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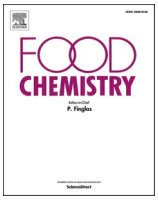

In vitro antioxidant analysis of flavonoids extracted from *Artemisia argyi* stem and their anti-inflammatory activity in lipopolysaccharide-stimulated RAW 264.7 macrophages

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ABSTRACT

In this study, flavonoids were successfully extracted from *Artemisia argyi* stem, and their yield reached 15.3 mg/g dry *A. argyi* stem. The flavonoid extract from *A. argyi* stem had a purity of 88.58 % (w/w), meanwhile, which also contained 1.57 % (w/w) carbohydrates, 2.04 % (w/w) proteins and 7.81 % (w/w) polyphenols, respectively. *In vitro* antioxidant activity analysis showed the increased scavenging effects of flavonoid extract from *A. argyi* stem on 1,1-diphenyl-2-picrylhydrazyl, 2,2′-azinobis-di-(3-ethyl-benzothiazolin-6-sulfonic acid) diammonium salt, hydroxyl, and superoxide radicals in a concentration-dependent manner. Furthermore, the flavonoid extract from *A. argyi* stem exerted protective effects on lipopolysaccharide-stimulated RAW 264.7 macrophages via inhibiting the levels of tumor necrosis factor-alpha, interleukin-6, interleukin-1 beta, and nitric oxide free radicals. Overall, this work will provide guidance and help in the utilization of edible *A. argyi* as plant-based diet and its bioactive flavonoid extract as antioxidant and anti-inflammatory ingredients to improve the function, nutrition, and healthiness of foods.

1. Introduction

An increasing number of scholars around the world are focusing on the improvement of the function, nutrition, and healthiness of foods via changing their structure and composition, especially with the addition of edible foods ingredients or their bioactive substances. As the second largest number of metabolites in plants, flavonoids not only have good biological activities and have frequently been used for the prevention and treatment of diseases, but can also serve as motivation for healthy foods development (Shen et al., 2022). Hao, Li, Li, Sun-Waterhouse, and Li (2022) observed that the isovitexin and vitexin extracted from mung bean hull can inhibit skin aging and damage via alleviating oxidative injury and autophagy. Duan et al. (2021) verified that with the regulation of bile acid metabolism and gut microbiota by administration of whole-grain oat flavonoids, the hyperlipidemia symptom of high fat diet-induced mice was alleviated. Wang et al. (2021) reported that the depression-like behavior of mice can be alleviated by supplementation of soy isoflavones, which affect the gut microbiota. Hu et al. (2022) revealed that dietary flavonoids can ameliorate androgen profiles and disorders. Wei et al. (2022) reported that the marijuana-induced vascular inflammation can be attenuated by the administration of genistein. Furthermore, Huang, Wu, and Chen (2022) indicated that dandelion flavonoids can improve the physicochemical properties of cooked potato starch and enhance its *in vitro* digestibility. Therefore, the application of flavonoids and flavonoid-rich edible ingredients in foods must be developed and expanded for human nutrition and healthiness.

Artemisia argyi not only has more than 500 species and belongs to *Asteraceae* family but is also an edible and medicinal plant distributed especially in Asia (Song et al., 2019). To date, numerous bioactive substances have been extracted from *A. argyi*. Ruan et al. (2022)

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observed that the acidic polysaccharides extracted from *A. argyi* leaves have scavenging capability for 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radicals. Zhang, Wang, Jia, Scarlett, and Sheng (2019) revealed that the sesquiterpenoids extracted from *A. argyi* leaves have inhibitory activities against cyclooxygenases. Huang, Wang, Yih, Chang, and Chang (2012) reported that the *A. argyi* leaf essential oil showed anti-melanogenic and antioxidant activities. Melguizo-Melguizo, Diaz-de-Cerio, Quirantes-Piné, Švarc-Gajić, and Segura-Carretero (2014) verified that the phenolic compounds extracted from *A. vulgaris* leaves have radical-scavenging capability. Meanwhile, Lv, Li, and Zhang (2018) suggested that the flavonoids extracted from *A. argyi* leaves have anticoagulation activities. However, the active compounds reported above are mainly extracted from *A. argyi* leaves, and the stem is often ignored and discarded, thus causing waste of resources and environmental pollution.

As an edible and medicinal plant, *A. argyi* is often used as a whole. Therefore, we hypothesized that the stem, which accounts for most of *A. argyi*, also contains several bioactive substances. Hence, in the present work, *A. argyi* stems were used for flavonoid (*A. argyi* stem flavonoids (AASFs)) extraction and analysis. On the one hand, the components and types of AASFs were broadly identified. On the other hand, the *in vitro* antioxidant effects of AASFs on 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis-di-(3-ethyl-benzothiazolin-6-sulfonic acid) diammonium salt (ABTS), hydroxyl, and superoxide radicals were assayed. Finally, the protective effects of AASFs on lipopolysaccharide-induced RAW 264. 7 cells were investigated. This work may contribute to the utilization of *A. argyi* as a plant-based diet constituent and its bioactive AASFs as antioxidant and anti-inflammatory additives to improve the function, nutrition, and healthiness of foods.

2. Materials and methods

2.1. Materials and chemicals

Artemisia argyi (planted in Yuzhou of Henan province and harvested in September 2018; product lot number: 201801) was purchased from Yuzhou Baicaohui Pharmaceutical Co. Ltd. (Xuchang, China), and its leaves and roots were removed to obtain the stem. Standard flavonoids, including catechin, epicatechin, rutin, vitexin, luteolin, quercetin, apigenin, kaempferol, naringenin, naringenin chalcone, (+)-dihydrokaempferol, (+)-dihydroquercetin, isorhamnetin, dihydromyricetin, kaempferol-3-*O*-glucoside, and quercetin 3-*β*-D-glucoside, were analytical grade and bought from Sigma-Aldrich (Shanghai, China). RAW 264.7 macrophages were preserved and cultured as previously reported (Wang et al., 2021). AB-8 macroporous resin (M0042), lipopolysaccharide from *Escherichia coli* 055:B5 (LPS, L8880, purity ≥ 98 %), streptomycin (S8290, potency: 650–850 mcg/mg), penicillin (P8010, potency: 1580 units/mg), high-glucose Dulbecco's Modified Eagle's Medium (DMEM, D6570), fetal bovine serum (FBS, S9020), DPPH (D9370, purity ≥ 97 %), and ABTS (A9590, purity ≥ 98 %) were bought from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Enzyme-linked immunosorbent assay kits for the detection of cell proliferation and cytotoxicity (Cell Counting Kit-8 (CCK-8, CA1210)), tumor necrosis factor-alpha (TNF-α, SEKM-0034, sensitivity:7 pg/mL), interleukin-6 (IL-6, SEKM-0007, sensitivity: 4 pg/mL), interleukin-1 beta (IL-1β, SEKM-0002, sensitivity: 4 pg/mL), and nitric oxide (NO, BC1475, sensitivity: 0.2 μmol/L) free radical levels were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Ethanol, ascorbic acid (Vc), $NaNO_2$, $AlCl_3$, NaOH, methanol, acetonitrile, acetic acid, pyrogallic acid, $FeSO_4$, H_2O_2, potassium persulfate, *ortho*-hydroxybenzoic acid, and other chemicals were of analytical grade and bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Extraction of AASFs

AASFs were extracted in accordance with the method reported by Yang et al. (2022) with several modifications. First, *A. argyi* stems were cut into about 10 cm pieces and dried in an oven until constant weight at 60 °C. The dried *A. argyi* stem pieces were shattered by a muller (RT-34, Hongquan Pharmaceutical Machinery Ltd., Hong Kong, China), collected with a 40 mesh sieve, and used for AASFs extraction using the ethanol refluxing method as follows: Moderately sieved stems and three volumes of 70 % (V: V) ethanol were added into a Florence flask and heated at 80 °C for reflux leaching (DH2800, Dehoo Chuangrui scientific instruments Co., Ltd., Qingdao, China) for 2 h, after which the filtered liquor was collected by vacuum filtration (SHZ-DIII, Shanghai Yuhua Instrument Equipment Co., Ltd., Shanghai, China), and the residue was re-extracted twice with ethanol refluxing method. The distillate was re-dissolved in 70 % (V:V) ethanol with magnetic stirring (WH280-NH, WIGGENS Co., Ltd., Straubenhardt, Germany) under room temperature for 1 h and centrifuged (5424R, Eppendorf Co., Ltd., Hamburg, Germany) at 10,000 r/min for 10 min to collect the supernatant. Then, the supernatant was absorbed with AB-8 macroporous resin (the column was 1.8 cm × 50 cm, and the loading height was 30 cm) at 1.0 mL/min at room temperature. Finally, the column was re-rinsed with 30 % ethanol (V: V), and the eluant was collected, concentrated, evaporated at 45 °C under vacuum, and lyophilized (ModulyoD-230, Thermo Fisher Scientific, Waltham, USA) to obtain AASFs.

2.3. AASFs content and composition determination

The content of flavonoid in AASFs was determined using the chemical coloration method (Chen et al., 2020). Briefly, 0.1 mg AASFs were dissolved in 1.0 mL methanol and pumped at 60 °C for 4 h. Then, 0.5 mL crude extract was added to 4.5 mL methanol, followed by the addition of 0.3 mL $NaNO_2$ (5 %, W/V). Then, the mixture was kept at room temperature for 5 min. Afterward, 0.3 mL 0.1 mol/L $AlCl_3$ was added, and the mixture was kept at room temperature for another 6 min. Then, 4.0 mL 1.0 mol/L NaOH was added, and the reaction system was added with distilled water to a volume of 10 mL and kept at room temperature for 15 min. Finally, a microplate reader (Multiskan MK3, Thermo Fisher Scientific, Waltham, USA) was used to detect the absorbance of the mixture at 510 nm, and the mixture without the addition of $AlCl_3$ was used as control. Meanwhile, contents of carbohydrates, proteins, and polyphenols in AASFs were detected using the phenol sulfuric acid (Ruan et al., 2022), Coomassie brilliant blue (Wang et al., 2021), and Folin–Ciocalteu (Škerget et al., 2005) methods, respectively.

Furthermore, flavonoid types in AASFs were detected using the liquid chromatography (LC)–mass spectrometry (MS) reported by Zhou et al. (2019) with slight modifications. Approximately 100 mg AASFs were extracted in 2 mL 70 % aqueous methanol (V/V) using ultrasonic waves for 30 min at a time for a total of 2 h. Then, the mixed extracts were concentrated to near dryness on a rotary evaporator at 35 °C under reduced pressure. Before analysis, the residue was dissolved in 200 μL 50 % aqueous methanol (V/V) and transferred to insert-equipped vials. The sample extracts were analyzed using an ultraperformance LC (UPLC)-Orbitrap-MS system (UPLC, Vanquish; MS, QE). The analytical conditions were as follows: UPLC, column, Waters HSS T3 (50 × 2.1 mm^2, 1.8 μm); column temperature, 40 °C; flow rate, 0.3 mL/min; injection volume, 2 μL; solvent system, water (0.1 % acetic acid): acetonitrile (0.1 % acetic acid); gradient program, 90:10 V/V at 0 min, 90:10 V/V at 2.0 min, 40:60 V/V at 6.0 min, 40:60 V/V at 8.0 min, 90:10 V/V at 8.1 min, and 90:10 V/V at 12.0 min. HRMS data were recorded on a Q Exactive hybrid Q-Orbitrap mass spectrometer equipped with a heated electrospray ionization (ESI) source (Thermo Fisher Scientific, Waltham, USA) utilizing the SIM-MS acquisition methods. The ESI source parameters were set as follows: spray voltage, −2.8 kV; sheath gas pressure, 40 arb; aux gas pressure, 10 arb; sweep gas pressure, 0 arb; capillary temperature, 320 °C; aux gas heater temperature, 350 °C. Data were

acquired on the Q-Exactive using Xcalibur 4.1 (Thermo Fisher Scientific, Waltham, USA) and processed using TraceFinder™4.1 Clinical (Thermo Fisher Scientific, Waltham, USA).

2.4. In vitro antioxidant activity analysis

AASFs were dissolved in sterilized water to a concentration of 1.0 mg/mL and stirred at 100 r/min for 1 h at room temperature. Then, the solution was centrifuged at 10,000 r/min for 10 min to collect the supernatant, and its concentration was identified as 1.0 mg/mL. Next, the supernatant was diluted to 0.8, 0.6, 0.4, and 0.2 mg/mL with sterilized water, after which the *in vitro* scavenging effects of AASFs on DPPH, ABTS, hydroxyl, and superoxide radicals were detected. Meanwhile, Vc (ascorbic acid) was dissolved in sterilized water to 1.0, 0.8, 0.6, 0.4, and 0.2 mg/mL concentrations and used as positive control.

2.4.1. DPPH radical-scavenging activity

The DPPH radical-scavenging activity of AASFs was detected following the method reported by Wang et al. (2019). In brief, 4 mg DPPH was dissolved in 100 mL ethanol in a brown-glass volumetric flask. Then, 2 mL AASFs solutions of various concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL) were added with 2 mL alcoholic DPPH solution. Next, the system was mixed fully in the dark for 30 min at room temperature and measured for absorbance at 517 nm. DPPH radical-scavenging activity (%) = $[1 - (A_i - A_j)/A_0] \times 100$ %, where A_0 is the absorbance of the control group without AASFs solution, A_i is the absorbance of AASFs solution, and A_j is the absorbance of background without the DPPH radical.

2.4.2. Superoxide anion-scavenging activity

The superoxide anion-scavenging activity of AASFs was detected by pyrogallic acid method (Wang et al., 2021). In brief, 2 mL AASFs solutions of various concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL) were added with 4.5 mL 1 mol/L Tris-HCl buffer (pH = 8.2). The mixtures were reacted in a 50 °C water bath (HH-8, Changzhou Yineng Experimental Instrument Co., Ltd., Jiangsu, China) for 30 min. Then, 1 mL 2 mmol/L pyrogallic acid was added, and the reaction was continued in a 50 °C water bath for another 10 min. Finally, the reaction was quenched by the addition of 0.5 mL HCl, and absorbance was measured at 380 nm. Superoxide anion-scavenging activity (%) = $(1 - A/A_0) \times 100$ %, where A is the absorbance of AASFs solution, and A_0 is the absorbance of the control group without AASFs solution.

2.4.3. Hydroxyl radical-scavenging activity

For detection of hydroxyl radical-scavenging activity of AASFs, the method reported by Wang et al. (2019) was applied with several modifications. Briefly, 2 mL AASFs solutions of various concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL), 2 mL $FeSO_4$ (9 mmol/L), and 2 mL H_2O_2 (9 mmol/L) were added into a 25 mL centrifuge tube. Afterward, the system was mixed well and reacted at room temperature for 10 min. Then, 2 mL *ortho*-hydroxybenzoicacid (9 mmol/L) was added and mixed with the system, and the reaction was continued for another 30 min at room temperature. Finally, absorbance of the mixture was measured at 510 nm. Hydroxyl radical-scavenging activity (%) = $1 - [(A_i - A_j)/A_0] \times 100$ %, where A_0 is the absorbance of AASFs solution replaced by distilled water, A_i is the absorbance of AASFs solution, and A_j is the absorbance of H_2O_2 replaced by distilled water.

2.4.4. ABTS radical-scavenging activity

The ABTS radical-scavenging activity of AASFs was detected in accordance with a method reported previously (Wang et al., 2020). Before the experiment, ABTS was added to 0.2 mol/L phosphate buffer (pH = 7.4) to a concentration of 7.4 mmol/L. Then, 2.6 mmol/L potassium persulfate was added, and the compounds were mixed and reacted in the dark at room temperature for 16 h. Afterward, the mixture was diluted with phosphate buffer (pH = 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm. Then, 0.2 mL AASFs solutions of various concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL) and 2.0 mL ABTS radical solution were added into a 5 mL centrifuge tube. After the system was mixed vigorously and reacted in the dark at room temperature for 20 min, the absorbance was measured at 734 nm. ABTS radical-scavenging activity (%) = $[1 - (A_i - A_j)/A_0] \times 100$ %, where A_0 is the absorbance of the control group without AASFs solution, A_i is the absorbance of the AASFs solution, and A_j is the absorbance of background without ABTS radical.

2.5. Toxicity of AASFs on RAW 264.7 macrophages

The toxicity of AASFs on RAW 264.7 macrophages was detected following a previously reported method (Wang et al., 2021) with slight modifications. Meanwhile, in accordance with the work of Yang et al. (2022), AASFs were dissolved in DMEM solution before analysis with 100, 200, 300, 400, 500, and 600 μg/mL concentrations. Briefly, RAW 264.7macrophages were cultured in DMEM with 10 % (V/V) FBS, 100 U/mL penicillin, and 100 U/mL streptomycin at 37 °C in a humidified 5 % CO_2 incubator. Afterward, RAW 264.7 macrophages with a concentration of 2×10^4 cells/mL were transferred to 96-well plates and incubated at 37 °C in 5 % CO_2 for 24 h. Then, 100 μL different concentrations of AASFs (100–600 μg/mL) were also added to the wells for 24 h. Next, 10 μL CCK-8 solution was added to each well, and the plates were incubated in the dark for another 1 h. Lastly, the microplate reader (Multiskan MK3, Thermo Fisher Scientific, Waltham, USA) was used to detect the absorbance of supernatant in each well at 450 nm to analyze the toxicity of AASFs, and DMEM without dissolved AASFs was used as control.

2.6. Anti-inflammatory analysis of AASFs in LPS-induced RAW 264.7 macrophages

The protective effect of AASFs on LPS-induced RAW 264.7 macrophages was detected in accordance with a previously reported method (Wang et al., 2021). In brief, RAW 264.7 macrophages with a concentration of 2×10^4 cells/mL in 96-well plates were divided into control, LPS-stimulated model, and administration groups. The control group contained normally cultured macrophages with DMEM, and the LPS-stimulated model group was cultured with 1 μg/mL LPS for 24 h. For the administration group, RAW 264.7 macrophages were first treated with 100, 200, and 400 μg/mL AASFs DMEM solutions for 2 h and then stimulated with 1 μg/mL LPS for another 24 h. Finally, the levels NO, TNF-α, IL-6, and IL-1β in the supernatants of RAW 264.7 macrophages were detected following the kits' instructions.

2.7. Statistical analysis

Data were expressed as mean ± standard deviation with triple repeats. Data were subjected to one-way analysis of variance (ANOVA), and significant differences were analyzed using SPSS version 19.0 (IBM Company, USA).

3. Results and discussion

3.1. AASFs component determination

Given that *A. argyi* is an edible and medicinal plant, reports on bioactive compounds mainly focused on its leaves, whereas the stem is often ignored and discarded. In the present work, after being extracted by 70 % (V:V) ethanol and with AB-8 macroporous resin purification, flavonoids (AASFs) were extracted from *A. argyi* stems. Given the difference in the extraction process (Li, Zhou, Yang, & Meng, 2018) and *A. argyi* species (Song et al., 2019), the yield of AASFs in the present work was 15.3 mg/g dry *A. argyi* stem, which was considerably lower

than the flavonoid content of *A. argyi* (Song et al., 2019). Meanwhile, component determination results showed that the purity of AASFs was 88.58 % (w/w), and 1.57 % (w/w) carbohydrates, 2.04 % (w/w) proteins, and 7.81 %(w/w) polyphenols were detected. Yang et al. (2022) reported that in addition to the 80.09 ± 0.60 % flavonoid present in the flavonoids extracted from fruits of *Lycium barbarum* (LBFs), they detected 4.09 ± 0.63, 4.78 ± 0.82, and 11.04 ± 1.25 % proteins, carbohydrates, and polyphenols, respectively. Although the flavonoid content in LBFs is lower than that in AASFs, LBFs also contain four components, namely, flavonoids, carbohydrates, proteins, and polyphenols, with polyphenols accounting for the second largest proportion of LBFs after flavonoids due to their high solubility in water and ethanol (Li et al., 2014).

3.2. AASFs composition detection

Table 1 shows that catechin, epicatechin, rutin, vitexin, luteolin, quercetin, apigenin, kaempferol, naringenin, (+)-dihydrokaempferol, (+)-dihydroquercetin, isorhamnetin, kaempferol-3-*O*-glucoside, and quercetin 3-*β*-D-glucoside were detected in AASFs, but naringenin chalcone and dihydromyricetin were not. Meanwhile, rutin, vitexin, quercetin 3-*β*-D-glucoside, kaempferol-3-*O*-glucoside, and luteolin were the main flavonoid types in AASFs, and their contents were higher than 50 mg/g AASFs. Furthermore, the total flavonoid content was 651.3158 mg/g AASFs and lower than the purity of 88.58 % (w/w), which indicated that there might some types of flavonoid were not detected. Given that different organs are used for flavonoid extraction, the composition of flavonoids can vary greatly. Han et al. (2017) observed that 5,7,4′,5′-tetrahydroxy-6,3′-dimethoxyflavone, 7,3′,5′-tetrahydroxy-6,4′-dimethoxyflavone, casticin, centaureidin, and hispidulin were present in the flavonoids extracted from *A. argyi* leaves. Lee et al. (2018) verified that homoeriodictyol, 5,7,3′,4′-tetrahydroxyflavone and 3-dihydroisorhamnetin were purified from *A. argyi* leaves. Li et al. (2018) reported that quercetin, isoquercetin, kaempferol, and rutin are the main types of flavonolS in *A. argyi* leaves. In addition, several differences were observed in the flavonoid types in different plants. Zhang et al. (2019) observed that the typical flavonoid species in *Lycium barbarum* include rutin, hyperoside, kaempferol-3-*O*-rutinoside, scopoletin, quercetin, and isorhamnetin. Zhao, Zhang, and Shi (2021) reported the extraction of flavonols, namely, kaempferol, quercetin, isorhamnetin, and isoquercetin, from wolfberry fruit. This study is the first report on *A. argyi* stem flavonoids, and their activity was analyzed in the present work.

3.3. In vitro antioxidant activity analysis of AASFs

Radicals are closely related to body health; excessive radical levels can cause oxidative injury to large bio-molecules, including DNA, lipids, and proteins, thus resulting in diseases, such as diabetes, cardiovascular and cerebrovascular diseases, obesity, inflammation, and aging (Masisi, Beta, & Moghadasian, 2016). Dietary antioxidants from foods or their ingredients represent an efficient strategy to counteract or attenuate this situation. As shown in Fig. 1A and C, the scavenging effects of AASFs on ABTS radical and superoxide anions increased when their concentration increased from 0 mg/mL to 0.4 mg/mL, and at 0.4 mg/mL, the scavenging effects against ABTS radical and superoxide anions were 88.39 ± 2.16 % and 84.74 ± 3.01 %, respectively. Fig. 1B and D show that the scavenging effects of AASFs on DPPH and hydroxyl radicals increased from 0 mg/mL to 0.8 mg/mL, and their scavenging effects on DPPH and hydroxyl radicals at 0.8 mg/mL were 95.13 ± 3.82 % and 92.07 ± 2.48 %, respectively. Yang et al. (2022) reported that LBFs not only have scavenging effects on DPPH, ABTS, hydroxyl, and superoxide radicals but also ferric-reducing and total antioxidant capacities. The phenolic hydroxyl groups in flavonoids have a reducing property and can stabilize free radicals (Lou, Hsu, & Ho, 2014), which may be the reason for the antioxidant effect of flavonoids, which has been verified by numerous researchers (Hwang, Yoon, Lee, Cha, & Kim, 2014; Peng, Yang, Zhou, & Pan, 2015). Furthermore, the antioxidant effects of flavonoids are important for the life of people. Khan et al. (2017) observed that wogonin can activate the reactive oxygen species/extracellular signal-regulated kinase/Nrf2 pathways to mitigate and prevent osteoarthritis. Hao et al. (2022) suggested that the isovitexin and vitexin extracted from mung bean hull inhibit skin aging and damage via alleviating oxidative injury and autophagy. This work indicated that AASFs or edible *A. argyi* can be potentially used in foods to prevent and mitigate oxidative stress diseases of the human body.

Table 1
Identification of AASFs (data are means ± standard deviation, n = 3).

Flavonoid type	Content (mg/g of AASFs)
Catechin	0.3232 ± 0.0009
L-Epicatechin	0.4725 ± 0.0017
Dihydromyricetin	/
Rutin	148.2109 ± 0.0093
Vitexin	63.319 ± 0.0023
Quercetin 3-*β*-D-glucoside	136.3682 ± 0.0011
(+)-Dihydroquercetin	5.3633 ± 0.0007
Kaempferol-3-*O*-glucoside	75.2468 ± 0.0015
(+)-Dihydrokaempferol	0.7975 ± 0.0014
Luteolin	118.4234 ± 0.0129
Quercetin	11.1547 ± 0.072
Naringeninchalcone	/
Apigenin	36.5785 ± 0.0046
Naringenin	33.0674 ± 0.0113
Kaempferol	10.0011 ± 0.0008
Isorhamnetin	11.9893 ± 0.0062
Total flavonoid content	651.3158

Note: "/" means not detected.

3.4. Toxicity of AASFs on RAW 264.7 macrophages

The toxicity of AASFs toward the cell viability of RAW 264.7 macrophages was detected with CCK-8 kit, and the results are shown in Fig. 2. As displayed in Fig. 2, when the concentration of AASFs increased from 100 μg/mL to 400 μg/mL, the cell viability of RAW 264.7 macrophages was hardly affected, indicating the security of AASFs under detected concentrations (100–400 μg/mL). However, when the AASFs concentration increased to 500 μg/mL, the cell viability of RAW 264.7 macrophages decreased to 68.31 ± 1.75 %, and it further reduced to 39.04 ± 2.24 % when the AASFs concentration increased to 600 μg/mL; thus, the safe AASFs concentration was 0–400 μg/mL. Notably, Yang et al. (2022) also verified that LBFs showed no toxicity on RAW 264.7 macrophages at the concentration of 100–400 μg/mL, which was in agreement with the toxicity results in the present work. Considering the accuracy of experiments, 100, 200, and 400 μg/mL AASFs concentrations were used in the following anti-inflammatory tests.

3.5. Anti-inflammatory activity analysis of AASFs

Inflammation is the first bio-response to the immune system of infection or irritation, and prolonged inflammation may lead to fever, atherosclerosis, asthma, arthritis, and cancer. The hydroxyl (Lee, Kim, & Kim, 2014), methoxy (Chen, Tait, & Kitts, 2017), epoxy (Takano-Ishikawa, Goto, & Yamaki, 2006), and isopentene groups (Paoletti et al., 2009) in flavonoids can endow or enhance anti-inflammatory activity. Therefore, AASFs and flavonoid-rich *A. argyi* are expected to be used in foods to alleviate the body's inflammatory response via a plant-based diet. As shown in Fig. 3, compared with those in the control group, NO, TNF-α, IL-1β, and IL-6 levels in LPS-stimulated RAW 264.7 macrophages increased from 3.83 ± 0.48 μmol/L, 135.27 ± 19.03, 15.31 ± 2.12, and 5.95 ± 0.33 pg/mL to 35.26 ± 0.79 μmol/L, 1251.85 ± 33.89, 227.04 ± 3.38, and 43.82 ± 0.58 pg/mL after being treated by 1 μg/mL LPS for 24 h, respectively. When RAW 264.7 macrophages were first treated with different concentrations of AASFs for 2 h and then with 1 μg/mL LPS for another 24 h, the NO, TNF-α, IL-1β, and IL-6 levels in LPS-

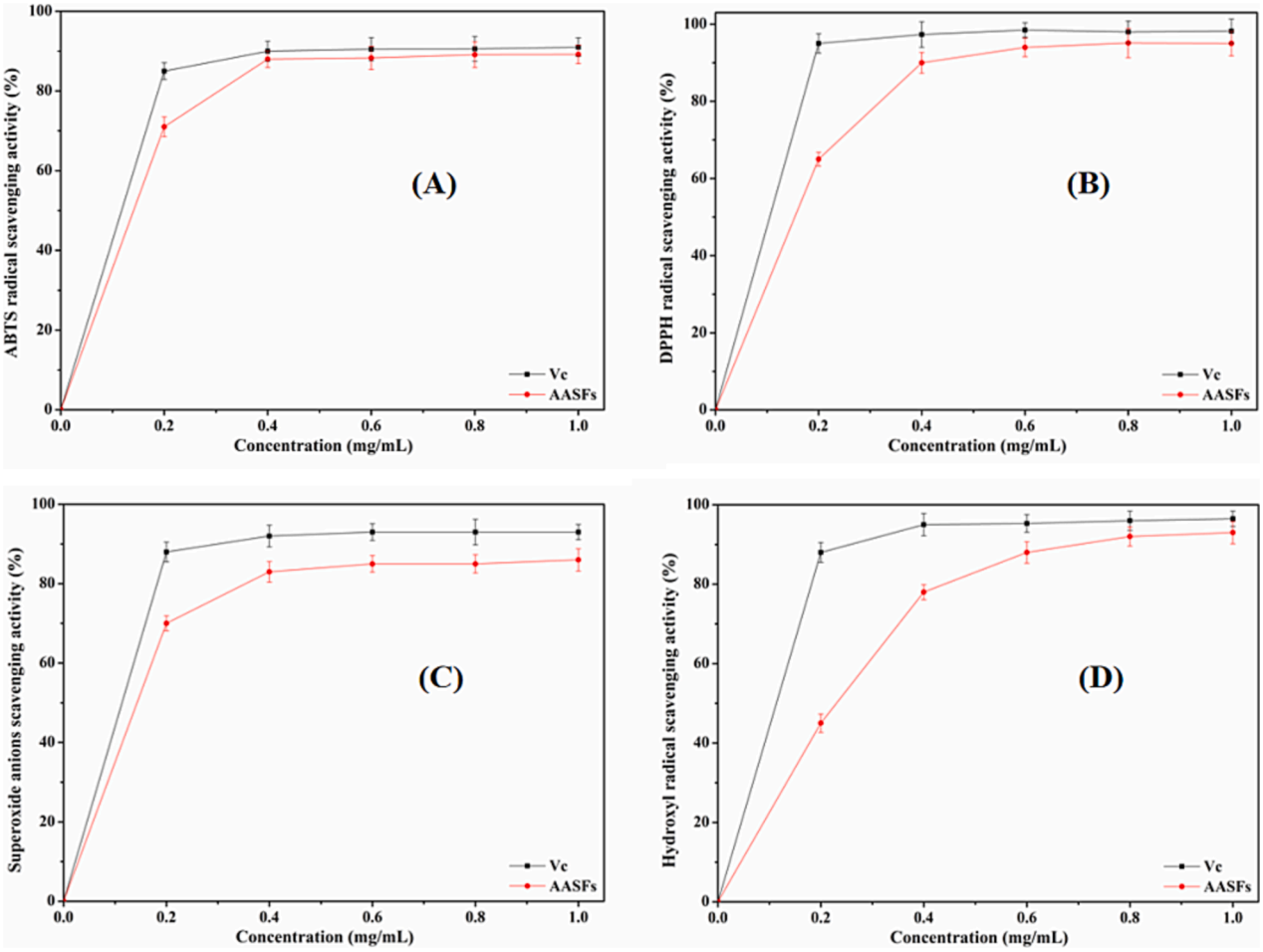

Fig. 1. Antioxidant effect analysis of AASFs *in vitro*. ABTS radical-scavenging activity (A), DPPH radical-scavenging activity (B), superoxide anion-scavenging activity (C), and hydroxyl radical-scavenging activity (D).

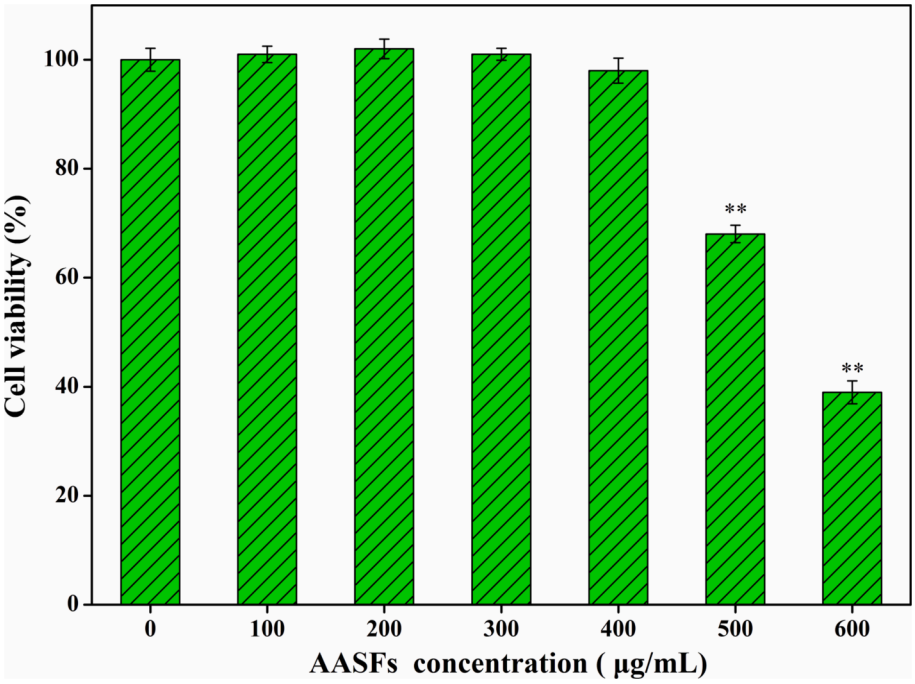

Fig. 2. Effect of AASFs on cell viability of RAW 264.7 macrophages (significance was determined through ANOVA; $^{*}P < 0.05$; $^{**}P < 0.01$, and $^{***}P < 0.001$).

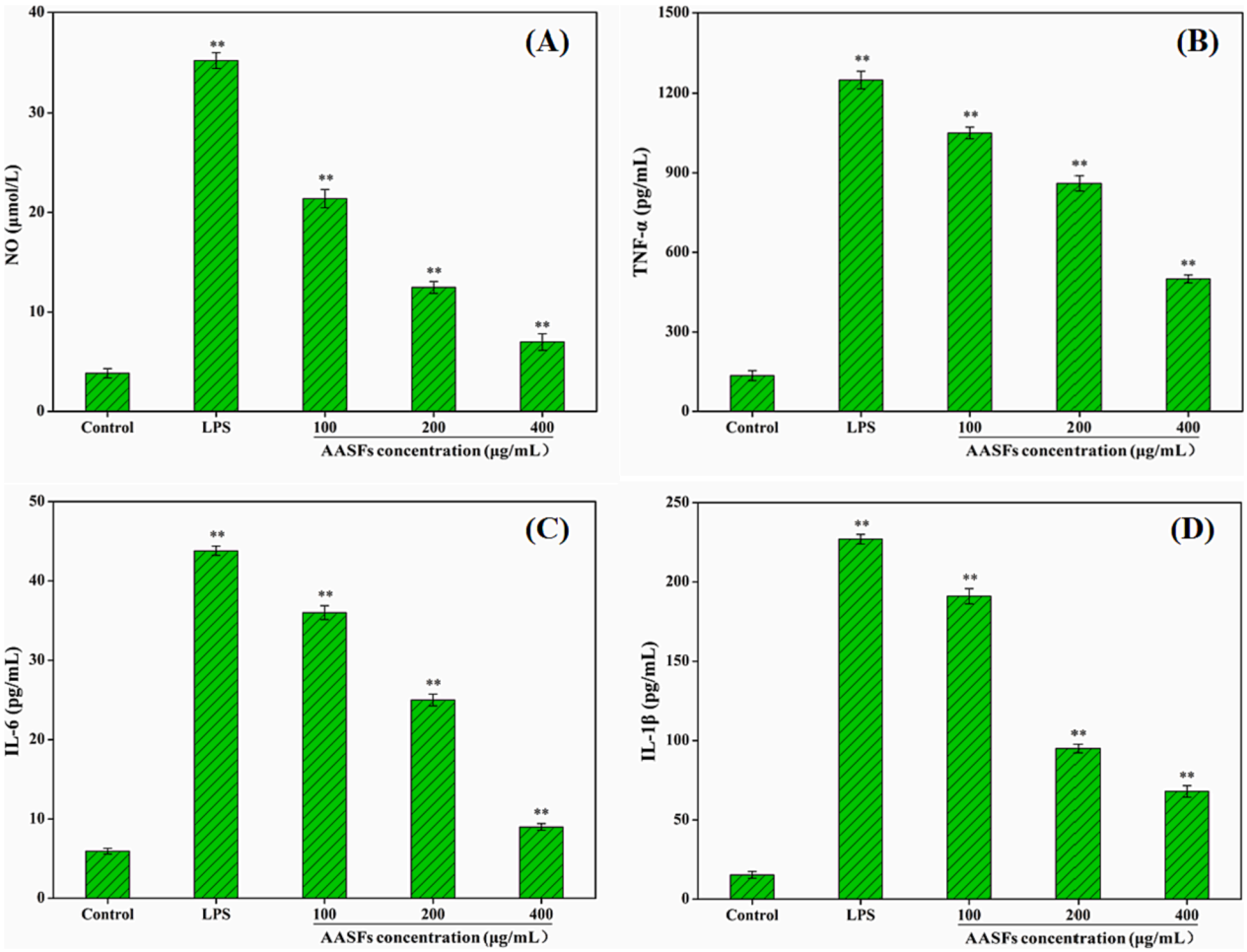

Fig. 3. Effects of AASFs on cytokine production of RAW 264.7 macrophages during inflammation. (A) NO, (B) TNF-α, (C) IL-6, and (D) IL-1β (significance was determined through ANOVA; $^{*}P < 0.05$; $^{**}P < 0.01$, and $^{***}P < 0.001$).

stimulated RAW 264.7 macrophages showed a dose-dependent decrease. Thus, AASFs exhibited a concentration-dependent anti-inflammatory activity on LPS-stimulated RAW 264.7 macrophages. When the treatment concentration of AASFs was 400 μg/mL, the NO, TNF-α, IL-1β, and IL-6 levels in LPS-stimulated RAW 264.7 macrophages measured 6.98 ± 0.83 μmol/L, 501.92 ± 15.77, 68.21 ± 3.63, and 9.04 ± 0.42 pg/mL, respectively. These results suggest that AASFs can effectively alleviate the inflammation of LPS-stimulated RAW 264.7 macrophages. A number of researchers obtained anti-inflammatory effects of flavonoids that are similar to those observed in the present work. Dong, Yin, Zhang, Fu, and Lu (2017) reported that ononin can alleviate LPS-stimulated inflammatory responses of RAW 264.7 macrophages via inhibiting nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) pathways. Tian et al. (2019) also revealed that flavonoids extracted from *Abutilon theophrasti* Medic. Leaves protected LPS-stimulated acute lung injury of mice via inhibiting the NF-κB and MAPK pathways. Coppin et al. (2013) showed that flavonoids from *Moringa oleifera* inhibited NO production in LPS-stimulated RAW 264.7 macrophages. Rong et al. (2021) verified that nobiletin can alleviate inflammatory responses of LPS-stimulated RAW 264.7 macrophages by activating the IL-6/signal transducer and activator of transcription 3/forkhead box O3a-mediated autophagy. Meanwhile, strawberry extract (Gasparrini et al., 2017), Capuli cherry extract (Alvarez-Suarez et al., 2017), and Manuka honey (Afrin et al., 2018; Gasparrini et al., 2018) also showed protective effects on LPS-induced damage in RAW 264.7 macrophages. Although AASFs can be potentially used as an ingredient in foods to attenuate and reduce the inflammation condition of the human body, their anti-inflammatory mechanism remains unclear.

4. Conclusion

In the present work, flavonoids were extracted from *A. argyi* stem (AASFs). Their yield reached 15.3 mg/g dry *A. argyi* stem, and the purity was 88.58 % (w/w). Meanwhile, the contents of carbohydrates, proteins, and polyphenols in AASFs were 1.57 % (w/w), 2.04 % (w/w), and 7.81 % (w/w), respectively. AASFs not only showed excellent antioxidant effects on DPPH, ABTS, hydroxyl, and superoxide radicals *in vitro* but also a protective effect on LPS-stimulated RAW 264.7 macrophages via inhibiting the NO, TNF-α, IL-1β, and IL-6 levels. Although the edible *A. argyi* and its bioactive flavonoids can be potentially used as antioxidant and anti-inflammatory ingredients to improve the function, nutrition, and healthiness of foods, the mechanism and structure–activity relationship of AASFs in exerting their antioxidant and anti-inflammatory activities are still unclear.

CRediT authorship contribution statement

Zichao Wang: Writing – original draft, Conceptualization. **Lu Wang:** Methodology, Resources. **Hongtao Huang:** Data curation, Software. **Qiuyan Li:** Formal analysis, Visualization. **Xiaoyuan Wang:** Conceptualization, Methodology. **Qi Sun:** Supervision, Writing – review & editing. **Qi Wang:** Writing – review & editing, Funding acquisition. **Na Li:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Further reading

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