

Anti-neuroinflammatory activity of Shenqi Fuzheng Injection and its main active constituents

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SUMMARY Enhancement of alternative activation (M2) in microglia is a promising therapeutic target for microglia-mediated neuroinflammation. Shenqi Fuzheng Injection (SFI) is a clinical adjuvant treatment for cancer to reduce the side effects during cancer treatment, including boosting mood and improving appetite. However, the mechanism of SFI's effects on central symptoms is not clear. Therefore, using arginase 1 (*Arg1*) and transforming growth beta-1 (*Tgfb1*) as markers for M2 microglia activation, we found that compounds **1**, **5**, **12**, **14**, and **15** are the major M2-promoting constituents in SFI, which significantly upregulated *Arg1* or *Tgfb1* gene expression. Our results suggested that these compounds in SFI may promote M2 microglial activation and have potential uses in modulating microglial activation and alleviating neuroinflammation.

Keywords Neuroinflammation, Shenqi Fuzheng Injection (SFI), Arginase 1 (*Arg1*), Transforming growth factor beta-1 (*Tgfb1*), microglia

1. Introduction

Neuroinflammation contributes to most, if not all, neurodegenerative diseases, such as multiple sclerosis (1-3), Parkinson's disease (4,5), Alzheimer's disease (6,7), and Huntington's disease (8,9). As the resident innate immune cell in the central nervous system (CNS), microglia surveils brain parenchyma's homeostatic environment by phagocytosing the cell and myelin debris and facilitating neuronal tissue repair. In recent studies, elevated microglial activity is also recognized in psychiatric disorders (10,11), such as major depression (12,13), bipolar disease (14,15), and schizophrenia (16,17). Under various physiopathological conditions, microglia respond to the stress signals and serves as a critical mediator in local neuroimmune regulation.

The rapid activation of microglia is heterogeneous and can be generalized into two major phenotypes: the classic pro-inflammatory M1 phenotype and the alternative protective M2 phenotype. M1 microglial cells release pro-inflammatory cytokines and reactive oxygen species, resulting in impaired mitochondrial function, disrupted bioenergetic homeostasis, and aggravated neuroinflammation. Elevated expression of inducible nitric oxide synthase (iNOS) and NADPH

oxidase in M1 activation can further mediate local neurons and glia' cell death (15,18). Therefore, inhibiting M1 microglia activation is under investigation as a therapeutic target for neurodegenerative diseases and psychiatric disorders.

While chronic activation of M1 microglia is a hallmark of neuroinflammation, M2 polarization is considered to take the neuroprotective role by producing anti-inflammatory factors, such as arginase 1 (ARG1) and transforming growth factor beta-1 (TGFB1) (19). ARG1 is one of the canonical activation markers of M2 microglia. It blocks nitric oxide production by competing with iNOS for the same substrate L-arginine, thus reducing the cytotoxic effects during pathological events in the CNS. Alteration in arginine metabolism has been implicated in patients with schizophrenia and Alzheimer's disease in clinical studies (20). By converting L-arginine to the downstream neurotrophic L-ornithine and polyamines (18), ARG1 has been associated with neuroprotective functions, such as neurite growth, tissue repair, and secretion of anti-inflammatory cytokines. TGFB1 is another anti-inflammatory cytokine and an activation marker of M2 microglia. It has a dampening effect on activated pro-inflammatory immune cells to reduce

the cytotoxicity effects and protect neurons and glial cells (21,22). TGF- β is a highly conserved multipotent cytokine and has three isoforms: TGF- β 1, TGF- β 2, and TGF- β 3. All belong to the TGF- β superfamily, which contributes to the CNS neurogenesis, homeostasis, and response to tissue injury (23,24). *In vitro* treatment with TGF- β 1 reduces excitatory neuronal injury, and *in vivo* pretreatment with TGF- β 1 before vascular occlusion reduced ischemic areas, suggesting that TGF- β 1 plays a protective role against neuroinflammation (25). Besides, TGF- β also promotes neuronal survival and proliferation. For example, TGF- β 2 perfusion can reduce the death of mature motor neurons caused by injury, which is more potent than glial cell-derived neurotrophic factor (GDNF) or brain-derived neurotrophic factor (BDNF) (26). Hence, promoting microglia to the M2 phenotype provides potential therapeutic benefits and is an attractive alternative strategy against neuroinflammation.

Shenqi Fuzheng injection (SFI) is an herbal extract of Dangshen (*Radix Codonopsis*), and Huangqi (*Radix Astragali Mongolici*) developed from Traditional Chinese Medicine (TCM) and has been widely used as adjuvant therapy for cancer patients in chemotherapy and radiation therapy. For example, SFI reduced radiation-induced brain injury by modulating inflammatory factors to achieve neuroprotective effects (27,28). The alleviation of cancer-related depression-like behaviors after SFI treatment was found in a murine model (29). Many clinical cases also support that SFI has significant effects on ameliorating some of the side effects of cancer treatment, including boosting mood, improving appetite, and strengthening immunity (30-33). These studies indicate that SFI may be associated directly or indirectly with neuroprotective roles against CNS-related inflammation during cancer treatment.

In the present study, we evaluated the effects of SFI using a murine microglial cell line (BV-2 cell) based on the interleukin-4 (IL-4) induced alternative activation of M2 microglia. By evaluating the gene expression levels of M2-activation markers *Arg-1* and *Tgfb1*, we explored the bioactive compounds in SFI that might have anti-neuroinflammatory effects *via* the M2 microglia activation.

2. Materials and Methods

2.1. Materials

SPE Bond Elut Plexa was from Agilent (Santa Clara, CA, USA). Fetal bovine serum (FBS) was purchased from Gibco Ltd. (Grand Island, NY, USA). Dulbecco's modified Eagle's medium (DMEM) was from Hyclone (Logan, UT, USA). Lipopolysaccharides (LPS) from *Escherichia coli* O55:B5 was from Sigma-Aldrich Co. (St. Louis, MO, USA). Recombinant Mouse IL-4 was from R & D system (Minneapolis, MN, USA). CCK-

8 was from meilunbio (Dalian, Liaoning, China). RNAprep pure Cell/Bacteria Kit, FastKing gDNA Dispelling RT SuperMix, and SuperReal PreMix Plus (SYBR Green) were purchased from TIANGEN (Beijing, China). SFI was provided by Livzon Pharmaceutical Group Inc. (Zhuhai, Guangdong, China). Compounds **1-15** (purities $\geq 98\%$) were purchased from Nautre-Standard (Shanghai, China). 800TS Microplate Reader was from Bio-Tek (Winooski, VT, USA); Q5000 UV-Vis Spectrophotometer was from Quawell Technology, Inc. (San Jose, CA, USA); S1000 Thermocycler was from Bio-Rad (Hercules, CA, USA); Quantstudio 6 Flex Real-Time PCR System was from ThermoFischer Scientific (Waltham, MA, USA).

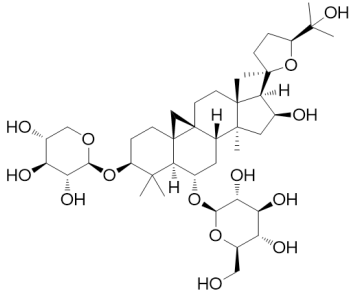
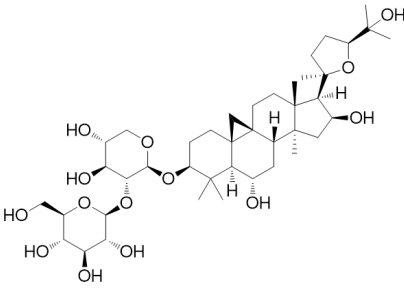
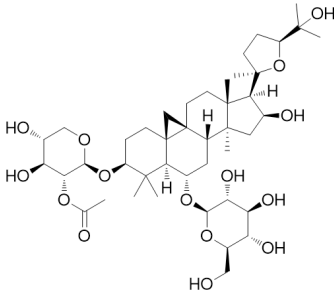
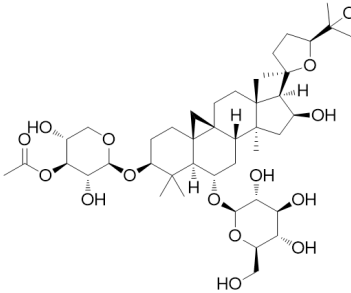
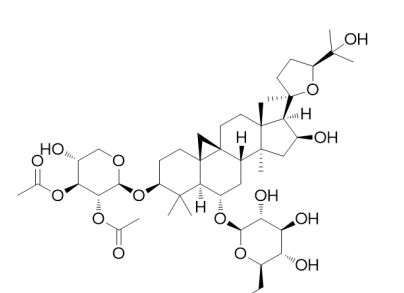
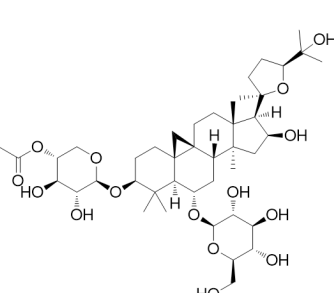
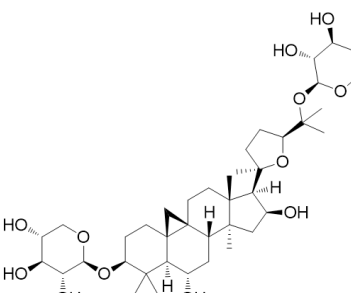
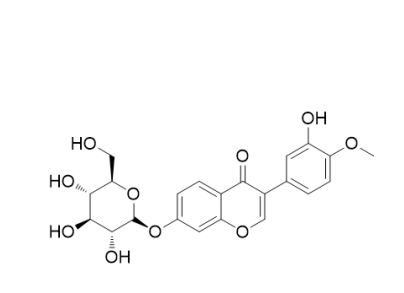
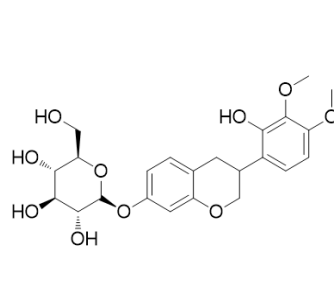
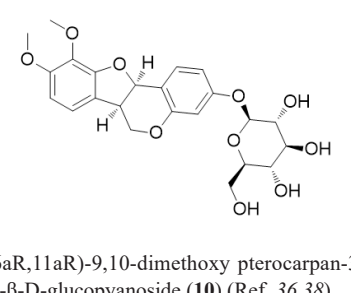
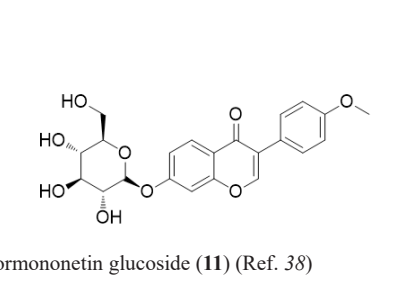
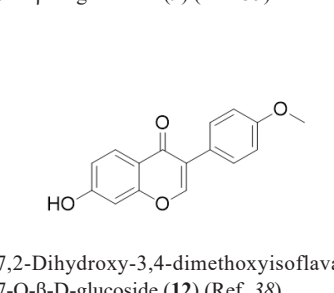
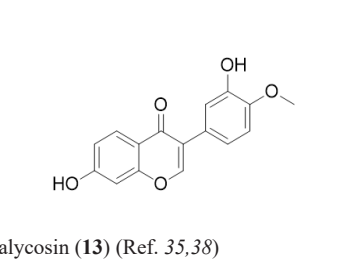
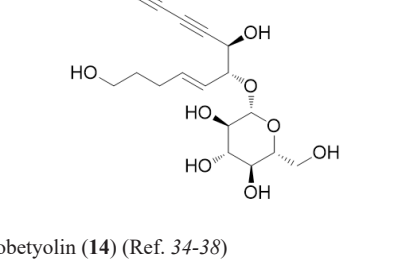
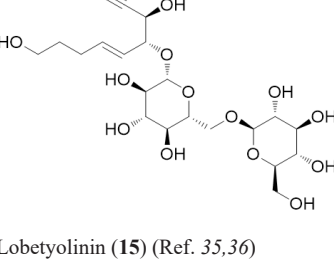
2.2. The preparation of AW and WW

The SPE column (filler: non-polar diethylene phenyl neutral polymer adsorbent, specification: 6 mL/200 mg, 45 μ m) was conditioned with methanol and water, and 15 mL SFI was added to the column and eluted with 10 mL water. The elute at this step was collected as the WW component. The column was further eluted with 6 mL methanol. The methanol was evaporated under heat, and the residue was resuspended in water and set to 5 mL in a volumetric flask. The resuspended portion was filtered and used as the AW component. The final solution passed through a 0.22 μ m filter before used in the cell culture. Compounds **1-15** are derived from AW components of SFI, which has been reported in previous literature (Table 1) (34-38). Table 1 was made using ChemOffice software (ver. 14.0).

2.3. Cell culture and treatment

Murine microglial cell line BV-2 was a kind gift from Professor Linyin Feng at the Shanghai Institute of Materia Medica. Cells were cultured in DMEM with 10% FBS, 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO₂. When reaching 80-90% confluency, they were passaged at a culture ratio of 1:5 and used during their logarithmic growth phase in the following biological experiments. Only cells under 15 passages were used. BV-2 microglia were pretreated with 10% (v/v) SFI, SFI alcohol- or water-washing components 10% (v/v), or compounds (200 nM) for 1 h before polarization was induced. LPS (100 ng/mL) was applied to induce the M1-polarization of microglia, and IL-4 at 20 ng/mL was added to induce M2-type activation. For M1 microglia related experiment, aspirin (1.2 mM) was included as a positive control for its anti-inflammatory activity. Pretreatment with IL-4 (20 ng/mL) was included as a positive control for enhancement of M2-polarization. An equal volume of 1% BSA in phosphate buffer saline, normal saline, and DMEM was used as the vehicle control for SFI, IL-4, and SFI compounds, respectively.

Table 1. The main components and their structures of SFI (Structure)

		
Astragaloside IV (1) (Ref. 34-38)	Astragaloside III (2) (Ref. 34-38)	Astragaloside II (3) (Ref. 34-38)
		
Isoastragaloside II (4) (Ref. 34-38)	Astragaloside I (5) (Ref. 35,38)	Cyclocephaloside II (6) (Ref. 35-37)
		
Isoastragaloside IV (7) (Ref. 34)	Calycosin-7-O- β -D-glucoside (8) (Ref. 34-38)	7,2-Dihydroxy-3,4-dimethoxyisoflavan 7-O- β -D-glucoside (9) (Ref. 35)
		
(6aR,11aR)-9,10-dimethoxy pterocarpin-3-O- β -D-glucopyranoside (10) (Ref. 36,38)	Formononetin glucoside (11) (Ref. 38)	7,2-Dihydroxy-3,4-dimethoxyisoflavan 7-O- β -D-glucoside (12) (Ref. 38)
		
Calycosin (13) (Ref. 35,38)	Lobetyolin (14) (Ref. 34-38)	Lobetyolinin (15) (Ref. 35,36)

2.4. Cell viability assay

Cells were seeded in 96-well culture plates at a density of 8,000 cells/well. After overnight incubation, the culture medium was replaced with fresh medium containing 5% FBS per well. Cells were pretreated and stimulated as mentioned in Section 4.3. The cells were incubated in a humidified atmosphere of 95% air and 5% CO₂ for 24 h. Before the viability assessment, the culture medium was replenished with 100 µL of serum-free culture medium, and 10 µL CCK-8 was added per well. The cells were further cultured at 37°C, and 5% CO₂ for 30 min and the absorbance (OD) was measured on a microplate reader at 450 nm. The cell viability of BV-2 cells was calculated using the following formula:

$$\text{Cell viability}\% = \frac{(\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}})}{(\text{OD}_{\text{control}} - \text{OD}_{\text{blank}})} \times 100\%$$

2.5. Real-Time Quantitative PCR (qPCR)

In the gene expression studies, BV-2 microglia were pretreated with 10% (v/v) SFI or its components, or SFI compounds at indicated concentrations for 1 h, followed by 4 h stimulation of IL-4 (20 ng/mL). At the end of the treatment, the culture medium was removed, and cells were washed twice with cold PBS. Cells were collected, and total RNA extraction and RT-qPCR analysis were performed according to the manufacturer's instructions. Briefly, total RNA was isolated using RNeasy Pure Cell/Bacteria Kit. Total RNA concentrations and purity was determined by a microvolume spectrophotometer. 1 µg of RNA was reverse transcribed to cDNA in a 20 µL mixture in the thermocycler, and cDNA was used as templates for qPCR on a Quantstudio 6 Flex Real-Time PCR System using a final reaction volume of 20 µL. Ct values were converted to $\Delta\Delta\text{Ct}$ values using *Gapdh* as the reference gene and normalized to the vehicle control group for each probed gene. The primers were synthesized by Beyotime Biotechnology. Primers for qPCR sequences are as follows (from 5' to 3'):

Arg1:

forward 5'-CTCCAAGCCAAAGTCCTTAGAG-3'
and reverse 5'-GCATCCACCCAAATGACACAT-3'.

Tgfb1:

forward 5'-GGTCCTTGCCCTCTACAACC-3'
and reverse 5'-CCACGTAGTAGACGATGCGC-3'.

Gapdh:

forward 5'-CTCCACTCACGGCAAATTC AACG-3'
and reverse 5'-AGGGGCGGAGATGATGACCC-3'.

2.6. Statistical analysis

The results are presented as the means \pm standard error (SD). Data were analyzed by one-way analysis

of variance (ANOVA) of the differences among treatments, and Dunnett's multiple comparisons test was applied for comparison between the treatment groups with the mode group using GraphPad Prism 9.0 (San Diego, CA, USA). Differences with P values less than 0.05 was considered statistically significant.

3. Results

3.1. Chemistry

Since the SFI showed anti-neuroinflammatory activity, we analyzed its components. By solid phase extraction (SPE) column chromatography, the extracts were divided into alcohol-washing (AW) part and water-washing (WW) part. The activity test showed that the active ingredients were mainly in the alcohol washing part. Further composition study revealed that fifteen compounds were the main components of alcohol washing (Table 1). Among the fifteen compounds, compounds 1-13 were from *Radix Astragali Mongolici* and belong to saponins and flavonoids, while compounds 14 and 15 were from *Radix Codonopsis* and belong to alkynyl glycosides. These compounds are obtained through commercial purchase.

3.2. Biological evaluation

First, the effect of SFI on the viability of BV-2 microglia was measured by the Cell Counting Kit-8 (CCK-8) assay, and the results showed that SFI did not alter the viability of LPS-stimulated M1 or interleukin-4 (IL-4)-stimulated M2 microglia (Figure 1). These results demonstrated that SFI did not interfere with the cell proliferation or inhibition of BV-2 microglia.

Then, the effects of SFI on LPS-induced M1 microglia were investigated. The cells were pretreated with SFI (10% v/v) for 1 h before LPS (100 ng/mL) stimulation for 4 h. Aspirin was used as the positive control for its anti-inflammatory activity. The results showed that tumor necrosis factor- α (*Tnf*) and interleukin-6 (*Il6*) expression levels (Figure 2A-B) in BV-2 microglia were significantly upregulated after LPS-stimulation. SFI significantly upregulated

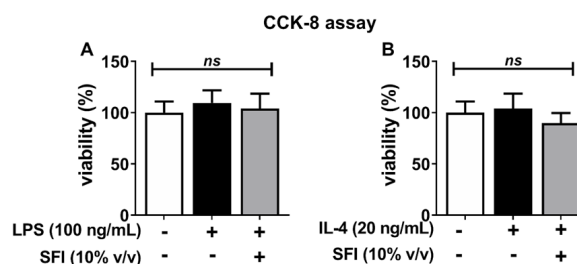


Figure 1. Effects of SFI (10% v/v) on cell viability of M1 and M2 microglia after 24 h treatment. All values were presented as mean \pm SD.

Il6 expression level (Figure 2B) but did not alter *Tnf* expression at 4 h (Figure 2B). Then, to further evaluate the effect of SFI on the function of M2 microglia, we pretreated BV-2 cells with SFI (10% v/v) for 1 h, and stimulated the cells with IL-4 (20 ng/mL) for 4 h. We measured the transcription levels of *Arg1* and *Tgfb1*, and the results showed that IL-4 significantly enhanced *Arg1* ($P < 0.0001$) and *Tgfb1* ($P < 0.01$) expression in BV-2 cells (Figure 2C-D). The combination of SFI and IL-4 significantly increased the transcriptional expression of *Arg1* and *Tgfb1* in BV-2 cells compared with IL-4 alone ($P < 0.05$) (Figure 2C-D). These results indicated that the anti-neuroinflammatory and neuroprotective effects of SFI may be achieved

via enhancing alternative activation (M2) rather than inhibiting the pro-inflammatory classic activation (M1).

To explore the material basis for the anti-neuroinflammatory compounds in SFI, we separated SFI into AW and WW components using an SPE column. The effects of both components on M2 microglia were evaluated, respectively. The results showed that the AW and WW components of SFI did not affect the cell viability of M2 microglia (Figure 3A). The AW components significantly increased the transcriptional expression of *Arg1* in BV-2 cells ($P < 0.05$), while the WW group did not affect the transcriptional expression of *Arg1* and *Tgfb1* (Figure 3B-C). These results demonstrated that for SFI, compounds with significant M2-promoting activity were in the AW component. The up-regulation of *Tgfb1* expression level was absent in the AW component (Figure 3B). It is possible that different compounds in the AW component have various regulatory effects on *Tgfb1* expression level in BV-2 cells, and the combined effects on *Tgfb1* expression level were not manifested. Therefore, the regulatory effect of the individual compound is worth further exploration.

As the AW component demonstrated M2-promoting activity, 15 compounds from the AW component were selected for further activity screening. None of the compounds showed inhibitory effects on M2 microglia cell viability at the concentration of 200 nM (Figure 4A). The 15 compounds demonstrated various capabilities in regulating transcription levels of *Arg1* and *Tgfb1*. Compounds 5 and 14 augmented the expression levels of both *Arg1* and *Tgfb1* ($P < 0.01$), while compounds 4, 6, and 7 posted an inhibitory effect on the expression of the M2 markers. Compounds 1, 9, 10, and 14 only increased the transcription level of *Arg1* ($P < 0.01$), while compounds 11, 12, and 15 only increased *Tgfb1* transcriptional expression ($P < 0.001$) (Figure 4B-C).

To measure the compounds' potency, compounds 1, 5, 12, 14, and 15 (Figure 5) were further examined for their regulatory effects on *Arg1* and *Tgfb1* expression level at doses of 12.5 nM, 50 nM, and 200 nM. Compounds 5 and 14 increased the expression

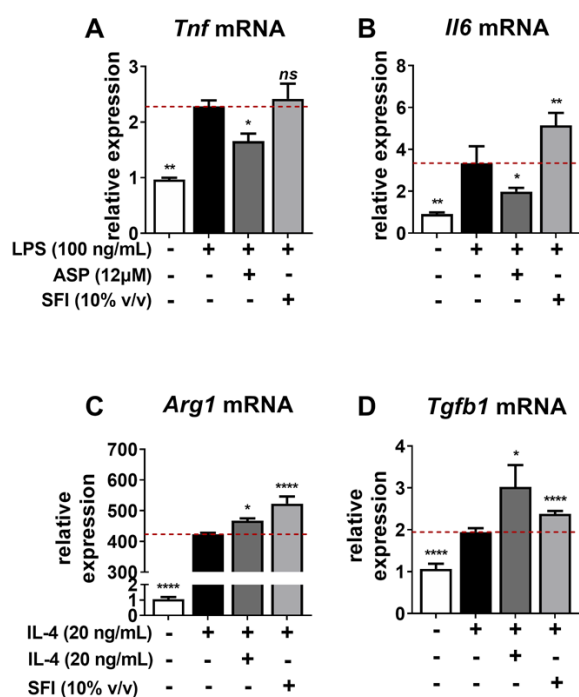


Figure 2. Effects of SFI on BV-2 microglia gene expression levels of M1- (A and B) and M2- (C and D) polarization markers. SFI showed significant promoting effects on the gene expression levels of BV-2 M2-microglia markers, arginase-1 (*Arg1*) (C) and transforming growth factor- β (*Tgfb1*) (D). * $P < 0.05$, ** $P < 0.01$, **** $P < 0.001$ compared with IL-4 group. All values were presented as mean \pm SD.

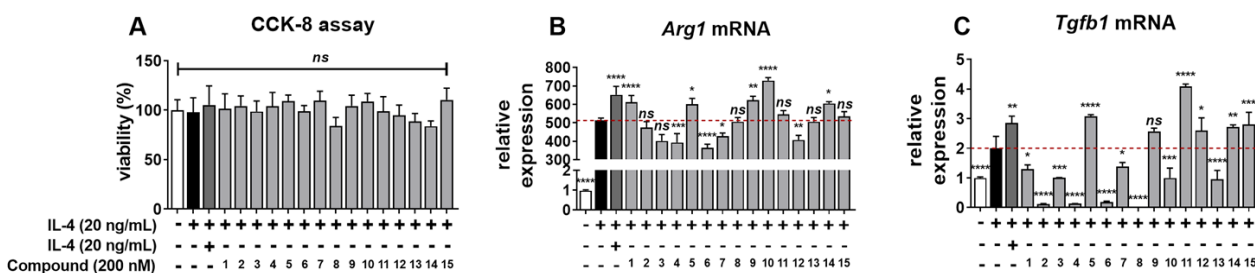


Figure 4. Effects of 15 compounds derived from AW component of SFI on BV-2 cell viability and gene expression levels of *Arg1* and *Tgfb1*. The 15 compounds did not alter cell viability at the concentration of 200 nM, and they demonstrated different regulatory effects on the expression level of *Arg1* and *Tgfb1*. ** $P < 0.01$, **** $P < 0.001$ compared with IL-4 group. All values were presented as mean \pm SD.

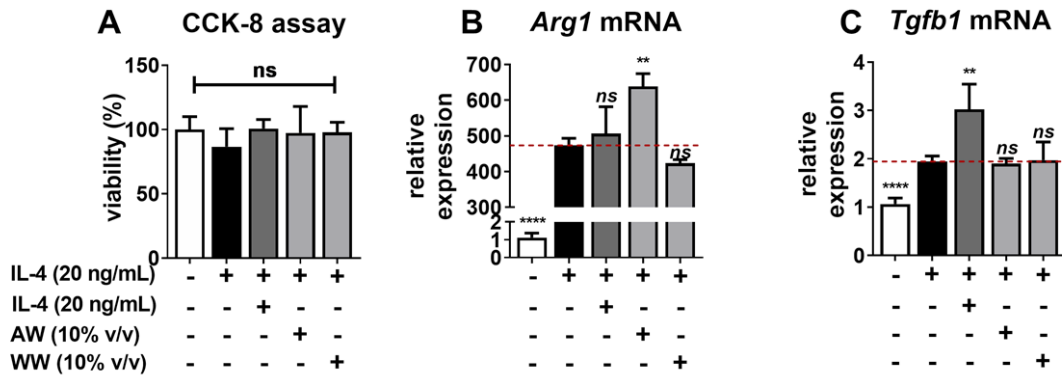


Figure 3. The regulatory effects of AW component and WW component derived from SFI. AW component of SFI significantly upregulated the gene expression level of Arg-1. ** $P < 0.01$, **** $P < 0.001$ compared with IL-4 group. All values were presented as mean \pm SD.

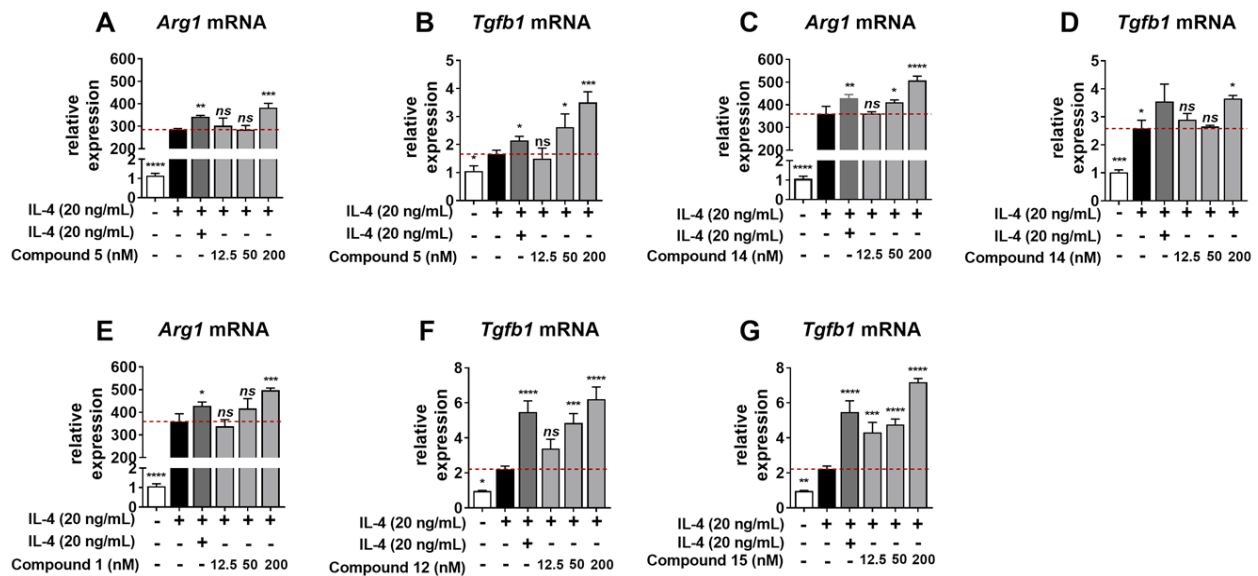


Figure 5. The regulatory effects of compounds 5 (A and B), 14 (C and D), 1 (E), 12 (F), and 15 (G) on the gene expression levels of *Arg1* (B) and *Tgfb1* (C) in IL-4-stimulated BV-2 cells. Cells were pretreated with compounds at indicated concentrations for 1 h before stimulation with 20 ng/mL IL-4 for 4 h. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with IL-4 group. All values were presented as mean \pm SD.

levels of *Arg1* and *Tgfb1* in a dose-dependent manner in the range of 12.5 nM to 200 nM. Compounds 5 upregulated the expression of *Arg1* at the concentration of 200 nM (Figure 5A), while compound 14 showed a promoting effect on *Arg1* at a lower concentration of 50 nM (Figure 5C). The expression level of *Tgfb1* upregulated by compounds 5 and 14 at 50 nM and 200 nM (Figure 5B and D), respectively. Other compounds demonstrated limited enhancing activity on the chosen M2 markers. For example, compounds 1 only enhanced the expression of *Arg1* at 200 nM (Figure 5E), while compounds 12 and 15 upregulated *Tgfb1* at 50 nM (Figure 5F) and 12.5 nM (Figure 5G), respectively. These results indicated that different types of compounds in SFI showed good potency in regulating the expression level of *Arg1* and *Tgfb1*, but their effective concentrations were not the same.

4. Discussion

The aim of this study was to explore the bioactive constituents of SFI and their anti-neuroinflammatory effects. By using a murine microglial cell line (BV-2 cell) based on the IL-4 induced alternative activation of M2 microglia, the gene expression levels of M2-activation markers *Arg1* and *Tgfb1* were measured to indicate the anti-neuroinflammatory effects via the M2 microglia activation. We demonstrate that SFI augments the IL-4 mediated M2 microglia activation, characterized by upregulated gene expression of M2 markers *Arg1* and *Tgfb1*. Compounds 1, 5, 12, 14, and 15 in the AW components SFI are the major bioactive constituents that have regulatory effects on enhancing M2 microglia activation.

Our studies showed that the M2 microglia

promoting activity of SFI was found mainly in the alcohol washing components (Figure 3). It is in accordance with our findings that the characteristic compounds in Dangshen (*Radix Codonopsis*) and Huangqi (*Radix Astragali Mongolici*) (Table 1) were mainly in the alcohol washing component, and the water washing components are mainly composed of amino acids, nucleosides, and oligosaccharides (data not published), which may not be the major contributor to the microglia/macrophage alternative activation.

At the concentration of 200 nM, the regulatory effects of the 15 characteristic compounds on *Arg1* and *Tgfb1* were not identical (Figure 4). For example, Compounds **1** and **10** had promoting effects on *Arg1* but inhibitory effects on *Tgfb1*; Compounds **5** and **14** had the same up-regulatory effects on both genes as SFI (Figure 5A-D); Compounds **4**, **6**, and **7** had the opposite effect on both indexes. Studies have shown that the regulatory mechanism are quite different between *Arg1* and *Tgfb1*. As a significant anti-inflammatory cytokine, *Tgfb1* signaling plays a critical role in IL-4-induced M2 macrophage activation through co-signaling to Akt, as well as MAPK pathway, manifested by synergic upregulation with IL-4 on *Arg1* expression level in an autocrine and/or paracrine fashion (39,40). Another study showed that Compound **1** (astragaloside IV) were associated with promoting microglia polarization to M2 and improve tissue regeneration in peri-infarct regions in a rat cerebral ischemia/reperfusion injury model, which may be mediated by PPAR γ pathway (41). The exact molecular mechanism of how Compound **1** and other compounds regulate microglia polarization is to be identified, but our study indicates that the two herbal ingredients Dangshen and Huangqi may contain compounds with immune-modulatory effects. Made from natural herbs, the complex constituents pose challenges in understanding the pharmacological action mechanisms of SFI. Therefore, more studies are required to determine the accurate concentration of each compound in SFI to further compare their contribution to the final efficacy. Also, compounds action mechanisms and their possible synergistic effects in regulating M2 microglial activation are worth further exploration.

In summary, the current study explores the bioactive constituents of SFI and their anti-neuroinflammatory effects. We demonstrate that SFI augments the IL-4 mediated M2 microglia activation, characterized by upregulated expression of M2 markers *Arg1* and *Tgfb1*. Compounds **1**, **5**, **12**, **14**, and **15** in the AW components SFI are the major bioactive constituents that have regulatory effects on enhancing M2 microglia activation. These results indicated that SFI might have protective and modulatory effects against neuroinflammation by polarizing microglia toward an anti-inflammatory M2 phenotype, and thus these bioactive compounds may provide beneficial effects in neuroinflammation-related CNS disorders.

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Conflict of 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.

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