Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Compounds Isolation and Immunomodulatory Activity of *Vicia amoena* Fisch. var. *Angusta*. Freyn Aerial Parts

Yuli Sang, Lei Shi, Hao Pan, Fangyan Guo, Yu Wang, Naizhi Zhang, Guangwen Fan, Yanjun Hao[1], Li Bai[2], Jianzhong Qi[3]

School of Pharmaceutical Sciences, Liaoning University, Shenyang, [1]Liaoning College of Traditional Chinese Medicine, Traditional Chinese Medicine of Liaoning University, Shenyang, [2]Market Service Center, Anshan Qianshan District Market Supervision and Administration Bureau, Anshan, [3]China Resources Chenzhou Pharmaceutical Co., Ltd. Chenzhou, China

Submitted: 16-Jan-2021 Revised: 27-Mar-2022 Accepted: 27-Apr-2022 Published: 23-Nov-2022

ABSTRACT

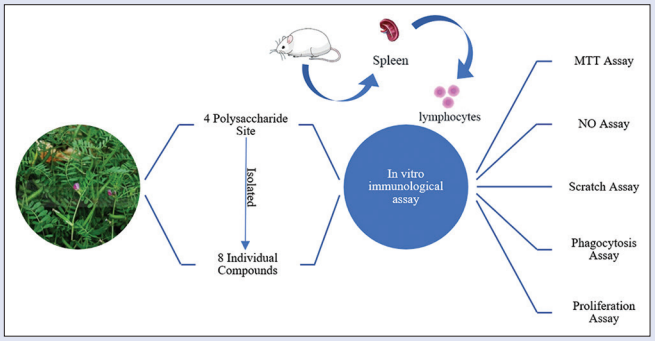
Background: *Vicia amoena* Fisch. var. *Angusta*. Freyn, also known as tuogucao, is a traditional medicinal plant in China. Early researches of *V. amoena* Fisch. var. *Angusta*. Freyn were focused on biosystematical studies, and only a few were concerned with chemical constitutes and bioactivities. **Objectives:** We aimed to isolate chemical constitutes in aerial parts of *V. amoena* Fisch. var. *Angusta*. Freyn and study the immunomodulatory activity of these chemical components via immune cells. **Materials and Methods:** Polysaccharide was obtained via hot water extraction and alcohol precipitation at different concentrations. Individual compounds were obtained after several chromatographic techniques, and the structures of all individual compounds were characterized by physical-chemical process, ^{1}H, $^{13}CNMR$, UV techniques and by comparing their NMR data with those discovered in the literature. *In vitro* immunological activity of tested samples was evaluated using MTT assay, NO assay, scratch assay, phagocytosis assay and proliferation assay of spleen lymphocytes in mice. **Results:** The four polysaccharide sites including 30%, 50%, 70% and 90% were obtained. Eight pure chemical constitutes were isolated and identified as: isoquercitrin, quercetin, quercitrin, hyperin, chlorogenic acid, kaempferol, kaempferitrin and wogonin. Four polysaccharide sites and individual compounds displayed different levels in improving proliferation and phagocytosis of RAW264.7 macrophages and lymphocytes. **Conclusion:** Compounds were isolated from the herb that possessed immunomodulatory function, and it is likely that the extract from the herb via the TLR-4-mediated signalling pathway to achieve immunomodulatory effects provided a scientific rationale of the traditional uses of the herb.

Key words: Immunomodulation, nitric oxide, polysaccharide, RAW macrophage, spleen lymphocyte, *Vicia amoena*

SUMMARY

- In this work, we evaluated immunomodulatory activity of 4 polysaccharide sites and eight compounds isolated from the aerial parts of *Vicia amoena* Fisch. var. *Angusta*. Freyn, a folk medicine widely used in treating rheumatic diseases in Inner Mongolia, Liaoning and other place in China, via immune cells to reveal underlying mechanism of rheumatic diseases. In addition, this is first time an immunomodulatory activity in RAW264.7 macrophage and mouse splenic lymphocytes of chemical constitutes extracted and isolated from *V. amoena* Fisch. var. *Angusta*. Freyn aerial parts has been found.

Abbreviations used:
ConA: Concanavalin A; DMSO: Dimethyl sulfoxide; FBS: Fetal Bovine Serum; LPS: Lipopolysaccharide; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO: Nitric oxide; NMR: Nuclear magnetic resonance; ODS: Octadecylsilyl; PHPLC: Preparative high performance liquid chromatography; P/E: Petroleum ether and ethyl acetate; PBS: Phosphate-buffered saline; SPF: Specific pathogen-free; TMS: Tetramethyl silane; TLC: Thin Layer chromatography; UV: ultraviolet; *V. amoena* Fisch. var. *Angusta*. Freyn; *Vicia. amoena* Fisch. var. *Angusta*. Freyn; *V.* L, *Vicia*. L; *v/v*: Volume per volume.



Correspondence:

Dr. Yanjun Hao, Traditional Chinese Medicine of Liaoning University, 79 Chongshan East Road Huanggu District, Shenyang - 110847, China.
E-mail: haoyanjun@sina.com
Li Bai, Anshan Qianshan District Market Supervision and Administration Bureau, 170 Jiubao Road Qianshan District, Anshan - 114041, China.
E-mail: 1215580911@qq.com
Jianzhong Qi, China Resources Chenzhou Pharmaceutical Co., Ltd. Chenzhou 423000, China.
E-mail: kingbeII@999.com.cn
DOI: 10.4103/pm.pm_24_21

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Vicia amoena Fisch. var. *Angusta*. Freyn, a variety of traditional Chinese medicine, belongs to the genus *Vicia*, and is commonly known as tuogucao in China.[1] It mainly grows in the regions of Asia at an altitude of 80–7500 m above sea level, especially in the northeast of China which is extremely rich in resources.[2] At present, many kinds of chemical constituents including terpenoids, flavonoids, amino acids, and coumarins have been isolated and identified from the *V. amoena*.[3] Meanwhile, some species of the *V. amoena* also have been reported to have antioxidant, antibacterial, anticancer, hypoglycemic, liver protection, anti-Parkinson's disease and other bioactivities. For instance, Sinha[4] found that the extract of polyflavonoid from *V. faba* L was capable of eliminating free radicals. The chemical compounds of diethyl phthalate and 4-hydroxybenzoic acid isolated from *V. villosa* can effectively inhibit grape phylloxera and bacillus subtilis.[5] The extract of

Cite this article as: Sang Y, Shi L, Pan H, Guo F, Wang Y, Zhang N, *et al*. Compounds Isolation and Immunomodulatory Activity of *Vicia amoena* Fisch. var. *Angusta*. Freyn Aerial Parts. Phcog Mag 2022;18:1000-10.

polyphenol from *V. Sativa* L has an anti-proliferative activity to THP-1 leukemia cells.[6]

V. amoena Fisch. var. *Angusta*. Freyn (tuogucao), as traditional herbal medicine, is listed in Chinese pharmacopoeia.[7] Although it is applied in the treatment of many diseases such as scabies and skin infections, the herb is especially recommended for the treatment of rheumatic diseases. Previous studies on the *V. amoena* Fisch. var. *Angusta*. Freyn (tuogucao) mainly focus on biosystematical studies and quantitative determination of compounds.[1,8–10] At present, there are few available investigations for the research of chemical constituents and bioactivities of *V. amoena* Fisch. var. *Angusta*. Freyn. To reveal its underlying mechanisms in treating rheumatic diseases, this study was designed to investigate the chemical constitutes that were extracted and isolated from *V. amoena* Fisch. var. *Angusta*. Freyn aerial parts and their immunomodulatory activity in RAW264.7 macrophage and mouse spleen lymphocytes for the first time.

MATERIALS AND METHODS

Plant materials

The aerial parts of *V. amoena* Fisch. var. *Angusta*. Freyn were collected in September 2015 at Xiao Ta Zi village in Chaoyang city, Liaoning province, China. The plant was identified by pharmacist Wang W.N. (Liaoning institute for food control, China) and the voucher specimen was deposited at the Herbarium of Liaoning institute for food control, China (specimen number 1593/LIFC).

Animals and chemicals

Experiments were performed using 6–8-week-old specific pathogen-free (SPF) male mice, weighing 18-22 g. Animals were obtained from the animal breeding facility of the resource center of experimental animals, Liaoning province, China. Most of the chemicals such as FBS, RPMI-1640, ConA, LPS, MTT, PBS and DMSO were purchased from Solarbio Science Co., Ltd (Beijing, China). Other chemicals such as ethyl alcohol absolute and acetic acid were of analytical grade and were purchased from Yongda Chemical Reagent Co., Ltd (Tianjin, China).

Extraction and isolation

Leaves and stems (5 kg), dried in a cool place at room temperature, were crushed and extracted with 50 vol. of hot water boiled for thrice. The aqueous extract was concentrated to 10% of the original volume with a rotary evaporator at 50°C under vacuum. The undiluted solution was extracted by petroleum ether to remove interfering compounds such as pigments. Then alcohol (95%, *v/v*) was added, and the solution was further precipitated to 24 hrs. After filtered and centrifuged, the precipitates were dried under vacuum at −40°C to obtain the polysaccharides of 30%, 50%, 70% and 90%. Four polysaccharide sites were lyophilized. The freeze-dried powder was obtained, weighed, and placed in a desiccator and set aside. The filtrate was extracted by ethyl acetate (EtOAc) and evaporated under reduced pressure to obtain crude extract (81.3 g). The extract was separately applied to a silica gel column chromatography (160–200 mesh, 10.0 × 33 cm) by gradient elution with P/E (100:0–0:100). Fractions were further purified by Sephadex LH-20 gel column chromatography, octadecyl-silica (ODS) column chromatography and preparative high performance liquid chromatography (PHPLC). The same fractions were combined based on Thin Layer chromatography (TLC).

General producers

Nuclear magnetic resonance (NMR) spectroscopy was performed on DMSO-d_6 on a Bruker Avance III 400 Hz. One-dimensional ^{1}H and ^{13}C-NMR spectra were obtained using standard pulse sequences and parameters, respectively. Column chromatography was carried out on silica gel and thin layer chromatography (TLC) analysis was performed on silica gel GF_{254} plates (Merck, Germany). Spots were visualized by UV lamp (254 nm and 365 nm) or by spraying with 50% H_2SO_4/H_2O solution. The isolated compounds were elucidated with a NMR spectrometer with $CDCl_3$ as the solvent and TMS as an internal standard.

Cell culture of macrophages and the preparation of lymphocytes

RAW264.7 cell line was obtained from Peking Union Medical College Hospital (Beijing, China) and inoculated to RPMI 1640 medium supplemented with 8% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in an incubator with a humidified atmosphere of 5% CO_2 at 37°C. Splenocytes were isolated from the spleen of specific pathogen-free (SPF) male mice. The preparation of peritoneal splenocytes was performed as was done previously.[11]

Assessment of cell viability

The cytotoxicity of the polysaccharides of 30%, 50%, 70% and 90% was tested using MTT assay based on the methods described by Brahmi, with some modification.[12] 3×10^4 of RAW264.7 cells were seeded in triplicates in 96-welled plates, then cultured with polysaccharides at different concentrations of 0.004 μg/mL, 0.015 μg/mL, 0.06 μg/mL, 0.23 μg/mL, 0.94 μg/mL, 3.75 μg/mL, 15 μg/mL, and 60 μg/mL for 24 hrs. Subsequently, 10 μL of MTT solution (5 mg/mL) was added to each well, and the cells were incubated for 3 hrs at 37°C. The supernatant was discarded and 150 μL of dimethyl sulfoxide (DMSO) was added to each well. The absorbance value was estimated at 490 nm by a microplate reader (Tecan, Austria). Macrophages can be activated by lipopolysaccharide (LPS) (1 μg/mL) which was regarded as a positive control. Cell viability was calculated using the following equation:

Cell viability (%) = A2/A1 × 100

where A1 is the absorbance value of the blank control group, and A2 is the absorbance value of the treatment group.

Griess reaction method

The effect of different concentrations (0.004–60 μg/mL) of polysaccharides on NO level in RAW264.7 cells was evaluated in the medium using the Griess reaction method.[13] Briefly, the cells were treated with polysaccharides at different concentrations (0.004 μg/mL, 0.015 μg/mL, 0.06 μg/mL, 0.23 μg/ml, 0.94 μg/mL, 3.75 μg/mL, 15 μg/mL, 60 μg/mL) in the presence or absence of LPS (1 μg/mL) for 24 hrs. 50 μL of cultured medium and an equal volume of Griess reagent (50 mg naphthyl ethylenediamine in 50 mL distilled water and 1% sulfanilamide in 5% phosphoric acid) were mixed and incubated for 20 min at room temperature. The absorbance at 540 nm was measured with a microplate reader (Tecan, Austria). Nitrite levels were measured by a standard curve prepared from sodium nitrite.[14]

Scratch assay

The effect of 30%, 50%, 70% and 90% polysaccharides on the migration ability of macrophages was detected via scratch assay. The method was measured as described.[15] Macrophages were treated with a series of concentrations, of which 30% polysaccharide and 50% polysaccharide were treated with 0.06 μg/mL, 0.23 μg/mL, 0.94 μg/mL, 3.75 μg/ml. 70% and 90% polysaccharides were tested with 0.94 μg/mL, 3.75 μg/mL, 15 μg/mL and 60 μg/mL, respectively. LPS (1 μg/mL) was regarded as positive control. Scratch widths were measured and images were taken at the same reference points along with the scratches. The scratch healing rate was calculated by the following formula: Scratch healing rate (%) = [(0 hr scratch width – 24- or 48-hr scratch width)/0 hr scratch width]

× 100. It was photographically recorded, and cell confluence area was measured to calculate the cell migration.[16]

Phagocytosis assay

The phagocytosis of macrophages of test samples including four polysaccharide sites and individual compounds were evaluated using the neutral red uptake (NRU) assay.[17] RAW264.7 cells were seeded in 96-welled plates (3×10^5 cells/well). After an incubation of 2 hrs, the supernatant was discarded. RAW264.7 cells were pre-treated with a test sample at different concentrations in the absence of LPS (1 μg/ml) for 24 hrs. Moreover, RAW264.7 cells were pre-treated with the polysaccharides of 30% and 50% (0.06–3.75 μg/mL) and the polysaccharides of 70% and 90% (0.94–60 μg/mL). RAW264.7 cells were pre-treated with individual compound at different concentrations (0.05 μmol/ml, 0.5 μmol/ml, 5 μmol/ml, and 50 μmol/ml). After the treatment for 24 hrs, the supernatant was removed and washed with phosphate-buffered saline (PBS). Then 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (0.05%, w/w) was added into each well and incubated for another 0.5 hr. The supernatant was discarded and the cells were repeatedly washed twice with PBS. Afterwards, the cells were lysed for 0.5 hr at room temperature with 100 μL cell lysis buffer ($V_{ethanol}$:$V_{acetic\ acid}$ = 1:1). Finally, after 45 min optical density was measured at 540 nm in a microplate reader (Tecan, Austria).

The proliferation of splenic lymphocytes

Isolated splenic lymphocytes were induced using a T-cell (Con A, 7.5 μg/mL) or B-cell (LPS, 1 μg/mL) mitogen. 2.5×10^6 of spleen lymphocytes in mice cells were seeded in triplicates in 96-welled plates, 100 μL per well. The lymphocytes were treated with four polysaccharide sites and individual compounds at a series of concentrations, respectively. The specific concentrations of tested samples are referenced in the above experiments. Cells were incubated in RPMI at 37°C in a humidified atmosphere of 5% CO_2. After culturing for 48 hrs, cell proliferation was evaluated using the MTT method.[18]

Statistical analysis

Data are expressed as mean ± standard deviation (x̄±SD). Data analysis was performed using SPSS statistical software. Comparison between groups was performed by independent sample *t* test or Dunnett's test in one-way analysis of variance (ANOVA). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

RESULTS

Weight after freeze-drying of polysaccharide

The crude polysaccharide was isolated from the aerial part of *V. amoena* Fisch. var. *Angusta*. Freyn via hot water extraction and ethanol precipitation to obtain the polysaccharides of 30%, 50%, 70% and 90%. Then four polysaccharide sites were dried and weighed; the weight after freeze-drying of the polysaccharides is shown in [Table 1].

Table 1: The weight after freeze-drying of polysaccharide

Polysaccharide (%)	Weight (g)
30	2.261
50	7.172
70	5.740
90	5.612

The crude polysaccharide was isolated from the aerial part of *Vicia amoena* Fisch. var. *Angusta*. Freyn by hot water extraction and ethanol precipitation to obtain the polysaccharides of 30%, 50%, 70% and 90%. Then four polysaccharide sites were dried and weighed, the weight after freeze-drying of polysaccharide is shown

The structural analysis of isolated individual compounds

Eight pure compounds were obtained and identified as isoquercitrin, quercetin, quercitrin, hyperin, chlorogenic acid, kaempferol, kaempferitrin and wogonin via physical-chemical process. The spectrum data and the molecular structures of these constituents are presented in Figure 1.

Isoquercitrin (1), light yellow powers, $C_{21}H_{20}O_{12}$, the reactions of Mg-HCl and Molish were positive. UV (MeOH) λ (max): 254 nm, 356 nm; ^{1}H-NMR (400 MHz, DMSO-d6) δ (ppm): 6.20 (1H, d, J = 1.79 Hz), 6.40 (1H, d, J = 1.79 Hz), 7.60 (1H, d, J = 1.66 Hz), 6.85 (1H, dd, J = 1.66, 8.48 Hz), 6.41 (1H, d, J = 8.48 Hz); ^{13}C-NMR (150 MHz, DMSO-d6), δ (ppm): 156.7 (C-2), 133.7 (C-3), 177.8 (C-4), 101.1 (C-1′′), 74.5 (C-2′′), 76.9 (C-3′′), 70.3 (C-4′′), 78.0 (C-5′′), 61.3 (C-6′′). The analytical data were consistent with the reported data.[19]

Quercetin (2), light yellow powers, $C_{15}H_{10}O_7$, the reactions of Mg-HCl and Molish were positive. UV (MeOH) λ (max): 255 nm, 370 nm; ^{1}H-NMR (400 MHz, DMSO-d6) δ (ppm) 6.19 (1H, d, J = 1.94 Hz) 6.40 (1H, d, J = 1.94 Hz), 7.69 (1H, d, J = 2.14 Hz), 7.55 (1H, dd, J = 2.14, 8.48 Hz), 6.90 (1H, d, J = 8.48 Hz). The analytical data were consistent with the reported data.[16]

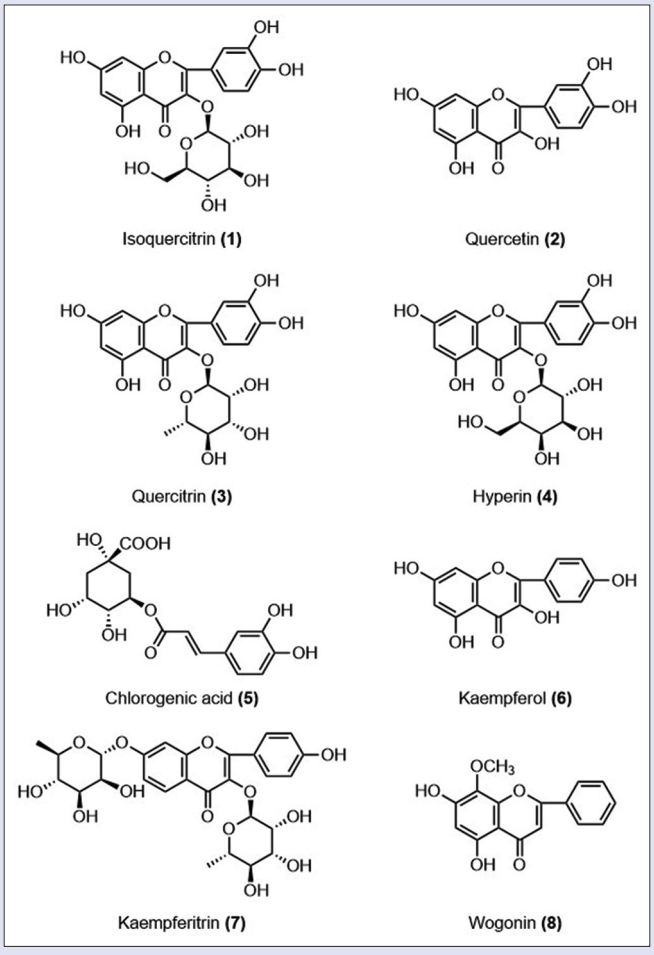


Figure 1: Compounds isolated from the *V. amoena* Fisch. var. *Angusta*. Freyn. Eight pure compounds were obtained and identified as isoquercitrin (1), quercetin (2), quercitrin (3), hyperin (4), chlorogenic acid (5), kaempferol (6), kaempferitrin (7) and wogonin (8) by physical-chemical process and spectrum data. The molecular structures of these constitutes are presented

Quercitrin (3), yellow powers, $C_{21}H_{20}O_{11}$, the reactions of Mg-HCl and Molish were positive. UV (MeOH) λ (max): 255 nm, 371 nm; ^{1}H-NMR (400 MHz, DMSO-d6) δ (ppm): 6.41 (1H, d, J = 1.79 Hz), 6.78 (1H, d, J = 1.79 Hz), 7.72 (1H, d, J = 1.66 Hz), 7.58 (1H, dd, J = 1.66, 8.48 Hz), 6.89 (1H, d, J = 8.48 Hz) 5.32 (1H); ^{13}C-NMR (150 MHz, DMSO-d6), δ (ppm): 47.9 (C-2), 136.1 (C-3), 176.0 (C-4), 98.4 (C-1′′), 70.3 (C-2′′), 70.1 (C-3′′), 71.6 (C-4′′), 69.9 (C-5′′), 17.9 (C-6′′). The analytical data were consistent with the reported data.[20]

Hyperin (4) yellow powers, $C_{21}H_{20}O_{12}$, the reactions of Mg-HCl and Molish were positive. UV (MeOH) λ (max): 254 nm, 358 nm; ^{1}H-NMR (400 MHz, DMSO-d6) δ (ppm) 6.20 (1H, d, J = 1.79 Hz), 6.41 (1H, d, J = 1.79 Hz), 7.53 (1H, d, J = 2.21 Hz), 7.68 (1H, dd, J = 2.21, 8.48 Hz), 6.83 (1H, d, J = 8.48 Hz), ^{13}C-NMR (150 MHz, DMSO-d6), δ (ppm): 156.6 (C-2), 133.9 (C-3), 177.9 (C-4), 102.2 (C-1), 71.6 (C-2′′), 73.6 (C-3′′), 68.3 (C-4′′) 76.2 (C-5′′), 60.5 (C-6′′). The analytical data were consistent with the reported data.[16]

Chlorogenic acid (5) white crystals, $C_{16}H_{18}O_{9}$, the reactions of bromocresol purple was positive. ^{1}H-NMR (400 MHz, DMSO-d6) δ (ppm) 7.41 (1H, d, J = 15.87 Hz), 6.14 (1H, d, J = 15.87 Hz), 7.02 (1H, d, J = 2.03 Hz), 6.97 (1H, dd, J = 8.15, 2.03 Hz), 6.76 (1H, d, J = 8.15 Hz); ^{13}C-NMR (150 MHz, DMSO-d6), δ (ppm): C174.8 (7-COOH), 165.0 (C-9′), 148.3 (C-4′), 145.5 (C-3′), 125.5 (C-1′), 121.3 (C-6′), 115.6 (C-2′), 114.7 (C-5′), C144.9 (C-7′), 114.2 (C-8′). The analytical data were consistent with the reported data.[21]

Kaempferol (6) light yellow powers, $C_{15}H_{10}O_{6}$, the reactions of Mg-HCl and Molish were positive. UV (MeOH) λ (max): 255 nm, 370 nm; ^{1}H-NMR (150 MHz, DMSO-d6) δ (ppm): 6.19 (1H, d, J = 1.90 Hz) 6.44 (1H, d, J = 1.90 Hz), 12.48 (1H, s), 10.79 (1H, s), 10.11 (1H, s), 9.38 (1H, s), 6.92 (2H, d, J = 8.86 Hz), 8.04 (2H, d, J = 8.86 Hz); ^{13}C-NMR (150 MHz, DMSO-d6), δ (ppm): 115.8 (C-3', 5'), 129.9 (C-2′, 6′). The analytical data were consistent with the reported data.[19]

Kaempferitrin (7) light yellow powers, $C_{27}H_{30}O_{14}$, the reactions of Mg-HCl and Molish were positive. UV (MeOH) λ (max): 264 nm, 343 nm; ^{1}H-NMR (400 MHz, DMSO-d6) δ (ppm): 6.46 (1H, d, J = 2.09 Hz), 6.79 (1H, d, J = 2.09 Hz), 10.26 (1H, s), 6.93 (2H, d, J = 8.88 Hz), 7.80 (2H, d, J = 8.88 Hz); ^{13}C-NMR (150 MHz, DMSO-d6), δ (ppm): 156.5 (C-2), 134.9 (C-3), 178.3 (C-4), 162.1 (C-7), 98.8 (C-6), 95.0 (C-8), 102.2 (C-1′′), 99.8 (C-1′′′), 71.9 (C-4′′), 70.5 (C-4′′′), 71.1 (C-3′′), 70.7 (C-3′′′), C70.6 (C-2′′), 70.5 (C-2′′′), 70.4 (C-5), 69.2 (C-5′′′), 18.3 (C-6′′), 7.9 (C-6′′′). The analytical data were consistent with the reported data.[22]

Wogonin (8) light yellow needle, $C_{16}H_{12}O_{5}$, the reactions of Mg-HCl and Molish were positive. UV (MeOH) λ (max): 264 nm, 343 nm; ^{1}H-NMR (400 MHz, DMSO-d6) δ (ppm): 7.00 (1H, s), 12.50 (1H, s), 10.82 (1H, s), 8.07 (2H, m), 7.62 (3H, m); ^{1}H-NMR (150 MHz, DMSO-d6) δ (ppm): 156.2 (C-5), 126.3 (C-3′, 5′). The analytical data were consistent with the reported data.[23]

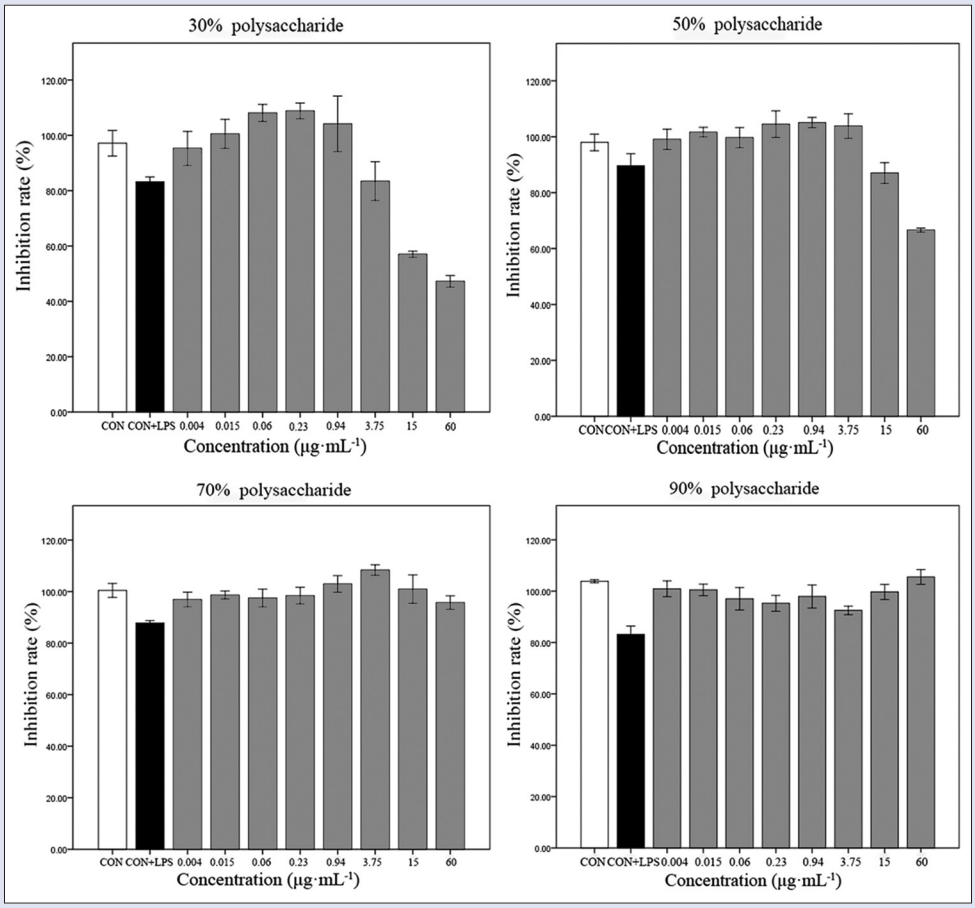


Figure 2: Effects of four polysaccharide sites on RAW264.7 cell viability macrophages cells; data is expressed as mean ± SD (*n* = 3). Within 0.06–3.75 μg/mL, the polysaccharides of 30% and 50% did not cause any significant cell damage compared with control group, which showed that the polysaccharides of 30% and 50% were non-cytotoxic to macrophages within that concentration range

Effects of polysaccharides cell viability of RAW264.7 cells

MTT colorimetric method is a common measure to response the number of viable cells and cell viability by detecting the activity of mitochondrial dehydrogenase of live cells.[24] Figure 2 shows that within 0.06–3.75 μg/mL, the polysaccharides of 30% and 50% do not cause any significant cell damage compared with the control group, which showed that the polysaccharides of 30% and 50% were non-cytotoxic to macrophages within that concentration range. Additionally, cell viability of RAW264.7 cells, which were treated with the polysaccharides of 30% and 50%, increased at doses of 0.06 μg/mL, 0.23 μg/mL, 0.94 μg/mL and 3.75 μg/mL. However, the polysaccharides of 30% and 50% at the high concentrations inhibited cell proliferation; this effect could be associated with mitochondrial dysfunction such as the loss of transmembrane mitochondrial potential.[12] The polysaccharides of 70% and 90% were non-cytotoxic to macrophages in the concentration range of 0.004–60 μg/mL. And the cell viability improved at doses of 0.94 μg/mL, 3.75 μg/mL, 15 μg/mL and 60 μg/mL.

Effect of polysaccharide treatment on nitric oxide

It is known that nitric oxide (NO) is a messenger involved in immunomodulation, inflammatory response, signal transduction

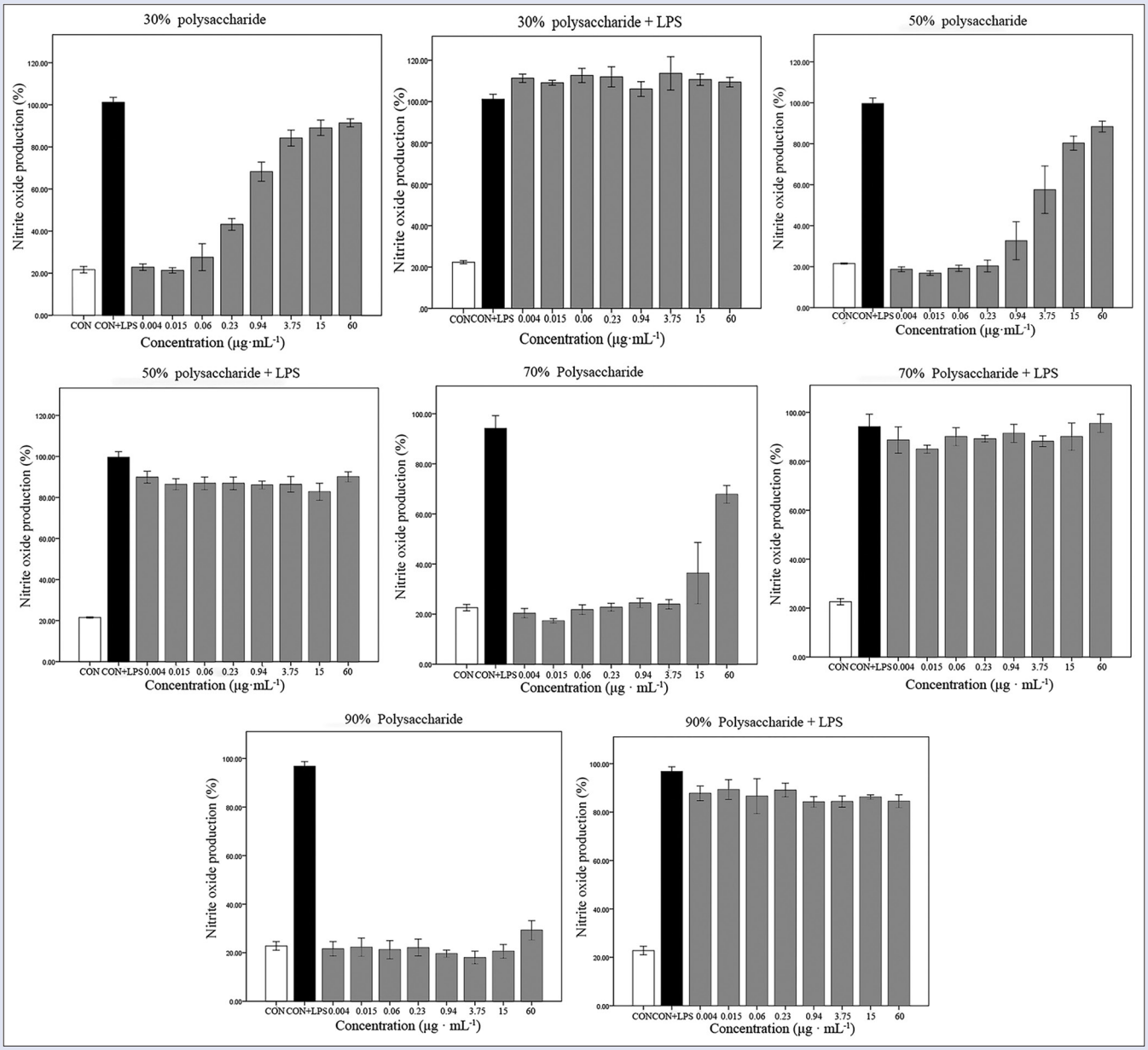


Figure 3: Effects of polysaccharides and polysaccharides + LPS on the production of NO in macrophages RAW264.7 cells; data is expressed as mean ± SD (n = 3). The RAW264.7 macrophages that were treated with the polysaccharides of 30%, 50% and 70% presented a significant dose-dependent increase in macrophages nitric oxide production at 0.004–60 μg/mL, as compared with negative control. The results showed that the polysaccharides of 30%, 50% and 70% could promote RAW264.7 macrophages to release NO. The production of NO in LPS-stimulated RAW264.7 macrophages pre-treated with the polysaccharides of 30%, 50%, 70% and 90% were higher than that of RAW264.7 macrophage treated with the polysaccharides of 30%, 50%, 70% and 90% at the concentration range of 0.004–60 μg/mL, which indicated a synergistic effect on the production of NO

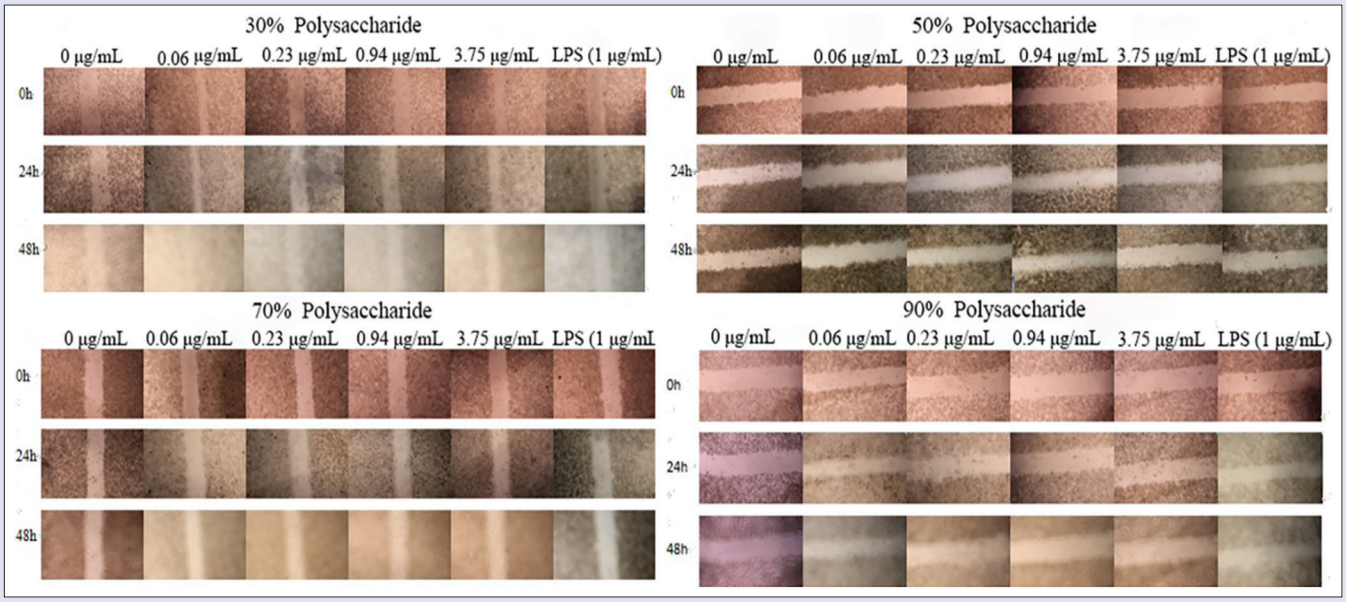


Figure 4: Microscopic view of the effect of four polysaccharide sites from *V. amoena* Fisch. var. *Angusta* on scratch healing of macrophages. The width of the scratch did not significantly changed at 24rs or 48 h. Trherefore, we chose to use Image J software to analyze the experimental results that there was no significant difference in the healing rate of the scratch of macrophages in the four polysaccharide sites

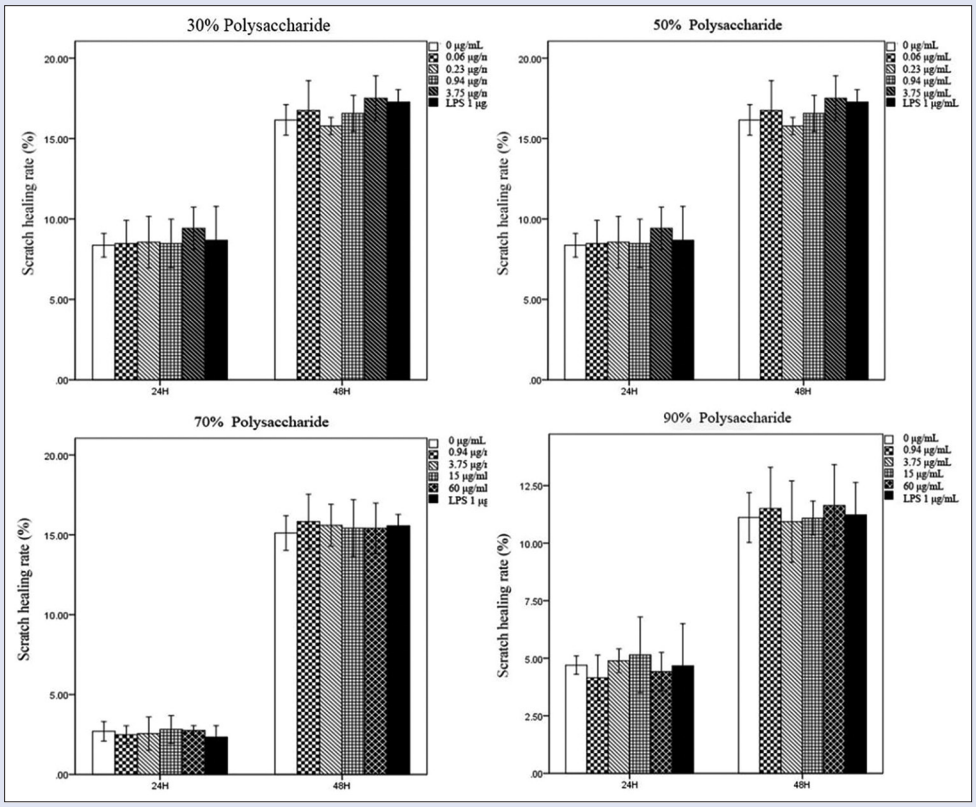


Figure 5: Effect of four polysaccharide sites from *V. amoena* Fisch. var. *Angusta* on the ability of scratch healing of macrophages. The polysaccharides of 30% and 50% were dealt with a series of concentrations (0.06 μg/mL, 0.23 μg/mL, 0.94 μg/mL and 3.75 μg/mL). The polysaccharides of 70% and 90% were treated with the concentrations of 0.94 μg/mL, 3.75 μg/mL, 15 μg/mL and 60 μg/mL. And the width of scratch did not significantly change at 24 or 48 hrs. There was no significant difference in the healing rate of the scratch of macrophages in the four polysaccharide sites. There was no effect of polysaccharide isolated from *Vicia amoena* Fisch. var. *Angusta* on cell migration

and vasodilatation. Macrophages can be activated by cytokines, LPS and microbial antigens to release NO to eradicate microbes, parasites and tumor cells.[25] The nirtric oxide production of RAW264.7 macrophages treated with 30%, 50% and 70% polysaccharides

increased significantly in a dose-dependent manner at 0.004-60 μg/mL, compared with negative control [Figure 3]. s shown in Figure 3, the RAW264.7 macrophages were treated with the polysaccharides of 30%, 50% and 70% presented a significant dose-dependent increase in macrophages nitric oxide production at 0.004-60 μg/mL, as compared with negative control. The results showed that the polysaccharides of 30%, 50% and 70% could promote RAW264.7 macrophages to release NO. The production of NO in LPS-stimulated RAW264.7 macrophages pre-treated with the polysaccharides of 30%, 50%, 70% and 90% were higher than that of RAW264.7 macrophages treated with the polysaccharides of 30%, 50%, 70% and 90% at a concentration range of 0.004–60 μg/mL, which indicated a synergistic effect on the production of NO. The nitrite concentration of NO production was even higher than LPS when the cells were exposed to LPS after pre-treatment with the polysaccharides of 30% [Figure 3]. The effect of 90% polysaccharide activated macrophages to release NO was not obvious [Figure 3]. These results indicated that four polysaccharide parts could increase immune effect by stimulating RAW264.7 macrophages to secrete NO at different levels.

TLR4 is a newly discovered transmembrane receptor of polysaccharides, and its discovery greatly promotes the knowledge of LPS or polysaccharide signal transduction pathways. At present, some reports have confirmed that some polysaccharides from natural plants acted on macrophages via TLR4 signal pathway.[26–28] Therefore, it is likely that when polysaccharides recognize TLR4 receptor on the surface of RAW264.7 macrophages, the cells were triggered to release iNOS, which in turn activated the release of NO.

Effect of polysaccharides on cell migration

Scratch wound healing assay was used to determine cell migration. Based on the data of MTT and NO assay, the polysaccharides of 30% and 50% were dealt with a series of concentrations (0.06 μg/mL, 0.23 μg/mL, 0.94 μg/mL, and 3.75 μg/mL). The polysaccharides of 70% and 90% were treated with concentrations of 0.94 μg/mL, 3.75 μg/mL, 15 μg/mL, and 60 μg/mL. The results are presented in Figures 4 and 5. From Figure 4, the width of scratch did not significantly change at 24 or 48 hes. Therefore, we chose to use Image J software to analyze the experimental results which are demonstrated in Figure 4. The figure shows that there was no significant difference in the healing rate of the scratch of macrophages in the four polysaccharide sites. Therefore, the polysaccharides isolated from *V. amoena* Fisch. var. *Angusta*. Freyn aerial parts have no effect on cell migration.

Effect of polysaccharides on phagocytosis of macrophages

The NRU assay was used for the evaluation of phagocytosis by RAW264.7 macrophages. Phagocytes play an important role in immunity by eliminating pathogenic bacteria, fungi and malignant cells and maintaining the healthy physiological state.[29,30] Phagocytosis was regarded as the key indicator to assess the activities of macrophages.[16] When tissues and organs become impaired, activated macrophages exhibit phagocytosis leading to destruction of foreign substances, and play an important part in the body's immune system.[31] As shown in Figure 6, compared with the control group, the phagocytosis of RAW264.7

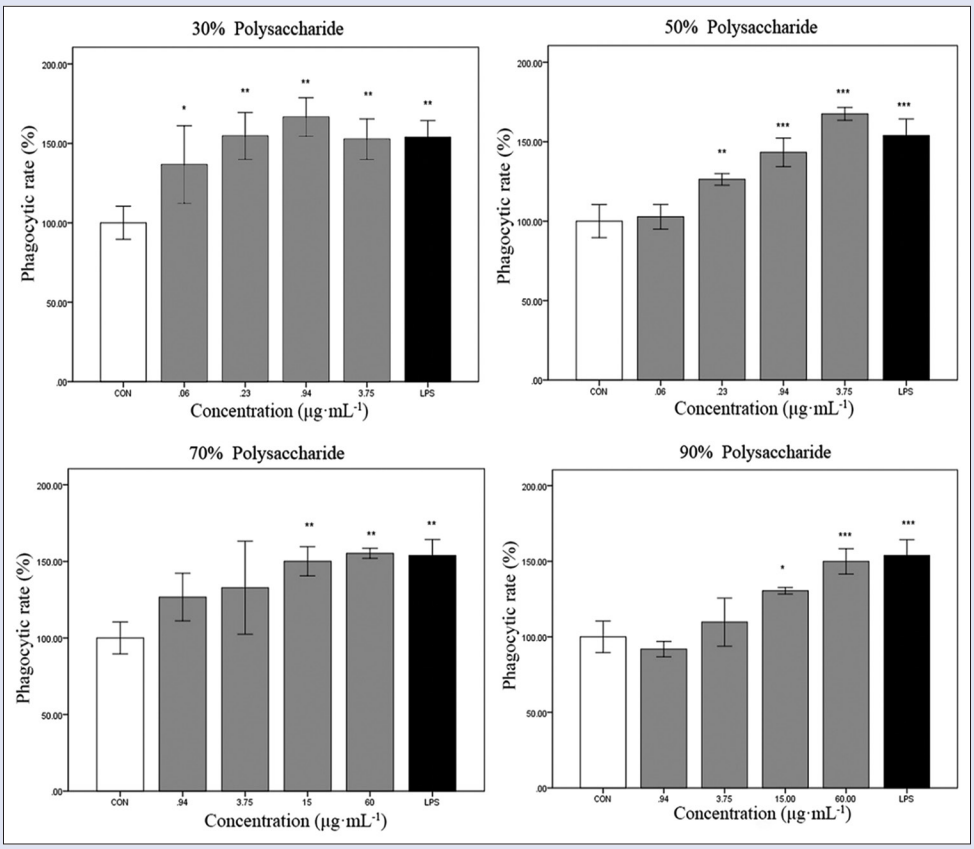


Figure 6: Effect of four polysaccharide sites on phagocytosis of RAW264.7 macrophages cells; data is expressed as the mean ± SD (n = 3), * P < 0.05; ** P < 0.01; *** P < 0.001. Compared with the CON group, the phagocytosis of RAW264.7 macrophages treated with the polysaccharides of 30%, 50%, 70% and 90% were increased in a dose-dependent manner at the test concentrations, which showed that the four polysaccharide sites had abilities to enhance phagocytic activity of RAW264.7 macrophages

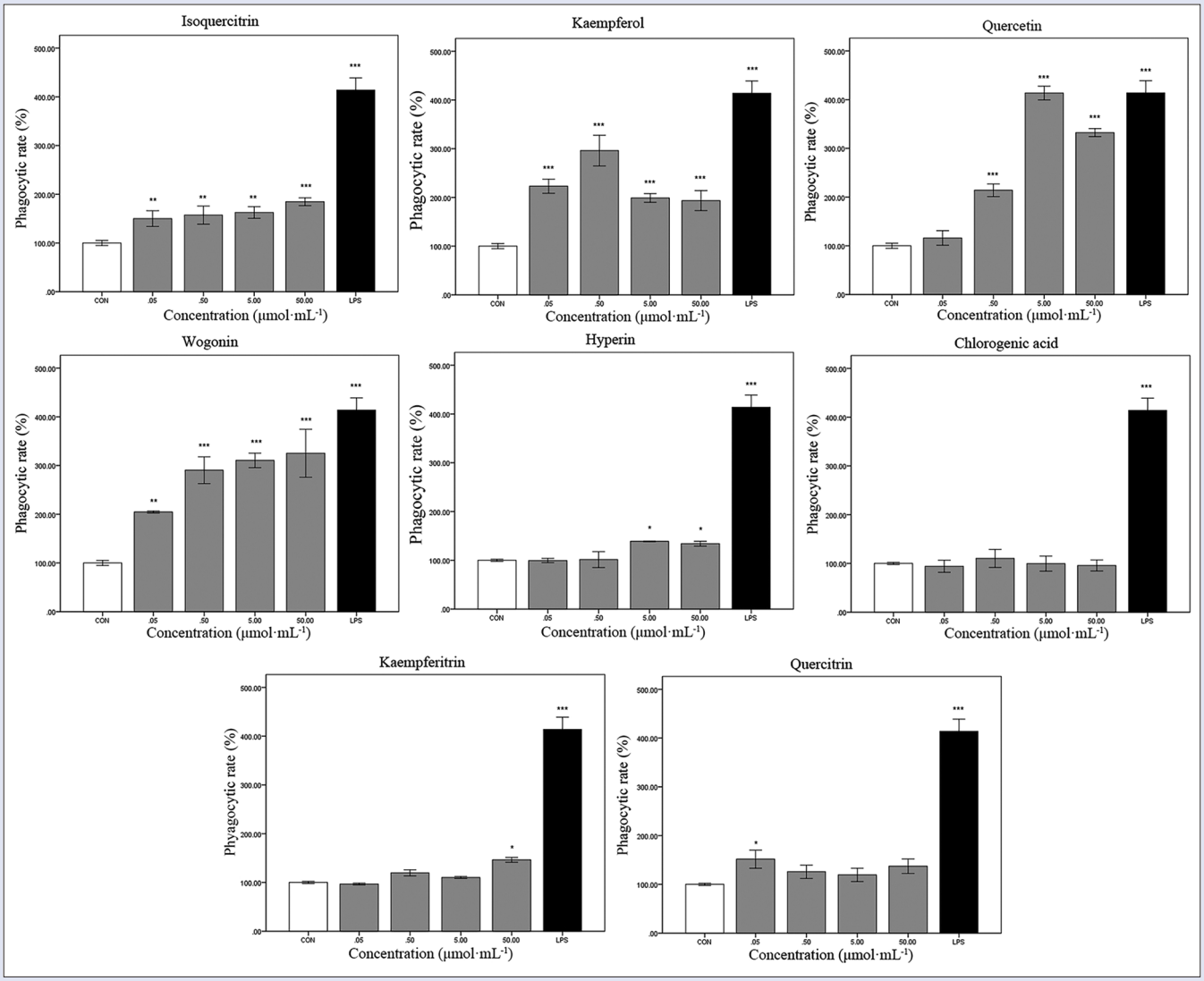


Figure 7: Effect of individual compounds on phagocytosis of RAW264.7 macrophages cells. Data is expressed as the mean ± SD ($n = 3$), *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Eight individual compounds except chlorogenic acid had the abilities to enhance phagocytic activity of macrophages. Furthermore, the phagocytosis rates of isoquercitrin, kaempferol, quercetin and wogonin in the concentration range of 0.05–50 μmol/mL were higher than those in other compounds

macrophages treated with polysaccharides of 30%, 50%, 70% and 90% increased in a dose-dependent manner at the test concentrations, which showed that the four polysaccharide sites had abilities to enhance phagocytic activity of RAW264.7 macrophages. For example, cells were treated with polysaccharides of 30% at a dose of 0.94 μg/mL and 50% at a dose of 3.75 μg/mL; the phagocytic rates were even higher than the positive control (LPS). These results indicated that polysaccharides could improve immune function by activating RAW264.7 macrophages to phagocytose foreign substances. Furthermore, polysaccharides might recognize TLR4 receptor to activate RAW264.7 macrophages, which was consistent with the release of NO result.

Effect of individual compounds on phagocytosis of macrophages

Eight chemical components isolated from *V. amoena* Fisch. var. *Angusta*. Freyn except chlorogenic acid were flavonoids. The basic structures of flavonoids have a common carbon skeleton of two benzene rings (ring A and B), joined by a three-carbon bridge (C_6-C_3-C_6).[32] Based on previous literature, flavonoids possessed a series of anti-inflammatory, antioxidants, anticancer and prevented the immunological and chronic disorders biological functions.[33] As shown in Figure 7, eight individual compounds except chlorogenic acid had the ability to enhance phagocytic activity of macrophages. Furthermore, the phagocytosis rates of isoquercitrin, kaempferol, quercetin and wogonin in the concentration range of 0.05–50 μmol/mL were higher than those in other compounds. However, the phagocytosis rate of quercitrin can be only improved at the dose of 0.05 μmol/mL, which indicated that high concentrations might induce cytotoxicity. Collectively, the above results confirmed that flavonoids can enhance immune response by increasing phagocytic activity of macrophages.

Effect of polysaccharide on splenic lymphocyte proliferation

The spleen is an important immune organ containing B lymphocytes and T lymphocytes.[34] B lymphocytes (60%) and T lymphocytes (40%)

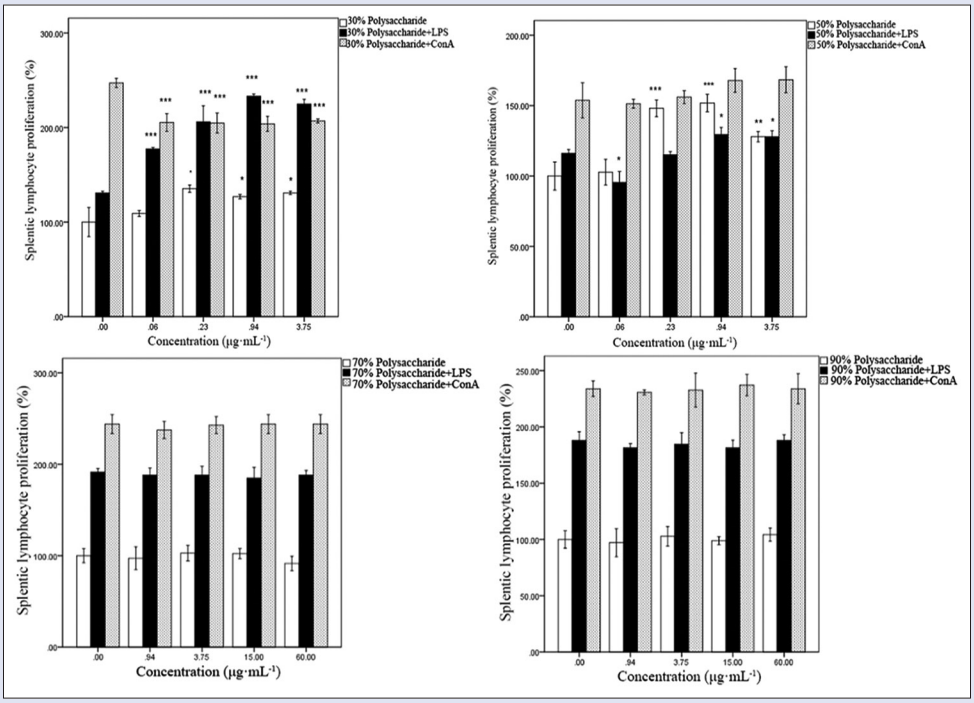


Figure 8: Effect of individual compounds on proliferation of RAW264.7 macrophage cells. Data is expressed as mean ± SD ($n = 3$), * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The polysaccharide of 30% and 50% can improve the proliferation ability of spleen lymphocytes in mice to varying degrees, and 50% polysaccharide can increase the proliferation ability of spleen lymphocytes in mice better than 30% polysaccharide. The result indicated that the polysaccharides of 30% and 50% played an active role in the immune regulation of the body. The proliferation rate was clearly suppressed ($P < 0.01$) when the cells were exposed to ConA after pre-treatment with the polysaccharides of 30% at 0.06–3.75 μg/mL, while the proliferation rate was increased in a dose-dependent manner ($P < 0.01$) when the cells were exposed to LPS after pre-treatment with the polysaccharides of 30% at 0.06–0.94 μg/mL. The above results showed that there could be an exertion of a synergistic effect about polysaccharides of 30% and LPS. On the contrary, there could be an exertion of an antagonistic effect about polysaccharides of 30% and ConA. The lymphocytes were treated with the polysaccharides of 50% at 0.06–0.94 μg/mL, and the proliferation rate increased in a dose-dependent manner. The cells were exposed to LPS and ConA after pre-treatment with the polysaccharides of 50% at 0.06–3.75 μg/mL, and the proliferation rate markedly improved. The polysaccharides of 70% and 90% had no effect on the proliferation of spleen lymphocytes in mice

can be stimulated by LPS and Concanavalin A (ConA), respectively.[35] Lymphocyte proliferation is the most direct indicator of host immune status.[36] It was reported that the imbalance of functions and numbers of specific T cell subsets is critical to pathogenic derangements in systemic rheumatic diseases.[37] Although the pathogenesis of rheumatic diseases are poorly understood, it is worth noticing that these antibodies and self-reactive lymphocytes are capable of influencing immune responses and can eventually trigger tissue and organ damage.[38] In this experiment, the lymphocytes were treated with the polysaccharides of 30% and 50% at 0.06–3.75 μg/mL, due to fact that the proliferation activity was most effective without affecting cell viability. And the lymphocytes were treated with the polysaccharides of 70% and 90% at 0.94–60 μg/mL. As shown in Figure 8, the polysaccharide of 30% and 50% can improve the proliferation ability of spleen lymphocytes in mice to varying degrees, and 50% polysaccharide can increase the proliferation ability of spleen lymphocytes in mice better than 30% polysaccharide. The result indicated that the polysaccharides of 30% and 50% played an active role in the immune regulation of the body. The proliferation rate was clearly suppressed ($P < 0.01$) when the cells were exposed to ConA after pre-treatment with the polysaccharides of 30% at 0.06–3.75 μg/mL, while the proliferation rate increased in a dose-dependent manner ($P < 0.01$) when the cells were exposed to LPS after pretreatment with the polysaccharides of 30% at 0.06–0.94 μg/mL. The above results showed that there could be an exertion of a synergistic effect about polysaccharides of 30% and LPS. On the contrary, there could be an exertion of an antagonistic effect about polysaccharides of 30% and ConA. The lymphocytes were treated with the polysaccharides of 50% at 0.06–0.94 μg/mL; the proliferation rate increased in a dose-dependent manner. The cells were exposed to LPS and ConA after pre-treatment with the polysaccharides of 50% at 0.06–3.75 μg/mL, and the proliferation rate markedly improved. The polysaccharides of 70% and 90% had no effect on the proliferation of spleen lymphocytes in mice.

Effect of individual compounds on splenic lymphocyte proliferation

Previous studies reported that flavonoids could exert cytoprotective effects, restoring lymphocyte proliferation and preventing apoptosis.[39] As known in Figure 9, the proliferation rates significantly improved when lymphocytes separately were treated with isoquercitrin, quercetin, wogonin, quercitrin, kaempferitrin and kaempferol; the results showed that they could exert the effect of immune regulation. The cells were exposed to LPS after pretreatment with each tested samples. All samples except for isoquercitrin had a considerable proliferation ability on lymphocytes and there were obvious differences between groups of quercetin, quercitrin and wogonin. The proliferation ability had improved to varying degrees when the cells were exposed to ConA after pre-treatment with isoquercitrin, wogonin, quercitrin, kaempferitrin and kaempferol. The proliferation rate in ConA-stimulated cells pretreated with isoquercitrin was

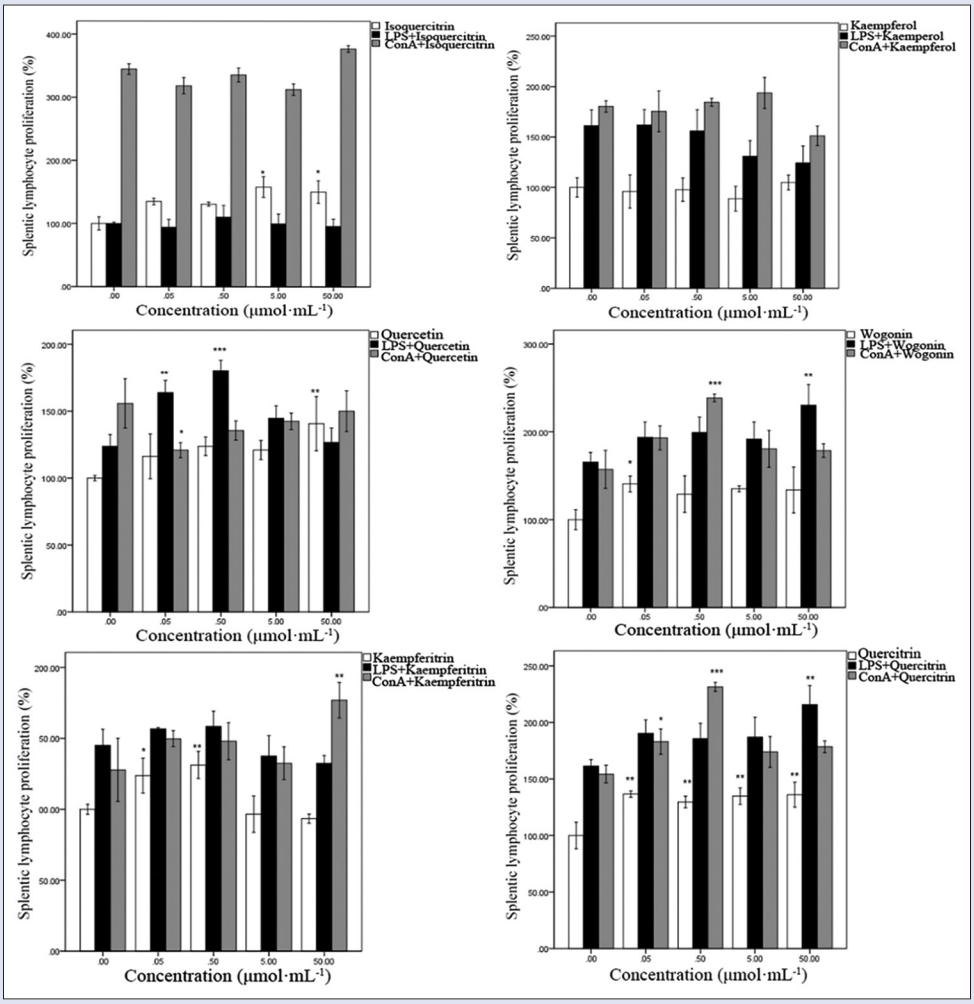


Figure 9: Effect of individual compounds on proliferation of RAW264.7 macrophage cells. Data is expressed as mean ± SD (n = 3), * P < 0.05; ** P < 0.01; *** P < 0.001. The proliferation rates significantly improved when lymphocytes separately were treated with isoquercitrin, quercetin, wogonin, quercitrin, kaempferitrin and kaempferol; the results showed that they could exert the effect of immune regulation. The cells were exposed to LPS after pre-treatment with each tested samples. All samples except for isoquercitrin had a considerably proliferation ability on lymphocytes and there were obvious differences between groups of quercetin, quercitrin and wogonin. The proliferation ability had been improved to varying degrees when the cells were exposed to ConA after pre-treatment with isoquercitrin, quercetin, wogonin, quercitrin, kaempferitrin and kaempferol. The proliferation rate in ConA-stimulated cells pre-treated with isoquercitrin was higher than other tested groups, while quercetin can decrease the proliferation rate

higher than other tested groups, while quercetin could decrease the proliferation rate. Li[40] suggested that flavonoids exerting their immunomodulatory effect might be through regulating TLR signal pathways, especially the expression of TLR2 and TLR4.

Previous literatures have reported the chemical constitutes and bioactivities of some species from the genus of *Vicia*. However, there are few reports about the chemical constituents and bioactivities from the species of *V. amoena* Fisch. var. *Angusta.* Freyn. In this study, our team investigated the immunological activities of four polysaccharide sites and individual constituents isolated from *V. amoena* Fisch. var. *Angusta.* Freyn aerial parts for the first time. The results showed that there presented a positive effect of the four polysaccharide sites and individual constituents on immune-regulation. Meanwhile, the four polysaccharide sites and individual constituent showed different results about immune-regulation. Additionally, compared to individual constitutes, polysaccharides had a more obvious effect on improving immune activity.

DISCUSSION

All results demonstrated that four polysaccharide sites and individual compounds isolated from *V. amoena* Fisch. var. *Angusta.* Freyn aerial parts was able to enhance the immune responses in proliferation, NO production and phagocytosis of macrophages, increasing splenic lymphocyte proliferation. Hence, our results might help in preliminarily applying the use of *Vicia. amoena* Fisch. var. *Angusta.* Freyn traditional medicine to treat rheumatic diseases, and it is likely that the extract from the herb might be via the TLR-4-mediated signaling pathway to achieve immunomodulatory effects.

CONCLUSION

In summary, we not only provide a scientific rationale of the traditional uses of the herb, but also that the natural compounds, especially polysaccharides and polyphenols, from *V. amoena* Fisch. var. *Angusta.* Freyn can be considered as a novel therapeutic entity for immune-regulation in humans.

Acknowledgements

The authors gratefully acknowledge the Establishment of Preparation Technology System and High-Quality Preparation Standard of Mongolian Classic Formula Zhachong Shisanwei Pill and Anshen Buxin Liuwei Pill (2018YFC1708205), and 2022 Project of the Education Department of Liaoning Province (Servicing for local enterprise), and 2021 Ministry of Education Industry-University Cooperation Collaborative Education Project.

Author's contributions

Fang-Yan Guo and Yu Wang designed the study. Fang-Yan Guo, Yu Wang, Lei Shi, Li Bai and Jing-Yu Liu performed all the experiments. Yu Wang, Nai-Zhi Zhang and Guang-Wen Fan designed and performed the biological experiments. Li Bai and Lei Shi wrote the manuscript with the input from other authors. Hao Pan checked the manuscript. Lei Shi reviewed and submitted this manuscript. Yu-Li Sang and Yan-Jun Hao provided the guidance throughout the experiment.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Liu Y, Zhang JM, Wang XG, Liu F, Shen ZB. Genetic diversity in Vicia amoena (Fabaceae) germplasm resource in China using SRAP and ISSR markers. Biochem Syst Ecol 2013;51:86-93.
2. Constance RBL. Flora Reipublicae Popularis Sinicae. Taxon 1992;41:626.
3. Xu XY, Li AP, Kang ZM, Zheng KB. Research progress of chemical constituents and pharmacological activities of Vicia genus. Chinese Sci Bull 2015;31:74-80.
4. Sinha SK, Kumar M, Kumar A, Bharti S, Shahi VK. Antioxidant Activities of Different Tissue Extract of Faba Bean (Vicia Faba L.) Containing Phenolic Compounds. LEGUME RES. 2013;36:496-504.
5. Islam MT, Ahn SY, Cho SM, Yun HK. Isolation of antibacterial compounds from hairy vetch (Vicia villosa) against grapevine crown gall pathogen. Hortic Environ Biotechnol 2013;54:338-45.
6. Megias C, Pastor-Cavada E, Torres-Fuentes C, Giron-Calle J, Alaiz M, Juan R, *et al.* Chelating, antioxidant and antiproliferative activity of Vicia sativa polyphenol extracts. Eur Food Res Technol 2009;230:353-9.
7. Pharmacopoeia of The People's Republic of China (Part 1). 2021.
8. Chang A, WWN, Hao YJ, Yang YY, Xu L, Kang TG. Simultaneous determination of six compounds in Vicia amoena var. Angusta by HPLC. Chin J Exp Tradit Med Form 2016;9:48-51.
9. GUO, Fang yan, WANG, Yu, HAO, Yan jun, *et al.* Determination of four flavonoids in Vicia amoena Fisch. and Vicia amoena Fisch. var. angusta Freyn. by HPLC. Chinese J Pharm Anals .2017,37:432-7.
10. Li RJ, Liu, XJ, Liu M, Liu MY. Biosystematical Studies on Northeast China VICIA L.VI.-Karyotype and cytogeography of VICIA venosa complex and its allied species. Bull Bot Res 1991;4:77-82.
11. Bonacorsi C, Raddi MS, Carlos IZ, Sannomiya M, Vilegas W. Anti-Helicobacter pylori activity and immunostimulatory effect of extracts from Byrsonima crassa Nied. (Malpighiaceae). BMC Complement Altern Med 2009;9:2.
12. Brahmi F, Hadj-Ahmed S, Zarrouk A, Bezine M, Nury T, Madani K, *et al.* Evidence of biological activity of Mentha species extracts on apoptotic and autophagic targets on murine RAW264.7 and human U937 monocytic cells. Pharm Biol 2017;55:286-93.
13. Benevides Bahiense J, Marques FM, Figueira MM, Vargas TS, Kondratyuk TP, Endringer DC, *et al.* Potential anti-inflammatory, antioxidant and antimicrobial activities of Sambucus australis. Pharm Biol 2017;55:991-7.
14. Park EK, Shin YW, Lee HU, Kim SS, Lee YC, Lee BY, *et al.* Inhibitory effect of ginsenoside Rb1 and compound K on NO and prostaglandin E2 biosyntheses of RAW264.7 cells induced by lipopolysaccharide. Biol Pharm Bull 2005;28:652-6.
15. Asmus SE, Batey KG, Gale-Butto SN, Schmitt BL. Using the scratch assay to study cell migration in an inquiry-based cell biology lab. J Bio Educ 2019;53:157-64.
16. Luo C, Wang X, An C, Hwang CF, Miao W, Yang L, *et al.* Molecular inhibition mechanisms of cell migration and invasion by coix polysaccharides in A549 NSCLC cells via targeting S100A4. Mol Med Rep 2017;15:309-16.
17. Yu Y, Shen M, Wang Z, Wang Y, Xie M, Xie J. Sulfated polysaccharide from Cyclocarya paliurus enhances the immunomodulatory activity of macrophages. Carbohydr Polym 2017;174:669-76.
18. Monmai C, You S, Park WJ. Immune-enhancing effects of anionic macromolecules extracted from Codium fragile on cyclophosphamide-treated mice. PLoS One 2019;14:e0211570.
19. Lei L, Li ZQ, Ma GY. Flavonoids and spiro lactones from Hypericum hookerianum. Chin Tradit Herbal Drugs 2005:17-20.
20. Alhozaimy GA, Al-Sheddi ES, Ibrahim TA. Biological activity and isolation of compounds from stem bark of plumeria acutifolia. Pharmacogn Mag 2017;13(Suppl 3):S505-11.
21. Shen SM, Shen LG, Lei QF, Si JY, Liu CM, Lu H. [Chemical constituents contained in aerial parts of Emilia sonchifolia]. Zhongguo Zhong Yao Za Zhi 2012;37:3249-51.
22. Gohara AA, Elmazar MMA. Isolation of hypotensive flavonoids from Chenopodium species growing in Egypt 1997;11:564-7.
23. Xiao LH, Wang HY, Song SY, Zhang SJ, Song GP, H.X., *et al.* Isolation and identification of the chemical constituents of Roots of Scutellaria amoena C.H.Wright. J Shenyang Pharm Univ. 2003:181-183+193.
24. Xiong L, Ouyang KH, Jiang Y, Yang ZW, Hu WB, Chen H, *et al.* Chemical composition of Cyclocarya paliurus polysaccharide and inflammatory effects in lipopolysaccharide-stimulated RAW264.7 macrophage. Int J Biol Macromol 2018;107:1898-907.
25. Shreshtha S, Sharma P, Kumar P, Sharma R, Singh SP. Nitric oxide: Its role in immunity. J Clin Diagn Res 2018;12:BE01-5.
26. Li M, Chen L-X, Chen S-R, Deng Y, Zhao J, Wang Y, *et al.* Non-starch polysaccharide from Chinese yam activated RAW 264.7 macrophages through the Toll-like receptor 4 (TLR4)-NF-κB signaling pathway. J Funct Foods 2017;37:491-500.
27. Luan A, Tang F, Yang Y, Lu M, Wang H, Zhang Y. Astragalus polysaccharide attenuates isoproterenol-induced cardiac hypertrophy by regulating TNF-α/PGC-1α signaling mediated energy biosynthesis. Environ Toxicol Pharmacol 2015;39:1081-90.
28. Park MJ, Ryu HS, Kim JS, Lee HK, Kang JS, Yun J, *et al.* Platycodon grandiflorum polysaccharide induces dendritic cell maturation via TLR4 signaling. Food Chem Toxicol 2014;72:212-20.
29. Lim JJ, Grinstein S, Roth Z. Diversity and versatility of phagocytosis: Roles in innate immunity, Tissue remodeling, and homeostasis. Front Cell Infect Microbiol 2017;7:191.
30. Mallat M, Marin-Teva JL, Cheret C. Phagocytosis in the developing CNS: More than clearing the corpses. Curr Opin Neurobiol 2005;15:101-7.
31. Liu X, Xie J, Jia S, Huang L, Wang Z, Li C, *et al.* Immunomodulatory effects of an acetylated Cyclocarya paliurus polysaccharide on murine macrophages RAW264.7. Int J Biol Macromol 2017;98:576-81.
32. Aherne SA, O'Brien NM. Dietary flavonols: Chemistry, food content, and metabolism. Nutrition 2002;18:75-81.
33. Daubney J, Bonner PL, Hargreaves AJ, Dickenson JM. Cardioprotective and cardiotoxic effects of quercetin and two of its *in vivo* metabolites on differentiated h9c2 cardiomyocytes. Basic Clin Pharmacol Toxicol 2015;116:96-109.
34. Lori A, Perrotta M, Lembo G, Carnevale D. The Spleen: A hub connecting nervous and immune systems in cardiovascular and metabolic diseases. Int J Mol Sci 2017;18:1216.
35. Quakyi EK, Frasch CE, Buller N, Tsai CM. Immunization with meningococcal outer-membrane protein vesicles containing lipooligosaccharide protects mice against lethal experimental group B Neisseria meningitidis infection and septic shock. J Infect Dis 1999;180:747-54.
36. Sun Y. Structure and biological activities of the polysaccharides from the leaves, roots and fruits of Panax ginseng C.A. Meyer: An overview. Carbohydrate Polymers 2011;85:490-9.
37. Gizinski AM, Fox DA. T cell subsets and their role in the pathogenesis of rheumatic disease. Curr Opin Rheumatol 2014;26:204-10.
38. Feng Y, Li B, Li XY, Wu ZB. The role of autophagy in rheumatic disease. Curr Drug Targets 2018;19:1009-17.
39. Carrero P, Ortega H, Martinez-Botas J, Gomez-Coronado D, Lasuncion MA. Flavonoid-induced ability of minimally modified low-density lipoproteins to support lymphocyte proliferation. Biochem Pharmacol 1998;55:1125-9.
40. Li X, Liu HC, Yao QY, Xu BL, Zhang SC, Tu CT. Quercetin protects mice from ConA-induced hepatitis by inhibiting HMGB1-TLR expression and down-regulating the nuclear factor Kappa B pathway. Inflammation 2016;39:96-106.