AMERICAN JOURNAL OF BIOCHEMISTRY AND BIOTECHNOLOGY

RESEARCH ARTICLE

Immunomodulatory and Antioxidant Effects of *Stachys japonica* Miq. Ethanol Extract on Cyclophosphamide-Induced Immunosuppressed Mice and its Phytochemical Composition

Xueping Jiang[1, 2, 3]*, Jianglong He[3], Yanjun Chen[1], Fang Wang[2]

[1]Anhui Engineering Research Center for Eco-agriculture of Traditional Chinese Medicine, West Anhui University, Lu'an, Anhui, 237012, China
[2]Anhui Dabieshan Academy of Traditional Chinese Medicine, Lu'an, Anhui, 237012, China
[3]School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang, Jiangsu, 212100, China

*Corresponding Author: jiang1025@mail.vinhelp.com

Abstract: *Stachys japonica* Miq. is an edible wild botanical drug and a traditional medicine in China. This study focused on examining the phytochemical profile and antioxidant properties of the ethanol extract of *S. japonica* Miq (SJEE) in vitro, as well as its immunomodulatory and antioxidant activities in cyclophosphamide (Cy)-induced immunosuppressed mice. The SJEE was analysed by UPLC-HRMS/MS. 36 Polyphenols, including flavonoids, cinnamic acid derivatives, alcohols, and polyolsphenylethaoids, and hydroxycoumarins were identified. Total phenolics (102.06 ± 2.35 mg GAE/g extract) and total flavonoids (59.77 ± 1.09 mg RE/g extract) contents were measured. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, hydroxyl radical scavenging activity, superoxide anion scavenging activity, and reducing ability tests demonstrated that SJEE possesses remarkable antioxidant properties. In Cy-treated mice, intragastric administration of SJEE (100, 200, and 400 mg/kg•d^{-1}) resulted in upregulation of immune organ indices, mitigation of immune organ injury, and restoration of impaired immune function. SJEE significantly enhanced splenocyte proliferation, strengthened macrophage phagocytosis, and substantially improved hemolysin levels while restoring delayed-type hypersensitivity (DTH) responses. It also promoted body weight recovery in immunosuppressed mice. Furthermore, the high-dose SJEE treatment resulted in increased liver enzyme activities of superoxide dismutase, glutathione peroxidase, and catalase, accompanied by reduced malondialdehyde levels, indicating potent in vivo antioxidant effects. These results suggest that SJEE has considerable immunomodulatory potential, capable of shielding the immune system from oxidative stress and helping maintain internal homeostasis. Consequently, SJEE holds promise for development as a functional food or immune adjuvant to support human health.

Keywords: Stachys Japonica Miq., UPLC-HRMS/MS, Immunostimulatory Activity, Antioxidant, Cyclophosphamide

Received: 20-01-2025 | **Revised:** 12-05-2025 | **Accepted:** 28-08-2025 | **DOI:** 10.3844/ajbbsp.2026.22.01.003

Introduction

The innate immune system is the most powerful and effective structure in the human body to protect organisms from antigens and pathogens, so improving the body's immunity is crucial for health [1, 2]. Immunomodulators can restore the immune response of the human body to the normal level, thus alleviating and treating various diseases caused by low immune function, such as Rheumatoid arthritis, AIDS and so on [3, 4]. Although immunomodulators such as chemosynthetic drugs and mammalian proteins have been developed, toxic side effects remain a serious concern. In the past few decades, traditional Chinese medicine has become a research hotspot in the development of functional food and new drugs in recent years, due to its abundant resources, low cost, rich in a variety of bioactive metabolites, low toxicity and no residue, and its effects of enhancing immunity, antioxidant and antibacterial [5-7].

Stachys japonica Miq. is a perennial medicinal plant of the Lamiaceae, classified under the genus Stachys, and is predominantly found in ditches, riversides, and other wetland habitats. Within the Lamiaceae, Stachys is a large genus widely distributed worldwide, except in Australia and New Zealand. About 300 Stachys species are recorded, in which 18 species and 11 subspecies are distributed in China [8]. Many Stachys taxa such as *S. lavandulifolia*, *S. inflata*, *S. parviflora*, *S. brachyclad*, and *S. annuahave* been reported to have antimicrobial [9], antiproliferative properties [10], anti-hyperglycemic [11, 12], antioxidant [13], anti-inflammatory, antiallergic [14], anxiolytic activities [15]. Immunomodulatory activity of Stachys has been reported [16].

Traditionally, *S. japonica*, first recorded in the "Shennong Materia Medica Classic," is used for treating whooping cough, tonsillitis, and pharyngitis [8]. Furthermore, in the Dabie Mountains of China, *S. japonica* rhizomes were commonly eaten by the local people. Phytochemical studies have shown that this genus contains phenols, flavonoids, anthraquinones, tannins and polysaccharides [17]. Particularly, flavonoids and phenols are important secondary metabolites in plants, with antibacterial, anti-inflammatory, and anticancer activities [18]. They are also natural antioxidant agents that can effectively reduce and eliminate free radicals, delay aging, improve immune function, and participate in disease prevention [19]. In our previous study, we found that the ethanol extract from *S. japonica* Miq rhizomes contains flavonoids, phenols, anthraquinones, tannins polysaccharides, and other bioactive compounds by preliminary qualitative experiment [20].

However, while many reports have detailed the chemical composition, immune function, and antioxidant activity of various Stachys species [21], comprehensive studies focusing specifically on *S. japonica* Miq. is notably scarce. There is a clear need for in-depth chemical characterization of *S. japonica* Miq. extract using advanced hyphenated techniques like Ultra-Performance Liquid Chromatography coupled with High-Resolution Mass Spectrometry (UPLC-HRMS/MS), which can provide a detailed understanding of its metabolite composition [22]. Furthermore, rigorous evaluation of its immunomodulatory and antioxidant potential, especially through well-designed in vivo studies, is largely missing from the current literature for this particular species.

Accordingly, this study sought to bridge the gap by undertaking a detailed phytochemical characterization of the ethanol fraction of *S. japonica* Miq. (SJEE) employing UPLC-HRMS/MS. Subsequently, we aimed to evaluate its antioxidant activity in vitro and, more critically, to investigate its immunoregulatory and antioxidant effects in vivo using a Cyclophosphamide-induced immunosuppressed mouse model. This research aims to provide a robust experimental basis for the potential development and utilization of the wild botanical drug *S. japonica* Miq. in functional foods and as a source for novel immunomodulatory and antioxidant agents.

Materials and Methods

Materials

Rutin standard was purchased from Sigma-Aldrich (Sigma, USA). Gallic acid, Cyclophosphamide (Cy), Levamisole hydrochloride was purchased from Shanghai Macklin Biochemical Co. (Shanghai, China), the same batch was used for all experiments. The assay kit for superoxide dismutase (SOD), malondialdehyde (MDA), Glutathione peroxidase (GSH-Px) and Catalase (CAT) were purchased from Shanghai yuanye Biotechnology Co. (Shanghai, China). India Ink, 2,4-dinitrofluorobenzene (DNFB), Nitrotetrazolium Blue chloride (NBT), Phenazinemethosulfate (PMS), Nicotinamide adenine dinucleotide (NADH), saline, potassium ferricyanide, Vitamin C (Vc) were purchased exclusively from Aladdin Co. (Shanghai, China). Other reagents employed, which met analytical grade standards, were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Plant Material and Extraction

The rhizomes and root of *S. japonica* Miq. were collected in October 2022 from Huoshan(N 31°.03',E 115º,52'), Anhui, China. The plant material was identified and authenticated by Dr. Naifu Chen of Biotechnology and Pharmaceutical Engineering, West Anhui University. The rhizomes and root of *S. japonica* Miq. were washed, dried and powdered using a pulverizer (60 mesh) and were used for extraction. Exactly 100 g of powder was extracted with 50% ethanol (1000 mL) for 3 h, two times (3 h for each time) at 78 ℃ [22]. The extracts were collected, combined, and evaporated under low pressure by using a rotary evaporator and finally subjected to lyophilization in a freeze dryer to obtain the SJEE. The ethanol extract yielded 22.3% (w/w). The SJEE was stored at −20 ℃ until it was utilized for further experiment. After dissolution in distilled water, SJEE was adjusted to 400, 200, and 100 mg/kg•d^{-1} and administered intragastrically; the treated animals were categorized into SJ-H (high), SJ-M (medium), and SJ-L (low) dose groups.

Experimental Animals

The animal experiments conducted in this study were approved by the Ethics Committee of Experimental Animals of West Anhui University (IACUC No. 2022-0032, 2022.10.11).Healthy male Kunming mice (18–20 g,3-week-old) were obtained from Anhui Medical University Experimental Animal Center. Animals were accommodated in the departmental animal facility under sanitary conditions, with regulated temperature (22 ± 2 ℃), humidity (55 ± 10%), and a 12-hour light/dark schedule. A standard pellet diet and clean water were supplied ad libitum. After 5 days of adaptive feeding, the experiment began. All experiments used mice from the same supplier to minimize genetic variability. According to the preliminary experimental results, the experimental grouping and administration are shown in Table 1. At the conclusion of the trial, cervical dislocation was conducted to euthanize mice.

Table 1: Grouping and administration of mice (n=10)

Groups	Treatment
Normal contro l(NC)	Normal saline
Model group (MC)	Normal saline + Cy
Low-dose group (SJ-L)	SJEE (100 mg/kg) + Cy
Mid-dose group (SJ-M)	SJEE (200 mg/kg) + Cy
High-dose group (SJ-H)	SJEE (400 mg/kg) + Cy
Positive control group (PC)	Levamisole hydrochloride(10mg/kg)

Determination of Total Flavonoids and Polyphenols in SJEE

Determination of Total Phenolics (TPC)

The phenolic content of the extract was quantified using the Folin-Ciocalteu procedure [23], with gallic acid serving as the reference compound. Briefly, varying volumes of gallic acid solution (0.1 mg/mL) were combined with 0.5 mL of Folin-Ciocalteu reagent. After a 5 min incubation, 5 mL of 20% Na2CO3 was added. The mixture was allowed to stand for 30 min at ambient temperature to enable color development, and absorbance was subsequently recorded at 750 nm using a spectrophotometer. These steps were repeated with a sample instead of gallic acid standard solution to calculate the content of total phenolic. The measurement results were expressed as mg of gallic acid equivalents (GAE) per gram of dried material.

Determination of Total Flavonoid (TFC)

Flavonoid quantification was carried out in line with the method reported by [24]. Different volumes of rutin standard solution (0.256 mg/mL, 0.4–2.0 mL) were mixed with 1 mL of $NaNO_2$ (5%). After 6 min, 1 mL of 10% Al $(NO_3)_3$ was shaken and stood for 6 min, and 10 mL of NaOH (4%) was added to the mixture. Finally, the mixture was adjusted to 25 mL with 50% ethanol. Absorbance readings were obtained at 517 nm with a spectrophotometer. These steps were repeated with a sample instead of rutin standard solution according to the formula to calculate the content of total flavonoid. Results were reported in terms of rutin equivalents (RE, mg) per gram of sample dry weight.

DOI: 10.3844/ajbbsp.2026.22.01.003

Antioxidant Capacity of SJEE in Vitro

DPPH Free Radical Scavenging Test

The DPPH assay was conducted according to a modified procedure reported by [15]. SJEE dissolved in water was diluted into different concentrations of the sample solution (0, 15.625, 31.25, 62.5, 125, 250, 500, 1000 μg/mL). Each sample solution (2 mL) was combined with 2 mL of 0.1 mmol/L DPPH prepared in ethanol. The mixture was thoroughly shaken and subsequently incubated in the dark at room temperature for 30 minutes, after which absorbance was recorded at 517 nm. Ultrapure water served as the blank, while vitamin C (Vc) acted as the positive reference. The scavenging activity against DPPH radicals was determined using the following formula:

$$\text{DPPH scavenging effect}\ (\%) = \left[1 - \frac{A_i - A_j}{A_c}\right] \times 100\% \quad (1)$$

The absorbance values were designated as follows:A_i for the extract sample, A_j for the control sample, and $A_c$for the blank.

Hydroxyl Radical Scavenging Test

Hydroxyl radical scavenging activity was measured according to Lee et al. [25]. Accurately pipette 1 mL of 9 mmol/L $FeSO_4$solution and 1 mL of salicylic acid ethanol solution of the same concentration into a colorimetric tube. Add 0.5 mL of each sample solution to be tested, mix well, let stand for 10 min, and then add 1 mL of 8 mmol/L H_2O_2 solution. Samples were mixed well and incubated for 30 minutes at 37℃. Measurement of absorbance was carried out at 510 nm, using Vc as the positive standard. Ultrapure water as a blank control. Calculate the OH clearance rate according to the formula:

$$Hydroxylradicalscavengingability\ (\%) = \left[1 - \frac{A_1 - A_2}{A_0}\right] \times 100\% \quad (2)$$

The absorbance values were designated as follows:A_i for the extract sample, A_j for the control sample, and $A_c$for the blank.

Superoxide Anion Radical Scavenging Test

The hydroxyl radical scavenging activity of SJEE was measured according to Chaves et al. [26]. For the superoxide scavenging test, 0.5 mLNBT(0.2 mM), 0.5 mLNADH(0.5 mM), and 1 mL SJEE were combined, followed by 0.5 mLPMS(25 μM) addition. After incubation at 25℃ for 10 min, absorbance was measured at 570 nm. Ultrapure water acted as blank and Vc as positive control. Reduced absorbance indicated enhanced scavenging activity. Superoxide radical scavenging activity was calculated using the equation below:

$$Superoxideradicalscavengingeffect(\%) = \left[1 - \frac{A_{sample}}{A_{control}}\right] \times 100\% \ (3)$$

Where $A_{control}$ was the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} was the absorbance of the test compound.

Reducing Power Test

According to the protocol reported by Napolitano et al., the reducing properties of the samples were measured [27]. Samples (2 mL) were incubated with buffer and potassium ferrocyanide (2 mL, 0.2 M, pH 6.6) at 50 ℃ for 20 min, followed by trichloroacetic acid addition (2 mL, 1%) and centrifugation. The supernatant was reacted with ferric chloride, left for 10 min, and absorbance determined at 700 nm. Ultrapure water acted as blank, Vc as positive control.

UPLC-HRMS/MS Analysis

UPLC-Orbitrap-MS (Vanquish UPLC, HFX MS) was applied for extract profiling. Separation was performed on a Waters HSS T3 column (100 × 2.1 mm, 1.8 μm) at 40 ℃, with a flow rate of 0.3 mL/min and 2 μL injection volume. Mobile phases consisted of water (0.1% formic acid, A) and acetonitrile (0.1% formic acid, B), using a gradient from 0% B (0–1 min) to 95% B (1–12 min), held at 95% B (12–13 min), then returned to 0% B (13–17 min). Samples (5 μL) were injected via an autosampler at 4 ℃ after preparation by dissolving 10 mg SJEE in 1 mL methanol, centrifugation (12000 r/min, 10 min), and filtration (0.22 μm nylon) [28].

HRMS analysis was conducted on a Thermo Q Exactive HFX Hybrid Quadrupole-Orbitrap with heated ESI, operating in both positive and negative ion modes. Data acquisition and processing were performed using Thermo Xcalibur software. Source parameters: spray voltage −2.8/3.0 kV, sheath gas 40 arb, aux gas 10 arb, sweep gas 0 arb, capillary 320 ℃, and aux heater 350 ℃ [29].

The acquired raw mass spectrometry data were processed using Progenesis QI, Xcalibar and Compound Discoverer 3.0software (Waters Corporation, Milford, USA), including baseline correction, peak detection and integration, retention time alignment, and chromatographicpeak matching, ultimately generating a three-dimensional data matrix comprising retention time (RT), m/z values, and signal intensity. Compound identification was primarily performed using the following databases: MassBank (https://massbank.eu/), METLIN (https://metlin.scripps.edu/)MassBankand other public metabolomics databases, supplemented by an in-house library of authentic standards for further validation.

Immunomodulatory Activities

Immune Organ Indexes

Immune organ indexes were assessed as described by He et al. [30]. Sixty mice (20±2 g) were randomly divided into six groups (each group n=10). They were the model group (MC), normal control (NC), positive control group (PC), SJEE low-dose group (100 mg/kg·d^{-1}) (SJ-L), SJEE mid-dose group (200 mg/kg·d^{-1}) (SJ-M) and SJEE high-dose group (400 mg/kg·d^{-1}) (SJ-H), respectively. The normal control and model group were given normal saline, the positive group was given levamisole hydrochloride (10 mg/kg), and the drug group was given SJEE by gavage once a day for 21 days. From the 7th day, MC, SJ-L, SJ-M, SJ-H and PC were given intraperitoneal injection of 80 mg/kg·d^{-1} cyclophosphamide for 3 consecutive days. Meanwhile, 1 mL/kg saline was given to NC mice every day by intraperitoneal injection. After 1 h of the last dose of SJEE, the mice were euthanized by cervical dislocation, and the thymus and spleen of each mouse were removed, flushed, dried with filter paper, and weighed. The thymus and spleen indexes were calculated as follows:

$$\text{Spleen index} = \frac{W_{spleen}}{\text{W}} \quad (4)$$

$$\text{Thymus index} = \frac{W_{thymus}}{\text{W}} \quad (5)$$

Where W expresses the weight of the mouse (g), W_{spleen} expresses the weight of the mouse's spleen (mg), W_{thymus} expresses the weight of the mouse's thymus (mg).

Carbon Clearance Test

Phagocytic activity in vivo was estimated by using the carbon clearance test. According to the method of Oluwole et al. [31]. The same method was utilized in the "Immune organ indexes" section. Mice were injected with Indian ink (0.1 mL/10 g) via the tail vein 1 h after the last SJEE administration. Blood samples were taken at 2 and 10 min, mixed with sodium carbonate solution, and absorbance recorded at 670 nm. Meanwhile, all thymus and spleen of animals were taken out and weighed. The clearance index K and phagocytic index α were calculated using the following equation:

$$\text{K} = \frac{\lg OD_1 - \lg OD_2}{t_2 - t_1} \quad (6)$$

$$\alpha = \frac{\text{W}}{W_{liver} + W_{spleen}} \sqrt[3]{\text{K}} \quad (7)$$

Where $OD_1$and OD_2 express the absorbance at 2 and 10min, respectively. $t_2$was 10 min, t_1 was 2 min.

Determination of Serum Hemolysin(HC_{50})

The grouping and administration of mice were the same as above. On the 18th day of administration, each mouse was intraperitoneally injected with 0.2 mL of 2% (v/v) Sheep red blood cell (SRBC) for immune stimulation. On the day of immunization, except for the normal control group, each group was intraperitoneally injected with 80 mg/kg of cyclophosphamide for three consecutive days. On the 21st day after administration, mouse serum was taken and diluted 500 times with normal saline for standby. Take 1 mL of diluted serum, add SRBC (0.5 mL, 5%) and complement (1 mL, 10%) in sequence, keep it in a constant temperature water bath at 37 ℃ for 10 min, and then terminate the reaction in an ice bath. Centrifuge the supernatant and measure the OD value at 540 nm. Take another 0.25 mL of 5% SRBC and add 4 mL of DuPont reagent as a half hemolytic blank control tube for co incubation [3]. Measure the OD value using the same method and calculate the half hemolytic value (HC_{50}).

$$HC_{50} = \frac{D(\lambda)\text{sample}}{D(\lambda)\text{SRBC half hemolytic}} \times W \quad (8)$$

Where $d(\lambda)$and $D(\lambda)\text{SRBC half hemolytic}$ were the absorbance of sample and half hemolysis, respectively. W was the dilution ratio.

DNFB-Induced Delayed-Type Hypersensitivity Reaction (DTH)

Sixty mice weighing 20 ± 2 g were allocated at random into six groups, with ten animals per group. SJEE (400, 200, and 100 mg/kg·d^{-1}) was administered for 9 days. sensitization was performed by administering 50 µL of 1% DNFB (1-fluoro-2,4-dinitrobenzene) dissolved in acetone-sesame oil (3:1, v/v) onto a 3×3 cm area of shaved abdominal skin. The next day, the same amount of DNFB to strengthen again was used. At the same time, MC, SJ-L, SJ-M, SJ-H and PC groups were given intraperitoneal injection of cyclophosphamide 80 mg/kg for 3 consecutive days. The immunosuppressive model was prepared. The mice were sensitized after 5 days. About 10 µL of 1% DNFB solution was smeared evenly on the mice ears on both sides. After 24 h, animals were euthanized via cervical dislocation. Circular tissue sections (8 mm in diameter) were excised from both ears using a borer, and each sample was weighed [32]. The degree of auricular swelling was calculated using the following equation: Ear swelling weight =B−A, Percentages swelling = [B−A]×100%, where A expresses the weight of the mouse's left ear and B expresses the weight of the right ear.

Determination of Antioxidant Capacity in Vivo

Take an appropriate amount of liver, and prepare 10% tissue homogenate with precooled PBS buffer solution, Centrifugation was performed (3000 r/min, 10 min) to separate and collect the tissue supernatant. The catalase, superoxide dismutase, malondialdehyde, glutathione peroxidase enzyme concentration were measured using a quantitative ELISA kit (Shanghai yuanye, China) following the manufacturer's instructions for a catalase, superoxide dismutase, malondialdehyde, glutathione peroxidase assay.

Statistical Analysis

All the data from the experiments were conducted in triplicate. In this study, results are presented as mean values accompanied by Standard Deviation (SD). Statistical analyses were performed using SPSS software (version 19.0). Differences among groups were assessed through one-way ANOVA followed by Tukey's post hoc test. The data satisfies the assumptions of homogeneity of variance and normal distribution. The test results of undergo normality test, homogeneity test and hypothesis evaluation. A difference was regarded as statistically significant when $p<0.05$. Significance levels are denoted as *$p<0.05$ and **$p<0.01$.

Results

Phytochemical Composition Analysis

The ethanolicextracts was investigated by high-resolution UPLC- HRMS/MS analysis, focusing on the plant metabolites of the extracts. Figure 1 presents the total ion current chromatograms of SJEE. Initial identification of SJEE compounds was performed through comparison of ion m/z values, MS/MS spectra, and retention behavior with literature references and database records (MassBank Europe, PubChem). In the current investigation, 36 polyphenols were preliminarily identified in the ethanol extract, including 14 flavonoids, 6 methoxyphenols, 5 Benzoic acids and derivatives, and other metabolites such as gallic acid, neochlorogenic acid, hydroxycoumarins, etc. (Table 2). Figure 2 demonstrates the representative ESI-MS/MS ions m/z of some polyphenols and flavonoids. Overall, most phenolic acids were eluted before 9.31 min. The elution time of most of the flavonoids was between 7.35 to 11.45 min.

DOI: 10.3844/ajbbsp.2026.22.01.003

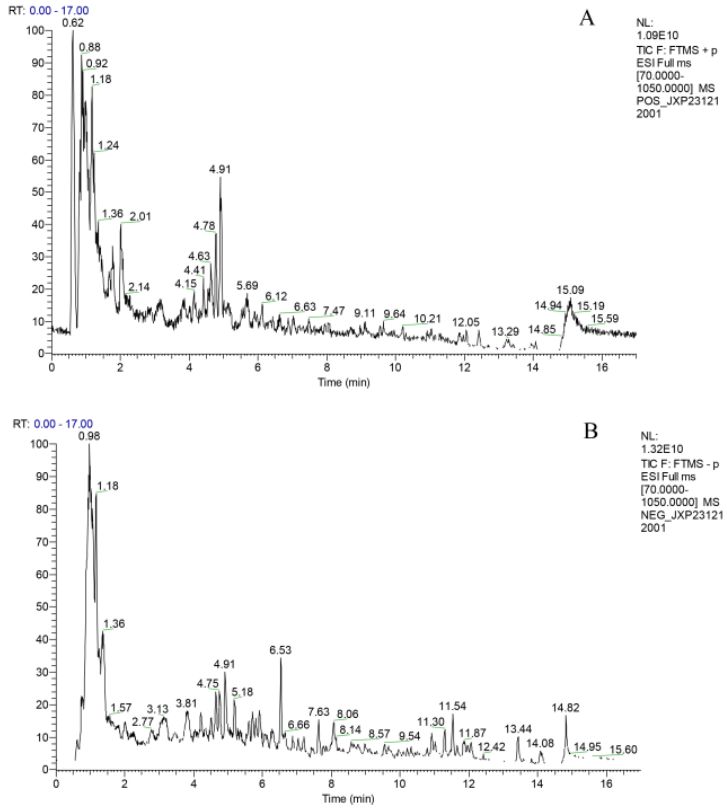

Fig. 1: Total ion current chromatograms of SJEE. (A) [M+H]+, (B) [M-H]-

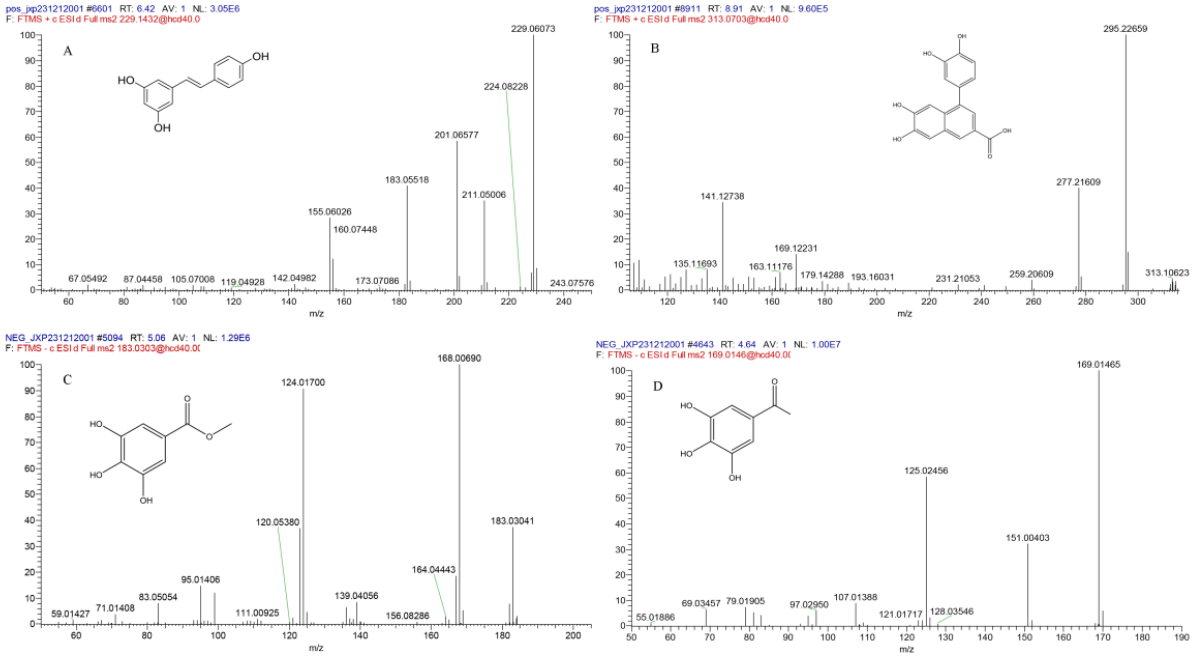

Fig. 2: Representative ESI-MS/MS ions m/z of some important polyphenols and flavonoids. (A) resveratrol, (B) 4-(3,4-dihydroxyphenyl)-6,7-dihydroxynaphthalene-2-carboxylic acid, (C) Methyl gallate, (D) Gallic acid

DOI: 10.3844/ajbbsp.2026.22.01.003

Table 2: Identification of SJEE by UPLC-HRMS/MS

Compound No.	RT (min)	Formula	Molecular ion (m/z)	Fragment ions(m/z)	Mass error (ppm)	Precursor type	Identification	Compound class
1	3.16	$C_7H_6O_3$	139.0388	93.4888 121.6227	-1.4384	$[M+H]^+$	Salicylic acid	Benzoic acid and analogues
2	3.70	$C_8H_8O_4$	169.0495	151.0389 95.08604	-0.1496	$[M+H]^+$	Orsellinic acid	Benzoic acid and analogues
3	4.30	$C_6H_6O_3$	127.0389	99.0446 127.0393	0.0787	$[M+H]^+$	1,3,5-Trihydroxybenzene	Benzoic acid and analogues
4	4.92	$C_9H_{10}O_3$	167.0702	167.0698 149.05977 121.06501	-0.4830	$[M+H]^+$	Paeonol	Organooxygen compounds
5	5.29	$C_8H_{10}O_3$	155.0700	109.0658 137.0611	-1.2942	$[M+H]^+$	3,4-Dihydroxyphenylethanol	Tyrosols and derivatives
6	5.65	$C_9H_{10}O_2$	151.0753	105.0742 133.0687	-0.1985	$[M+H]^+$	2-Methoxy-4-vinylphenol	Methoxyphenols
7	5.74	$C_{27}H_{30}O_{16}$	611.1548	287.0562 449.1070	-9.5901	$[M+H]^+$	Sophoraflavonoloside	Flavonoids
8	5.90	$C_7H_8O_2$	125.0599	125.0599 93.6329	1.3593	$[M+H]^+$	Mequinol	Methoxyphenols
9	5.96	$C_8H_8O_3$	153.0545	93.033	-0.4619	$[M+H]^+$	Vanillin	Methoxyphenols
10	6.07	$C_9H_{10}O_4$	183.0655	95.0495 123.0446 155.0706	2.1014	$[M+H]^+$	Syringaldehyde	Methoxyphenols
11	6.56	$C_{11}H_{12}O_4$	209.0804	177.0545 149.0596 121.0649	-1.7954	$[M+H]^+$	Sinapaldehyde	Methoxyphenols
12	7.35	$C_{16}H_{12}O_3$	253.0836	210.0788 238.0734	-9.170	$[M+H]^+$	6-Methoxyflavone	Flavonoids
13	7.69	$C_{27}H_{32}O_{15}$	597.1803	435.1279 597.1803	-1.8362	$[M+H]^+$	Eriocitrin	Flavonoids
14	7.90	$C_{15}H_{10}O_5$	271.0598	271.0598 197.0581 153.0194	-1.0695	$[M+H]^+$	apigenin	Flavonoids
15	7.94	$C_{14}H_{12}O_3$	229.0878	211.0501 119.0492	8.2466	$[M+H]^+$	resveratrol	Stilbenes
16	8.07	$C_6H_6O_2$	111.0443	93.0328 111.044	2.4674	$[M+H]^+$	Pyrocatechol	Benzenediols
17	8.91	$C_{17}H_{12}O_6$	313.0703	295.2269 277.2161	-1.1646	$[M+H]^+$	4-(3,4-dihydroxyphenyl)-6,7-dihydroxynaphthalene-2-carboxylic acid	Naphthalenecarboxylic acids and derivatives
18	9.31	$C_{17}H_{26}O_4$	295.1898	137. 0596 151. 1117	-1.8151	$[M+H]^+$	6-Gingerol	Methoxyphenols
19	9.40	$C_{16}H_{12}O_5$	285.0757	270.0521 243.0659 257.0815	-0.6665	$[M+H]^+$	acacetin	Flavonoids
20	10.84	$C_{21}H_{22}O_9$	419.1332	389.0864 371.0758	-1.2136	$[M+H]^+$	5-Hydroxy-3,6,7,3',4',5'-Hexamethoxyflavone	Flavonoids
21	11.45	$C_{20}H_{20}O_8$	389.1227	359.0761 374.0989 341.0654	-0.9097	$[M+H]^+$	5-O-Demethylnobiletin	Flavonoids
22	1.58	$C_9H_{10}O_5$	197.0453	153,0197 124.0406 109.0295	-0.9135	$[M-H]^-$	Ethyl gallate	Benzoic acids and derivatives
23	4.45	$C_{16}H_{18}O_9$	353.0885	135.0453 191.0565	1.9675	$[M-H]^-$	Neochlorogenic acid	Alcohols and polyols
24	4.64	$C_7H_6O_5$	169.0147	151.0040 125.0245	2.6625	$[M-H]^-$	Gallic acid	Benzoic acids and derivatives
25	5.06	$C_8H_8O_5$	183.0304	124.0170 183.0304 168.0069	2.8050	$[M-H]^-$	Methyl gallate	Benzoic acids and derivatives
26	5.14	$C_9H_6O_4$	177.0197	177.0197 133.0297 105.0346	2.0788	$[M-H]^-$	Esculetin	Hydroxycoumarins
27	5.19	$C_9H_8O_4$	179.0353	107.0542	1.9996	$[M-H]^-$	caffeic acid	Cinnamic acids and derivatives
28	5.84	$C_{27}H_{30}O_{16}$	609.1467	285.0407 284.0308 255.0285	1.1197	$[M-H]^-$	Kaempferol- 3-gentiobioside	Flavonoids
29	6.98	$C_{15}H_{10}O_6$	285.0408	285.0408 117.0347 255.0291	1.3987	$[M-H]^-$	Scutellarein	Flavonoids
30	7.74	$C_{10}H_{10}O_3$	177.0557	177.0557 162.0325 133.0299	-0.0424	$[M-H]^-$	Coniferaldehyde	Methoxyphenols
31	7.05	$C_{16}H_{12}O_6$	299.0566	117.03458 136.9884	1.4669	$[M-H]^-$	Hispidulin	Flavonoids
32	8.28	$C_{16}H_{12}O_6$	299.0567	284.0352 227.0364 255.0315	1.8682	$[M-H]^-$	Diosmetin	Flavonoids
33	9.22	$C_{18}H_{16}O_7$	343.0835	328.0596 343.0835 313.0358	3.3342	$[M-H]^-$	Santin,5,7-Dihydroxy-3,6,4'-trimethoxyflavone	Flavonoids
34	9.38	$C_{19}H_{18}O_8$	373.0938	343.0469 358.0701 328.0231	2.4639	$[M-H]^-$	Skullcapflavone II	Flavonoids
35	9.40	$C_{16}H_{12}O_5$	283.0617	268.0378 283.0617 240.0429	1.9194	$[M-H]^-$	Genkwanin	Flavonoids
36	11.91	$C_{15}H_{10}O_4$	253.0508	209.1916 181.1604	0.5845	$[M-H]^-$	Nordalbergin	Flavonoids

These compounds have been confirmed that they have antioxidant activity. Therefore, TPC and TFC of SJEE were measured in SJEE. TPC and TFC of SJEE were determined, with values of 102.06 ± 2.35 mg GAE/g extract and 59.66 ± 1.09 mg RE/g extract, respectively (Table 3). The results suggest that the rich-phenolic and flavonoid plants can be good sources of antioxidants.

Table 3: Phenolic and flavonoid content of SJEE

SJEE properties	Total amounts
TPC (mg)	102.06 ± 2.35
TFC (mg)	59.66 ± 1.09

1 SJEE: the ethanol extract of *S. japonica* Miq; TPC: total phenolic content; TFC: total flavonoid content; GAE: gallic acid equivalent; RE: rutin equivalent. Data are presented as mean ± SD. (n = 3)

Antioxidant Activity of SJEE in Vitro

Analysis of DPPH Radical Scavenging Activity

Absorbance changes were used to assess radical scavenging and antioxidant activity. As shown in Figure 3(A), SJEE exhibited strong DPPH scavenging. At 1000 μg/mL, its removal rate peaked at 87.58 ± 1.89%, lower than vitamin C. This may be because with the increase of SJEE mass concentration, the number of antioxidants in SJEE increases, and these antioxidants can be paired with the DPPH single electrons present in the system. This reduces the number of free radicals and shows an enhanced scavenging ability for DPPH. The scavenging abilities of SJEE on DPPH radicals were a little weaker than ascorbic acid. Experimental results revealed that SJEE had superior activity in eliminating DPPH radicals and certain antioxidant effects in vitro, confirming its efficacy in DPPH radical suppression.

Analysis of Hydroxyl Radical Scavenging Activity

The scavenging ability of SJEE on ·OH was provided in Figure 3 (B). SJEE has a certain concentration dependence on its ability to scavenge OH. The clearance rate rose sharply between 15-500 μg/mL, then leveled off. At 1000 μg/mL, SJEE achieved 80.00 ± 2.51% clearance, lower than ascorbic acid (95.28 ± 0.27%).

Analysis of Superoxide Anion Radical Scavenging Activity

In this experiment, the NADH-PMS-NBT system was applied to evaluate the superoxide anion scavenging capacity of the extracts. As shown in Figure 3 (C). With the increase of concentration, the clearance rate of SJEE reached 74.17%, and showed a certain dose-dependent. The results showed that compared with 500 μg/mL SJEE, the clearance rate of 1000 μg/mL SJEE increased from 70.42% to 74.17%, but the rising trend slowed down significantly. At this time, the clearance rate of 1000 μg/mL Vc was 93.97%. The results showed that the optimal removal concentration was 1000 μg/mL of SJEE.

Analysis of Reducing Power

The reducing ability of SJEE was assessed by the potassium ferricyanide method. According to Figure 3(D), the reducing strength of SJEE and Vc increased progressively with concentration in a certain range. The absorbance at 700 nm (1000 μg/mL) values for SJEE and Vc were 3.01 ± 0.01, 2.1 ± 0.10, respectively. The suggest that the reducing powers of SJEE was significantly lower than the Vc.

Effect of SJEE on Body Weight in Cy-Treated Mice

In this study, no mice died during the entire experimental period. We recorded the state and weight changes. As shown in Figure 4 (A). After receiving Cy treatment, mice from all groups exhibited a notable decrease in body weight. While the NC group continued to increase, indicated that Cy can significantly inhibit the weight of mice. After the end of Cy modeling, the weight of mice in each group slowly increased, while the speed of weight recovery in SJ-L, SJ-M, SJ-H and PC groups was significantly higher than that in MC groups. In particular, the weight recovery rate of SJ-H group was close to that of PC group.

Effect of Sjee on Immune Organ Indexes in Cy-Treated Mice

Thymus and spleen indices serve as markers of immune status. According to Figure 4(B) and 4(C), the MC group showed a notable decrease relative to the NC group ($p < 0.05$), reflecting suppressed immunity after 3 days of Cy exposure. Meanwhile, the thymus index remained stable in SJEE-treated animals, implying that SJEE administration did not negatively

DOI: 10.3844/ajbbsp.2026.22.01.003

influence thymus function. But the administration of the SJEE-treated group of immunosuppressive animals (SJ-M, SJ-H) significantly enhanced the spleen indexes compared with the Cy-treated immunosuppressive MC group ($p<0.05$). The organ index of the SJ-H group was close to that of the PC group. The results indicate that SJEE is capable of partially counteracting the immunosuppressive effects of Cy and promoting immune function.

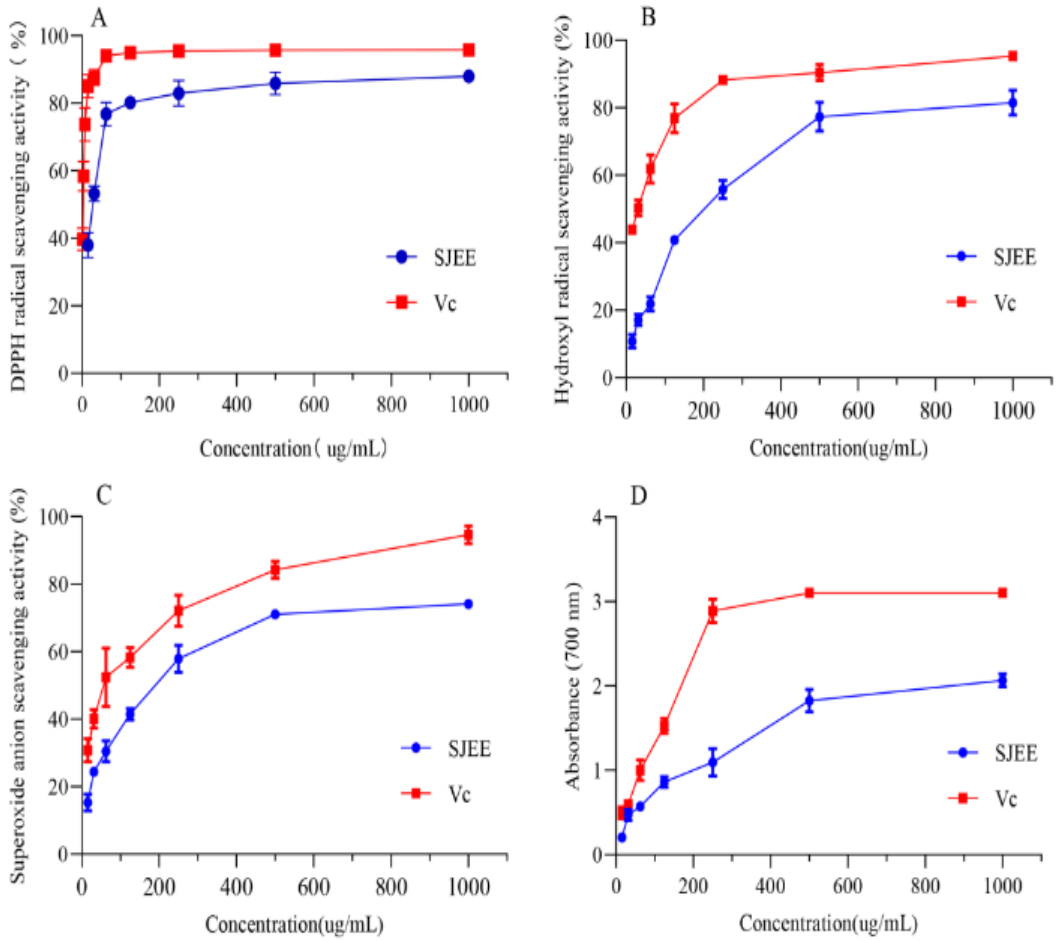

Fig. 3: In vitro antioxidant activities of SJEE and Vc. A: DPPH radical scavenging activity; B: Hydroxyl radical scavenging activity; C: Superoxide radical scavenging activity; D: Reducing power; Note: Values are expressed as the mean ± SD (n = 3)

Effect of Sjee on Clearance Index and Phagocytic Index in Cy-Treated Mice

Ethanol extract of *S. japonica* Miq. rhizomes possess macrophage stimulatory activity as evidenced by increased clearance index (K) and phagocytic index (α) in the carbon clearance test. The effect on specific and Non-specific immune has been shown in Figure 4 (D) and Figure 4 (E). The clearance index (K) of the NC, MC, SJ-L, SJ-M, SJ-H and PC groups were 0.09 ± 0.0074, 0.06 ± 0.0080, 0.07 ± 0.0066, 0.07 ± 0.0041, 0.09 ± 0.0075 and 0.09 ± 0.0089, respectively. The phagocytic index (α) of the NC, MC, SJ-L, SJ-M, SJ-H and PC groups were 6.42 ± 0.21, 5.15±0.14, 5.54 ±0.11, 5.64 ± 0.32, 6.40 ±0.28and 6.12 ± 0.15, respectively. The Clearance index and phagocytic index in the model groups (MC) was significantly decreased compared with that in the normal untreated NC group ($p<0.01$). The data indicated that the immunosuppressive model was made successfully. Compared with the MC group, the clearance index and phagocytic index of SJ-H group and PC group were significantly increased ($p<0.01$) and the clearance index of SJ-M group was also significantly increased ($p<0.01$). There was a slight upward trend in the clearance index (K) and phagocytic index (α) after the administration of SJ-L, but which was not significant.

Effect of Sjee on Serum Hemolysin Content in Cy-Treated Mice

The effect of SJEE on serum hemolysin levels in mice treated with cyclophosphamide was shown in Figure 4 (F), the half hemolysis values of the NC, MC, SJ-L, SJ-M, SJ-H and PC groups were 215.52 ± 6.28, 133.95 ± 0.74,169.39 ± 9.53, 195.22 ± 11.86 and 196.93 ± 16.86, respectively. After an intramuscular injection of Cy, the serum half hemolysin level markedly reduced ($p<0.01$) in immune suppressed mice compared with the control group. However, the serum half hemolysin level of the SJ-M, SJ-H and PC groups all markedly raised ($p<0.01$). This result indicated that SJEE can promote the division, proliferation, differentiation, and maturation of B lymphocytes into antibody forming cells that secrete SRBC antibodies.

Effect of SJEE on the DNFB-Induced Delayed-Type Hypersensitivity Reaction in Cy-Treated Mice

The DTH response to DNFB was evaluated by measuring the degree of earlap swelling. As shown in Figure 4 (G), the ear swelling of mice in the NC, MC, SJ-H, and PC groups was 4.72 ±0.25, 3.63 ± 0.14, 4.81 ± 0.24 and 4.59 ± 0.23 mg, respectively. The delayed-type hypersensitivity test showed that the ear swelling degree was significantly lower in the immunosuppressive MC group than in the NC group ($p<0.01$). These indicated that the mice model of immunosuppressive was successful. Meanwhile, the ear swelling degree of the SJ-H and PC groups of immunosuppressive mice were significantly increased compared with immunosuppressive model group ($p<0.01$). These results indicated that SJEE have obvious enhance cellular immune response in mice.

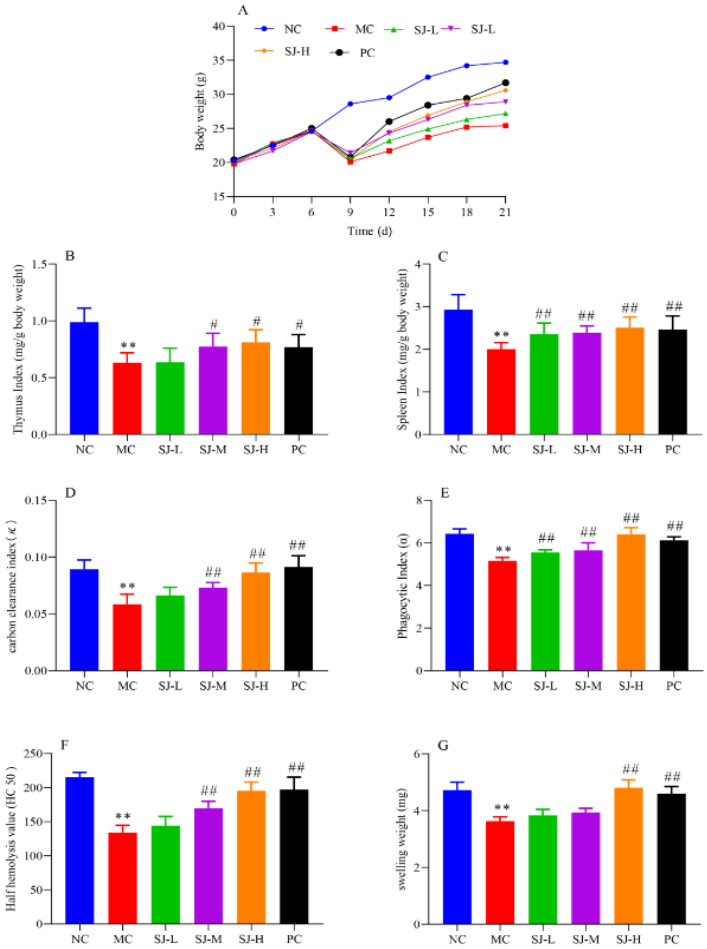

Fig. 4: Immune effects of SJEE on Cy-induced immunosuppressed mice. (A) Body weight, (B) Thymus index, (C) Spleen index, (D) Carbon clearance index, (E) Phagocytic index, (F) Half he-molysis value (HC50), (H) Swelling weight. Note: Values were expressed as means ± SD (n = 10). *p<0.05 compared with NC group, **p<0.01 compared with NC group, #p<0.05, ##p<0.01 compared with MC group

The Effect of SJEE on the Content of SOD, CAT, MDA, GSH-Px in the Liver Tissues of Cy-Treated Mice

We examined Reactive Oxygen Species (ROS) content or activity in the liver of Cy mice. As shown in Figure 5 (A)(B)(C)(D), compared with the normal control group, the liver CAT/GSH-Px and SOD activity in the model control group were significantly reduced ($p<0.05$), while the MDA content was significantly increased ($p<0.01$). It showed that cyclophosphamide destroyed the oxidation antioxidant homeostasis in mice. Compared with the model group, the CAT/GSH-Px and SOD levels in the SJ-M, SJ-H and PC group were significantly increased, while the MDA content was significantly reduced ($p<0.05$). Moreover, the MDA concentration in the SJ-L group was also significantly decreased ($p<0.05$). And all showed significant concentration dependence.

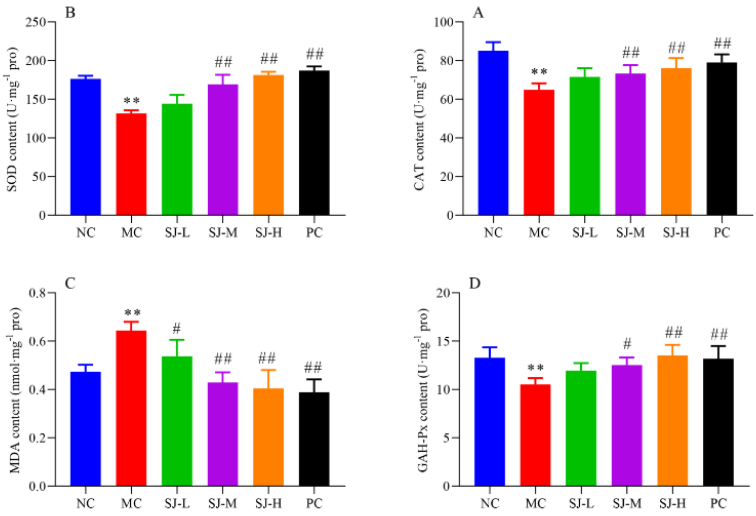

Fig. 5: In vitro antioxidant activities of SJEE on Cy-induced immunosuppressed mice. (A) Catalase (CAT) activity, (B) Superoxide dismutase (SOD) activity, (C) Malondialdehyde (MDA) activity, (D) Glutathione peroxidase (GAH-Px) activity, Note: Values were expressed as means ± SD (n = 10). *p<0.05 compared with NC group, **p<0.01 compared with NC group, #p<0.05, ##p<0.01 compared with MC group

Discussion

The therapeutic efficacy of medicinal plants is intrinsically linked to their rich profile of bioactive secondary metabolites. Consequently, a detailed investigation into the phytochemical composition of plants like *S. japonica* Miq. (SJEE) is a foundational step for evaluating their biological properties [19, 33-34]. Our UPLC-HRMS/MS analysis successfully led to the putative identification of 36 compounds in SJEE, with a significant representation of polyphenols and flavonoids. Notably, compounds such as 14 and 28 (flavonoids) have been previously documented in *S. annua* [19] and *S. lavandulifolia* [34]. Similarly, compounds 9, 24, and 27 are reported in *S. cretica subspecies* [35, 36], and compound 20 has been identified in other Stachys species including *S. beckeana*, S. zepcensis, and *S. alpina* subsp. Dinarica [37]. The prevalence of these specific polyphenols and flavonoids in SJEE likely reflects common biosynthetic pathways within the Stachys genus, although species-specific enzymatic activities and environmental factors could influence their relative abundance, an area warranting further investigation.

The total phenolic content (TPC) of SJEE was determined to be 102.06 ± 2.35 mg GAE/g extract, and the total flavonoid content (TFC) was 59.66 ± 1.09 mg RE/g extract. For comparison, Kirkan reported lower TPC (57.65 ± 2.33 mg GAE/g) and TFC (40.24 ± 1.38 mg RE/g) in methanolic extracts of *S. cretica* subsp. Vacillans [35]. In contrast, Laggoune et al. found substantially higher TPC (470.21±1.22 mg GAE/g) and TFC (189.05±0.72 mg RE/g) in *S. arvensis* (L.) L [38]. These comparisons indicate that *S. japonica* Miq. contains a moderate yet significant concentration of bioactive compounds compared to other Stachys species, providing a chemical basis for its observed biological activities.

Cyclophosphamide is a widely utilized alkylating agent for establishing immunosuppressive models, primarily through its detrimental effects on rapidly dividing cells, including lymphocytes, leading to immune organ atrophy [39]. Consistent with previous findings, our study confirmed that Cyclophosphamide treatment significantly reduced the thymus and spleen indices in mice [15]. Notably, administration of medium (200 mg/kg) and high (400 mg/kg) doses of SJEE significantly counteracted this Cyclophosphamide-induced atrophy ($p<0.05$). This protective effect on vital immune organs is likely attributable to the rich array of identified polyphenols and flavonoids in SJEE, which are known for their cytoprotective, anti-inflammatory, and antioxidant properties, thereby potentially mitigating Cyclophosphamide-induced cellular damage and apoptosis.

The mononuclear phagocyte system constitutes a critical component of the body's non-specific immunity [40], and its efficacy can be reliably assessed using the carbon particle clearance test [15]. Previous research by Benmebarek et al. demonstrated that Stachysmialheside Noé extract enhanced phagocytic activity in a dose-dependent manner, attributing this to its rich content of natural metabolites such as terpenoids, flavonoids, and phenolic compounds [41]. Our findings align with this, showing that SJEE significantly improved both the phagocytic index and clearance index in Cyclophosphamide-treated mice. This enhancement of macrophage activity is a key immunomodulatory effect likely mediated by the diverse flavonoids and polyphenols present in SJEE.

Humoral immunity, primarily mediated by B lymphocytes and their antibody production, was assessed by measuring serum hemolysin (HC_{50}) levels [3]. Rahimi Khoigani et al. reported that dietary supplementation with *S. lavandulifoliaVahl* extract increased serum hemolysin HC_{50} in rainbow trout [34]. In our study, medium and high doses of SJEE significantly elevated serum hemolysin HC_{50} values in Cyclophosphamide-induced immunosuppressed mice. This indicates that SJEE can antagonize cyclophosphamide-induced humoral immunosuppression and promote the recovery of B-cell function. This effect is likely mediated by specific immunomodulatory flavonoids or polyphenols in the extract, which may stimulate B-cell proliferation and antibody synthesis.

Cellular immunity, predominantly driven by T cells, was evaluated using the delayed-type hypersensitivity (DTH) response to DNFB [42]. The ear swelling in the model group was significantly suppressed, but treatment with medium and high doses of SJEE markedly enhanced this T-cell mediated response. This finding is consistent with Tae-young Shin, who showed that Stachysriederi aqueous extract inhibited allergic reactions, also involving T-cell modulation [43]. The enhancement of DTH by SJEE further underscores the immunomodulatory potential of its polyphenolic constituents, which can influence T-cell proliferation and cytokine production.

The immune system's functionality is intricately linked to the body's redox balance [44]. Oxidative stress, induced by agents like Cyclophosphamide, can impair immune cell function, particularly phagocytes, which produce ROS themselves [7, 30]. Therefore, supplementing antioxidants can restore this balance and enhance immunity [45]. Our in vivo results demonstrated that SJEE treatment significantly increased the levels of crucial antioxidant enzymes (SOD, CAT, GSH-Px) and reduced MDA (a marker of lipid peroxidation) in the liver tissue of Cyclophosphamide-immunosuppressed mice. These findings clearly indicate that SJEE mitigates Cyclophosphamide-induced oxidative stress. This effective antioxidant activity has been confirmed in both in vivo and in vitro experiments (for example, the significant scavenging effect on DPPH radicals), which is largely attributed to the abundant polyphenols and flavonoids in SJEE. These compounds are well-documented for their ability to scavenge free radicals, chelate metal ions, and upregulate antioxidant enzyme expression [46]. By mitigating oxidative damage through these mechanisms, they contribute to cellular protection and enhanced immune function. While this study did not include direct comparisons to benchmark antioxidants like Vitamin C, the observed potent effects strongly suggest SJEE as a significant source of natural antioxidants.

This study is the first comprehensive evaluation of *S. japonica* Miq. extract (SJEE), combining phytochemical analysis with in vitro and in vivo (cyclophosphamide-induced immunosuppressed mice) assessments. We identified 36 polyphenols/flavonoids, linking its chemical profile to observed antioxidant and immunomodulatory effects. Our research findings are consistent with those of other Stachys species, but this work specifically validates these characteristics of *S. japonica* Miq. And highlight its unique plant chemical characteristics.

Of course, we should acknowledge certain limitations. UPLC-HRMS/MS analysis provides identification, but it is difficult to confirm all compounds using real standards. Furthermore, the specific contribution of each individually identified compound to the overall observed biological effects still needs to be elucidated. The selection of doses (100, 200, 400 mg/kg) is based on literature precedents of similar extracts and preliminary experimental results, but further dose optimization research would be beneficial. Importantly, although this study suggests that SJEE has the potential to serve as a functional food or plant-

based drug source. But important translational studies are needed, including comprehensive preclinical toxicology, pharmacokinetic studies, and final human clinical trials, to validate these applications.

Conclusion

This first systematic study on *S. japonica* Miq. extract (SJEE) revealed significant total phenolic and flavonoid contents, with UPLC-HRMS/MS identifying 36 metabolites, mainly polyphenols and flavonoids, as likely contributors to its bioactivity. SJEE demonstrated robust vitro antioxidant properties and, crucially, potent immunomodulatory effects in a cyclophosphamide-induced immunosuppressed mouse model. Treatment with SJEE improved immune organ indices, enhanced phagocytic activity, and bolstered both humoral and cellular immune responses. Furthermore, SJEE effectively mitigated cyclophosphamide-induced oxidative stress by boosting antioxidant enzyme levels and reducing lipid peroxidation. These findings establish *S. japonica* Miq. as a promising natural source of antioxidants and immunostimulants, providing a theoretical basis for its further development.

Authors Contributions

Fang Wang: Resources, supervision, designed the project. Xueping Jiang: Conceptualization, methodology, writing – original draft, Jianglong He and Yanjun Chen were responsible for analysis, investigation, and visualization. All authors were involved in the work and endorsed the submitted manuscript.

Acknowledgment

This work was supported by the Open Fund of Anhui Engineering Research Center for Eco-agriculture of Traditional Chinese Medicine, West Anhui University (WXZR202429 and WXZR202027), Natural Science Fund for Colleges and Universities in Anhui Province (grant number KJ2020A0635), Anhui Province University Outstanding Young Talents Support Project (grant number gxyq2022064). Key laboratory of plant protection equipment, ministry of agriculture and rural affairs (ZBZB202507). Funding for the platform of Traditional Chinese Medicine Institute of Anhui Dabie Mountain.

Ethics

Authors followed the guidelines of the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No.8023, revised 1978). The animal study was approved by Experimental Animal Ethics Committee, Institute of Clinical Pharmacology, Anhui Medical University.

Data Availability

All datasets and materials that underpin the conclusions of this research are fully presented within the article itself.

Conflict of Interest

The authors confirm that there are no competing interests or potential conflicts related to this work.

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