistically significant.

2.2 Scoping review

2.2.1 Approach

The scoping review adhered to the Joanna Briggs Institute Methodological Guidelines (19) and reporting of this review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analysis extension for Scoping Reviews (PRISMA-ScR) checklist (20).There was no need for ethical approval because this was a literature research.

2.2.2 Data sources and search strategy

Two experienced researchers (YJL and LJJ) independently conducted comprehensive searches for studies on the immune mechanism effects of HM, using the PubMed and CNKI databases from their inception until April 10, 2024. The following keywords were used for the possible mechanisms of immunology: ("Chinese herbal medicine" OR "herbal medicine" OR "Traditional Chinese medicine" OR "Chinese Drug" OR "Korean Medicine" OR "East Asia Medicine") AND ("immune response" OR "immune dysregulation" OR "immunologic mechanism" OR "Immunological reaction" OR "Immune Processes"). both in English and Chinese. There is no limitation on language or publication type. We also screened the references of included studies to ensure that no eligible studies were missed by the search strategy (Supplementary Data Sheet 2).

2.2.3 Inclusion and exclusion criteria

In our study, two team members (YJL and WJY) collaborated on literature screening. We meticulously planned the full-text screening process. Each researcher independently evaluated abstracts and methodologies to find relevant articles. Selected literature underwent a rigorous full-text review, advancing only if meeting our criteria. We synthesized the screening results and illustrated them with concise flowcharts. In case of disagreements, we discussed until reaching a consensus to ensure the integrity of our decisions.

For inclusion in the scoping review, articles must focus on the mechanisms of HM in treating ALS, with a particular emphasis on immune mechanisms. The search will encompass all types of qualitative, quantitative, and mixed-method studies, irrespective of their design, and will not be restricted by language. Publications were excluded if they did not discuss the results of ALS or HM. Additionally, publications focusing solely on WM treatment and not on the mechanism of herbal treatment for ALS were excluded. Finally, publications meeting the aforementioned criteria but reporting non-primary research, such as editorials, letters, concept papers, review articles, unpublished literature, dissertations, books, and book studies, were also excluded.

2.2.4 Data extraction

We collected data using specially designed extraction forms. The following information was recorded for each study: (a) author, (b) year of publication,(c) journal, (d) country, (e) type of studies, (f) Involved mechanisms (g)Involved HMs Two researchers performed the data extraction and synthesis processes independently (YJL and LJJ). A third researcher resolved any disagreement.

3 Results

3.1 Systematic analysis

3.1.1 Study inclusion

For the preclinical evidence research section, we initially identified 2026 papers through systematic searches across six databases. After removing duplicates, 1718 records persisted. Upon careful examination of titles and abstracts, 1679 articles were excluded for one or more of the following reasons: (1) the article constituted a review, case report, comment, abstract-only, or editorial; (2) the article was not related to animal studies; (3) the article did not focus on research related to ALS; (4) the article did not focus on the therapeutic effects of HM. After thorough examination of the full text of the remaining 39 articles, 21 articles were excluded for one or more of the following reasons: (1) the primary outcome measures were not survival time and onset time, (2) incomplete outcome measure data, (3) primarily cell-based studies, and (4) intervention involving acupuncture at Zusanli (ST36) acupoint (Figure 1).

3.1.2 Characteristics of included studies

We ultimately selected 18 studies (21–39) involving 19 comparisons, comprising 8 Chinese (22–28, 38) and 10 English (21, 29, 31–37, 39) publications. One study did two different comparisons because of the way they were treated (38). All 19 comparisons used $SOD1^{G93A}$ mice as the experimental group, with each group exclusively receiving HM treatment. Fourteen comparisons (21–24, 26, 28, 29, 31, 33, 34, 36, 38, 39) included a blank control group, which was regularly administered saline or distilled water. Meanwhile, Six comparisons established a positive control group, five (24, 25, 32, 38, 39) of these used riluzole and one (38) used edaravone as the positive controls. Among these comparisons, seven utilized male animals exclusively (21, 22, 24, 32, 33, 36, 37), one utilized female animals exclusively (19), and three used an equal mix of males and females (25, 26, 28), eight comparisons did not specify the gender of the animals. Among the included comparisons, 6 employed a concentration gradient of HMs (26–28, 32, 38, 39). Nine comparisons administered treatments via oral gavage (22, 24, 26, 29, 32, 37–39), six via oral administration (21, 28, 29, 33, 34), two via intraperitoneal injection (36, 38) one via bilateral subcutaneous injection (33), and one did not specify the method of administration (25). Ten comparisons simultaneously recorded onset time and survival time as outcome measures (22, 23, 25–29, 32, 34, 39) For outcome measures, when a concentration gradient was employed, the highest concentration group was recorded (Table 1).

3.1.3 Study quality and risk of bias

3.1.3.1 CAMARADES

The quality scores of the studies ranged from 4 to 8, with a total score of 10. One study received a score of 4 (25); two studies received a score of 5 (29, 38); four studies received a score of 6 (23, 24, 37, 39); seven studies received a score of 7 (23–25, 28, 29, 32, 33), and four studies received a score of 8 (21, 22, 33, 34). All included records were peer-reviewed publications, and all studies utilized

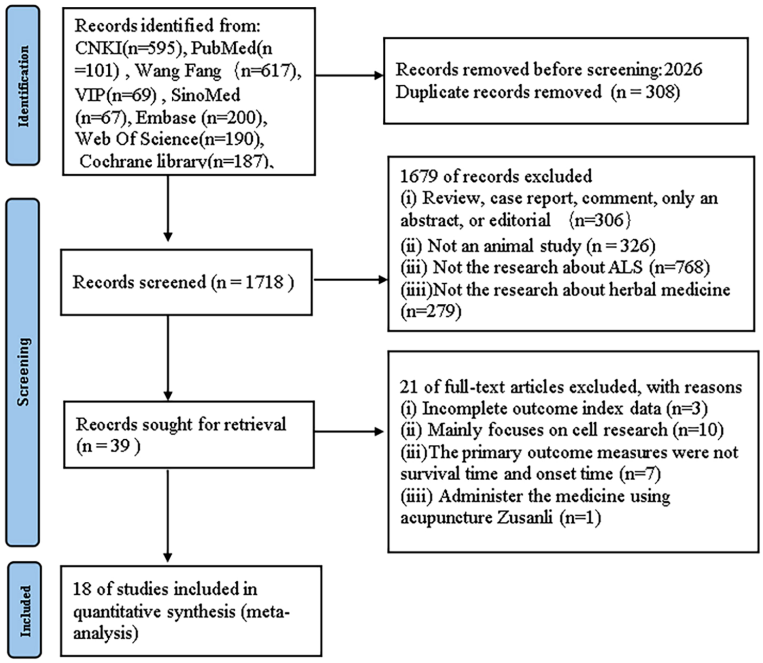

FIGURE 1
PRISMA diagram flaw.

appropriate animal models without the use of anesthetics with marked intrinsic properties. Twelve studies mentioned random allocation of animals into treatment and control groups (21–23, 25–28, 31–33, 36, 39), the methods mentioned in the two studies for specific randomization included random number table and sequential numbering (26, 29). three studies reported blinded outcome assessment (22, 33, 34), however, no studies reported sample size calculations. Twelve studies described temperature control (21, 22, 24, 26–28, 38, 39), twelve studies reported compliance with animal welfare regulations (22, 24, 26–28, 31, 33–37, 39), and seven studies declared no potential conflicts of interest (21, 29, 32–36) (Table 2).

3.1.3.2 SYRCLE

The SYRCLE's ROB is currently the only tool specifically designed for evaluating the internal validity of animal experiments. The risk of bias scores of the studies ranged from 3 to 7, with a total score of 10. Two studies received a score of 3 (38, 39); Five studies received a score of 4 (24, 25, 34, 35, 37); Seven studies received a score of 5 (23, 26, 29, 31, 33); Three studies received a score of 6 (22, 28, 35), One studies received a score of 7 (36). It is developed based on the Cochrane Bias Risk Assessment Tool and are additional items. As shown in Table 3, within the 10 items: A. Sequence generation: two studies used the "random number table method" for grouping, rated as "low risk"; Ten studies only mentioned "random" without detailed explanation, and six studies did not mention the grouping method, rated as "uncertain risk" (the quality assessment table still needs modification). B. Baseline characteristics: three studies mentioned baseline comparison of mice, rated as "low risk." The remaining fifteen studies mentioned only one or more of the age, gender, weight, or species of rats, and did not provide baseline values of relevant outcome indicators in the experiment, hence rated as "uncertain risk." C. Allocation concealment: two studies mentioned "random" or "random number table," rated as "low risk"; the remaining sixteen studies did not mention concealment of allocation or the provided information was insufficient to achieve the unpredictability of the random sequence, hence rated as "uncertain risk." D. Random housing: fifteen studies indicated placing mice in individually housed environments with free access to water, similar temperature, humidity, etc., rated as "low risk." 3 studies did not mention housing conditions, rated as "uncertain risk." E. Performance bias (Blinding): All studies did not describe blinding of animal caregivers, researchers, and outcome assessors, hence rated as "uncertain risk." F. Outcome assessment: three studies mentioned "random" selection of mice for outcome assessment, rated as "low risk"; fifteen studies did not mention it, hence rated as "uncertain risk." G. Detection bias (Blinding):one study mentioned blinding in evaluating experimental results, rated as "low risk"; seventeen studies did not mention it, hence all rated as "uncertain risk." H. Incomplete outcome data: one study had missing data during the experiment, but did not provide any explanation on whether the missing data affected the final result's authenticity, hence rated as "high risk"; four studies only reported the data range, making it impossible to determine if there was data missing, hence rated as "uncertain risk." I. Selective outcome reporting: All studies did not find incomplete data reporting, rated as "low risk." J. Other sources of bias: All studies did not find other sources of bias, hence rated as "low risk." (Supplementary Data Sheet 3).

TABLE 1 Basic features of the included studies.

Study (year)	Animal model (age)	number(sex)		treatment			Route of administration Followup period	outcome index	Intergroup differences
		Trail (male/ female)	Control (male/ female)	intervention groups (mg/kg·d)	Positive control groups	Control			
Liu 2006 (28)	SOD1^{G93A} 70D	T1:18(8/10) T2:18(8/10)	C:19(9/10) WT:26(13/13)	T1: madecassoside (61.1 ± 11.0) T2: madecassoside (185.6 ± 18.7)	NO	C: Ordinary feed WT: Ordinary feed	Oral administration until death	1.onset time 2.Survival time 3.athletic ability	1 P>0.05 2.3 P<0.05
Cai 2019 (31)	SOD1^{G93A} 8W	T:24(UK/UK)	C:24(UK/UK) 24(UK/UK)	T2. Bojungikgi-tang 1000	NO	C: Ordinary feed WT: Ordinary feed	Oral administration 6W	1.Survival time 2.athletic ability	1 P<0.05 2. P<0.01
Kook 2017 (34)	SOD1^{G93A} 13W	T2:UK	C:12(0/12) WT:11(0/11)	T: KCHO-1 250	NO	C: NS WT: NS	Oral administration 4W	1.athletic ability 2.onset time 3.survival time	1.2.3 P<0.05
Cai and Yang 2019 (33)	SOD1^{G93A} 2M	T1:11(11/0) T2:11 (11/0)	C:8(8/0)	T1: Ordinary feed T2: Bojungikgi-Tang treated 1	NO	C: Ordinary feed WT : Ordinary feed	Oral administration 6W	1.athletic ability 2.survival time	1.2 P<0.05
Zhou 2016 (27)	SOD1^{G93A} 8W	T1:6(UK/UK) T2:6(UK/UK) T3:6(UK/UK)	6(UK/UK)	T1: Jianpi Yifei formula 28.6 T2: Jianpi Yifei formula 57.2 T3: Jianpi Yifei formula 114.4	NO	WT: NS	oral gavage until death	1.onset time 2.Survival time 3.athletic ability	1.2 P<0.01 3. P<0.05
Sujimoto 2021 (36)	SOD1^{G93A} 87D	T1:18(18/0) T2:18(18/0)	C1:18(18/0) C2:18(18/0)	T1: NS T2: Shenqi Fuzheng Injection 3	NO	C1: NS C2: Shenqi Fuzheng Injection 40ml/kg 3times a week	intraperitoneally injected until death	1.onset time	1. P<0.1
Shang 2021 (22)	SOD1^{G93A} 64D	T:19(19/0)	C:19(19/0) WT:19(19/0)	T: Bailing Capsule 0.01	NO	C: NS WT: NS	oral gavage 56D	1.Onset time 2.survival time	1. P>0.05 2. P<0.05
Xian 2018 (26)	SOD1^{G93A} 8W	T1:6(3/3) T2:6(3/3) T3:6(3/3)	C:6(3/3) WT:6(3/3)	T1: Jianpi Yifei formula 2.288 T2: Jianpi Yifei formula 1.144 T3: Jianpi Yifei formula 0.5522	NO	C: NS WT : NS	oral gavage until death	1.Onset time 2. delayed survival 2.athletic ability	1.2 P<0.01 3. P<0.05
Xu 2023 (23)	SOD1^{G93A} 90D	T1:16(0/16) T2:16(0/16)	C1:16(0/16) C2:16(0/16)	T1: NS T2: Lycium barbarum polysaccharide-glycoprotein 20	NO	C1: NS C2: Lycium barbarum polysaccharide-glycoprotein 20mg/kg·d	oral gavage 80D	1.Onset time 2.survival time	1. P>0.05 2. P<0.01
Winter 2018 (29)	SOD1^{G93A} 60D	T:9(UK/UK)	C:9(UK/UK) WT:9(UK/UK)	T: anthocyanin-enriched 2	NO	C: Ordinary feed WT: Ordinary feed	Oral administration until death	1. survival 2.Onset time 3.athletic ability	1. P<0.001 2.3. P<0.05
Yang 2022 (21)	SOD1^{G93A} 8W	T:8(8/0)	C:8(8/0) WT:8(8/0)	T: herbal formula extract 1	NO	C: distilled water WT:distilled water	Oral administration 6W	1.athletic ability 2.pathological manifestations	1.2 P<0.05

(Continued)

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TABLE 1 Continued

Study (year)	Animal model (age)	number(sex)		treatment			Route of administration Followup period	outcome index	Intergroup differences
		Trail (male/ female)	Control (male/ female)	intervention groups (mg/kg·d)	Positive control groups	Control			
Dutta 2018 (37)	SOD1^{G93A} 50D	T1:25(UK/UK)	C2:25(UK/UK)	T1: Withania somnifera	NO	C: NS	oral gavage UK	1.athletic ability 2.survival time	1. P<0.01 2. P<0.05
Yang 2011 (35)	SOD1^{G93A} 98D	T1:11(11/0) T2:11(11/0)	C:10(10/0) C2:11(11/0)	T: Melittin	NO	C: NS	subcutaneously injected bilaterally 18D	1.Survival time 2.athletic ability	1. P>0.05 2. P<0.01
Li 2022 (38)	SOD1^{G93A} 12W(male) 13W (female)	T1:16 T2:17 T3:17 T4:18 T5:17	C1:12 C2:12	T1: NS T2: TBN 10 T3: TBN 30 T4: TBN 60	T5: Edaravone	C1: NS C2:TBN 30 mg/kg	intraperitoneal injection 4W	1.survival time 2.athletic ability	1. P>0.05 2. P<0.001
Li 2022 (38)	SOD1^{G93A} 6W	T1:17 T2:19 T3:20 T4:20	C:24	T1: NS T2: TBN 30	T3: Riluzole T4: Riluzole + TBN	C1: NS	oral gavage 14W	1.survival time	1. P<0.01
Yan 2022 (24)	SOD1^{G93A} 30D	T1:3*6(3*6/0) T2:3*7(3*7/0) T3:3*7(3*7/0)	3*7(3*7/0)	T1.NS T2.Jianpi Tongluo formula 630	T3. Riluzole	NS	oral gavage 30/70/110 D	1.athletic ability	1. P<0.05
Sekiya 2009 (39)	SOD1^{G93A} 6W	UK	UK	T1: Water T2: Wen-Pi-Tang 100 T3: Wen-Pi-Tang 200	T4: Riluzole	C1: Water	oral gavage UK	1. survival time 2.Onset time 3.athletic ability	1. P>0.05 2.3 P<0.05
Zhu 2017 (25)	SOD1^{G93A}	T1:8(3/5) T2:8(4/4)	C8(3/5)	T1: Flavored Sijunzi decoction	T2: Riluzole	C: NS	UK UK	1.onset time 2. delayed survival 2.athletic ability	1.2 P<0.001 2. P<0.05
Zhou 2018 (32)	SOD1^{G93A} 57D	T1:9(9/0) T2:9(9/0) T3:9(9/0) T4:9(9/0) T5:9(9/0) T6:9(9/0)	C:9(9/0)	T1: HLSJ 30 T2: HLSJ 45 T3: HLSJ 60 T4: HLSJ 75 T5: Glucose	T6: Riluzole	C: double distilled water	oral gavage until death	1. survival time 2.Onset time	1. P>0.05 2. P<0.01

T, Experimental group; T1;2…, Specific group; C, Control group; C1;2…, Specific group; WT, Wild type; UK, Unknown; TBN, ligustrazine derivatives HLSJ, Huolingshengji Formula; NS, Normal saline.

10.3389/fimmu.2024.1433929

TABLE 2 Quality assessment of included studies.

Studys	A	B	C	D	E	F	G	H	I	J	TOTAL
liu 2006 (28)	√	√	√	√	×	√	√	×	√	×	7
Zhou 2016 (27)	√	√	√	√	×	√	√	×	√	×	7
Cai 2019 (31)	√	√	√	√	×	√	√	×	√	×	7
Kook 2017 (34)	√	√	√	√	√	√	√	×	√	√	8
Sujimoto 2021 (36)	√	×	√	√	√	√	√	×	√	×	7
Li 2022 (38)	√	√	×	√	×	√	√	×	×	×	5
Shang 2021 (22)	√	√	√	√	√	√	√	×	√	×	8
Xian 2018 (26)	√	√	√	√	×	√	√	×	√	×	7
Xu 2023 (23)	√	×	√	√	√	√	√	×	×	×	6
Cai and Yang 2019 (33)	√	√	√	√	×	√	√	×	√	√	8
Winter 2018 (29)	√	×	×	√	×	√	√	×	×	√	5
Sekiya 2009 (39)	√	√	×	√	×	√	√	×	√	×	6
Yang 2011 (35)	√	√	×	√	×	√	√	×	√	√	7
Zhu2017 (25)	√	×	×	√	×	√	√	×	×	×	4
Dutta 2018 (37)	√	×	√	√	×	√	√	×	√	√	6
Zhou 2018 (32)	√	√	√	√	×	√	√	×	×	√	7
Yan 2022 (24)	√	√	×	√	×	√	√	×	√	×	6
Yang 2022 (21)	√	√	√	√	×	√	√	×	√	√	8

A. Publication in a peer-reviewed journal; B.Statement of temperature control; C. Randomization to treatment group; D. Allocation concealment; E. Blinded assessment of outcome; F. Avoidance of anesthetics with known notable intrinsic neuroprotective properties; G. Use appropriate ALS animal models; H. Sample size calculation; I. Compliance with animal welfare regulations; J. declared any potential conflict of interest.

3.1.4 Effectiveness

3.1.4.1 Onset time

3.1.4.1.1 Meta analysis

Twelve studies were included (22, 23, 25–29, 34, 36, 38, 39), with a total sample size of 267animals, including 134 animals treated with HM and 133 animals in the control group, with individual study sample sizes ranging from 5 to 25. Heterogeneity test results showed I^2= 75%, P < 0. 01. A random-effects model was used. The overall effective rate Standardized Mean Difference(SMD) was 1.75, 95% Confidence Interval(CI) (1.14 ~ 2.36), Z = 5.60, P < 0.01, indicating that HM treatment was effective and superior to the control group (P < 0.01) (Figure 2).

3.1.4.1.2 Sensitivity analysis

Further sensitivity analyses comparing HM with conventional feeding regimens (12 trials (22, 23, 25–29, 32, 34, 36, 38, 39) with 267 participants) showed that Chinese HM was more beneficial in terms of overall mean reduction in onset time, with no significant heterogeneity between studies (Figure 3).

3.1.4.2 Survival time

3.1.4.2.1 Meta analysis

Seventeen studies were included (22–29, 31–39), with a total sample size of 385 animals, including 192 animals treated with HM and 193 animals in the control group, with individual study sample sizes ranging from 5 to 25. Heterogeneity test results showed I^2 = 85%, P < 0.01. A random-effects model was used. The overall effective rate SMD was 1.42, 95% CI (0.79 ~ 2.04), Z = 4.44, P < 0.01, indicating that HM treatment was effective and superior to the control group (P < 0.01) (Figure 4).

3.1.4.2.2 Sensitivity analysis

Further sensitivity analyses comparing HM with conventional feeding regimens (17 trials (22–29, 31–39) with 385animals) showed that HM was more beneficial in terms of overall mean reduction in survival time, with no significant heterogeneity between studies (Figure 5).

3.1.4.3 Stride length

Four studies were included (20, 23, 30, 32), with a total sample size of 89 animals, including 44 animals treated with HM and 45 animals in the control group, with individual study sample sizes ranging from 5 to 25. Heterogeneity test results showed I^2= 62%, P = 0.05. A random-effects model was used, and further subgroup analysis was conducted, dividing the samples into two groups based on sample size: n > 10 and n < 10. In the n < 10 group, three studies were included, with a total sample size of 41 animals, including 20 animals treated with HM and 21 animals in the control group. The overall effective rate SMD was 2.80, 95% CI (0.90 to 4.70), Z = 2.89, P = 0.004, indicating that HM was effective in treating ALS in the n < 10 group and its efficacy was superior to the control group (P < 0.05). In the n > 10 group, one study was included (18), with a total sample size of 48 animals, including 24 animals treated with HM and 24 animals in the control group. The overall effective rate SMD was 1.90, 95% CI (1.21 to 2.59), Z = 5.39, P < 0.01, indicating that HM was effective in treating motor neuron diseases compared to the control group in the n > 10 group (P < 0.01) (Figure 6).

3.1.4.4 Duration time

In the 18 studies included, 3 studies (22, 32, 36) assessed the therapeutic effect by calculating the duration of disease in ALS mice, with records of disease duration in both the treatment and control groups. Meta-analysis was conducted on the durations of the two groups to evaluate the efficacy of HM in treating ALS animal models. In the 3 studies, both the treatment and control groups included 25 animals. The heterogeneity test showed P =0.004, I^2 = 82%, indicating statistical significance in heterogeneity between

TABLE 3 Basic features of the included studies.

Author	Year	Journal	Nation	Experimental method	Involved mechanism	Involving berbs
Ding Huang,Tang San et al. (40)	2019	Chinese Pharmacology Bulletin	China	Animal experiment	1	Borneol, Astragaloside, Total notoginseng
Xu Lu,Zhang Taijun et al. (41)	2016	Information Journal of Chinese Medicine	China	Cell experiment	1	Borneol
Wang Jian,Qu Xiaolan et al. (42)	2018	Chinese Journal of Hospital Pharmacy	China	Animal experiment	1	Borneol,safflower
Tang Bingrong,Li Hua et al. (43)	2019	Journal of Chinese Experimental Formulae	China	Animal experiment	1	Sijunzi Decoction
Rao Xiao,Tang Tibo et al. (44)	2014	Information Journal of Chinese Medicine	China	Animal experiment	1	Buyang Huanwu Decoction
Liu Yangbo,Chen Haodong et al. (45)	2023	Chinese Journal of Pharmacy	China	Animal experiment	1	Ginsenoside Rg1
Zhong Xiaoqin,Luo Chuanjin et al. (46)	2019	Experimental and Therapeutic Medicine	China	Cell experiment	1	Breviscapine
Zhang Qiuyan,Wang Lingling et al. (47)	2011	Journal of Intractable Diseases	China	Animal experiment	1	Jiweiling lyophilized powder
Li Yinghui,Liu Shaobo et al. (48)	2017	Acta Physiologica Sinica	China	Cell experiment	2	Tripterygium wilfordis
Yang Eun Jin,Kim Seon Hwy et al. (35)	2011	Journal of Neuroinflammation	Korea	Animal experiment	2	Bee venom,Melittin
Lee Kang-Woo,Ji Hye Min et al. (49)	2013	Journal of Ethnopharmacology	Korea	Cell experiment	2	Human placenta extract
Wu Jie,Wang Bin et al. (50)	2019	European Journal of Pharmacology	China	Cell experiment	2	Gardenia, Geniposide (GEN)
Shi Ruili,Qi Ruifang et al. (51)	2017	New Chinese medicine and clinical pharmacology	China	Cell experiment	2	Melon seed gold
Kook Myung Geun,Choi Soon Won et al. (34)	2017	Journal of Veterinary Science	Korea	Animal experiment	2	Turmeric,Danshen root, Tall gastrodia tuber, Common floweringqince fruit
Hsu Chien-Chin,Kuo Ting-Wei et al. (52)	2020	Journal of Neuroimmune Pharmacology	China	Animal experiment	2	Astragaloside IV, Total saponins of Japanese ginseng, Wogonin
Zhang Xuejun,Yi Tingjin et al. (53)	2011	Research and development of natural products	China	Pharmacological experiment	3	Polysaccharide component
Zeng Min,Xu Huifang et al. (54)	2018	Modern Chinese medicine research and practice	China	Pharmacological experiment	3	Aucklandia
Ruan Shunan,Lu Yan et al. (55)	2013	Chinese journal of traditional Chinese Medicine	China	Pharmacological experiment	3	Cablin patchouli herb
Shen Lulu,Lu Yan et al. (56)	2013	Chinese Traditional and Herbal Drugs	China	Pharmacological experiment	3	Giant knotweed rhizome
Du Dongsheng,Cheng Zhihong et al. (57)	2017	Chinese journal of traditional Chinese Medicine	China	Pharmacological experiment	3	Tokyo violet herb
Jin Jiahong,Cheng Zhihong et al. (58)	2012	Journal of Chinese Pharmaceutical Sciences	China	Pharmacological experiment	3	Dayflower herb
Yazdani Solmaz, Seitz Christina et al. (59)	2022	Nature Communications	Sweden	Clinical trials	4	No

(Continued)

TABLE 3 Continued

Author	Year	Journal	Nation	Experimental method	Involved mechanism	Involving berbs
Ding Xuping,Wang Xue et al. (60)	2021	World Science and technology - TCM modernization	China	Cell experiment	4	Trichosanthes
Murdock Benjamin J,Zhou Tingting et al. (61)	2017	JAMA Neurology	America	Clinical trials	5	NO
Garofalo Stefano,Cocozza Germana et al. (62)	2020	Nature Communications	Italy	Clinical trials and Animal experiment	5	NO
Li Jianming,Peng Lishan et al. (63)	2022	Journal of Shenyang Pharmaceutical University	China	Animal experiment	5	Futenge mixture, Futenge, milkvetch root, red ginseng
Cheng Qi,Li Ning et al. (64)	2013	Journal of Ethnopharmacology	China	Animal experiment	5	Fuzheng huayu
Jin Mei Ling,Park Sun Young et al. (65)	2013	Environmental Toxicology and Pharmacology	Korea	Cell experiment	6	Acanthopanax extract
Mahesh Ramalingam,Jung Hyo Won et al. (66)	2012	Phytotherapy research: PTR	Korea	Cell experiment	6	Cryptotanshinone
Sun Meng-Meng, Bu Hui et al. (67)	2009	Neurological Research	China	Pharmacological experiment	7	Allicin
Kanekura Kohsuke,Hashimoto Yuichi et al. (68)	2005	Journal of Biological Chemistry	Japan	Pharmacological experiment	6	Cryptotanshinone
Guo Yansu,Zhang Kunxi et al. (69)	2011	Brain Research	China	Animal experiment	7	Allicin
Li Shu-Yan,Jia Yu-Hong et al. (70)	2010	Free Radical Biology and Medicine	China	Animal experiment	8	Chuan Xiongqin
Callewaere Céline,Banisadr Ghazal et al. (71)	2006	Proceedings of the National Academy of Sciences of the United States of America	France	Pharmacological experiment	8	Chuan Xiongqin
Chen Zhao,Pan Xueke et al. (72)	2013	Cancer Letters	China	Cell experiment	8	Chuan Xiongqin

The numbers in the Related mechanism column indicate the following mechanisms 1.Blood-brain barrier protection 2.Anti-neuroinflammation 3.Inhibition of complement system activation 4.Regulate T lymphocytes 5.Regulates natural killer cells 6.Excitatory amino acids toxicity 7.Oxidative stress 8.Cytotoxicity of calcium.

groups. Therefore, a random-effects model was used to combine the effect sizes of disease duration. The results showed that the disease duration in the treatment group was shorter than that in the control group (MD=6.79, 95% CI [-0.28, 13.87]), but the difference between the two groups was not statistically significant (P =0.06). Regarding the treatment of ALS mice, using recorded disease duration for efficacy evaluation suggests that HM has no effect in delaying the progression of ALS compared to the control group (Figure 7).

3.2 Scoping review

3.2.1 Study inclusion

For the scoping review section on the immune mechanisms of HM in the treatment of ALS, a total of 3702 articles were extracted from the initial search carried out in two databases. After removing the duplicates, 3694 articles were selected for the first analysis by title and abstract. The full-text analysis included 75 articles, of which 35 were considered for this scoping review (Figure 8).

3.2.2 Characteristics of included studies

This scoping review encompasses a total of 35 studies, with 25 from China, 5 from Korea, 1 from Japan, and 4 from other Western countries. The included studies range from as early as 2005 to as recent as 2023. There are 13 studies on animal experiments, 10 on cell experiments, 3 on clinical trials, and 9 on pharmacological experiments. Five immune modulation pathways are covered, along with three other mechanisms. The literature on blood-brain barrier protection is the most abundant, with 8 studies. In total, 26 different single drugs and compound formulations are involved (Table 3).

3.2.3 Mechanisms of HM in the treatment of ALS

ALS is an incurable neurodegenerative disease that affects the upper and lower motor neurons of the spinal cord, the cerebral cortex, and the spinal cord. The etiology and pathogenesis of ALS remain unknown at present (1). Here, some studies indicates that HMs show promising potential in combating oxidative stress, excitatory amino acid toxicity, nerve inflammation, and calcium cytotoxicity, offering hope in the treatment of ALS.

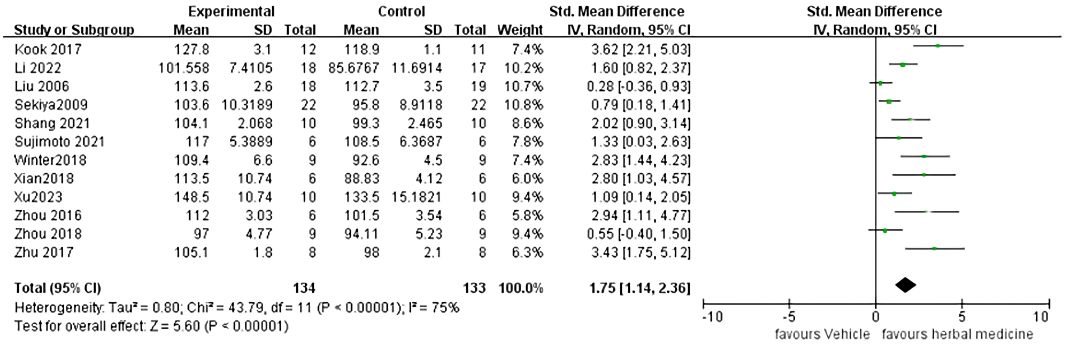

FIGURE 2
ALS onset time Meta analysis.

3.2.3.1 Excitatory amino acids toxicity

In 1957, Lucas and Newhouse's (73) pioneering research demonstrated the lethal effects of glutamate on neurons in the central nervous system (CNS).Following this, The molecular mechanisms underlying neuronal injury due to excessive glutamate receptor stimulation are starting to be unraveled, indicating that glutamate may exert toxicity on neurons through multiple pathways (74). The excitotoxicity resulting from the abnormal elevation of extracellular excitatory neurotransmitter glutamate, including the generation of free radicals and lipid superoxide, can induce spontaneous dissolution and degeneration of neurons, contributing to the development of ALS. Numerous experimental findings demonstrate the ability of cryptotanshinone to counter glutamate-induced cytotoxicity and safeguard neurons, indicating its potential utility in mitigating the onset of ALS. The pivotal involvement of the PI3K/Akt signaling pathway in cell survival against glutamate-induced toxicity has been underscored (68). several Chinese herbs exhibit the capability to inhibit amino acid toxicity and consequently shield neurons. For instance, Acanthopanax extract elevates heme oxygenase (HO)-1 expression, thereby curbing the generation of NO/ROS induced by LPS. Notably, HO-1 expression serves to safeguard cells against glutamate-induced neuronal demise (65). Additionally, Cryptotanshinone-mediated neuroprotection combats glutamate-induced toxicity by activating the PI3K/Akt pathway and averting the downregulation of Bcl-2 within the anti-apoptotic protein family. Furthermore, Mahesh's research revealed cryptotanshinone's capacity to hinder nerve cell apoptosis induced by sodium nitroprusside (SNP), thus exhibiting neuroprotective properties (66).

3.2.3.2 Oxidative stress

Oxidative stress is caused by an imbalance in the production and removal of Reactive oxygen species (ROS) and Reactive nitrogen

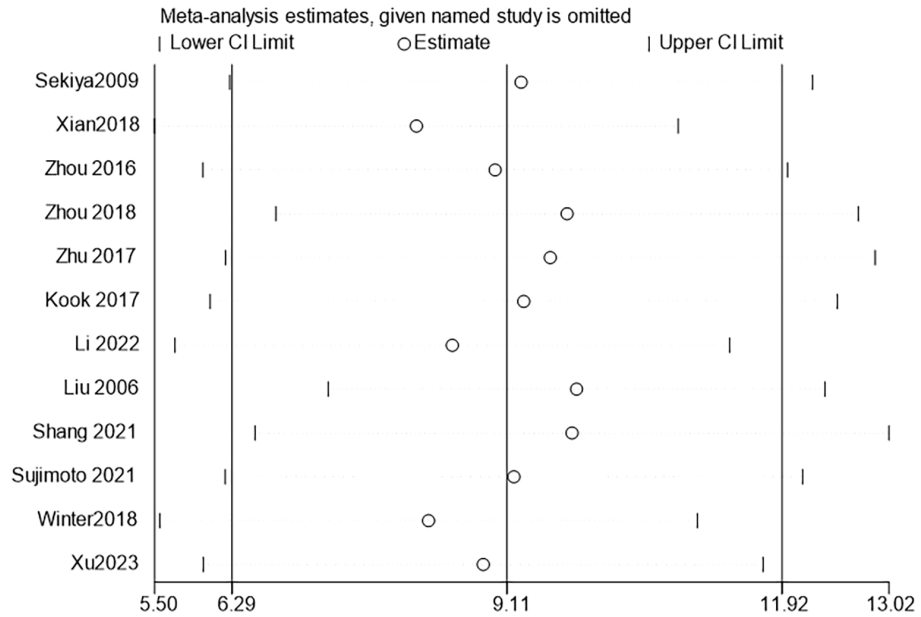

FIGURE 3
ALS onset time sensitivity analysis.

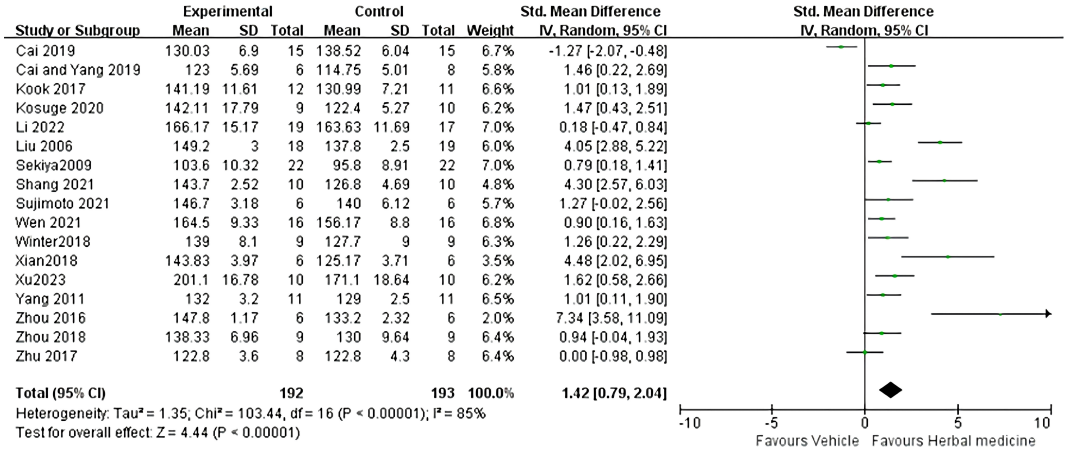

FIGURE 4
ALS survival time Meta analysis.

species (RNS) (75). Oxidative stress can cause oxidative modification of bioactive molecules such as proteins, lipids, sugars, nucleic acids, etc., so that they lose their original structure and function, affect the normal physiological function of cells, and finally lead to cell degeneration and necrosis. Under normal conditions, free radicals do not cause pathological changes in the body, because the body has enzymes to fight free group damage, such as SOD1, glutathione peroxidase (GSH), catalase (CAT) and non-enzymatic system defense system. Such as non-enzymatic antioxidants carotenoids, tocopherols and vitamin C, as well as free metals and heme-binding proteins. They can suspend the free radical chain reaction, or turn the free radical into a less active substance, so that the production and removal of free radicals are in balance. If the production of free radicals exceeds the body's ability to remove them, the body will experience oxidative stress (76). Allicin, the primary compound in garlic oil, has demonstrated its ability to induce phase II enzymes, thereby enhancing antioxidant activity and protecting ALS neurons from oxidative stress (67). Administering allicin orally with DATS to SOD1-G93A mice at clinical onset induced the expression of HO-1 in the lumbar spinal cord, directly influencing oxidative damage. These findings suggest that oral administration of DATS significantly extends the lifespan of mice (69).

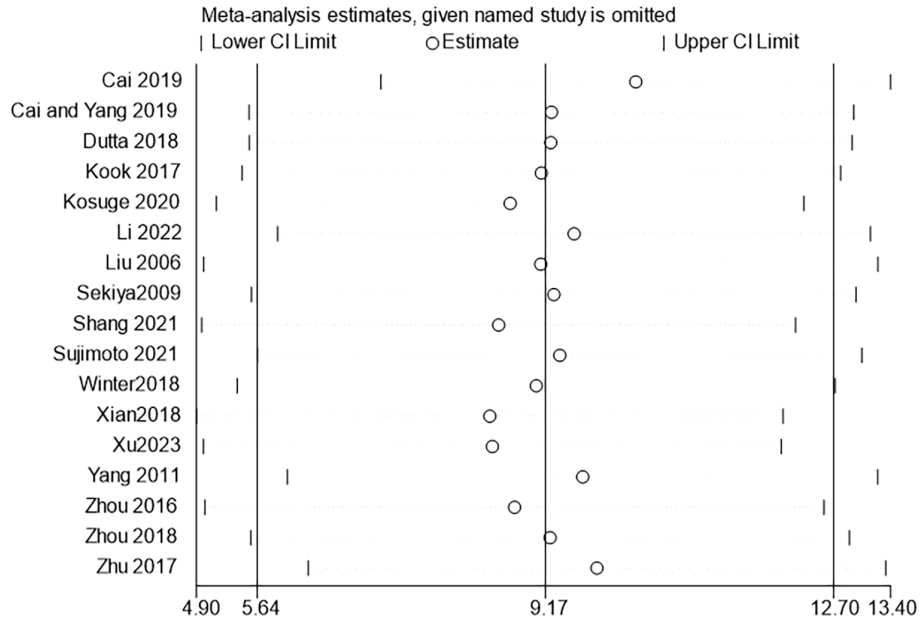

FIGURE 5
ALS onset time sensitivity analysis.

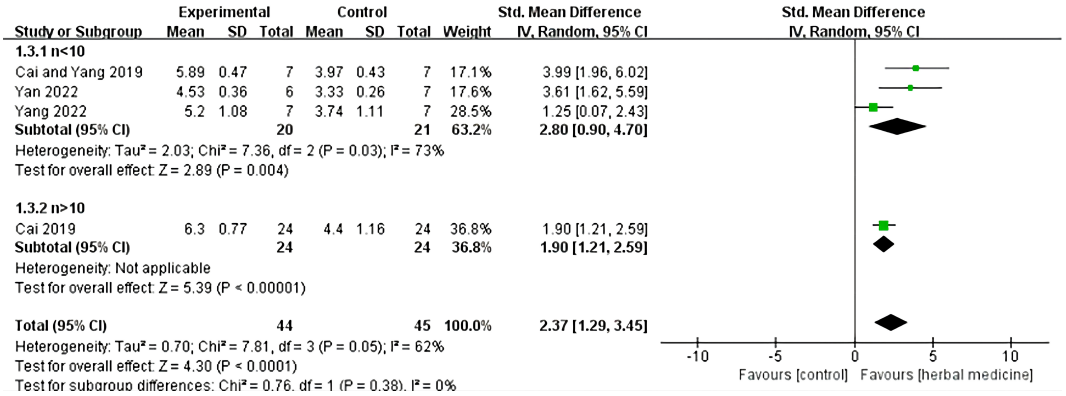

FIGURE 6
ALS stride length Meta analysis.

3.2.3.3 Cytotoxicity of calcium

In classical acute excitatory toxicity, the influx of Na+ and Cl- disrupts intracellular Ca2+ homeostasis, triggering a cascade of detrimental biochemical processes. Opening of voltage-gated calcium channels leads to a surge in calcium ions, resulting in excessive release of the excitatory amino acid glutamic acid. This influx of calcium ions through NMDA/AMPA receptors, metabolic glutamic acid receptors, and voltage-dependent calcium channels activates enzymes such as proteases, lipases, kinases, nucleases, and NOS. The generation of free radicals and synthesis of NO further exacerbate neuronal damage, ultimately leading to programmed cell death via apoptosis gene activation. Neuroprotective drugs are thought to primarily act by preventing calcium influx, regulating excitatory amino acid toxicity, and modulating microvascular inflammatory responses. Studies have shown that an extract from the Chinese herb Chuan Xiongqin, can protect nerve cells by lowering intracellular calcium levels and inhibiting glutamate release (70). Callewaere et al. (71) investigated the protective effect of ligustrazine by stimulating nerve cells with stromal cell-derived factor (SDF-1), which elevates intracellular calcium levels, and then treating them with ligustrazine. They observed a significant decrease in intracellular calcium levels in the ligustrazine-treated group exposed to SDF-1, indicating ligustrazine's ability to mitigate calcium cytotoxicity and serve as a neuroprotective agent (72).

3.2.3.4 Other relevant mechanisms

Some studies have also found that the incidence of ALS is related to neurotrophic factor deficiency, metal and trace element imbalance, cell apoptosis, viral infection and abnormal neurofilament aggregation.

3.2.4 The possible immunological mechanisms of HM in treating ALS

HM has better anti-inflammatory properties and extensive immunomodulatory effects, and can have better curative effect in the diseases of immune system disorders. ALS can be alleviated and treated by protecting the blood-brain barrier, anti-neuroinflammation, inhibiting the activation of complement system, inhibiting natural killer cytotoxicity and regulating T cells (77).

3.2.4.1 Blood-brain barrier protection

Blood central nervous system barrier includes blood-brain barrier and blood-spinal barrier, which can control the transport of substances on both sides of the barrier, thus protecting the relatively stable environment in nervous tissue. It is an important immune barrier for ALS, which can effectively prevent toxic substances from infiltrating into the central nervous system from the blood and pump toxins outward (78). The target of HM protecting blood brain barrier is closely related to tight junction protein. Tight junction protein is composed of three peripheral cytoplasmic proteins, namely, occludingprotein, closure protein and attachment molecule, and closed small cyclic proteins (ZO-1, ZO-2 and ZO-3).Both borneol and astragaloside can increase the expression of atretic zonule 1 and closure protein 5, and their combination with total notoginseng can also inhibit the downregulation of atretic zonule 1 and occlusion protein, thus

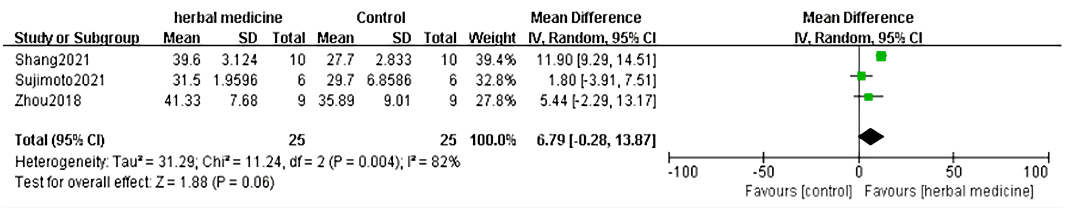

FIGURE 7
ALS duration time Meta analysis.

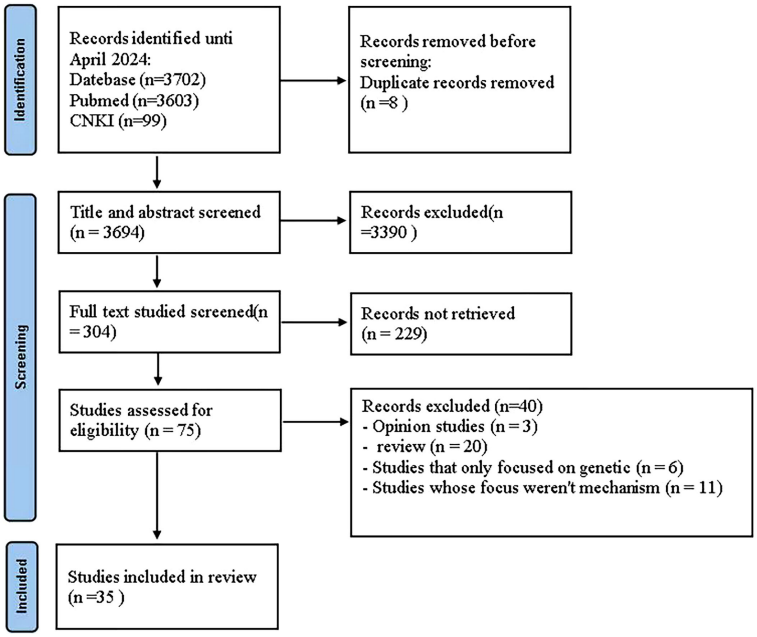

FIGURE 8
PRISMA diagram flaw.

significantly improving the permeability of the blood-brain barrier (40). If borneol was administered with safflower, the expression of MMP-2 and MMP-9 could be decreased, and the expression of ZO-1 and closure protein 5 could be increased (42). Sijunzi Decoction can increase the expression levels of occlusal protein, ZO-1, closure protein 1 and their mRNA (43). The expression of von Willeophilia factor in serum, vascular endothelial growth factor, MMP-9 and MMP-2 in brain tissue decreased after the use of Buyang Huanwu Decoction, indicating the protective effect of the Blood-brain barrier (44). Ginsenoside Rg1 can also up-regulate the levels of atretic zonolin-1 and occlusion-protein, and down-regulate the expression of matrix metalloproteinase-2 and matrix metalloproteinase-9 to restore the integrity of the Blood-brain barrier (45). Breviscapine mainly plays its role by up-regulating the expression of CD63 and the blood-brain barrier tight junction proteins claudin5, occludin and ZO-1 (46). Jiweiling related preparations such as Jiweiling lyophilized powder can significantly improve blood-brain barrier score, delay neuronal edema and reduce blood-brain barrier permeability in damaged mice (47). By regulating the permeability of the blood-brain barrier, the active components of Chinese medicine can restore the integrity of the immune barrier in ALS and enhance its self-protection.

3.2.4.2 Regulates microglia

Neuroinflammation is an important host defense mechanism that protects the brain from infection or injury and restores normal structure and function (79, 80), chronic inflammation can induce cytotoxicity and worsen the severity of different neurodegenerative diseases, such as Parkinson's disease (PD) (81), multiple sclerosis (MS) (82), and ALS (83). Dysregulation of the inflammatory response, characterized by abnormal activation of microglia and overabundance of pro-inflammatory cytokines, leads to the neurodegeneration observed in ALS (84). Chinese HM has rich historical background, remarkable curative effect and minimal adverse reactions. It acts by regulating microglia activation and polarization, inhibiting inflammatory responses, and by mediating microglia and various related pathways such as NF-κB signaling pathway, Toll-like signaling pathway, Notch signaling pathway, AMPK signaling pathway, MAPK signaling pathway, MAPK signaling pathway, etc (85).Tripterygium wilfordis extract (Tripterygium wilfordis) can regulate the phosphorylation of kinase 1/2 and nuclear factor kB by blocking extracellular signals to reduce the production of pro-inflammatory factors and nitric oxide, so as to inhibit the anti-inflammatory effect of autoimmune (86). As resident macrophages of the central nervous system, microglia play a key role in maintaining brain homeostasis (87), but if microglia are over-activated, it will lead to the release of many pro-inflammatory factors and neurotoxic substances, aggravating the damage. Melittin, an effective component extracted from bee venom, can directly reduce the activity of microglia or indirectly reduce the secretion of inflammatory factors and the phosphorylation level of P38 mitogen-activated protein kinase in brain stem and spinal cord, significantly regulate the inflammation of ALS mice and delay the development of the disease (35). The extract can inhibit C-JUN amino-terminal kinase signaling pathway, reduce the expression of microglia-induced nitric oxide synthase and cyclooxygenase-2, and play an anti-inflammatory role (49). The up-regulated expression of TLR4 is a key receptor involved in the activation and function of microglia. The active ingredient Geniposide (GEN) in Gardenia can effectively reduce the expression of TLR4, MyD88, p-IκB, NF-κB, p-ERK1/2 and p38 proteins, thereby playing an anti-inflammatory role and inhibiting

the activation of microglia by down-regulating the TLR4-dependent pathway of MyD88 (50). The triterpenoid saponin compound polyhemigosaponin F (PGSF) extracted from melon seed gold of Polygala can effectively counteract the up-regulation of toll-like receptor 4 (TLR4) in microglia, and down-regulate the expression of nitric oxide synthase (iNOS) and circular cell enzymase-2 (COX-2) induced by cellular inflammatory proteases. Improve the overactivation of microglia and the production of neurotoxic factors, and reduce the damage of nerve cells (35). KCHO-1, a natural ethanol extract obtained from turmeric, salvia miltiorrhizae, Tianma, papaya and other herbs, can reduce oxidative stress by decreasing gp91phox subtype expression of NADPH oxidase, down-regulating the level of induced-nitro oxide synthase, and alleviating the phosphorylation of P38 mitogen-activated protein kinase and the activation of extracellular signal-regulated kinase 1/2. Inhibit microglia proliferation and activation (34). Astragaloside IV, total saponins and baicalin can regulate microglia polarization and improve brain tissue inflammation by mediating MAPK signaling pathway. Calycosin has the ability to reduce TNF-α-containing microglia populations by activating the BDNF/TrkB signaling pathway, thereby reducing inflammation and neuronal damage (52).

3.2.4.3 Inhibition of complement system activation

The complement system is a group of activated enzymatically active proteins in human serum and interstitial fluid. It is composed of more than 30 kinds of soluble proteins and membrane binding proteins synthesized by the liver. The complement consists of nine components, named C1, C2, C3,… and C9 (88). Normal activation of complement is beneficial to enhance immunity, but excessive activation can cause inflammation, tissue damage and various immune hemolysis reactions (89). Complement activation has long been implicated in the pathogenesis of ALS, and many clinical and animal studies have shown that complement factors, including C1q and C3, have a strong upregulation in the dead regions of motor neurons (90). HM has a complex mechanism of action and a wide range of effects. According to research, polysaccharides in natural HM are important components of HM to regulate complement activity (91). C1r, C1s, C3, C4 were the main targets of the crude polysaccharide extract of S. mellowsis, which inhibited the activity of the complement system (92). The polysaccharide component PsEULl3 of Eucommia ulmoides has very high anti-complement activity against the classical pathway (53). The APS-2 glucomglycan isolated and purified from the plant has good anti-complement activity, and its targets are Clq, C2, C3, C5 and C9 (54). Lentinan can decompose complement C3 into allergen C3a, the mechanism of which may be that the recognition spot on complement protein can recognize the structure of polysaccharide and activate the complement system. The main targets of quercetin 7,3',4' -trimethyl ether from patchouli were C1q, C2, C5 and C9 (55). We often know that Chinese herbs often act on the classical pathway of complement to inhibit the activity of the complement system, but in addition, the active ingredients of Chinese medicines can also act on the side pathway. Among them, C1q, C2, C4 and C9 are the main targets of the charming chun components extracted from Knotweed (56), The extract components can be adjusted by acting on different complement targets or multiple targets together (57). The (+) -catechin-3-0-b-D-1 (2-cinnamyl) -glucoside isolated and identified from the Chinese herb Anagardia showed different degrees of inhibition on the classical and bypass pathways of the complement system (58).

3.2.4.4 Regulates T lymphocytes

In the central nervous system, CD4+T cells are believed to have neuroprotective effects, which can promote neuroprotection of glial cells and delay the progression of diseases by changing the morphology of glial cells (93). CD4+T cells can differentiate into regulatory and effector T cells, with the former regulating the proliferation of the latter. In the T-cell-mediated immune response, the imbalance of effector and regulatory T cells will trigger neuroinflammation and eventually lead to neuronal degeneration and necrosis (94). Regulatory T cells are responsible for regulating the immune response of the body, and usually play an important role in maintaining self-tolerance and avoiding excessive immune response damage to the body. In the early stage of ALS, the level of regulatory T cells increases and thus plays an anti-inflammatory role in the central nervous system, while in the late stage of rapid progression, the level of regulatory T cells decreases and shows the deterioration of neuroinflammation. Effector T cells are cells that proliferate and differentiate after T cells receive antigen stimulation, and have the function of releasing lymphocytes and can actively respond to stimulation. Observation of patients with ALS also showed that increased effector T cells in blood and cerebrospinal fluid were associated with decreased survival, while increased regulatory T cells in blood were associated with improved survival (59). Trichosanthin extracted from trichosanthes trichosanthes can directly increase the number of regulatory T cells, the level of immune index interleukin-10 and induce higher expression of forked head spiral transcription factor 3, thereby enhancing the immune regulatory ability of regulatory T cells (60). Dichloromethylamine, a soluble component of wheat zhai, can change AKT phosphorylation signaling, reduce the differentiation of helper T cells 17, and induce the proliferation of regulatory T cells to maintain balance (95). Zuogui pill can also up-regulate the immune index interleukin 10, so as to improve the immune response function of regulatory T cells (96).

3.2.4.5 Regulates natural killer cells

Natural killer cells are key components of adaptive immunity and active cells with significant toxicity. One study showed an increase in NK cells in the blood of ALS patients compared to controls (61). At present, there are few studies on the relationship between NK cells and the occurrence and development of ALS, and the relationship between the two is unclear. However, infiltration of NK cells and increased expression of NKG2D ligands on MN were found in the motor cortex and spinal cord of deceased ALS patients, and NK cells were toxic to MN expressing NKG2D ligands. NK cells also secreted IFN-γ to activate microglia and damage regulatory T cells in the spinal cord of mSOD1 mice (62). These results suggest that NK cells may affect the occurrence and development of ALS

through multiple immune mechanisms. Futenge mixture, which is composed of Futenge, Astragalus and red ginseng, can up-regulate CD2, CD95, PD-1 receptors and activated molecules on the surface of natural killer cells, and play an excellent regulatory role on natural killer cells (64).

All the above studies have shown that by influencing related proteins such as atretic zonule 1 and atretic protein 5, reducing the production of pro-inflammatory factors and nitric oxide, acting on complement targets, and upregating cell surface receptor molecules, HM can regulate the stability of the blood-brain barrier, inhibit the activation of microglia and complement system, weaken the toxicity of natural killer cells, and regulate the function of T cells. Thereby alleviating and treating ALS at the level of immune function (Table 4).

4 Discussion

4.1 Summary of results

ALS poses a significant challenge to the medical community due to its nature as a neurodegenerative disease. Adjunct therapies, such as HM treatment, have garnered attention as potential avenues for novel therapeutic approaches, particularly in efforts to prolong the progression of ALS. This study aims to investigate the efficacy of HM therapy in an ALS mouse model, probing its potential mechanisms of action and its impact on immune system regulation.

In this meta-analysis, we synthesized data from 18 studies involving a total of 443 animals, aiming to evaluate the effects of herbal treatments on a mouse model of ALS. By amalgamating findings from these investigations, we gained a more comprehensive understanding of the potential of herbal therapy for ALS management. The research demonstrated significant positive effects of herbal treatments in ALS, highlighting the role of their active constituents in neurogenic regulatory pathways crucial for delaying neurodegeneration. Our meta-analysis results showed notable effects of herbal medicine in ALS mice. However, we also observed that the heterogeneity (I^2 value) in most analyses was quite high, suggesting significant variations in experimental design, animal models, treatment doses, and duration across the included studies. This heterogeneity may impact the stability and interpretability of the meta-analysis results. Therefore, caution is needed when interpreting these findings. To address the issue of heterogeneity, we employed a random-effects model to account for variability across studies, aiming to minimize the impact of these differences. Additionally, we conducted sensitivity analyses to assess the influence of individual studies on the overall results. Despite these efforts, it is important to acknowledge that high heterogeneity may reduce the generalizability of these findings. Despite these challenges, this study provides valuable insights into the potential of herbal medicine in ALS treatment. Herbal treatments may protect motor neurons and slow disease progression. However, future research should be conducted under more consistent study designs to reduce heterogeneity and further validate these findings.

The scoping review involved 35studies. Immune disorders play a crucial role in the pathological process of ALS. Studies have found that the activation of microglia and astrocytes in the central nervous system, as well as the increase in the number of pro-inflammatory peripheral lymphocytes and macrophages, directly affect the occurrence and progression of ALS. In particular, genetic mutations associated with ALS, especially SOD1, are thought to further increase neuroinflammation levels, further confirming the imbalance of the immune system in ALS pathophysiology. Clinical studies have not only revealed the influence of genetic variants on immune disorders, but have found that even in the absence of significant genetic changes, immune disorders lead to impaired function of regulatory T lymphocytes and increased proinflammatory macrophages. This further underscores the importance of the immune system in the onset and progression of ALS and underscores the need to consider immune regulation in treatment. In the future, developing effective methods to monitor the pathophysiology and progression of inflammation-mediated diseases will be important. Chemokines, especially CXC chemokines, may play an important role in the pathophysiology of ALS. Understanding their role in the onset and progression of ALS, as well as their modulated treatment of the disease, may provide new directions for the treatment of ALS.

HMs plays an important role in ALS treatment because of its immunomodulatory properties. Whether it is a single Chinese medicine or a compound Chinese medicine, ALS can be treated by protecting the blood-brain barrier, anti-neuroinflammation, inhibiting the activation of the complement system, regulating the toxicity of natural killer cells, and regulating the immune response mediated by T cells. Based on a bone marrow transplant experiment to evaluate the contribution of the immune system to movement disorders, specifically the role of CD8 T cells. One study concluded that pathological Senataxin expression in the hematopoietic system is necessary for the development of motor phenotypes in mice, supporting the idea that dysfunction of the nervous system and hematopoietic/immune system contributes to the onset or progression of ALS disease. While existing treatment options offer hope to ALS patients, there is a need to further delve into the mechanisms of immune disease and explore more personalized and integrated treatment strategies to address this challenging disease. By targeting treatments for diseases of the immune system, it is possible to slow disease progression, improve patients' quality of life and extend their survival. These efforts will provide more hope and possibilities for future ALS treatments.

4.2 Study quality and risk of bias

Based on the evaluation results, the studies included in the review were assessed using CAMARADES and SYRCLE's ROB tools to evaluate their methodological quality and risk of bias.

For CAMARADES, the quality scores of the studies ranged from 4 to 8, with a total score of 10. Specifically, one study received a score of 4; two studies received a score of 5; four studies received a score of 6; seven studies received a score of 7, and four studies received a score of 8. These scores indicate a moderate to high level of methodological quality in the included studies.

TABLE 4 The pathological mechanism of ALS.

HM	mechanism of action	immunologic mechanism
Borneol, milkvetch root	ZO-1, Claudin-5↑	Improve the tight connection structure and reshape the blood-brain barrier
Borneol with safflower	MMP-2, MMP-9 ↓; ZO-1, Claudin-5↑	
Sijunzi decoction	occlusal protein, ZO-1, closure protein 1 ↑	
Buyang huanwu decoction	vWF, VEGF, MMP-9, MMP-2↓	
Ginsenoside Rg1	ZO-1, occlusion-protein↑; MMP-2, MMP-9 ↓	
Breviscapine	CD63, claudin5, occludin, ZO-1↑	
Tripterine	The phosphorylation of ERK1/2 and NF-κB ↓; NO ↓	Inhibit microglia activation
Melittin	P38 mitogen-activated protein kinase↓	
Human placenta	NO synthase, cyclooxygenase 2 ↓	
Cape jasmine fruit, Polygala	TLR4↓	
KCHO—1	gp91phox subtype of NADPH oxidase, iNOS, P38MAPK, ERK1/2 phosphorylation↓	
Calycosin	TNF-α	
Barbated skullcup herb	C1r, C1s, C3, C4	Inhibit abnormal activation of complement system
Cablin patchouli herb	C1q, C2, C5, C9	
Tokyo violet herb	Different complement targets or multiple targets	
Dolomiaea costus	Clq, C2, C3, C5, C9	
Polygonum cuspidatum	C1q, C2, C4, C9	
Trichosanthes kirilowii	Tregs, Foxp3, IL- 10↑	Regulates T cell function
Fringed pink	Th17 cells ↓; Treg↑	
Zuogui pill	IL-10↑	
Futeng Mixture	CD2, CD95, PD1 receptor ↑	Regulates natural killer cells

ERK1/2, Extracellular signal-regulated kinase 1/2; NF-κB, Nuclear factor kB; P38MAPK, P38 mitogen Activated protein kinase; ZO-1, zonula occludens protein 1. Claudin - 5,closed for protein; MMP, Matrix metalloprotein Enzyme; JNK, C - Jun amino terminal kinase; COX-2, cyclooxygenase 2; INOS, Induction type Nitric oxide synthase; Foxp3, Fork head screw transcription factor 3; Tregs, Regulatory T cells; Occludin, Occludin protein.; NO, Nitric oxide.

In contrast, for SYRCLE's ROB, the quality scores of the studies ranged from 3 to 7, with a total score of 10. Two studies received a score of 3; five studies received a score of 4; seven studies received a score of 5; three studies received a score of 6, and one study received a score of 7. These scores suggest a moderate level of risk of bias in the included studies.

4.3 Analysis and discussion of therapeutic effect

Many studies on the efficacy of HM in treating ALS have highlighted affirmative results, with most studies yielding positive outcomes. This study conducted a meta-analysis of 18 animal studies investigating the efficacy of HM in treating ALS to provide a more objective and comprehensive evaluation. Among the included studies, a limited number were deemed high quality based on bias risk assessment. While two studies provided detailed descriptions of randomization using random number tables, the methods were unspecified in the remaining ten. Six studies did not specify the allocation concealment method, and the blinding implementation was only mentioned in three studies, with the remainder not addressing blinding. The primary outcome measures varied across the trials, with the majority utilizing onset time and survival time as endpoints; specifically, 11 out of 18 studies used onset time, while 16 used survival time. Some studies employed measures of motor function (e.g., stride length) and neuron-related indicators as efficacy measures, but the evaluation methods were inconsistent. Three studies reported disease duration in experimental animals as an outcome measure. A remarkable effects of HM in ALS mice, including onset time SMD=1.75, 95% CI (1.14 ~ 2.36), Z = 5.60, $P < 0.01$), survival time(SMD = 1.42, 95% CI (0.79 ~ 2.04), Z = 4.44, P < 0.01), stride length SMD=1.90, 95% CI (1.21 to 2.59), Z = 5.39, P < 0.01) and duration time MD=6.79, 95% CI [-0.28, 13.87], Z=1.88, P =0.06), showing HM's certain efficiency in treating ALS mice.

4.5 Implication

This study employed a comprehensive multidimensional systematic evaluation approach to thoroughly investigate the efficacy of HM in treating ALS animal models. Through a comprehensive reanalysis of various outcome indicators, we aimed to provide evidence-based medical support for the clinical application of HM in ALS treatment. This study utilized a rigorous scientific statistical method—meta-analysis—which not only opens new avenues for clinical research on herbal medicine in our country but also promotes the integration of HM with modern evidence-based medicine.

Specifically, this study summarized recent research on HM treatment for ALS. Through systematic review, we analyzed the effects of HM on ALS onset time and survival time, identifying its potential efficacy in delaying disease progression and extending survival. However, while the systematic review provided overall

efficacy data from preclinical studies, it could not fully elucidate the underlying biological mechanisms. Therefore, we conducted a scoping review and found that HM plays a significant role in immunotherapy for ALS. HM modulates innate and adaptive immune processes by reshaping the blood-brain barrier, inhibiting natural killer cell activity, suppressing complement system activation, regulating microglial cell activity, and restoring T cell function, thereby protecting motor neurons from toxic damage. These mechanisms not only provide a biological explanation for the efficacy observed in the systematic review but also deepen our understanding of the mechanisms through which HM acts in ALS treatment. These findings lay the foundation for further understanding the immunomodulatory mechanisms of HM in ALS treatment.

Combining the systematic review with the scoping review allowed us to delve deeper into the immune mechanisms of HM based on quantitative analysis of its efficacy. This dual-pronged research approach not only provides more comprehensive insights into current ALS research but also guides future clinical applications and drug development.

The significance of this study lies in its ability to deepen our understanding of HM in ALS treatment while also providing scientific evidence for the integration of modern evidence-based medicine with traditional herbal medicine. By reviewing the immunomodulatory mechanisms of HM in ALS treatment, we provide a theoretical basis and new therapeutic strategies for this refractory disease. Future research should continue to explore the potential role of HM in ALS treatment, aiming to bring more effective treatment options to ALS patients.

This comprehensive analysis not only enhances our understanding of ALS treatment methods but also advances the clinical application of HM in ALS, paving the way for research on refractory diseases. Through rigorous scientific research design and multidimensional evaluation methods, we provide a solid foundation for future ALS research and offer important references for the application of HM in modern medicine.

4.4 Limitations

Currently, Trials on the treatment of ALS with HMs are relatively scarce. However, our study faced some limitations, including the limitation of being limited to publicly published literature searches. This means that we may have missed some relevant grey literature that may contain important information on the effectiveness of HM in treating ALS. Second, our search covers only English and Chinese studies, which may introduce a degree of linguistic bias as studies in other languages may exist but are not taken into account. In addition, we need to recognize the existence of publication bias, that is, negative study results are relatively less likely to be published, which may cause our analysis to be influenced by positive studies and overestimate the therapeutic effect.

These limitations and biases may lead to varying degrees of bias in our findings. First, the incomplete coverage of the literature may mean that we miss some potentially important studies, resulting in an insufficiently comprehensive overall assessment of the efficacy of HM in the treatment of ALS. Secondly, due to the limited number and varying quality of studies, the results of our analysis may lack robustness and have certain uncertainties. In addition, due to the insufficient sample size, our analysis may lack the statistical power to draw accurate conclusions or generalize to the entire ALS patient population. Therefore, in order to more fully evaluate the efficacy of HM in the treatment of ALS, future studies need to overcome these limitations and biases. This could include expanding literature searches to include studies in grey literature and other languages, as well as strengthening assessments of research quality and controlling the effects of publication bias. At the same time, efforts should be made to increase the sample size and improve the quality of studies to ensure more reliable and robust analysis results and provide more convincing evidence support for the treatment of ALS with HM. Thirdly, A limitation of our study is the lack of detailed exploration of the side effects of HMs. Since our study focused on a preclinical systematic review, the issue of side effects of HM is rarely addressed in the existing literature. Detailed side-effect studies usually need to be performed in clinical studies. Considering the herbal medicine is widely used in the treatment of understanding the potential side effects is very important to ensure patient safety. We expect that future clinical studies will explore this issue in depth and provide more comprehensive and reliable data for systematic evaluation of the safety of Chinese herbal medicines.

5 Conclusion

The preclinical evidence supports the utilization of HM as a conventional treatment for ALS mice. Growing evidence indicates that HM may potentially delay neurological degeneration in ALS by activating diverse signaling pathways, especially immune pathways.

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

YJ: Data curation, Writing – original draft, Writing – review & editing. JW: Data curation, Writing – original draft. JL: Data curation, Writing – original draft. GZ: Data curation, Writing – original draft, Formal analysis, Funding acquisition, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1433929/full#supplementary-material

SUPPLEMENTARY DATA SHEET 1
Specific search strategy.

SUPPLEMENTARY DATA SHEET 2
Specific search strategy.

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Advances in the study of artemisinin and its derivatives for the treatment of rheumatic skeletal disorders, autoimmune inflammatory diseases, and autoimmune disorders: a comprehensive review

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Artemisinin and its derivatives are widely recognized as first-line treatments for malaria worldwide. Recent studies have demonstrated that artemisinin-based antimalarial drugs, such as artesunate, dihydroartemisinin, and artemether, not only possess excellent antimalarial properties but also exhibit antitumor, antifungal, and immunomodulatory effects. Researchers globally have synthesized artemisinin derivatives like SM735, SM905, and SM934, which offer advantages such as low toxicity, high bioavailability, and potential immunosuppressive properties. These compounds induce immunosuppression by inhibiting the activation of pathogenic T cells, suppressing B cell activation and antibody production, and enhancing the differentiation of regulatory T cells. This review summarized the mechanisms by which artemisinin and its analogs modulate excessive inflammation and immune responses in rheumatic and skeletal diseases, autoimmune inflammatory diseases, and autoimmune disorders, through pathways including TNF, Toll-like receptors, IL-6, RANKL, MAPK, PI3K/AKT/mTOR, JAK/STAT, and NRF2/GPX4. Notably, in the context of the NF-κB pathway, artemisinin not only inhibits NF-κB expression by disrupting upstream cascades and/or directly binding to NF-κB but also downregulates multiple downstream genes controlled by NF-κB, including inflammatory chemokines and their receptors. These downstream targets regulate various immune cell functions, apoptosis, proliferation, signal transduction, and antioxidant responses, ultimately intervening in systemic autoimmune diseases and autoimmune responses in organs such as the kidneys, nervous system, skin, liver, and biliary system by modulating immune dysregulation and inflammatory responses. Ongoing multicenter randomized clinical trials are investigating the

effects of these compounds on rheumatic, inflammatory, and autoimmune diseases, with the aim of translating promising preclinical data into clinical applications.

KEYWORDS

artemisinin, autoimmune diseases, autoimmune disorders, artememisinin derivatives, NF-κB pathway, immune modulation

1 Introduction

Autoimmune diseases are inflammatory conditions where the immune system mistakenly attacks normal cells, leading to impaired immune function, abnormal immune responses, and ultimately tissue damage and organ dysfunction (1). Although the exact mechanisms of autoimmune diseases remain unclear, they always exhibit several distinct characteristics (2, 3): (1) The causes are often unknown, with some cases linked to bacterial or viral infections or certain medications; (2) The incidence is higher in women than in men, with a female-to-male ratio of 10:1; (3) The disease course is typically chronic, with recurrent flares and progressive symptoms; (4) There is a noticeable familial predisposition; (5) A single patient may suffer from two or more autoimmune diseases simultaneously. Epidemiological studies indicate that approximately 10% of the global population is affected by various autoimmune diseases (4, 5). These conditions often involve the joints, muscles, bones, and other soft tissues throughout the body, and if left untreated, can lead to widespread organ or system damage. Autoimmune diseases are associated with high rates of disability, increased mortality, and a significant reduction in quality of life (6). As complex, multifactorial diseases influenced by genetic and environmental factors, they typically have a slow onset, with patients often testing positive for autoantibodies years before showing any clinical signs or symptoms (7–9). Clinically, corticosteroids and disease-modifying antirheumatic drugs (DMARDs) are commonly used to manage these conditions, but long-term or lifelong medication is often necessary. While these treatments can control disease activity, many patients continue to experience symptoms, and prolonged use of steroids and immunosuppressants can lead to metabolic disorders, immunodeficiency, secondary infections, and other adverse effects (10, 11). This highlights the need to explore alternative therapeutic biologics or novel traditional medicine formulations, particularly immunomodulatory or immunosuppressive DMARDs, to alleviate symptoms, reduce side effects, and improve quality of life (12, 13). The immune mechanisms underlying autoimmune diseases are thought to involve abnormal lymphocyte responses to target organs. Even with strict central and peripheral immune tolerance, a small number of potentially autoreactive lymphocytes can "escape" into the periphery, where they are repeatedly exposed to self-antigens and activated by costimulatory signals, initiating an immune response. Under the influence of various cytokines, naïve lymphocytes can become activated and differentiate into various types of inflammatory cells, ultimately leading to inflammation and soft tissue damage (14–16).

Artemisinin, an effective antimalarial compound, is extracted from the plant *Artemisia annua* L. using low-temperature ether extraction. Its structure, based on a sesquiterpene lactone core with a unique peroxide bridge, has led to the synthesis of various artemisinin derivatives, such as dihydroartemisinin (DHA) and artesunate (17). These derivatives have been improved in terms of water solubility, bioavailability, and pharmacological properties, making them some of the most widely used and effective antimalarial drugs today. DHA, the primary active metabolite of artemisinin, is produced by reducing artemisinin with sodium borohydride (18). With its unique peroxide bridge structure, DHA is a crucial natural drug from *Artemisia annua* L., offering advantages such as rapid absorption, fast metabolism and excretion, wide distribution, high efficacy, and low toxicity. Its oral bioavailability is more than ten times higher than that of artemisinin, and its antimalarial efficacy is 4 to 8 times greater (19). Additionally, studies have revealed that DHA possesses anticancer, anti-inflammatory, and antiparasitic properties (20). Notably, numerous studies have demonstrated that artemisinin and its derivatives have the capacity to regulate inflammation and autoimmune responses, marking significant progress in the treatment of various rheumatic and inflammatory diseases (21). This review provides an overview of the research on the mechanisms by which artemisinin-based drugs alleviate autoimmune diseases, aiming to serve as a reference for further in-depth studies.

2 Artemisinin and its main derivatives

With the deepening of research on artemisinin, the modification of artemisinin derivatives and analogs has become a hotspot of research. Artemisinin and its derivatives are all sesquiterpene lactone compounds. It has been demonstrated that deoxyartemisinin can inhibit the growth and reproduction of Plasmodium falciparum, which is related to the peroxide bridge in its structure (22). Therefore, the structural modifications of artemisinin are conducted based on the preservation of the peroxide bridge, and modifications at positions C-9 and C-10 are most common (23).

2.1 Artemisinin

Artemisinin was isolated in the 1950s by a research team at the China Academy of Chinese Medical Sciences from *Artemisia annua L.*, becoming the second natural product used to treat malaria after quinine. Clinical studies have confirmed its exceptional antimalarial activity. In 1975, high-resolution mass spectrometry identified the compound as a sesquiterpene, and infrared spectroscopy, along with a quantitative reaction with triphenylphosphine, revealed the presence of a unique peroxide group. Subsequently, nuclear magnetic resonance and X-ray diffraction were used to determine the structure and relative configuration of artemisinin, while optical rotatory dispersion was employed to establish the absolute configuration of the lactone ring (24). The distinctive structure and outstanding antimalarial properties of artemisinin have since garnered ongoing scientific interest and research.

2.2 DHA

Reducing the C-10 carbonyl group in artemisinin's structure with sodium borohydride produces DHA, the simplest compound in the semisynthetic process of artemisinin derivatives. DHA serves as a precursor for synthesizing other artemisinin-like compounds. Studies have shown that after artemisinin and its derivatives are absorbed by the human body, their pharmacological effects are primarily mediated by conversion to DHA, the active substance. DHA exhibits 4-8 times greater antimalarial efficacy than artemisinin and has significantly improved oral bioavailability, over 10 times higher (19). Additionally, it has a lower recurrence rate during treatment, lower toxicity, and better water solubility. However, DHA is less stable than artemisinin, and its water solubility remains suboptimal. Consequently, researchers are focused on developing new drug delivery materials and formulations to enhance DHA's pharmaceutical properties and bioavailability, including sustained-release tablets (25), DHA nanoparticles (26–28), DHA liposomes (29), and magnetic DHA nanoliposomes (30).

2.3 Artesunate

Artesunate, also known as artemisinin succinate, is synthesized by esterifying DHA with succinic anhydride. Artesunate exhibits a range of pharmacological effects, including antimalarial, antiviral, anti-inflammatory, antitumor, and immunomodulatory properties. It is highly effective, fast-acting, and has low toxicity, making it less prone to drug resistance (31, 32). As a weakly acidic drug, artesunate primarily relies on simple diffusion for transport within the body, allowing it to easily pass through biological membranes. Its pKa values range from 3.5 to 5.5, indicating a low degree of ionization in acidic environments, though it is soluble in weak alkaline solutions. These characteristics enable artesunate to be formulated into various dosage forms, including injections, tablets, and suppositories, for intravenous, oral, or rectal administration (33).

2.4 Artemether and arteether

By replacing hydrogen atoms with alkyl groups at the hydroxyl positions on C-10 of DHA, artemisinin ether derivatives are produced. The most notable compounds in this category are artemether and arteether, both of which demonstrate higher activity than artemisinin. Although these ether derivatives have good lipid solubility, they suffer from poor water solubility, low bioavailability, and can cause irritation when administered by direct injection. To address these issues, some researchers have encapsulated artemether in aminopterin-modified targeted nanoliposomes. These liposomes have a uniform and stable structure. *In vitro* release studies have shown that this liposome system effectively sustains drug release *in vivo*, thereby enhancing the metabolism and bioavailability of artemether (34).

2.5 Other derivatives

Building on the unique peroxide bridge structure of artemisinin, researchers have synthesized various artemisinin analogs, polymers, and simplified structures, expanding the range of artemisinin-like compounds. Artemisinin dimers, which consist of two artemisinin monomers linked by a connector, are among these innovations. Common linkers include alkyl, ether, ester, and carbamate groups. Compared to artemisinin monomers, dimer compounds offer stronger pharmacological activity, fewer adverse reactions, and better physicochemical properties. Studies have demonstrated that artemisinin dimers exhibit excellent anticancer activity both *in vitro* and *in vivo*, with the type of linker significantly influencing their efficacy. Thus, optimizing the properties of artemisinin dimers can be achieved by modifying the linkers (35). Additionally, sodium artesunate, an alkaline salt derivative of artemisinin, offers good water solubility, rapid action, and high tolerance, making it suitable for intravenous or intramuscular injection (36).

3 *In vivo* and *in vitro* immunomodulatory and anti-inflammatory effects of artemisinin and its derivatives

3.1 Involvement in regulation of the innate immune system

Macrophages and dendritic cells (DCs) are key components of the human innate immune system. Macrophages can adopt different phenotypes depending on their microenvironment, while DCs are known for their strong antigen-presenting capabilities (37). Research in experimental colitis models has shown that artemisinin derivatives primarily target the innate immune system, modulating immune responses by acting on macrophages and DCs. When used alone or in combination with immunomodulators, these derivatives have demonstrated significant anti-inflammatory effects (37). Artesunate, for example, induces apoptosis in primary peritoneal

macrophages and bone marrow-derived DCs in a time-dependent manner via the caspase-9 pathway. It also reduces the secretion of IL-12 by DCs and tumor necrosis factor (TNF)-α by macrophages, thereby mitigating inflammatory responses. A key mechanism behind these effects is the inhibition of the NF-κB signaling pathway, which decreases TNF-α release by macrophages—a classical pathway through which artemisinin and its derivatives exert their anti-inflammatory actions (38). In the RAW264.7 cell model, artesunate also inhibits macrophage autophagy activation through the TRAF6-Beclin1-PI3KC3 signaling pathway, reducing the release of TNF-α and IL-6 (39). Further research has revealed that the suppression of IL-1β, IL-6, IL-8, and other pro-inflammatory cytokines by artemisinin and its derivatives is closely linked to the NF-κB signaling pathway (40).

Additionally, artemisinin and its derivatives inhibit pro-inflammatory factors by mediating key signaling pathways, including mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/AKT, Toll-like receptor (TLR), and nucleotide-binding oligomerization domain protein 2 (NOD2) pathways (41). Beyond inhibiting the production of pro-inflammatory mediators, artemisinin and its derivatives also promote the production of anti-inflammatory cytokines like IL-10, further contributing to their anti-inflammatory effects (42). As macrophages serve as a bridge between the innate and adaptive immune systems, their regulation often impacts adaptive immunity, including processes such as phagocytosis, internalization, and antigen presentation. For instance, evidence suggests that under the influence of IFN-γ secreted by T cells, macrophages and macrophage-like fibroblasts become activated, producing mediators like matrix metalloproteinases (MMPs) and nitric oxide (NO), which exacerbate tissue damage. These cells also form a positive feedback loop by secreting IL-12/IL-23, which enhances the Th1/Th17 response (43). Collectively, this evidence indicates that artemisinin and its derivatives can inhibit the activation of macrophages and DCs, regulate their quantity and functional levels, and participate in inflammation regulation, potentially influencing the adaptive immune system as well.

3.2 Involvement in regulation of the adaptive immune system

Current research indicates that artemisinin and its derivatives play a positive role in maintaining Th cells, regulatory T cells (Tregs), follicular helper T cells (Tfh), and follicular regulatory T cells (Tfr). First, artemisinin and its derivatives effectively inhibit T cell activation, suppressing Th1 and Th17 cells while enhancing Th2 function and differentiation. This helps maintain a balanced Th cell population and subtype ratio, influencing cytokine release and modulating immune function and inflammation levels (42, 44, 45). Second, artemisinin and its derivatives are involved in regulating Treg levels and function. Early studies found that artemether could effectively limit T cell proliferation and reduce IL-2 levels, thereby restricting Treg function (46). However, other derivatives, including SM934, artesunate, and dihydroartemisinin, have been shown to significantly increase Treg level and improve the Treg/Th17 balance (44, 47–52).

Third, artemisinin and its derivatives also regulate Tfh and Tfr levels, both of which play crucial roles in B cell function, germinal center (GC) formation, antibody affinity maturation, and memory B cell production. An imbalance, characterized by increased Tfh or decreased Tfr, is associated with excessive pathological autoantibody secretion and can promote disease progression (53, 54). Current research on the regulatory effects of artemisinin derivatives on Tfh and Tfr levels is inconsistent. In a systemic lupus erythematosus (SLE) model, artesunate was found to effectively reduce Tfh levels in the spleen of SLE mice, increase Tfr levels, maintain the Tfr/Tfh ratio, reduce SLE severity, and prolong survival (55). However, another study reported that artesunate regulated GC B cells without affecting Tfh levels (56). SM934, while not affecting the proportion of GC B cells, has been shown to exert immunosuppressive effects by inhibiting the TLR/MyD88/NF-κB signaling pathway, reducing pro-inflammatory mediator secretion by monocytes/macrophages, and thereby decreasing Tfh cell activation and Tfh/Th17 differentiation (57).

Artemisinin and its derivatives can inhibit the production of antibodies in various pathological conditions, providing important evidence for their involvement in humoral immune system regulation. To date, there is no research confirming the direct effects of artemisinin and its derivatives on B cells. Currently, more research suggests that the decreased antibody levels result from secondary changes mediated by T cell suppression. Artesunate, DHA, and SM934 have been shown to effectively reduce the levels of antinuclear antibodies (ANA) and double-stranded DNA (dsDNA), among other pathological antibodies, in SLE mice (44, 52, 55, 58). In rheumatoid arthritis (RA) models, artemisinin has been found to regulate B cell function by inhibiting GCB cell proliferation, limiting GC formation, and the production of autoantibodies, thereby preventing the occurrence and progression of RA (59). SM934 can directly inhibit splenic local B cell activation and plasma cell formation through the TLR/MyD88/NF-κB signaling pathway, reduce their proportions in B cells, and decrease inflammation levels (56).

4 Immunomodulatory and anti-inflammatory effects of artemisinin and its derivatives

4.1 Effects on inflammatory mediators

The process of inflammatory response is a protective mechanism that releases mediators to defend against damage and disease, resist foreign substances, and prevent infection (60, 61). Inhibiting inflammatory mediators is an effective strategy for treating acute or chronic inflammatory diseases (62). Cytokines, as mediators of intercellular communication, play important roles in the occurrence and maintenance of inflammatory diseases (63, 64). To date, most studies on the anti-inflammatory effects of DHA have focused on pro-inflammatory cytokines, including TNF-α and

various interleukins (IL-1, IL-5, IL-6, IL-8, IL-17, IL-18, IL-22, IL-23) (65). Several studies have demonstrated the anti-inflammatory and anti-fibrotic effects of DHA. Dai et al. (66) found that DHA reduced TNF-α levels in serum and lung tissues in a radiation-induced lung injury model, thereby reducing inflammation and lung fibrosis. Similarly, Wang et al. (67) reported that DHA decreased TGF-β1 and TNF-α levels in a mouse model of carbon tetrachloride (CCl4)-induced liver fibrosis, highlighting its anti-fibrotic properties. In a nephritis model, Wu et al. (68) observed that DHA significantly lowered TNF-α and IL-6 levels in serum and improved kidney pathology. Yi et al. (69) showed that combining DHA with ibuprofen reduced IL-1β and TNF-α expression in rats with adjuvant-induced arthritis, compared to ibuprofen alone. Liu et al. (70) discovered that DHA inhibited the proliferation of HaCaT cells, elevated IL-4 and IL-10 levels, and reduced IFN-γ, IL-17, and IL-23 levels, suggesting modulation of the IL-23/IL-17 axis. In models of dextran sodium sulfate (DSS)-induced colitis, both Lei et al. (71) and Liu et al. (72) demonstrated that DHA reduced TNF-α, IL-1β, and IL-23 levels, improved intestinal symptoms, and restored epithelial integrity, indicating its potential in treating inflammatory bowel disease (IBD). Additionally, Li et al. (73) found that DHA reduced TNF-α, IL-1β, and IL-6 levels while increasing IL-10 in macrophages treated with ultra-high molecular weight polyethylene (UHMWPE), showing a dose-dependent anti-inflammatory effect. Macrophages are involved in the overproduction of inflammatory mediators [including nitric oxide (NO) and prostaglandin E2 (PGE2)] and play an important role in inflammation (74). NO, produced by inducible nitric oxide synthase (iNOS), is an important molecule for regulating the biological activity of blood vessels, nerves, and the immune system. However, excessive release of NO during the infection process can cause damage to target tissues (75). Yin et al. (76) found that DHA, at concentrations of 10, 20, and 50 μmol/L, reduced TNF-α and PGE2 expression in endothelial cells, and inhibited IL-6 and IL-1β upregulation induced by TNF-α or PGE2, highlighting its potential in treating infantile vascular diseases. Similarly, Yu et al. (77) reported that DHA inhibited the release of TNF-α, IL-6, and NO in LPS-induced macrophages, and suppressed iNOS protein expression, demonstrating its anti-inflammatory effects.

4.2 Effects of artemisinin and its main derivatives on signaling pathways

The effects of artemisinin and its main derivatives on signaling pathways are summarized in Figure 1.

4.2.1 NF-κB signaling pathway

The NF-κB/Rel family members, as dimeric transcription factors, play important roles in regulating the expression of cytokines, growth factors, and anti-apoptotic genes. The NF-κB family consists of five related transcription factors, including Rel-C, NF-κB1 (p50/p105), NF-κB2 (p52/p100), Rel-A (p65), and Rel-B (79). NF-κB can be inhibited by NF-κB inhibitory protein (IκB) binding and suppression of the NF-κB/Rel proteins. The activation of the classical pathway of NF-κB affects various biological processes including immune response, inflammatory response, stress response, B cell development, and lymphoid organogenesis (80, 81), while the alternative pathway depends on the activation of IκB kinase (IKKα) (82). Sui et al. (83) established a radiation-induced lung injury model in Wistar rats and administered a single dose of 15 Gy radiation to both lungs of the treatment group rats, followed by oral administration of 60 mg/(kg·day) DHA. The results showed that DHA could inhibit the expression of TNF-α, IL-6, and NF-κB in lung tissues of rats, indicating the inhibitory effects of DHA on the expression of TNF-α and its anti-inflammatory effects in radiation-induced lung injury by regulating the NF-κB signaling pathway, thereby alleviating inflammation. Huang et al. (84) incorporated 80 mg/kg DHA into the basic diet of postpartum growth-retarded (IUGR) piglets. The study found that DHA activated nuclear factor erythroid 2-related factor 2 (Nrf2), which in turn inhibited the NF-κB pathway. This action suppressed the infiltration of inflammatory cells, enhanced myeloperoxidase activity, reduced oxidative stress, and decreased the production of IL-1β, TNF-α, and IL-6 induced by lipopolysaccharide (LPS), ultimately leading to a reduced inflammatory response. Yang et al. (85) divided rats into different groups: normal saline control group (NS group), bleomycin group, dexamethasone group, DHA-1, DHA-2, and DHA-3 groups. The bleomycin group received intratracheal instillation of bleomycin, the NS group received an equal amount of normal saline instead of bleomycin, and rats in the DHA-1, DHA-2, and DHA-3 groups received intraperitoneal injection of DHA (25, 50, and 100 mg/kg) in combination with intratracheal instillation of bleomycin. The results showed that compared with the bleomycin group, the number of neutrophils and macrophages in the lungs of rats in the DHA-1, DHA-2, and DHA-3 groups was significantly reduced, and the expression levels of TGF-β1, TNF-α, IL-1, alpha-smooth muscle actin (α-SMA), and NF-κB in lung tissues were significantly decreased, as well as the level ofcollagen synthesis. Liu et al. (86) pretreated male C57BL/6 mice with DHA (20 mg/kg) for 2 days and then induced sepsis-induced acute kidney injury (AKI) in mice by intraperitoneal injection of lipopolysaccharide (LPS) (10 mg/kg). The results showed that DHA deactivated the NF-κB signaling pathway in mice and inhibited the oxidative stress response elicited by LPS. You et al. (87) constructed a lupus model in MRL/lpr mice and studied the mechanism of DHA in treating lupus nephritis. The mice were divided into the normal control group, DHA group, prednisone group, and DHA+prednisone group, and were given 1 mL of normal saline, 60 mg/(kg·day) DHA, 9 mg/(kg·day) prednisone, or 60 mg/(kg·day) DHA + 9 mg/(kg·day) prednisone by gavage, respectively. The mRNA and protein expression levels of irregular chemokine fractalkine (FKN), NF-κB p65, and other markers in the renal cortex of mice were detected using reverse transcription-polymerase chain reaction and immunohistochemistry, respectively. The results showed that DHA reduced the expression levels of FKN, NF-κB p65 mRNA, and protein in the kidney cortex of mice after treatment, similar to the effects of prednisone treatment. Furthermore, the combination of DHA and prednisone exerted a more significant effect. The mechanism of

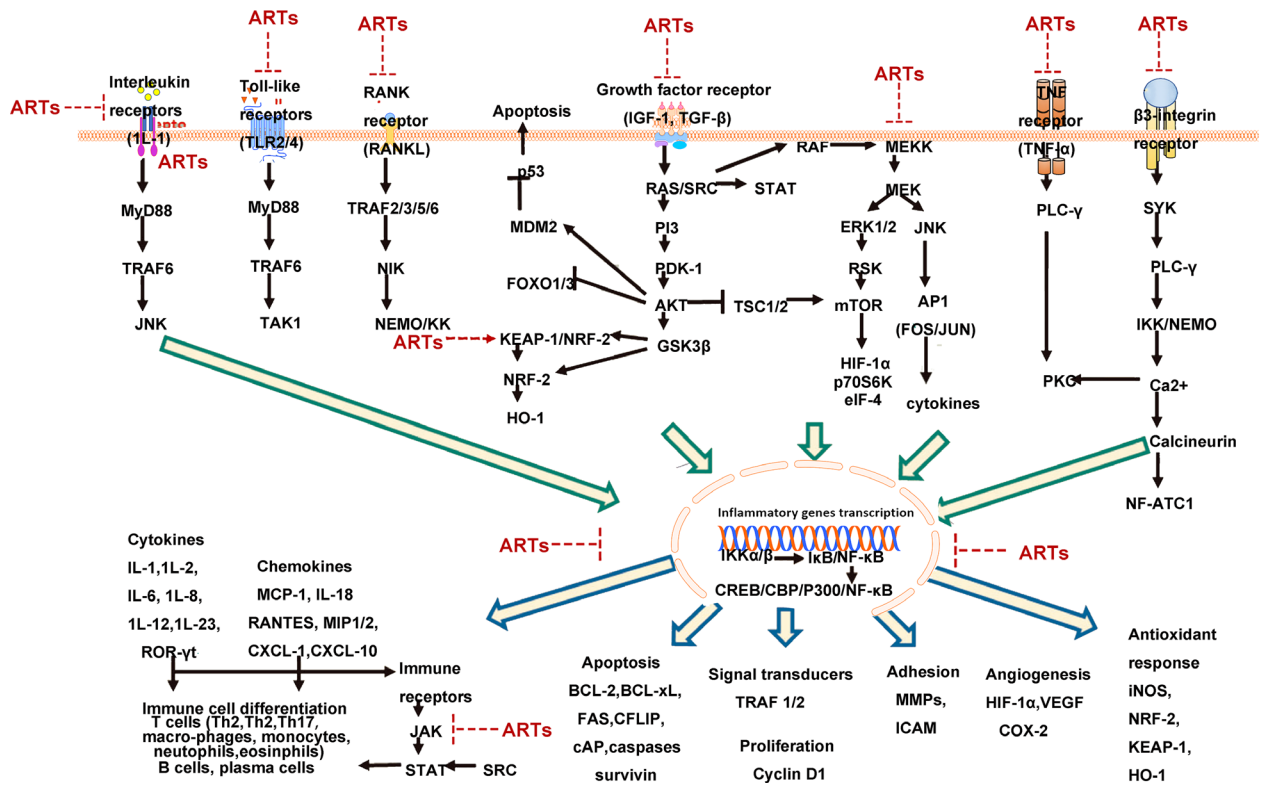

FIGURE 1
Hypothetical signaling network based on literature summary of immunosuppressive mechanisms of artemisinin and its derivatives [NF-κB plays a central role in the immunosuppressive activity of artemisinin and its derivatives (ARTs). The upstream mechanisms of NF-κB involve several receptor-driven signaling pathways, which are inhibited by artemisinin and its derivatives, subsequently suppressing numerous downstream mechanisms involved in inflammation and autoimmune diseases figures adapted from Efferth et al. 2021 (78), licensed under CC BY 4.0].

action of DHA was through the regulation of the NF-κB signaling pathway to modulate FKN expression. Dong et al. (88) constructed a lupus nephritis model in BXSB mice and found that DHA treatment at low, medium, and high doses (5, 25, 125 mg/kg) reduced the activation of NF-κB and the expression of p65 protein in kidney tissues, thus effectively inhibiting the deposition of various immunoglobulins (such as IgG, IgA, IgM) and complement components (such as C3, C1q) in renal tissues. Du et al. (89) established rat models of adjuvant-induced arthritis (AIA) and collagen-induced arthritis (CIA) and treated them with DHA (30 mg/(kg·day)). In addition, RAW264.7 macrophages were cultured *in vitro* and treated with different concentrations (0.5, 1, 2, 4, 8 μmol/L) of DHA. The results showed that DHA reduced the ankle swelling index in AIA/CIA model rats, decreased the spleen index and thymus index in AIA model rats, and lowered the serum IL-6 level. Furthermore, it suppressed RAW264.7 cell viability and nuclear translocation of NF-κB p65, suggesting its anti-inflammatory mechanism may be related to the NF-κB signaling pathway.

Protein kinase B (Akt) regulates the activity of downstream signaling molecule NF-κB, and it has been found that the enhanced expression of NF-κB is achieved through the activation of Akt, which controls various transcription factors (90). Xu et al. (91) found that DHA induced apoptosis in RA synovial cells through the Akt/NF-κB signaling pathway, exerting an anti-inflammatory effect. NF-κB is one of the downstream signaling molecules in the TLR4 signaling pathway, and the activation of the TLR4/NF-κB signaling pathway controls the expression of various cytokines and plays an important role in various inflammatory diseases. Qin et al. (92) also found that DHA inhibited the expression of iNOS, IL-1β, IL-6, and TNF-α mRNA in activated BV-2 cells, reduced the expression of TLR4 protein and cytosolic IκBα, and inhibited the nuclear translocation of NF-κB. The mechanism may involve the regulation of the TLR4/NF-κB signaling pathway to suppress the release of pro-inflammatory cytokines, thereby exerting anti-inflammatory effects.

The MAPK pathway is involved in the signal cascade induced by various extracellular stimuli to regulate inflammatory responses, including the production of inflammatory mediators. The MAPK family consists of three major components: ERK1/2 or p42/p44, JNK/SAPK, and p38MAPK (93). Activated MAPKs can bind to and activate other kinase targets, translocate to the cell nucleus, and initiate the transcription of pro-inflammatory genes, with NF-κB serving as a key downstream component of the MAPK signaling pathway (93). Wei et al. (94) studied the effects and mechanisms of low and high doses of DHA (25, 50 mg/(kg·d)) on skin inflammation in a psoriasis-like model in mice. The results showed that DHA significantly inhibited erythema, scaling, and skin thickness in mice, and the inhibitory effects were enhanced with increasing doses. Furthermore, DHA significantly reduced nail

psoriasis and infiltration of inflammatory cells. The study also found that DHA reduced the expression levels of IL-1β, IL-18, IL-6, and CXC chemokine ligand 1 (CXCL-1) in mouse skin lesions, inhibited the expression of NF-κB (p65) and phosphorylated p38 MAPK in a dose-dependent manner, and suggested that the mechanism of action may involve the MAPK/NF-κB signaling pathway, inhibiting excessive proliferation of keratinocytes and the secretion of cytokines.

4.2.2 AMPK/SIRT1 signaling pathway

The AMPK/SIRT1 signaling pathway is involved in various cellular metabolic processes. AMPK activation, indicated by phosphorylation, inhibits lipid synthesis, promotes fatty acid β-oxidation, and improves hepatic steatosis (95). SIRT1, a key enzyme involved in regulating glucose and lipid metabolism, plays a role in cellular differentiation and apoptosis (96). AMPK and SIRT1 can mutually regulate each other in physiological and pathological conditions and prevent the occurrence of inflammation (97). Zhao et al. (98) added 80 mg/kg DHA to the diet of IUGR piglets and compared them with piglets of normal body weight that did not receive DHA supplementation. The results showed that DHA increased the gene expression and activity of AMPK/SIRT1 signaling pathway in the liver of IUGR piglets, while the expression of inflammatory cytokine genes TNF-α, IL-1β, and IL-6 was suppressed. Zhao et al. (96) investigated the effects of adding 80 mg/kg DHA to the basal diet on hepatic inflammation and lipid metabolism in weanling piglets. The results showed that DHA treatment of LPS-induced hepatic inflammation model in piglets reduced the levels of triglycerides (TG), IL-1β, IL-6, TNF-α, and non-esterified fatty acids (NEFA) in the liver, inhibited the activity of fatty acid synthase (FAS), increased the mRNA expression of sterol regulatory element-binding protein 1c (SREBP-1c) and acetyl-CoA carboxylase β (ACCβ), and decreased the mRNA expression of carnitine palmitoyltransferase 1 (CPT1), SIRT1, AMP-activated AMPKα, and stearoyl-CoA desaturase (SCD), indicating the ability of DHA to alleviate hepatic inflammation and abnormal lipid metabolism through the AMPK/SIRT1 signaling pathway.

4.2.3 Mammalian target of mTOR/S6 kinase 1 pathway

The mTOR/S6K1 pathway regulates the activity of ribosomal S6 kinase (such as S6K1, S6K2) and eukaryotic initiation factor 4E (eIF4E). The mTOR/S6K1 pathway is an important cellular growth coordination pathway that plays a negative regulatory role in autophagy in mammals (99). Xi et al. (100) found that DHA treatment of IgA nephropathy mesangial cells significantly inhibited cell proliferation, downregulated the phosphorylation levels of mTOR/S6K1 signaling pathway, and increased the expression of autophagy-related protein LC3B, indicating that DHA has anti-inflammatory and autophagy-promoting effects in IgA nephropathy.

4.2.4 PI3K/Akt signaling pathway

The PI3K/Akt signaling pathway can be activated by various cellular stimuli or toxic injuries and regulates transcription, translation, proliferation, growth, and cell survival (100). Gao et al. (101) induced neuroinflammation in mice using LPS and found that treatment with DHA (40 mg/kg) improved the behavioral abnormalities in the LPS-induced neuroinflammatory mice and reduced the expression levels of glial fibrillary acidic protein (GFAP), IL-1β, IL-6, phosphorylated PI3K (p-PI3K)/PI3K, TNF, and phosphorylated protein kinase B (p-Akt)/Akt. The study demonstrated the inactivation of the PI3K/Akt signaling pathway in adult mice by DHA, protecting the hippocampus from neuroinflammatory damage.

4.2.5 MAPK signaling pathway

MAPKs are protein kinases that phosphorylate themselves on serine and threonine residues or are found phosphorylated on their substrates. They respond to various physiological signals such as hormones, cytokines, and growth factors (102). Artemisinin suppresses LPS-stimulated RAW264.7 macrophages from producing IL-12p40 by inhibiting JNK activity (103). L-A03, a DHA derivative, selectively inhibits the activation of JNK and promotes apoptosis in MCF-7 human breast cancer cells (104). DHA prevents dextran sulfate sodium-induced colitis by inhibiting NLRP3 inflammasome secretion and activation of the P38 MAPK signaling pathway (105). Artemisinin-based drugs have been used to treat concanavalin A (Con A)-induced autoimmunity in mice by suppressing the MAPK signaling pathway (106). Using single-cell transcriptome sequencing, Zhang et al. (107) constructed the first cellular transcriptome atlas of DHA-regulated immune cell subpopulations in the spleen. The study found that DHA significantly increased the proportion of Treg and cytotoxic CD8+IFN-γ+ T cells. It also revealed that Treg highly expressed inhibitory signaling molecules, such as CD73, CD274, and IL-10, while the expression of key effector molecules, including granzyme A and granzyme B, was downregulated in cytotoxic CD8+IFN-γ+ T cells. In terms of humoral immunity, DHA downregulated the expression of MHCII molecules in splenic follicular B cells and significantly decreased the expression levels of key molecules associated with B cell activation, such as CD79a, CD79b, and Ms4a1. These results suggest that DHA exerts different effects on humoral and cellular immunity. KEGG enrichment analysis and analysis of differential gene expression revealed that DHA significantly upregulated the expression of activating protein-1 (AP-1) transcription factors in various immune cells. The study confirmed the changes in AP-1 and its upstream kinase JNK expression levels regulated by DHA using flow cytometry and Western blot analysis. Superoxide dismutase 3 (SOD3), as a growth regulator, can drive the activation of AP-1. The study also used SOD3 knockout mice and confirmed that SOD3 knockout inhibited DHA-induced immune cell rearrangement and activation of the JNK-AP-1 signaling pathway. These findings indicate a close

relationship between the immunomodulatory function of DHA and the activation of the SOD3-JNK-AP-1 signaling pathway, laying the groundwork for further understanding and revealing the molecular mechanisms underlying the immunomodulatory function of DHA.

In the context of the ERK-MAPK signaling pathway, the ERK cascade can be activated by various extracellular factors, such as growth factors, hormones, and cellular stress, leading to processes like cell proliferation and differentiation (108–110). Ouyi et al. treated BEAS-2B cells, which had been exposed to influenza A virus, with varying concentrations of DHA (12.5, 25, 50 μmol/L) and observed changes in TNF-α and IL-6 mRNA and protein levels, as well as p-ERK protein expression. The results showed that DHA inhibited the expression of TNF-α, IL-6 mRNA, TNF-α, IL-6, and p-ERK protein through the ERK signaling pathway, with the inhibition displaying a dose-dependent effect (111). Similarly, Wei et al. studied the regulatory effects of DHA on inflammatory mediators in an ovalbumin (OVA)-induced asthma mouse model, administering DHA orally at a dose of 30 mg/kg (112). Their findings revealed that DHA suppressed the activation of p38 MAPK and ERK, suggesting that DHA alleviates OVA-induced asthma by inhibiting the p38 MAPK and ERK pathways. These studies highlight the potential therapeutic role of DHA in managing allergic inflammation (112).

4.2.6 Nrf2 signaling pathway

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor negatively regulated by Kelch-like ECH-associated protein 1 (KEAP1), which controls the transcription of anti-inflammatory genes and inhibits the expression of antioxidant enzymes, thus suppressing inflammation (113, 114). The p62-mediated degradation of KEAP1 promotes Nrf2 nuclear translocation and transcription of its target genes. DHA treatment increased the expression of Nrf2 and its target gene heme oxygenase-1 (HO-1) in myeloid-derived suppressor cells (MDSCs) in systemic lupus erythematosus (SLE) mice and delayed the aging of MDSCs (52). Artemether-lumefantrine activated Nrf2 by increasing p62 expression in mouse bone marrow macrophages and upregulated the expression of HO-1 and/or NAD(P)H:quinone oxidoreductase 1 (NQO1) (115). Similarly, DC32 promoted Nrf2/HO-1 signaling and enhanced p62 transcription or KEAP1 degradation in DBA/1 mice and mouse embryonic cells (116). In conclusion, artemisinin derivatives promote the activation of the Nrf2 signaling pathway, leading to increased expression of anti-inflammatory genes and the alleviation of inflammation.

4.2.7 JAK/STAT signaling pathway

Janus kinases (JAKs) and signal transducers and activators of transcription (STAT) proteins control the signal transduction of many cytokines and growth factors involved in cell growth, survival, and differentiation (117). DHA reduced STAT1 phosphorylation in human umbilical vein endothelial cells (HUVECs) stimulated with IFN-α and inhibited the phosphorylation of JAK2 and STAT3 in the kidneys of MRL/lpr mice, thereby alleviating the symptoms of lupus nephritis (55, 118).

4.2.8 Others

Based on the farnesoid X receptor (FXR) as a potential target for the treatment of alcoholic liver disease (ALD), Xu et al. (119) investigated the effects of DHA on ALD and its underlying mechanism. They constructed a rat model of alcoholic liver injury and administered DHA at a dose of 7 mg/kg by intraperitoneal injection, in combination with ursodeoxycholic acid (10 mg/kg) and orphenadrine (30 mg/kg). The results showed that DHA inhibited the expression and activity of FXR in the liver of rats with ALD, reduced the expression of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LD), improved liver injury, and suppressed the expression of inflammatory genes and infiltration of inflammatory cells. In conclusion, numerous studies have shown that artemisinin and its derivatives exert anti-inflammatory effects by modulating the levels of inflammatory cytokines through various signaling pathways, including the ERK, NF-κB, AMPK/SIRT1, ROS-JNK1/2, mTOR/S6K1, PI3K/Akt, Nrf2, JAK/STAT, and MAPK signaling pathways. See Figure 1.

5 Advances in the research on artemisinin and its derivatives in the treatment of autoimmune diseases

5.1 RA

RA is one of the most common immune-mediated diseases characterized by inflammatory arthritis, primarily affecting small joints of the hands and feet with symmetrical polyarthritis (120, 121). RA is a systemic disease with various co-existing diseases and extra-articular manifestations. The occurrence of synovial inflammation is a result of the interaction between genetic factors and specific environmental exposures (122). DHA is a derivative of artemisinin and one of the main active forms of artemisinin in the body (123). Du et al. (89) found that DHA had therapeutic effects on both AIA and CIA in rats, significantly reducing joint swelling and pathological scores, as well as the levels of the inflammatory cytokine IL-6 in RA rats. *In vitro* treatment of RAW264.7 macrophages with DHA for 24 hours resulted in a reduction in IL-6 in the culture supernatant and decreased nuclear expression of NF-κBp65, suggesting that the improvement of rheumatoid arthritis in rats may be related to the inhibition of the NF-κB signaling pathway by DHA. Another study found that DHA induced apoptosis in rheumatoid arthritis synovial cells by inhibiting the phosphorylation of Akt at serine 473 and the activation of NF-κB. These studies suggest the potential application of DHA in the treatment of rheumatoid arthritis (124). DC32, a derivative of DHA, has strong immune inhibitory properties. Studies have shown that DC32 can alleviate rheumatoid arthritis by activating the Nrf2/HO-1 antioxidant signaling pathway, increasing p62 protein transcription, and significantly reducing arthritis inflammation in CIA mice. *In vitro* intervention with DC32 promoted the degradation of Keap1 protein in NIH-3T3 embryonic fibroblasts and upregulated the expression of HO-1 and

p62, indicating that DC32 could alleviate rheumatoid arthritis by activating the Nrf2/p62/Keap1 pathway (125). Another study demonstrated that DC32 inhibited Th17 cells, elevated the proportion of immunosuppressive Tregs, restored the Treg/Th17 balance, suppressed synovial inflammation, and reduced arthritis symptoms in CIA mice (50). Artemether-lumefantrine, a combination drug derived from artemisinin, reduced experimental systemic lupus erythematosus (SLE) symptoms by inhibiting Th17 and upregulating Treg cells in the CIA model (126). Studies have also shown that DHA combined with Torch Ginger Root has a therapeutic effect on AIA rats, possibly by inhibiting the expression of VEGF, MMP-1, and MIF and reducing the levels of inflammatory cytokines (127). DHA combined with hydroxychloroquine (an analog of artemisinin) has a better treatment effect than monotherapy. The therapeutic effect of DHA and hydroxychloroquine in the treatment of rheumatoid arthritis was achieved by blocking the NF-κB pathway (128). Regarding B cells, artemisinin was found to eliminate germinal center B cells and inhibit autoantibody-mediated autoimmune arthritis (59).

DHA modulates the proliferation, apoptosis, and autophagy of chondrocytes in the rheumatoid arthritis mouse model through the PI3K/AKT/mTOR signaling pathway (126). *In vitro* studies have shown that artemisinin can inhibit the differentiation of osteoclast precursors and bone resorption in RANKL-induced RAW264.7 cells and mouse bone marrow-derived macrophages (129, 130). Dimeric artesunate phospholipid-conjugated liposomes, a novel amphiphilic artemisinin compound with low cytotoxicity, significantly improved ankle joint swelling and inflammatory response in AIA mice. It exhibited higher inhibitory effects on pro-inflammatory factors and improved the condition of AIA mice compared to artemisinin (131). In summary, artemisinin and its derivatives can reduce collagen content, decrease fibroblast viability and proliferation, and increase cell apoptosis by inhibiting various inflammatory-related signaling pathways in rheumatic diseases. These research findings suggest great potential for the use of artemisinin derivatives in the treatment of rheumatoid arthritis, providing new insights and drug targets for its treatment.

5.2 SLE

SLE is an autoimmune disease characterized by multi-organ inflammation and widespread production of autoantibodies. Autoantibodies, especially anti-dsDNA and anti-Sm autoantibodies, have high specificity in SLE and are involved in immune complex formation and inflammatory damage to various end organs, including the kidneys, skin, and central nervous system (CNS) (132–134). In recent years, studies have revealed that artemisinin-based drugs can modulate the immune system and alleviate SLE (135). Myeloid-derived suppressor cells (MDSCs) as immunosuppressive cells are reduced in autoimmune disease. Zhang et al. (136) found that DHA could delay the senescence of MDSCs and improve lupus symptoms in mice by activating the antioxidant-related Nrf2/HO-1 signaling pathway. Another study found that both 125 mg/kg and 25 mg/kg DHA could suppress the production of anti-dsDNA antibodies and reduce the levels of TNF-α in the serum of BXSB mice (recombinant inbred strain of female C57BL mice and male SB mice), significantly improving lupus nephritis in mice (137). Liang et al. (138) demonstrated that the artemisinin analogs hydroxychloroquine and quinacrine can reduce autoimmune antibody production, B lymphocyte proportions, and alleviate the symptoms of lupus nephritis by inhibiting the KLF15/NF-κB signaling pathway. Studies have also shown that chloroquine and quinacrine, artemisinin analogs, can reduce the production of autoantibodies, such as anti-DNA antibodies, and the levels of pro-inflammatory cytokines TNF-α, IL-8, IL-6, and IFN-α in peripheral blood mononuclear cells (PBMCs) of SLE patients. In addition, *in vitro* interventions with chloroquine and quinacrine in HEK293 cells transfected with TLR3, TLR8, and TLR9 demonstrated a dose-dependent decrease in IL-6 in the culture supernatant and the expression levels of the three proteins, indicating that artemisinin analogs such as chloroquine and quinacrine can alleviate SLE symptoms by inhibiting TLR signaling pathways (139). Feng et al. (118) found that artemisinin can regulate macrophage migration inhibitory factor (MIF) and inhibit macrophage migration and phosphorylation of STAT1, thus ameliorating systemic lupus erythematosus-related atherosclerosis in patients. A study demonstrated that treatment with a TAT-modified cationic liposome co-delivering DHA and HMGB1 siRNA effectively prevented lupus nephritis in experimental SS mice (140). DHA has been shown to protect against the aging of bone marrow-derived suppressor cells (MDSCs) in NOD/Ltj mice and improve salivary gland secretion (52). The therapeutic effect of artesunate in lupus-susceptible MRL/lpr mice is dependent on T follicular helper cell differentiation and activation of the JAK2-STAT3 signalling pathway. It can regulate T cell subset ratios, reduce the generation of pro-inflammatory lymphocyte subsets Th1, Th17, and Tfh, and increase the level of immunosuppressive Tregs in NOD/Ltj model mice (55).

5.3 Sjögren's syndrome

Primary Sjögren's syndrome (pSS) is an autoimmune disease characterized by dry eyes and dry mouth caused by inflammation in the salivary and lacrimal glands. Currently, effective treatment methods for pSS patients are lacking (141–143). Artesunate may inhibit disease progression in experimental Sjögren's syndrome and humanized Sjögren's syndrome mice by suppressing Th17 responses (144). A recent network pharmacological study revealed that artemisinin corresponds to 412 targets, while pSS corresponds to 1495 genes. There are 40 overlapping genes between artemisinin and pSS. According to KEGG analysis, artemisinin's therapeutic effect on pSS involves the IL-17 signaling pathway, HIF-1 signaling pathway, apoptosis signaling pathway, Th17 cell differentiation, PI3K-Akt signaling pathway, and MAPK signaling pathway (145). Molecular docking results further revealed that artemisinin molecules exhibited higher binding affinity to key nodes in the IL-17 signaling pathway. In *in vivo* experiments, artemisinin restored salivary gland secretory function in NOD/Ltj mice and improved glandular damage. It facilitated an increase in Treg levels

and a decrease in IL-17 secretion in an NOD mouse model. In terms of B cell intervention, studies have found that artemisinin analogs such as artemisinin-lumefantrine suppress autoimmune responses resembling Sjögren's syndrome by inhibiting TRAF6-mediated NF-κB signaling and excessive activation of B cells induced by BAP (B cell-activating factor) (146). Artemisinin-lumefantrine improves general conditions, increases salivary secretion, reduces fatigue, decreases the infiltration of lymphocytes in submandibular glands and lacrimal glands, inhibits the generation of pro-inflammatory lymphocyte subsets Th1, Th17, Tfh, and germinal center B cells, and increases the level of immunosuppressive Treg cells in NOD mice. Thus, it regulates the Th1/Th2 and Th17/Treg immunological balance. These actions may constitute specific mechanisms by which artemisinin-lumefantrine treats the dry symptoms of Sjögren's syndrome in NOD mice. In terms of B cell intervention, artemisinin improves SS-like symptoms in mice and modulates the TRAF6-NF-κB signaling pathway, which determines the survival and proliferation of B cells (147). A recent study found that DHA promotes Treg proliferation and inhibits the expansion of germinal center B cells, resulting in reduced numbers of circulating plasma cells and serum immunoglobulin levels. Overall, these findings demonstrate that artemisinin effectively suppresses disease progression in ESS and humanized SS mice by inhibiting Th17 responses. Additionally, the study identified a novel metabolic regulation function of artemisinin, involving the promotion of IRF4 proteasomal degradation, which inhibits the glycolytic pathway in Th17 cells. In summary, these findings suggest that artemisinin may be a promising therapeutic candidate for the treatment of pSS (148).

5.4 Inflammatory neurological diseases

CNS autoimmune diseases involve the immune system—comprising autoimmune cells, autoantibodies, and other immune molecules—attacking the nervous system, including neurons, glial cells, and myelin (149). Such diseases include neuromyelitis optica spectrum disorder (NMOSD), myelin oligodendrocyte glycoprotein-IgG associated disorders (MOGAD), multiple sclerosis (MS), acute disseminated encephalomyelitis (ADEM), autoimmune encephalitis (AE), and CNS vasculitis. The complex pathogenesis of these conditions is driven by immune-inflammatory reactions targeting the nervous system (150–152).

Research indicates that artemisinin helps to normalize behavioral deficits and protect hippocampal tissue in neuroinflammatory conditions. Artemisinin-based drugs reduce the release of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, MCP-1) and downregulate inflammatory signaling pathways such as NF-κB, TLR-4, ERK, and MAPK in mice (153). Zhang et al. (154) demonstrated that a 100 mg/kg dose of an artemisinin derivative significantly reduced the severity of experimental autoimmune encephalomyelitis (EAE). This effect was attributed to the inhibition of the TLR-4/NF-κB pathway, regulation of pro-inflammatory cytokine expression, and maintenance of intestinal barrier integrity by downregulating MUC2 and claudin-1 in colonic mucosal layers (155).

Further studies on oral delivery of chitosan-coated artesunate (CPA), another artemisinin derivative, showed significant inhibition of the TLR-4/NF-κB pathway, reduced pro-inflammatory cytokine mRNA levels, and increased IL-10 in RAW264.7 cells. CPA also protected zonula occludens-1 (ZO-1) in Caco-2 cells and modulated macrophage polarization by reducing CD86 and increasing CD206 and p-STAT6 expression. These effects were dependent on STAT6, as they were not observed in STAT6-deficient cells (156). In a UC mouse model, CPA treatment inhibited TLR-4/NF-κB activation, M1 macrophage polarization, and mucosal barrier damage, while promoting STAT6 phosphorylation, an effect reversed by an HO-1 inhibitor. These findings suggest that CPA reduces Th1 and Th17 cell generation through AP1 inhibition, thereby alleviating EAE severity and showing potential as a therapeutic agent for multiple sclerosis (157).

Additionally, artemisinin administration significantly improved EAE symptoms by reducing IL-6, IL-17, and IL-1 expression in the spinal cord. Both artemisinin and tehranolid treatments stimulated the expression of anti-inflammatory genes such as TGF-β, IL-4, IL-10, FOXP3, GATA3, MBP, and AXL, while reducing T-bet expression. Neither treatment affected the expression of IFN-γ, RORγt, nestin, Gas6, Tyro3, or Mertk mRNA in the spinal cord. These results indicate that artemisinin and tehranolid effectively regulate genes responsible for inflammation and myelination, with tehranolid showing greater efficacy than antiretroviral treatment, suggesting its potential as an alternative therapeutic approach in MS management (158).

5.5 IBD

IBD, including ulcerative colitis (UC) and Crohn's disease (CD), is a complex autoimmune disease with an unclear etiology (159, 160). Increasing evidence suggests that immune dysfunction, abnormal expression of inflammatory cytokines, and gut microbiome dysbiosis are major contributors to IBD pathogenesis (161, 162). NF-κB, a key transcription factor in M1 macrophages, induces the expression of various inflammatory factors, including TNF-α, IL-1β, IL-6, IL-10, IL-12, and COX-2. Dysregulation of NF-κB is closely linked to IBD development, making its inactivation crucial in IBD treatment (163, 164).

Artemisinin has been shown to inhibit the NF-κB signaling pathway, regulate inflammatory cytokine expression, and maintain intestinal barrier integrity by downregulating MUC2 and claudin-1 in the colonic mucosa, thereby suppressing DSS-induced colonic inflammation (165). CPA, another artemisinin derivative, significantly inhibited the TLR-4/NF-κB pathway and reduced pro-inflammatory cytokine mRNA levels while increasing IL-10 in RAW264.7 cells. CPA also protected ZO-1 in Caco-2 cells and modulated macrophage polarization by reducing CD86 and increasing CD206 and p-STAT6 expression, with STAT6 playing a critical role in these effects (166). Yang et al. (167) found that artemisinin reduced pro-inflammatory factors, M1 macrophages, and EMT-related proteins, while increasing M2 macrophages in colon tissues, improving disease activity index scores, and villus

structure in colitis mice, indicating its efficacy in IBD treatment. DHA may also exert therapeutic effects on DSS-induced IBD by regulating inflammatory cytokines, cell junction-related genes, and gut microbiota (168). The Th/Treg imbalance plays a crucial role in IBD pathogenesis, with excessive CD4+ T cell activation being a potential mechanism (169). HO-1, a rate-limiting enzyme in heme metabolism, has anti-inflammatory and antioxidant effects and can induce apoptosis in activated CD4+ T cells through the Fas/CD95-FasL pathway (170, 171). Yan et al. (51) found that DHA treatment improved colitis symptoms, reduced lymphocyte infiltration and tissue fibrosis, decreased Th1, Th17, Th9, and Th22 cells, and increased Tregs in TNBS- and OXA-induced colitis models. DHA also inhibited CD4+ T lymphocyte activation and induced apoptosis, promoting HO-1 production, which was accompanied by CD4+ T cell apoptosis and restoration of the Th/Treg balance—effects blocked by the HO-1 inhibitor tin-protoporphyrin IX (SnPPIX). These findings suggest that DHA is a promising candidate for IBD treatment and Th/Treg immune modulation.

Network pharmacology, an emerging discipline studying drug-target interactions, revealed that artemisinin acts on multiple targets and signaling pathways involved in IBD. Lei et al. (172) showed that DHA treatment tended to restore and upregulate E-cadherin and significantly increased the abundance of Bacteroidales_S24-7_group in the gut microbiome, which was decreased in the DSS group. In conclusion, artemisinin exerts anti-inflammatory effects through multiple targets and signaling pathways, repairing the intestinal barrier and providing insights for its use in IBD treatment. Yu et al. (173) also found that DHA-bear bile acid conjugates are potential therapeutic agents for IBD, reducing disease activity index, colonic damage, and splenomegaly in DSS-induced colitis mice.

5.6 Myasthenia gravis

MG is an autoimmune disease characterized by muscle weakness, primarily affecting the motor function. It has been found that MG is not limited to motor symptoms such as weakness in the eye muscles, respiratory muscles, and overall muscle weakness, but also involves non-motor symptoms. These non-motor symptoms can affect various target organs, presenting in different forms, including sleep disorders, emotional disorders, pain, special sensory disorders, cognitive impairment, and autonomic dysfunction. These symptoms severely impact the quality of life of patients and can even shorten their lifespan (174, 175). The production of high-affinity pathogenic anti-acetylcholine receptor (AChR) antibodies in MG requires the involvement of CD4+ T helper cells and their cytokines. Th17 and Treg cells, as subsets of CD4+ T cells, play critical roles in the development and progression of various inflammatory diseases, including autoimmune disorders. Th17 cells and their cytokines promote inflammation, while Treg cells and their cytokines exert anti-inflammatory effects. These cytokines are also essential in generating and regulating autoimmune responses. Differentiated CD4+ T cells are classified into various subsets based on the cytokines they produce, such as Th1 cells, which secrete pro-inflammatory cytokines IL-12 and IFN-γ, Th17 cells, which produce IL-17, and Treg cells, which secrete transforming growth factor-β (TGF-β) and IL-10 (172, 176). Artemisinin improves the symptoms of experimental autoimmune myasthenia gravis by regulating the balance of Th1 cells, Th17 cells, and Treg cells (177). Artemisinin-lumefantrine has therapeutic potential in experimental autoimmune myasthenia gravis by upregulating Treg cells and regulating the Th1/Th2 cytokines (178).

5.7 Autoimmune liver diseases

AILD are a group of liver-damaging diseases caused by autoimmune abnormalities, including autoimmune hepatitis (AIH), primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), and overlapping syndromes with simultaneous occurrence of two of these diseases (179). IL-17 is a pro-inflammatory cytokine belonging to the IL-17 family. IL-17A and IL-17F, mainly secreted by Th17 cells, can induce the secretion of IL-6 by hepatocytes, further activating Th17 cells in a positive feedback loop and promoting the release of other pro-inflammatory factors such as TNF-alpha (180, 181). A higher level of IL-17 has been found in the peripheral blood and liver tissues of AIH patients compared to healthy individuals, and it is positively correlated with disease activity and liver fibrosis (182). Studies have found that artemisinin may protect against Con A-induced autoimmune liver injury in mice by inhibiting the NF-κB signaling pathway (182). This study provides a scientific reference for the application of artemisinin in preventing autoimmune liver injury.It has been found that artemisinin effectively suppresses Con A-induced liver injury by inhibiting the activation of the TLR-4/NF-κB pathway and reducing the mRNA levels of pro-inflammatory cytokines while increasing the IL-10 level in RAW264.7 cells (106). Artemisinin treatment significantly improved the symptoms of Con A-induced experimental autoimmune hepatitis in mice. It reduced liver enzyme levels, inhibited the expression of inflammatory cytokines such as IFN-γ, TNF-α, IL-1β, IL-6, and IL-17, and increased the level of the anti-inflammatory cytokine IL-10. Additionally, artemisinin inhibited the phosphorylation of ERK, JNK, p38, IκBα, and NF-κB p65 in the liver, indicating its protective effect on autoimmune liver injury (183).

5.8 IgA nephropathy

IgA nephropathy is one of the most common forms of primary glomerulonephritis worldwide, characterized by the deposition of IgA immunoglobulin in the glomerular mesangium (184). Clinically, it often presents as microscopic hematuria and proteinuria, with 20% to 40% of patients progressing to end-stage kidney disease (ESKD) within 20 years of onset. The pathogenesis of IgA nephropathy, known as the "4 Hit" hypothesis, is closely linked to the immune system, involving B cells, T cells, TLRs, monocytes/macrophages, and the complement system (185–187). Activation of NF-κB-induced immune inflammation is a key factor in the disease's progression (188).

Studies have shown that artemisinin combined with hydroxychloroquine significantly enhances the secretion of extracellular vesicles in renal tubular epithelial cells in rat models of IgA nephropathy. These vesicles, when absorbed by glomerular mesangial cells, inhibit NF-κB signaling and NLRP3 inflammasome activation, thereby reducing the expression of related inflammatory proteins and suppressing inflammation (189). CMap analysis revealed that artemisinin can reverse the expression of differentially expressed genes in IgA nephropathy, identifying 87 potential targets, with AKT1 and EGFR showing the highest binding affinity with artemisinin. *In vivo*, artemisinin improved kidney damage and fibrosis, while *in vitro* studies showed it weakened LPS-induced oxidative stress and fibrosis by promoting AKT phosphorylation and Nrf2 nuclear translocation (190).

Artemisinin has also been shown to significantly improve kidney function, reduce mesangial matrix expansion, and decrease immune complex deposition in the kidneys of IgA nephropathy rats. Mechanistic studies indicated that artemisinin modulates the differentiation of CD4+ T cell subgroups, reducing the high proportion of Th2 and Th17 cells and increasing Th1 and Treg cells, suggesting an immunosuppressive effect (191). Further research demonstrated that artemisinin facilitates the secretion of extracellular vesicles in kidney tissues, which in turn inhibits NF-κB/NLRP3 signaling and related protein expression. This effect was blocked by GW4869, indicating the crucial role of extracellular vesicles in this process (192).

In terms of autophagy, DHA was found to inhibit the proliferation of mesangial cells in IgA nephropathy through the mTOR signaling pathway (193). To further explore the therapeutic potential, Chen et al. designed a randomized, double-blind, placebo-controlled clinical trial involving 120 patients with IgA nephropathy. Participants will receive either 100 mg or 50 mg of artemisinin or a placebo for six months, with changes in proteinuria and kidney function measured post-intervention. The study will also assess levels of Gd-IgA1 and anti-Gd-IgA1 to explore potential immune mechanisms (194).

5.9 Autoimmune skin diseases

Autoimmune skin diseases involve the immune system attacking its own antigens, leading to an immune imbalance that causes skin damage, with potential involvement of other tissues and organs. Traditional immunosuppressive agents often come with a range of adverse effects (195). Research has explored the potential of artemisinin derivatives in treating conditions such as general skin rashes, rosacea, and psoriasis.

In a mouse model of rash, artemisinin was shown to reduce or eliminate symptoms, including decreased dermatitis scores, reduced ear, spleen, and lymph node weight, thinner skin epidermis, lower IgE levels, reduced inflammatory cell infiltration, decreased Th7 cells, increased Treg cells, downregulation of Th17-related factors (such as IL-6, IL-17, and IL-23), and upregulation of TGF-β and SOCS-3 (196). A clinical trial in patients with erythematotelangiectatic rosacea demonstrated that those treated with a combination of artemisinin drugs had significantly lower papule and pustule scores compared to those receiving standard metronidazole therapy over four weeks, with similar erythema scores (78).

Artemisinin has also been effective in alleviating psoriasis-like dermatitis in mice induced by imiquimod (IMQ) (197, 198). It significantly improved acute skin lesions and reduced relapse in low-dose IMQ-induced psoriasis by decreasing the frequency and number of CD8 central memory T cells (TCM) and CD8+ resident memory T cells (TRM). Artemisinin mitigated histopathological changes and T cell infiltration in the skin, inhibited the expression of inflammatory cytokines such as IL-15 and IL-17, and other pro-inflammatory factors. Artemisinin also downregulated the expression of EGF and BCL-6 in CD8 T cells and suppressed CD8CLA, CD8CD69, or CD8CD103+ TRM cells in the skin (199).

Moreover, artemisinin prevented psoriasis relapse when administered at the onset, unlike methotrexate, which only reduced CD8+ TCM cells but not CD8+ TRM cells. Recombinant IL-15 or CD8 (rather than CD4) TCM cell transfer led to complete relapse in previously artemisinin-treated psoriasis mice. Additionally, artemisinin alleviated psoriasis-like lesions in humanized NSG mice while reducing CD8+ TCM and CD103+ TRM cells (199).

In IL-17A-induced HaCaT cells, artemisinin significantly inhibited excessive proliferation, migration, and the expression of inflammatory factors. It also suppressed FGFR1 expression, which is highly expressed in these cells. The addition of an FGFR1 agonist reversed artemisinin's inhibitory effects, suggesting that artemisinin targets FGFR1 to inhibit excessive proliferation and inflammation, presenting a new potential target for psoriasis treatment (200).

These findings highlight the potential applications and mechanisms of artemisinin in treating autoimmune disorders.

5.10 Autoimmune thyroiditis

AIT is a typical organ-specific autoimmune disease, characterized by immune imbalance leading to the production of specific antibodies against self-antigens, resulting in the onset of the disease under the influence of genetic and environmental factors (201). Several studies have confirmed the differentiation and functional imbalance of T cells in AIT patients, primarily characterized by an increase in Th1 and Th17 cells and a defect in Tregs (202, 203). Research has found that artemisinin treatment effectively decreases serum thyroglobulin antibody levels, improves thyroid lymphocyte infiltration and thyroid function, and reduces thyroid and spleen weights in AIT mice, indicating that artemisinin improves the condition of AIT mice by suppressing immune imbalance and lymphocyte infiltration (204).

5.11 Gout and gouty arthritis

Gout is a common and treatable disease caused by the deposition of monosodium urate (MSU) crystals in joints and non-articular structures. Hyperuricemia, an elevated level of uric acid in the blood, is the most important risk factor for gout (205). Gout is the most common inflammatory arthritis in adults. It is

characterized by sterile inflammation caused by the deposition of monosodium urate (MSU) crystals in the joints and periarticular tissues. It presents with rapid onset and can lead to arthritis, tophi, renal stones, and urate nephropathy (206, 207). Studies have found that artemisinin exhibits anti-inflammatory effects in gout patients through its ability to inhibit the interaction between NEK7 and NLRP3, thereby suppressing the activation of the NLRP3 inflammasome. Artemisinin also reduces intra- and extracellular potassium efflux in macrophages stimulated by LPS and MSU crystals, thus alleviating joint inflammation in gouty arthritis mice. Artemisinin treatment effectively reduces foot and ankle swelling in mice with MSU crystal-induced arthritis. The study suggests that artemisinin inhibits NLRP3 inflammasome activation by suppressing the interaction between NEK7 and NLRP3 in uric acid-induced inflammation (208).

6 Prospects

In summary, artemisinin and its derivatives exhibit unique mechanisms of action, making them promising candidates for the treatment of autoimmune diseases. These compounds effectively intervene in the overactive inflammation and immune responses characteristic of rheumatic and autoimmune diseases by modulating various signaling pathways, including the TNF, Toll-like, IL-6, RANKL, MAPK, PI3K/AKT/mTOR, JAK/STAT, and NRF2/GPX4 pathways. Notably, within the NF-κB signaling pathway, both systemic and localized application of artemisinin has been shown to significantly improve a range of autoimmune conditions, including systemic autoimmune diseases, autoimmune kidney diseases, neurological disorders, skin diseases, and autoimmune liver and gallbladder conditions (4, 209–212).

The regulatory mechanisms of artemisinin primarily involve inhibiting abnormalities in both the innate and adaptive immune systems, such as the excessive proliferation and activation of immune cells, abnormal differentiation of functional subsets, and dysregulated cytokine expression. Additionally, artemisinin helps suppress the cascade reactions triggered by changes in the phenotype and function of pro-inflammatory immune cells at various stages of autoimmune disease progression, exerting targeted effects on organs impacted by these conditions (58, 118, 213, 214).

Artemisinin's distinctive immunomodulatory effects focus on key nodes within pathological immune responses and cellular signaling cascades, promoting the restoration of immune homeostasis. This makes artemisinin and its derivatives particularly suitable for treating chronic rheumatic and autoimmune diseases with complex underlying mechanisms.

Clinically, several multicenter randomized controlled trials targeting rheumatic, inflammatory, and autoimmune diseases are currently underway, aiming to translate more preclinical findings into effective clinical treatments.

Author contributions

ZL: Conceptualization, Data curation, Formal analysis, Validation, Writing – original draft, Writing – review & editing. WaX: Conceptualization, Data curation, Formal analysis, Validation, Writing – original draft. WeX: Conceptualization, Data curation, Validation, Writing – original draft. YM: Conceptualization, Data curation, Formal analysis, Validation, Writing – original draft. QF: Conceptualization, Data curation, Formal analysis, Validation, Writing – original draft. BZ: Data curation, Formal analysis, Validation, Writing – original draft. LZ: Conceptualization, Data curation, Formal analysis, Validation, Writing – review & editing.

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