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Article

Scandoside exerts anti-inflammatory effect via suppressing NF-κB and MAPK signaling pathways in LPS-induced RAW 264.7 macrophages

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Abstract: The iridoids of *H. diffusa* play an important role in the anti-inflammatory process, but the specific iridoid with anti-inflammatory effect and its mechanism is lack of study. An iridoid compound named scandoside (SCA) was isolated from *H. diffusa* and its anti-inflammatory effect was investigated in lipopolysaccharide (LPS)-induced RAW 264.7 cells. Its anti-inflammatory mechanism was confirmed by *in intro* experiment and molecular docking analysis. As results, SCA significantly decreased the productions of nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) and inhibited the levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), TNF-α and IL-6 mRNA expression in LPS-induced RAW 264.7 cells. SCA treatment suppressed the phosphorylation of inhibitor of nuclear transcription factor kappa-B alpaha (IκB-α), p38, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). The docking data suggested that SCA had great binding abilities to COX-2, iNOS and IκB. Taken together, the results indicated that the anti-inflammatory effect of SCA is due to inhibition of pro-inflammatory cytokines and mediators via suppressing the nuclear transcription factor kappa-B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways, which provided useful information for its application and development.

Keywords: Scandoside; NF-κB; MAPK; Anti-inflammation

1. Introduction

Inflammation is a natural defense response when body invaded by bacteria, virus and fungus [1]. Lipopolysaccharide (LPS) that is a common endotoxin derived from the outer membrane of gram-negative bacterial can cause a series of inflammatory reactions. During the inflammatory process, macrophages can be recruited to inflammatory sites and plays an essential role by various signals that stimulate intracellular cascades [2]. Consequently, inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6, and inflammatory mediators such as nitric oxide (NO) and prostaglandin E_2 (PGE_2) were obviously increased [3-5]. Nuclear factor-Kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) are two crucial pathways to regulate the transcription of inflammatory cytokines and mediators during inflammatory process activated by LPS stimulation [3,6,7]. Once activated, NF-κB and MAPKs can induce collaboratively the expression of pro-inflammatory cytokine gene and the release of cytokine under the inflammatory process [8]. Therefore, molecule or chemical targeting NF-κB and/or MAPK signaling

pathways are considered to be a potential anti-inflammatory agent, which is strategy for the treatment of inflammation related disorders.

Hedyotis diffusa Willd as a famous traditional Chinese Medicine is widely distributed in South of China and other Asian countries [9]. Traditionally, it is used for the treatment of bronchitis, arthritis, rheumatism, urethral infection, appendicitis, sore throat, contusions, ulcerations and extension of malignancies [10]. Modern pharmacological studies have proved that *H. diffusa* have multiple effects, such as anti-inflammatory, anti-cancer, immunemodulating, neuroprotective and hepatoprotective activities [11]. It was found that the iridoid compounds possibly attributed to anti-inflammatory effect of *H. diffusa* [12-13]. In our previous study, the extract of *H. diffusa* treated group could significantly alleviate the inflammatory reaction and decrease the pro-inflammatory cytokines and mediators; meanwhile, ten iridoids were detectable in serum [14]. However, the specific iridoid with anti-inflammatory effect and its anti-inflammatory mechanism are still unclear. In this study, an iridoid compound named scandoside (SCA) (Figure 1) was isolated from *H. diffusa* and its anti-inflammatory effect was investigated using LPS-induced RAW 264.7 macrophage cells. Moreover, its anti-inflammatory mechanisms were further clarified.

2. Results

2.1 Effects of SCA on RAW 264.7 cell viability

The cytotoxicity of SCA on RAW 264.7 cells was examined by CCK-8 assay. As shown in Figure 2, the percentages of cell viabilities were from 98.38% to 103.48%. Cell viabilities were not significantly affected by various concentrations of SCA after 24 h treatment in the presence of 50 ng/mL LPS, indicating that SCA was non-toxic to RAW 264.7 cells below 400 μg/mL.

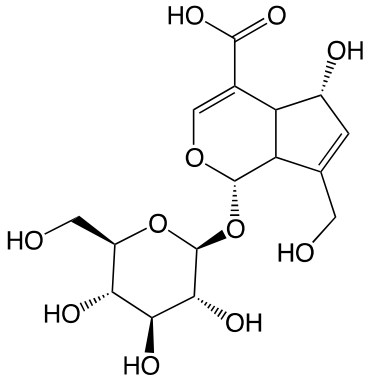

Figure 1. Chemical structure of scandoside

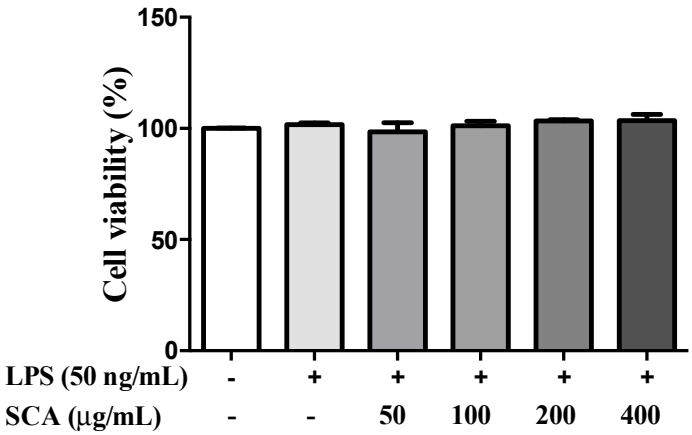

Figure 2. Effect of SCA on the viability of RAW 264.7 macrophage cells. RAW 264.7 cells were treated with SCA at the concentration of 0, 50, 100, 200 and 400 μg/mL, respectively, for 1 h, and then

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stimulated with 50 ng/mL LPS for 24 h. Cell viability was detected by CCK8 assay. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus LPS-only treatment group.

2.2 Effects of SCA on inflammatory mediators and inflammatory cytokines in RAW 264.7 cells.

As shown in Figure 3, the significant increases of inflammatory mediators (NO and PEG_2) and inflammatory cytokines (TNF-α and IL-6) in LPS-treatment group were observed when compared with the control group. Conversely, SCA treatment groups gave different behaviors. SCA treatment significantly reduced the productions of NO, PEG_2, TNF-α and IL-6 ($p<0.05$) at concentration-dependent manners.

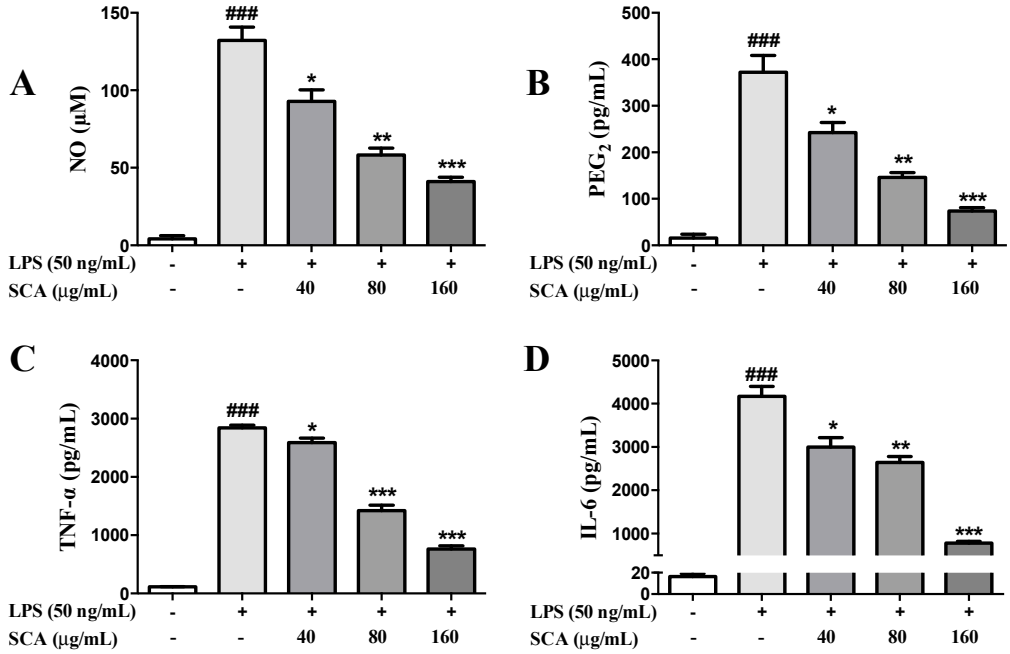

Figure 3. Effects of SCA on the productions of NO (A), PGE_2 (B), TNF-α (C) and IL-6 (D). RAW 264.7 cells were treated with SCA at the concentration of 40, 80 and 160 μg/mL, respectively, for 1 h, and then stimulated with 50 ng/mL LPS for 24 h. The concentrations in the cell-free culture were measured by ELISA. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus LPS-only treatment group; ###p < 0.001 versus control group.

2.3 Effects of SCA on TNF-α and IL-6 mRNA expression in LPS-induced RAW 264.7 cells

The mRNA expression of TNF-α and IL-6 were investigated to find out whether SCA could regulate their transcriptional levels. As shown in Figure 4, SCA treatment could obviously down-regulate the mRNA levels of TNF-α and IL-6 compared with LPS-treated group. The reduced mRNA levels of TNF-α and IL-6 were roughly consistent with their protein levels treated with SCA in trend, respectively.

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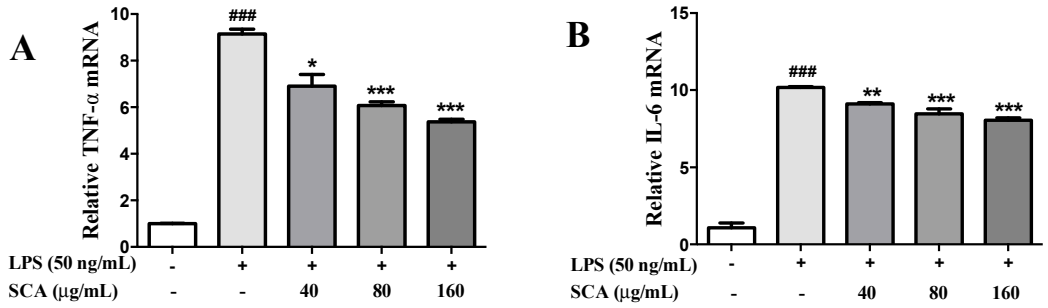

Figure 4. Effects of SCA on TNF-α (A) and IL-6 (B). RAW 264.7 cells were treated with SCA (40, 80 and 160 μg/mL) for 1 h and then stimulated with LPS (50 ng/mL) for 24 h. The TNF-α and IL-6 mRNA were analyzed by Real-time PCR. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus LPS-only treatment group; ###$p < 0.001$ versus control group.

2.4 Effects of SCA on iNOS and COX-2 proteins and mRNA expressions in LPS-induced RAW 264.7 cells

To study the issue whether SCA suppressed the productions of NO and PGE_2 via inhibiting the expression of their corresponding synthases of iNOS and COX-2, the protein and mRNA expression of iNOS and COX-2 were measured respectively. The results of RT-PCR analyses showed that LPS induced the significant up-regulation of the mRNA transcript levels of iNOS and COX-2. SCA treated group could significantly down-regulate the transcriptional levels of iNOS and COX-2 mRNA compared with the only LPS treated group, in a concentration-dependence (Figures 5A and 5B). SCA treatment concentration-dependently reduced the protein levels of iNOS and COX-2 (Figures 5C and 5D). The reduction of iNOS and COX-2 mRNA and protein levels were consistent with the reduced production of NO and PGE_2, respectively.

2.5 Effects of SCA on NF-κB and MAPK signaling pathways in LPS-induced RAW 264.7 cells

To study the potential mechanism of anti-inflammation, the effects of SCA on IκB-α phosphorylation and degradation were investigated in LPS-stimulated RAW 264.7 cells. As shown in Figure 6, LPS-induced IκB-α phosphorylation was significantly decreased after SCA treatment at the concentration-dependence. It was also examined to the regulatory effect of SCA on MAPK signaling pathway. As results, p38, Erk1/2, and JNK in RAW 264.7 cells stimulated by LPS were triggered a high phosphorylation. JNK phosphorylation was obviously inhibited by SCA at the concentration-dependence. For the effect on MAPK, SCA treatment remarkably decreased p38 and ERK1/2 phosphorylation at the highest concentration level (160 μg/mL).

2.6 Molecular docking analysis

The docking results of SCA with target proteins (iNOS, COX-2, PEG_2 and IκB) are shown in Figure 7 and Table 1. Total scores of complexes of SCA with iNOS, COX-2 and IκB were closed, but the obtained score of SCA with PEG_2 were much lower. In docking experiments on COX-2, His90, Tyr355, Tyr385and Ser530 formed hydrogen bonds. Sixteen hydrophobic interaction binding residues His90, Val349, Leu352, Ser353, Tyr355, Phe381, Leu384, Tyr385, Trp387, Phe518, Met522, Val523, Gly526, Ala527, Ser530 and Leu531 were found. For the docking experiments on iNOS, amino acid residues Trp295, Lys296, Asp303, Glu320, Ile321, Glu328 and Lys345 formed hydrogen bonds. Twelve common amino acid residues with hydrophobic interaction were Trp295, Lys296, Lys298, Phe302, Asp303, Val304, Glu320, Ile321, Pro323, Val326, Glu328 and Lys345. As for PEG_2, SCA could bind to the site Ala31, Arg38, Ala45, Asn46 and His72 of the crystal structure by hydrogen bonds. Besides, the hydrophobic interactions were found in the sites Thr34 and Leu69 of the complexes. Amino acid residues Arg73, Arg95, Arg96, Glu101, Asn137, Gln162 and Thr164 in crystal structure of IκB formed hydrogen bonds with SCA. Together with these amino acid residues,

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the other residues Phe99, Thr136 and Asn138 had the hydrogen interactions in the IκB-ligand complexes.

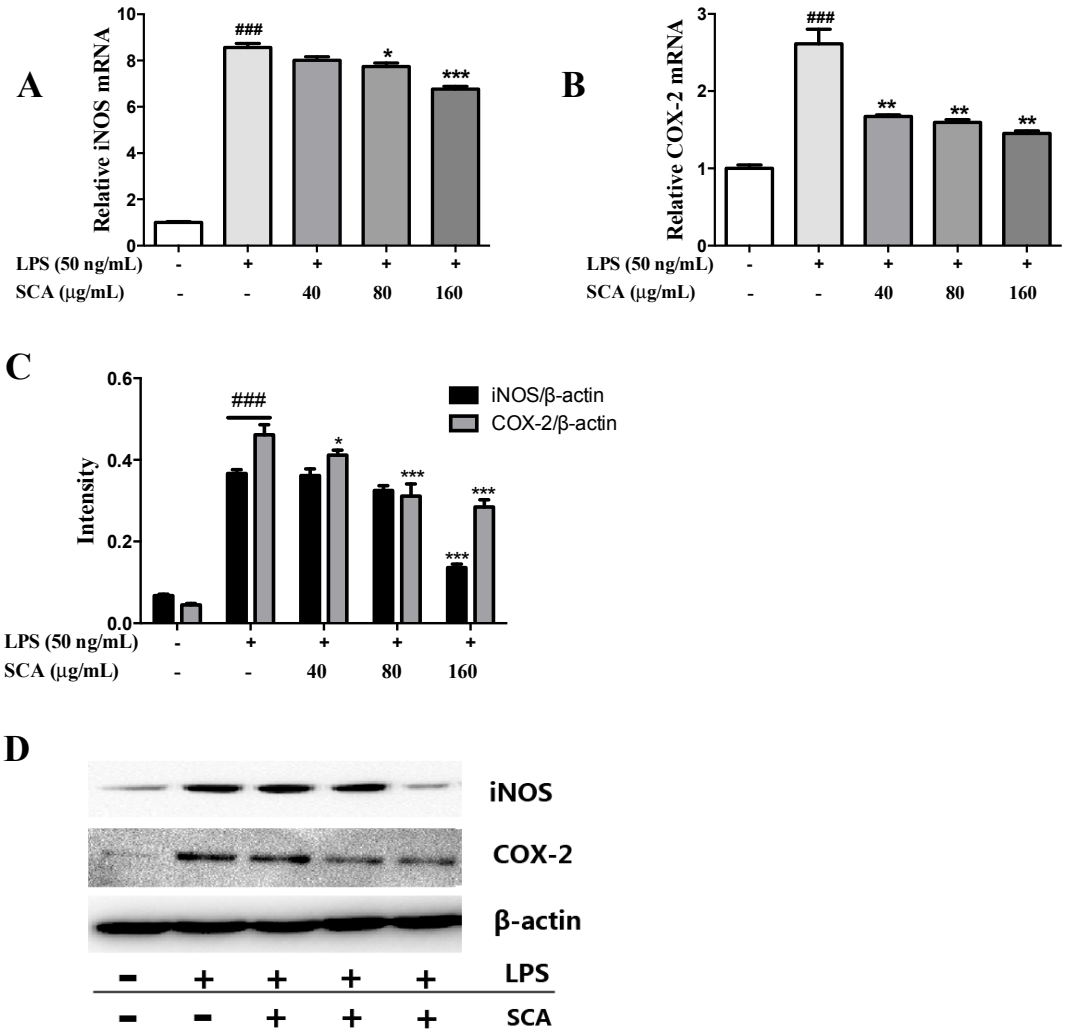

Figure 5. Effects of SCA on iNOS and COX-2 mRNA (A and B) and protein expressions (C and D). RAW 264.7 cells were treated with SCA (40, 80 and 160 μg/mL) for 1 h and then stimulated with LPS (50 ng/mL) for 24 h. The iNOS and COX-2 mRNA was analyzed by RT-PCR. The iNOS and COX-2 proteins were analyzed by Western blot. The bar chart shows the quantitative evaluation of iNOS and COX-2 protein band by densitometry. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus LPS-only treatment group; ### $p < 0.001$ versus control group.

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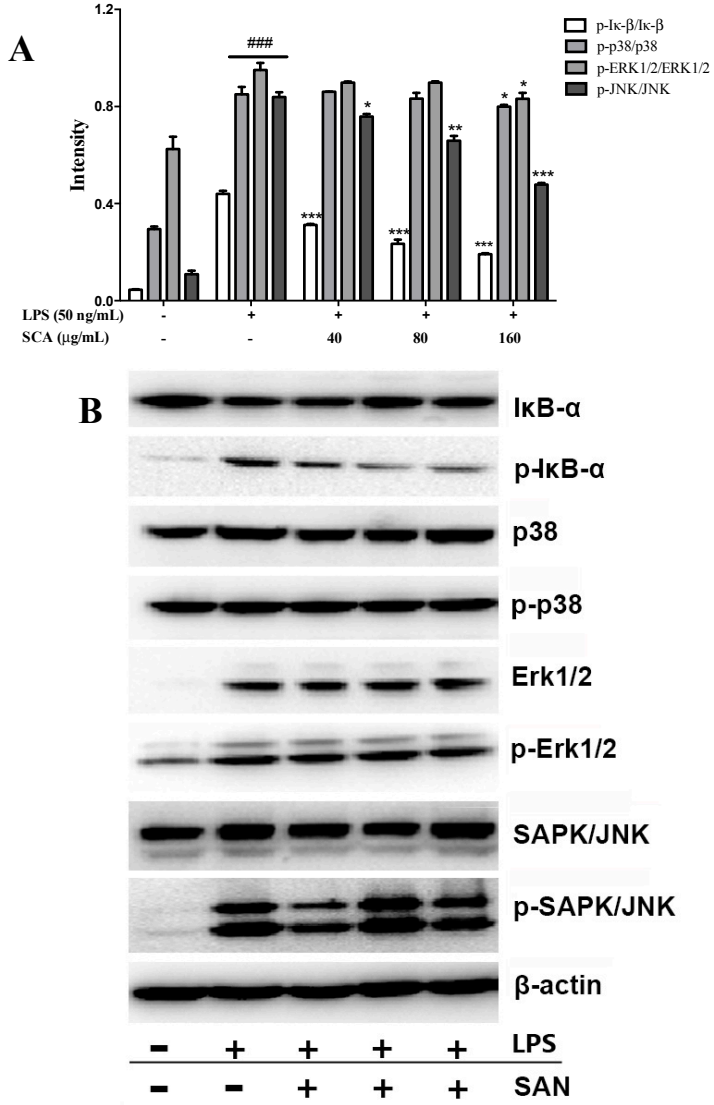

Figure 6. Effect of SCA on IκBα, p38, ERK1/2 and JNK phosphorylation. RAW 264.7 macrophage cells were treated with SCA (40, 80 and 160 μg/mL) for 1 h and then stimulated with LPS (50 ng/mL) for 24 h. The protein was analyzed by Western blot. The bar chart shows the quantitative evaluation of protein bands by densitometry. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus LPS-only treatment group; ###p < 0.001 versus control group.

Table 1. Virtual binding score values and interaction between the ligand and amino acid residues in scandoside protein-ligand complexes

Target Protein	Total score[1]	Crash[2]	Polar[3]	Hydrogen Bonds	Eletrostatic interaction	Hydrophobic interaction (0.5 Å)
COX-2(1CX2)	9.0084	-3.0357	2.9740	His90, Tyr355(3), Tyr385, Ser530	His90, Arg513	His90, Val349, Leu352, Ser353, Tyr355, Phe381, Leu384, Tyr385, Trp387, Phe518, Met522, Val523, Gly526, Ala527, Ser530, Leu531
iNOS(4NOS)	9.2757	-1.4618	7.6190	Trp295, Lys296, Asp303, Glu320, Ile321, Glu328, Lys345	Lys296	Trp295, Lys296, Lys298, Phe302, Asp303, Val304, Glu320, Ile321, Pro323, Val326, Glu328, Lys345
PEG_2(4AL0)	6.2647	-1.2700	5.0136	Ala31, Arg38(2), Ala45, Asn46, His72		Ala31, Thr34, Arg38, Ala45, Asn46, Leu69, His72
IκB(1NFI)	9.0953	-0.6933	9.9542	Arg73(2), [a]Arg95, Arg96(2), Glu101, Asn137(2), Gln162, Thr164(2)	[a] Arg95	Arg73, [a]Arg95, Arg96, Phe99, Glu101, Thr136, Asn137, Asn138, Gln162, Thr164

[1]Total score: total docking score expressed in –log (Kd) units to represent binding affinities. [2]Crash: the degree of inappropriate penetration by the ligand into the protein and of interpenetration between ligand atoms that are separated by rotatable bonds. [3]Polar: the effect of polar non-hydrogen bonding interaction to the total score. The number behind some residues stands for the number of hydrogen bond between the residue and the ligand. [a] Residues located in E subunit of IκB (1NFI) and other residues locate in A subunit.

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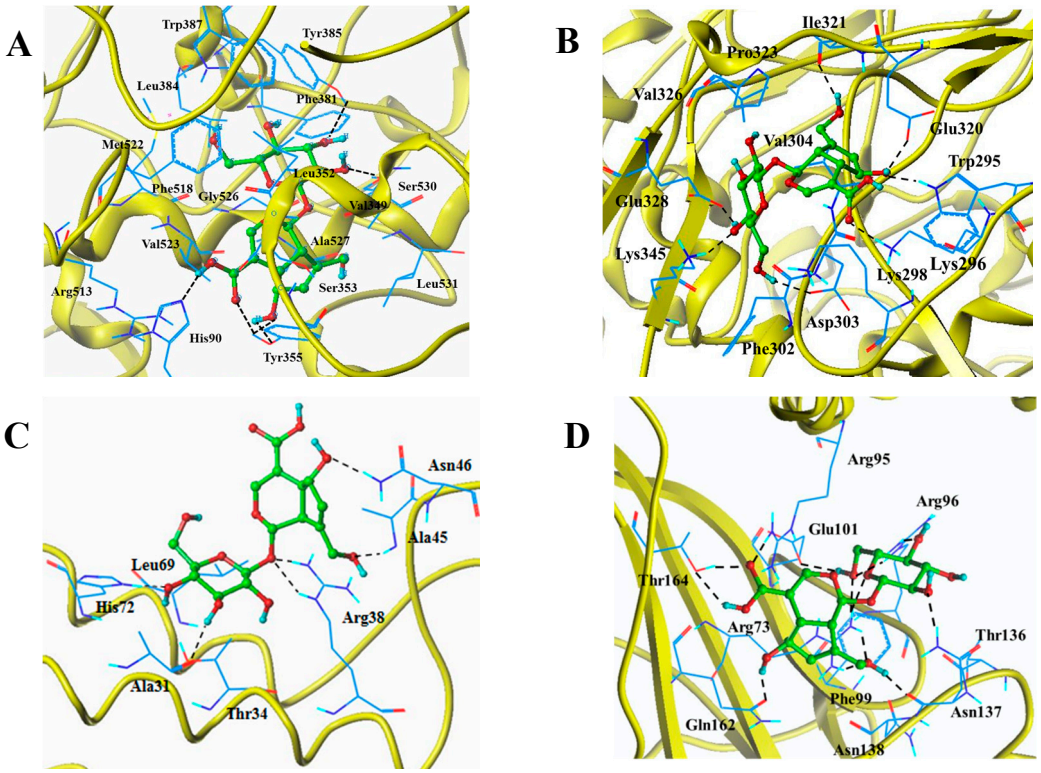

Figure 7. The best docked pose of SCA with target proteins. Docked orientation of SCA (colored in green) with corresponding secondary protein structure (colored in yellow) and amino acid residues (colored in blue) of COX-2 (1CX2) (A), iNOS (4NOS) (B), PEG_2 (4AL0) (C) and IκB (1NFI) (D).

3. Discussion

There are various natural products that defined as potential anti-inflammatory agents [15,16]. Among them, the iridoids attract much attention and some iridoid compounds have been proven to be of anti-inflammatory effect [17]. In this study, SCA, an iridoid compound, was successfully isolated from *H. diffusa* that usually used for the treatment of inflammation in clinic. SCA is not a main compound and of low content in *H. diffusa*, but it could be absorbed in blood and bind to the inflammatory tissues [14,18,19]. To elucidate the inflammatory effect of SCA, the pro-inflammatory cytokines and mediators in LPS induced RAW 264.7 cells were assayed. As results, SCA obviously showed the anti-inflammatory effect due to the significant reductions of NO, PGE_2, TNF-α and IL-6 after treatment.

The productions of inflammation related factors are regarded as the indicators for the inflammation reaction. Pro-inflammatory cytokines such as TNF-α and IL-6 play crucial roles in the development of inflammatory diseases and involve in the immunity and autoimmune diseases [20,21]. TNF-α and IL-6 mRNA expression in LPS-stimulated RAW 264.7 cells were measured. The results indicated that SCA could significantly suppress TNF-α and IL-6 mRNA expression. NO predominately produced by iNOS is a crucial indicator of inflammation reaction. PGE_2 synthesized by COX-2, is an important mediator due to the various biological effect associated with inflammation [3-5]. In RAW 264.7 cells, LPS effectively activated iNOS and COX-2 transcription leading to the overproductions of NO and PEG_2 [22]. The results demonstrated that SCA significantly decreased the productions of NO and PEG_2 by suppressing iNOS and COX-2, respectively, and at the same time markedly inhibited the iNOS and COX-2 mRNA expression.

NF-κB is a key signaling pathway related to regulating the transcription of numerous pro-inflammatory cytokines and mediators, including TNF-α, IL-6, iNOS and COX-2 [3,6]. The phosphorylation of IκBα by IKKα is a critical process for the activation of NF-κB. SCA were able to significantly inhibit the NF-κB activation via decreasing the phosphorylation of IκB. MAPKs,

including ERK, JNK and p38 also take part in the expression regulation of inflammation-related genes, leading to the overproduction of pro-inflammatory cytokines [23,24]. SCA obviously inhibited LPS-induced phosphorylation of p38, ERK and JNK in Raw 264.7 cells, displaying its anti-inflammatory effects and mechanism. It is reported that NF-κB and MAPKs signaling pathways can collaborate synergistically to promote the expression and release the target genes. As the results shown, SCA exerted the anti-inflammatory effect via suppressing NF-κB and MAPK signaling pathways.

The anti-inflammatory property of SCA was also confirmed by the molecular docking analysis. SCA bind to the COX-2 active sites (His90, Tyr355 Tyr385 Trp387, Met522, Val523 and Ser530) to form the complex, which was consistent with that of anti-inflammatory compounds [25]. Similarly, SCA could bind to the key amino acid of iNOS (Lys296 and Glu320), PEG_2 (His72) and IκB (Arg95) when forming enzyme-ligand complex [26]. These findings supported that SCA showed anti-inflammatory effect by affecting the activities of these proteins. These results also indicated that SCA was responsible for the anti-inflammatory effect of *H. diffusa*; as a stable and available natural product, it might be a valid drug candidate for treating inflammation. SCA should be considered as a crucial marker when studied on quality control, pharmacokinetics and drug development of herbaceous plants. Importantly, the other herbaceous plants containing SCA should be paid attention to the inflammatory effect. In view of the experimental results achieved *in vitro*, the further study on the anti-inflammatory effect of SCA is necessary to be performed *in vivo*.

4. Materials and Methods

4.1. Plant, chemicals and reagents

The materials of *H. diffusa* was purchased from Bozhou Chinese Medicine Processing Plant (Bozhou, Anhui, China) and identified by Doctor Jing Wang (School of Traditional Chinese Medicine, Southern Medical University). The reference substances of SCA (PubChem CID: 21602023) were isolated from *H. diffusa* and identified by comparison of its spectral data (MS, ^{1}H NMR and ^{13}C NMR). The purity of SCA was confirmed by HPLC-DAD to be over 97%.

Murine macrophage RAW 264.7 cells were purchased from Cell Bank of the Chinese Academy of Science (Shanghai, China). Dulbecco's modification of Eagle's medium (DMEM, No. 12430-054) and fetal bovine serum (FBS, No. 10099141) were purchased from Gibco (Thermo Scientific, USA). LPS was purchased from Sigma-Aldrich Co. LLC. (St. Louis, USA). The antibodies of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), nuclear transcription factor kappa-B alpaha (IκB-α), p38 and extracellular signal-regulated kinase (ERK)1/2 were purchased from Proteintech Group, Inc (Chicago, IL, USA). The antibodies of p-IκB-α, p-p38, p-ERK1/2, c-Jun N-terminal kinase (JNK) and p-JNK were purchased from Cell Signaling Technology, Inc. (MA, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan). The ELISA kits of IL-6, IL-β1 and TNF-α were purchased from Neobioscience Technology Company (Shenzhen, China). NO ELISA kit was purchased from Beyotime Biotechnology (Shanghai, China), and PEG_2 was purchased from Enzo Life Sciences (New York, USA). All other reagents were of analytical grade.

4.2. Sample preparation

The dried and ground *H. diffusa* (5.0 kg) were extracted with 50 L of 75% ethanol (v/v) at 80 °C for 1 h. The extraction was repeated once after filtrated. The filtrate was evaporated in vacuum (Tokyo Rikakikai Co., LTD., Japan) at 60 °C to yield the concentrate with the relative density of 1.15 g/mL. Adding 0.5% active carbon powder to the concentrate, the supernatant was obtained by centrifugation at 5000 rpm and then dried under vacuum. The extract was dissolved in methanol and loaded onto a macroporous resin column, and subsequently washed with water and 30% methanol. The 30% methanol extract was submitted to a YMC-Pack ODS-A column (20×250 mm, 5 μm). SCA was separated by semi-preparing liquid chromatography and identified by MS, ^{1}H NMR and ^{13}C NMR [27].

4.3. Cell line and culture

RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin and maintained in Thermo carbon dioxide incubator (Thermo Fisher Scientific, Waltham, USA) with a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

4.4. CCK8 assay for cell viability evaluation

RAW 264.7 cells were seeded in 96-well plates with a density of 1×10^4 cells/well and incubated for 24 h. The cells were treated with SCA at the concentrations of 0, 25, 50, 100, 200 and 400 μg/mL for 1 h, subsequently stimulated with 50 ng/mL LPS for 24 h. Finally, 10 μL of CCK8 were added in each plates, incubated for 1 h at 37 °C and then determined with the absorption wavelength at 450 nm using TECAN Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). The cell viability was measured by comparing the absorbance values of treatment groups with that of control group.

4.5 ELISA assay of NO, PGE_2, TNF-α and IL-6

The concentrations of NO, PGE_2, TNF-α and IL-6 were measured according to the manufacture's instruction of commercial ELISA kits. Briefly, RAW 264.7 cells were seeded in 96-well plates with a density of 1×10^4 cells/well and incubated for 24 h. Then the cells were treated with SCA (40, 80 and 160 μg/mL) for 1 h. After they were stimulated with 50 ng/mL LPS for 24 h, the cell supernatant were collected for the determination of NO, PGE_2, TNF-α and IL-6 levels by TECAN Microplate Reader. The absorption wavelengths were 540, 450, 450 and 450 nm for NO, PGE_2, TNF-α and IL-6, respectively.

4.6 Real-time PCR assay

RAW 264.7 cells were seeded in 6-well plates with a density of 2×10^5 cells/well and incubated at 37 °C for 24 h. A series of concentrations of SCA (40, 80 and 160 μg/mL) was used to treat the cells for 1 h and stimulated with LPS (50 ng/mL) for 24 h. Total RNA was extracted from these cells using RNAprep pure cell kit (Qiagen, Valencia, CA, USA). The RNA purity and content were determined by measuring the absorbance ratio at 260/280 nm. Subsequently, the total RNA was converted into cDNA with a reverse transcription system containing 4 μL 5x prime Script RT Master MIX (perfect Real Time), 0.5 μg total RNA, and 15.5 μL RNase-free water. The cDNA was used for Real-time PCR (RT-PCR) by an Applied Biosystems® 7500 Fast Real-time PCR System (Thermo Fisher Scientific, USA) for analysis of iNOS, COX-2, TNF-α and IL-6. The RT-PCR reaction system contained 10 μL SYBR Premix EX Taq(2x), 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM) and 8μL cDNA under the reaction conditions: 50.0 °C for 3min, 95.0 °C for 3min, followed by 40 cycles for 95.0 °C for 10 s and 60.0 °C for 30 s. The primers for iNOS, COX-2, TNF-α and IL-6 were used as shown in Table 2.

Table 2. The primers used for RT-PCR analysis

Cytokines	Sense primer sequence5′-3	Antisense primer sequence5′-3
TNF-α	GCGACGTGGAACTGGCAGAA	CAGTAGACAGAAGAGCGTGGTG
IL-6	GTTGCCTTCTTGGGACTGAT	CATTTCCACGATTTCCCAGA
iNOS	TGGAGCGAGTTGTGGATTGT	CTCTGCCTATCCGTCTCGTC
COX-2	ACCTGGTGAACTACGACTGC	TGGTCGGTTTGATGTTACTG
β-actin	TGCTGTCCCTGTATGCCTCTG	GCTGTAGCCACGCTCGGTCA

4.7 Western blot analysis

RAW 264.7 cells were seeded in 6-well plates with a density of 2×10^5 cells/well, after incubated at 37 °C for 24 h, the cells were treated with SCA (40, 80 and 160 μg/mL) for 1 h and then stimulated with LPS (50 ng/mL) for 24 h. Subsequently, the cells were collected for protein analysis. One

hundred microlitre of cell lysis buffer (10 mM Tris-HCl, 0.15 M NaCl, 5 mM EDTA, 1% Triton×100, 5 mM DTT and 0.1 mM PMSF) were added and incubated for 30 min at 4 °C, and then centrifuged at 12000 rpm for 10 min. After collecting the supernatant in a new tube, the protein concentration was measured by BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). All protein samples were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The immunoblot was blocked for 2 h with 10% nonfat milk at room temperature, and then incubated overnight with the primary antibodies, including iNOS (1:1000), COX-2 (1:1000), IκB-α (1:1000), p-IκB-α (1:1000), p38 (1:1000), p-p38 (1:1000), ERK1/2 (1:1000), p-ERK1/2 (1:1000), JNK (1:1000), p-JNK (1:1000) and β-actin (1:1000), at 4 °C. After washed for three times, the membranes were incubated with the secondary antibody (1:10000) for 1 h at room temperature. The blots were detected by ECL Chemiluminescence method plus Western Blotting Detection System (FluorChem R, ProteinSimple, USA).

4.8 Molecular docking

Docking experiments were performed using Sybyl.v 7.3 (Tripos, Inc., St. Louis, Massachusetts). Chemical structure of SCA was downloaded from Pubmed compound database (https://www.ncbi.nlm.nih.gov/pccompound/). Energy minimization was performed using the Tripos force field and Gasteiger-Huckel charges and the conjugate algorithm with a convergence criterion of 0.001 kcal/(mol A). X-ray crystal structures of target proteins of iNOS (PDB ID: 4NOS), COX-2 (PDB ID: 1CX2), PEG_2 (PDB ID: 4AL0) and IκB (PDB ID: 1NFI) were retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) and arranged using Sybyl.v 7.3 software suite. The protomol was generated using ligand mode for COX-2 by extracting SC-558 from the initial COX-2 crystal structure occupying the binding site [25]. While the protomols for iNOS, PEG_2 and IκB were generated using residue mode, based on the active residues reported [26]. Surflex-dock Program interfaced with Sybyl.v 7.3 was used in docking and both hydrogen and heavy atoms were chosen in result optimization.

4.9 Statistical analyses

All experiments were performed in triplicate. Data were analyzed by Graphpad prism (Graphpad Software, San Diego, CA) and presented as mean ± standard deviation (SD). The statistical differences ($p<0.05$) between groups were obtained by one-way ANOVA method.

5. Conclusions

SCA significantly decreased the productions of NO, PGE_2, TNF-α and IL-6 and inhibited the levels of iNOS, COX-2, TNF-α and IL-6 mRNA expression in LPS-induced RAW 264.7 cells. Its anti-inflammatory effect is associated to suppress NF-κB and MAPK signaling pathways. SCA might be a potential therapeutic agent for the treatment of inflammatory diseases.6. Patents

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/link.

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