Isolation and bioactivity screening of soy isoflavones from soybean glycolipids identifies daidzin as a promising anti-inflammatory agent

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Abstract
Background: Soybean has long been utilized in the realm of traditional Chinese medicine. One of its extracts, soybean glycolipids, serves as a vital by-product of soybean oil refining, but its chemical composition and pharmacological potential have yet to be fully elucidated.

Methods: In this study, the chemical components were isolated, and the inhibitory effects of these isolates were explored in different zebrafish inflammatory models by survival rate, Histological examination assay and quantitative Real-time PCR assay. The cytotoxicity of daidzin in RAW264.7 cells was evaluated by cell viability assay, and the effect of daidzin on the release of inflammatory cytokines in RAW264.7 cells was detected by enzyme linked immunosorbent assay (ELISA). Western blotting, immunofluorescence assay and alpha7 nicotinic acetylcholine receptors siRNA transfection assay were used to further explore the anti-inflammatory mechanism of daidzin.

Results: Four compounds (verticilloside, soya-cerebroside I, soya-cerebroside II and daidzin) were firstly isolated from the soybean glycolipids, among which verticilloside and daidzin inhibited the lipopolysaccharide, CuSO4, and tail cut-stimulated zebrafish inflammation. Noticeably, daidzin exhibited anti-inflammatory activities by increasing the survival rate, alleviating the inflammatory cells infiltration, and down-regulating the expression of pro-inflammatory cytokines and nuclear factor kappa-B, NF-kappa-B inhibitor alpha, and signal transducer and activator of transcription3 in zebrafish. Moreover, daidzin decreased the secretion of IL-6 and TNF-α, inhibited the nuclear translocations of nuclear factor kappa-B p65 and p-signal transducer and activator of transcription3 as well as the NF-kappa-B inhibitor alpha phosphorylation at Ser32 in RAW 264.7 cells. More importantly, it elevated the expression level of alpha7 nicotinic acetylcholine receptors in both zebrafish and RAW 264.7 cells, and the inhibitory effect of daidzin was attenuated after the addition of alpha7 nicotinic acetylcholine receptors siRNA.

Conclusion: Our study revealed that daidzin inhibited inflammation by activating the cholinergic anti-inflammatory pathway and further inhibiting the nuclear factor kappa-B and signal transducer and activator of transcription3 signaling. At the same time, it also promotes the recycling of crude soybean glycolipids and supports the potential use of daidzin as a functional food or natural dietary anti-inflammatory agent.

Keywords: soybean; daidzin; anti-inflammation; zebrafish; alpha7AchR

Author contributions
Fang XC, He XM and Zheng YR carried out the experiments, analyzed the data and wrote the manuscript. Li W, Yang TJ and Yu JT completed the statistical analysis and rendered figures. Li JL and Cai ZZ participated in experimental work and results writing. Wang Y, Yu LZ and Liu JS guided the experiment and provided funding for the research. All authors have read and agreed to publish this manuscript.

Competing interests
The authors declare no conflicts of interest.

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Abbreviations
α7nAchR, alpha7 nicotinic acetylcholine receptors; NF-κB, nuclear factor kappa-B; STAT3, signal transducer and activator of transcription3; IκBα, NF-kappa-B inhibitor alpha; LPS, lipopolysaccharide; Dex, dexamethasone; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; qRT-PCR, quantitative Real-time PCR assay.

Citation

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Highlights
Four compounds were firstly isolated from the soybean glycolipids and most of them showed significant anti-inflammatory activities in three zebrafish inflammatory models. Daidzin exerts the best anti-inflammatory effects in vitro and in vivo. Daidzin inhibits LPS-induced inflammation by activating the cholinergic anti-inflammatory pathway and subsequently inhibition of NF-κB and STAT3 pathways.

Medical history of objective
Soybean, a classical plant of Medicine Food Homology (MFH), is derived from Li-Shichen’s “The Compendium of Materia Medica – Volume 9” (1590 C.E.). In ancient times, soybean has the function of removing blood stasis (promoting blood circulation and eliminating blood stasis to alleviate pain), dredging the meridians (unblocking and promoting the flow of Qi (vital energy) and blood through the body’s meridians or channels) and reducing the swelling (promoting the removal of water from the body to relieve edema). Modern pharmacological studies have reported that it has a variety of bioactivities, such as cardiovascular protective, hepatoprotective, antioxidative, antiviral and anticancer effects.

Background
Soybean is the mature seed of Glycine max (L.) Merr., which has been a traditional Chinese medicine for thousand years in China. According to the Compendium of Materia Medica, soybean has the function of removing blood stasis, dredging the meridian, and reducing the swelling. In addition, modern pharmacological studies have reported that it has a variety of bioactivities, such as cardiovascular protective, hepatoprotective, antioxidative, antiviral, and anticancer effects due to the presence of isoflavones, lignans, sterols, and saponins [1–3]. Interestingly, previous studies suggested that soybeans attenuated inflammation by inhibiting the nuclear factor-kappa B (NF-κB) pathway [4]. Crude soybean glycolipids are valuable by-products obtained from the soybean oil [5]. They mainly consist of glycerolipid, glycosphingolipids, isoflavonoids, and other components. However, the chemical constituents of crude soybean glycolipids have yet to be clearly understood, and little is known about the pharmacological properties of this by-product.

Danio rerio (Zebrafish) as a model organism has received increasing attention in recent years [6]. Its popularity in the fields of inflammation research is primarily attributed to its physiological and genomic homology with humans as well as the capability for dynamically imaging inflammation [7]. Moreover, the zebrafish model provides a short and inexpensive route for rapidly screening anti-inflammatory agents when compared to rodents or in vitro cell models. Therefore, zebrafish was employed as an inflammatory model to screen the anti-inflammatory activity of drugs in this study.

The cholinergic anti-inflammatory pathway is a vagal immune circuit that modulates inflammation through vagus nerve stimulation. This stimulates the release of acetylcholine that interacts with alpha7 nicotinic acetylcholine receptors (α7nAChR). Recent studies have further highlighted the critical role of α7nAChR and related genes in a variety of diseases. Defects in α7nAChR function have been associated with a number of inflammation-related diseases, such as neuroinflammation and rheumatoid arthritis, suggesting that α7nAChR is essential for inhibiting inflammation [8]. NF-κB is a classical transcription factor essential for cellular inflammatory and immune responses. Studies have shown that the activation of the α7nAChR signaling pathway can inhibit NF-κB and STAT3 pathways, thus exerting anti-inflammatory effects [9, 10]. Therefore, ligands binding to α7nAChR may have the potential to suppress inflammatory responses [11].

In the present study, we investigated the chemical compositions of crude soybean glycolipids, leading to the isolation and identification of four compounds. These isolates were firstly assessed for their inhibitory effects on inflammation in vivo, and it is novel to clarify that compound 4 (daidzin) could activate the cholinergic anti-inflammatory pathway and then inhibit NF-κB and STAT3 signalings. Together, our study revealed that daidzin is a novel α7nAChR agonist, providing evidence as a natural anti-inflammatory agent. Additionally, it also promoted the recycling of the crude soybean glycolipids.

Materials and methods
Plant materials
Crude soybean glycolipids (Lot. 190386) were provided by Hisoya Biological Science & Technology Co., Ltd. (Guangzhou, China).

General experimental procedures
The 1H and 13C NMR spectra were determined on Bruker AV-600 (1H: 600 MHz, 13C: 150 MHz) spectrometers. The ESI-MS analysis was operated by using a Finnigan LCQ Advantage MAX mass spectrometer. High-performance liquid chromatography (HPLC) was recorded on an Agilent 1100 instrument, accompanied by a preparative Cosmosil C18 column (5 μm, 20 × 250 mm). Sephadex LH-20 (Pharmacia Biotech Co., Ltd., Uppsala, Sweden), silica gel (300–400 mesh, Qingdao Marine Chemical Inc., China) and pre-coated silica gel GF254 plates (Yantian Chemical Industry Research Institute, China) were applied in this study. All the solvents used in column chromatography were analytical (Guangzhou Chemical Reagent Factory, China) or chromatographic grade (Thermo Fisher Scientific Co., Ltd., Waltham, USA).

Extraction and isolation
The crude soybean glycolipids (100 g) were powdered and extracted three times with 0.8 L ethanol under reflux. The extract was concentrated under a vacuum and then dispersed in CHCl3. The CHCl3 extract was submitted to a silica gel column and eluted with MeOH/CHCl3 (100:0–50:50, v/v) to give six fractions (Fr. 1–Fr. 6). Fraction 2 was separated on a Sephadex LH-20 column with eluent MeOH-CHCl3 (1:1, v/v) to obtain compound 1 (25 mg). Fr. 4 was further separated by a Sephadex LH-20 column with MeOH-CHCl3 (1:1, v/v) and HPLC with MeOH-H2O (85:15, v/v) to provide compound 2 (12 mg) and 3 (9 mg). Fr. 5 was purified by a Sephadex LH-20 column using MeOH-CHCl3 mixture (1:1, v/v) and then recrystallized to obtain compound 4 (5 mg).

Zebrafish maintenance
All experimental operations involving zebrafish were consistent with the standard guidelines [12]. Transgenic zebrafish labeled with green fluorescent protein in neutrophils Tg (mpox: GFP) were maintained with a 14:10 h light/dark cycle at 28 ± 0.5 °C. The zebrafish embryos were gathered by natural mating of sexually mature zebrafish and cultured in chorion water, in which methylene blue (0.02 mg/mL) was included as a bacteriostatic agent. Then, the embryos that were developing normally were selected for the following experiments.

LPS-stimulated inflammation and drug treatments
Inflammatory response in zebrafish were elicited by LPS, as previously reported [13, 14]. Briefly, the yolk sac of zebrafish larvae was inoculated with 0.5 mg/mL of LPS (n = 20/well). Next, these larvae were divided stochastically into 12-well plates and incubated with dexamethasone (Dex, 5 μg/mL) or varying concentrations of isolated compounds (5 μM and 10 μM) for 12 h. The recruitment of neutrophils in these zebrafish was monitored with a fluorescence microscope from Olympus Co., Ltd. (MXVX10, Tokyo, Japan).

CuSO4-induced inflammation and drug treatments
CuSO4-induced inflammation assay was performed based on a previous report [14]. Briefly, 3 days post fertilization (dpf), healthy larvae were immersed in 20 μM CuSO4 solution containing different
isolated compounds (5 μM and 10 μM) or Dex for 2 h at 28.5 °C. Finally, the inflammatory neutrophil migration process was observed using a fluorescence microscope.

Tail cut-induced inflammation and drug treatments
3-dpf larvae were treated with cholinergic water containing 0.02% tricaine, and the caudal fin was amputated with a sterile scalpel blade [14]. After the injury, the zebrafish were recovered in fresh medium containing different isolated compounds (5 μM and 10 μM) or Dex for 4 h. The behavior of fluorescent neutrophils at the site of transection was monitored by a fluorescence microscope.

Measurement of the survival rate
To test the influence of daidzin on the survival rates in LPS-treated larvae, the zebrafish were inoculated with 0.5 mg/mL of LPS and further exposed to daidzin (10 μM) or Dex. The embryos were monitored daily until 72 h post injection (hpi). The dead zebrafish embryos were counted, and the mortality rate was measured [14, 15].

Histological examination
For histological examination, LPS and daidzin treated zebrafish were fixed with 4% paraformaldehyde, routinely processed, and sectioned at 4 μm slices after embedding in paraffin. Then, hematoxylin and eosin (H&E) staining was carried out. Finally, the zebrafish slides were imaged with an IX53 light microscope from Olympus Co., Ltd.(Tokyo, Japan).

RNA extraction and quantitative real-time PCR (qRT-PCR)
The total RNA extraction from zebrafish larvae was reverse transcribed to cDNA using a Takara PrimeScript™ RT Master kit (Takara Co., Ltd., Kyoto, Japan). TB Green™ Premix Ex Taq™ II (Takara Co., Ltd., Kyoto, Japan) was utilized to test the expression levels of related genes. The 2−ΔΔCt method was used to calculate the mRNA level of each sample relative to the control group. The primer sequences applied in the experiment are summarized in Table 1.

Cell viability determination
MTT assay was used to detect the cytotoxicity of daidzin to RAW 264.7 cells. In brief, RAW 264.7 macrophages were plated in 96-well plates (0.8 x 10^5 cells/well) and cultured at 37 °C for 24 h. Varying concentrations of daidzin (6.25, 12.5, 25, 50, 100, and 200 μM) were administered for another 24 h. Then, 20 μL of MTT dye (5 mg/mL) was added to the treated cells for an additional 4 h. Finally, the MTT solution was replaced with DMSO, and absorbance at 570 nm was determined.

Assessment of cytokine production
RAW 264.7 cells were pretreated with daidzin (25, 50, and 100 μM) or Dex (5 μM) for 12 h, and then LPS (100 ng/mL) was added for co-incubation. After 12 h, cell supernatants were gathered, and the concentrations of IL-6 and TNF-α were detected using ELISA kits (Dakewe Co., Ltd., Beijing, China) following the manufacturer's instructions.

Western blot analysis
Total protein or nuclear protein was extracted from daidzin-treated cells. Protein samples were electrophoresed on SDS-PAGE gels and then transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk in TBST, and indicated primary antibodies were added. The protein bands were probed with corresponding secondary antibodies for 2 h. Signals were detected by an ECL kit using the FluorChem™ system from ProteinSimple Co., Ltd. (San Francisco, CA, USA).

Immunofluorescence analysis
Treated cells were fixed with 4% formaldehyde and then permeabilized with 0.1% Triton X-100. This was followed by blocking with 5% skim milk followed by overnight incubation with NF-κB p65 or p-STAT3 antibody (1:100). Subsequently, the cells were stained with indicated secondary antibody. DAPI is utilized to label the nuclei. Images were visualized by a confocal microscope (LSM880, Carl Zeiss Co., Ltd., Oberkochen, Germany).

α7nAchR siRNA transfection
RAW 264.7 cells seeded in six-well plates were transfected with lipofectamine 2000 for 24 h, in which specific α7nAchR siRNA and negative control concentrations were both 120 nM. Cells were administrated with daidzin for 12 h before co-incubation with the addition of LPS. Finally, cells and supernatants were collected for western blot analysis and ELISA, respectively.

Statistical analysis
Data were obtained using the GraphPad Prism program 8.0 (San Diego, CA, USA). One-way analysis of variance was used to compare multiple groups. Data were presented as mean ± standard deviation of three triplicate determinations. When the P value is less than 0.05, it is regarded as statistically significant.

Results
Identification of isolated compounds
Four known compounds (1-4) were obtained from the crude soybean glycolipids, and the structures are shown in Figure 1. These compounds were identified as verticillolide (1), soya-cerebroside I (2), soya-cerebroside II (3) and daidzin (4) [16-18]. The detailed chemical information, as well as their NMR profiles, are displayed in Supplementary Figure S1–S8.

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**Table 1** Primer sequences for the qRT-PCR assay

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>Forward: ATGGATGAGGAAATCCGCTG</td>
</tr>
<tr>
<td></td>
<td>Reverse: ATGCAACCACTAACCCTCTG</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward: AGACCGCTGCTGCTAATCC</td>
</tr>
<tr>
<td></td>
<td>Reverse: TTTGATGTCGTTACACAGGA</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward: GCTGATCTCTCAAAAGTCGGGTGTA</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGGTAGTCTCAGACACTTCCATC</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Forward: GAGCCCTTTTTGCGAAGAGAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGGGATACGTTCCTCTGTTCC</td>
</tr>
<tr>
<td>IL-8</td>
<td>Forward: GCTGGAAAAGGACTCTGGAAGAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGGTAGTTAGGGAAGGTAAAGATG</td>
</tr>
<tr>
<td>STAT3</td>
<td>Forward: CCCCTGGACTAAGCTGGGCA</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGAGTTGACCGATCCCTCTCTTT</td>
</tr>
<tr>
<td>α7nAchR</td>
<td>Forward: TGGCTCATTGAGGCTGCTG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCGTCTACCTGGAACATTCCACAG</td>
</tr>
</tbody>
</table>
Anti-inflammatory effects of compounds 1–4 in zebrafish

Neutrophils are quickly recruited to sites of inflammation following tissue infection, which has been proved to be critical for host defense [19]. However, continuous neutrophil recruitment can cause a range of inflammation-related diseases. Therefore, it is significant to control excessive neutrophil recruitment or promote their elimination. To rapidly and conveniently screen for anti-inflammatory compounds isolated from soybean glycolipids, Tg (mpo: GFP) transgenic zebrafish were applied in this study, which allows a dynamic observation of neutrophil migration and recruitment during the inflammatory reactions.

LPS, an endotoxin obtained from Gram-negative bacteria, has been widely used as a potent trigger of inflammation in zebrafish [20]. Therefore, the LPS-stimulated inflammatory zebrafish model was first employed to detect the potential anti-inflammatory activities of the above compounds 1–4. In comparison with the control group, microinjection with LPS resulted in a significant aggregation of neutrophils presented in the zebrafish yolk sac. However, treatment with compound 4 distinctly decreased the accumulation of neutrophils in the yolk area. Correspondingly, 1 showed weaker inhibitory effects on migration and recruitment of neutrophils, while 2 and 3 were inactive (Figure 2).

CuSO₄ and tail amputation can chemically or physically induce the rapid migration of neutrophils into the neuromasts or injured tails and eventually lead to a robust acute inflammatory response in zebrafish [21, 22]. Then, the inhibitory effects of isolated compounds were further corroborated in CuSO₄ and tail transection-stimulated zebrafish models. Neutrophils recruited to the injured neuromasts or cut sites were markedly increased following CuSO₄ exposure or tail amputation. Compounds 1, 3, and 4 significantly inhibited the migration of neutrophils toward the injured neuromasts or tails, which further corroborates the anti-inflammatory effects of 1 and 4, especially 4 at the concentration of 10 μM (Figure 2).

Noticeably, all the above results demonstrated that compound 4 (daidzin) exhibits remarkable anti-inflammatory activities in all three kinds of inflammatory models. Thus, the protective effects of daidzin and its potential molecular mechanism were further elucidated.

Daidzin exerts protective effects in zebrafish stimulated by LPS

The survival curve is commonly used to intuitively reflect the protective effects of drugs. As shown in Figure 3A, the survival rate dramatically decreased to 20% by LPS microinjection at 60 hpi when compared to the control group. However, the survival rate of zebrafish in the daidzin group (10 μM) was higher than 40%, which indicated that daidzin greatly protects zebrafish larvae from inflammatory injury stimulated by LPS.

Infiltration of inflammatory cells is a key feature of inflammation [23]. H&E staining indicated the inflammatory cells in the LPS model group were densely infiltrated, whereas the histopathological features were significantly alleviated after the treatment of daidzin (10 μM), which further demonstrated that daidzin plays a protective role in reducing inflammation after LPS stimulation (Figure 3B).

Daidzin decreases the secretion of IL-6 and TNF-α in zebrafish

Pro-inflammatory cytokines are considerably secreted when inflammation is triggered by LPS [24]. To determine how daidzin exerts anti-inflammatory effects, IL-6 and TNF-α in zebrafish were examined using qRT-PCR. As illustrated in Figure 3C, 3D, LPS stimulation induced notable elevation of IL-6 and TNF-α levels compared to PBS injection. In contrast, daidzin significantly repressed the release of these cytokines.

Daidzin inhibits the activation of NF-κB and STAT3 in zebrafish

Both of NF-κB and STAT3 pathways have important functions in regulating inflammatory responses [25, 26]. To further elucidate whether daidzin influences these pathways, we employed a qRT-PCR assay to determine the pivotal transcription factors. The findings indicated the levels of NF-κB, IκBα, and STAT3 were remarkably elevated in the model group, while daidzin significantly decreased the upregulated mRNA expression in zebrafish (Figure 3E–3G). These data demonstrated that inhibition of the activation of NF-κB and STAT3 may partially contribute to the anti-inflammatory properties of daidzin.

Daidzin suppresses the IL-6 and TNF-α release in RAW 264.7 cells

Next, we explored whether the above in vivo findings obtained from zebrafish inflammatory models can be confirmed in an in vitro study. As depicted in Figure 4A, daidzin displayed no cytotoxicity to RAW 264.7 cells in the range of 200 μM.

To examine the in vitro effects of daidzin, the secretion levels of IL-6 and TNF-α were measured by ELISA kits (Dakewe Co., Ltd., Beijing, China). As illustrated in Figure 4B, 4C, LPS alone elicited a significant
elevation in IL-6 and TNF-α levels, while daidzin dose-dependently reversed their increases.

**Daidzin inhibits the activation of NF-κB and STAT3 pathways in RAW 264.7 cells**

Evidence suggests that phosphorylation of IκB results in nuclear translocation of isolated NF-κB dimers, ultimately leading to excessive secretion of proinflammatory cytokines [27]. Moreover, phosphorylation of STAT3 is known to lead to its translocation from cytoplasm to nucleus, DNA binding activities, and transcriptional activation of inflammatory genes [28]. Our above-mentioned studies have demonstrated that daidzin inhibits the LPS-induced NF-κB and STAT3 expression in zebrafish larvae. In order to gain a deeper understanding of the anti-inflammatory mechanisms at the cellular level, the expression levels of these two pathway-related proteins were detected. LPS promoted the nuclear translocations of NF-κB p65 and p-STAT3, which were remarkably inhibited after daidzin treatment (Figure 4D, 4F, 4G). Moreover, daidzin dose-dependently decreased the IκBα phosphorylation at Ser32 (Figure 4E).

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**Figure 2** Anti-inflammatory effects of compounds 1–4 (5 and 10 μM) in zebrafish inflammatory response induced by LPS, CuSO₄, and tail transection. (A) Representative images of zebrafish treated with different isolates in zebrafish inflammatory models induced by LPS, CuSO₄, and tail transection. (B) Neutrophils in the red region were quantitatively analyzed. Data were represented as mean ± SD from three independent experiments. *P < 0.01, **P < 0.001 vs. the model group. ***P < 0.001 vs. the control group. Ctrl, control group; Dex, dexamethasone group; M, model group.
DAIDZIN ATTENUATES INFLAMMATION THROUGH ACTIVATION OF THE CHOLINERGIC ANTI-INFLAMMATORY PATHWAY

As depicted in Figure 3H and Figure 5A, daidzin increased the α7nAChR expression level at both zebrafish and cellular levels. The knockdown of α7nAChR significantly attenuated the daidzin-mediated inhibition of TNF-α production. IL-6 also displayed the same tendency, but no significant difference was shown (Figure SB-SD). In addition, proteins related to NF-kB and STAT3 pathways were less impacted by daidzin after α7nAChR was silenced (Figure 5E, 5F). Collectively, these results indicated that daidzin suppressed inflammation by activating the cholinergic anti-inflammatory pathway and further inhibiting the NF-κB and STAT3 pathways (Figure 6).

Discussion

Inflammation is an immune response to harmful stimuli like pathogen invasion and tissue injury, but dysregulated and aberrant inflammation could result in the pathogenesis of various diseases [29, 30]. To date, pharmacotherapies of inflammatory disorders are mainly dependent on non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids (GCs). NSAIDs, however, might cause serious gastrointestinal toxicity and cardiovascular adverse effects [31], while the clinical application of GCs has also been hampered by various side effects, such as digestive ulcers, hypertension, and insulin resistance [32]. Here, we first showed that daidzin inhibited inflammation both in vitro and in vivo, suggesting its potential in inflammatory disease treatment.

Over the years, it has been known that intakeing medicinal and edible plants can reduce morbidity and mortality of multiple chronic diseases. A clinical study showed that supplementation with soy isoflavones had beneficial effects on both inflammation and nutritional status in End-stage renal disease (ESRD) patients on chronic haemodialysis (HD) [33]. Moreover, results from in vitro experiments demonstrated that soybean isoflavones were effective in reducing inflammation [34–36]. In addition to its medicinal value, as a primary source of plant proteins and edible oils for human consumption, soybean is also extensively used in food supplements due to its high nutritional value and health benefits [37]. The crude soybean glycolipids are a by-product of the soybean oil refining process. Previous literature suggests that a large fraction of soybean oil sediment is improperly discarded, which may lead to environmental pollution and ultimately threaten human health [38].
Figure 4 Daidzin inhibited the activation of NF-κB and STAT3 pathways in LPS-induced RAW 264.7 cells. (A) Daidzin (6.25–200 μM) had no significant cytotoxicity to RAW 264.7 cells. The cells were treated with different concentrations of daidzin (6.25, 12.5, 25, 50, 100 and 200 μM) for 24 h. The secretion levels of IL-6 (B) and TNF-α (C) in the culture supernatants were detected by ELISA kits. The cells were pretreated with daidzin (25, 50 and 100 μM) or Dex (5 μM) for 12 h and then further co-incubated with LPS (100 ng/mL) for another 12 h. The nuclear protein levels of NF-κB p65, p-STAT3 (Tyr705) (D) and the total protein expressions of IκBα, p-IκBα (Ser32), STAT3 (E) were determined by Western blot analysis. The subcellular distributions of NF-κB p65 (F) and p-STAT3 (Tyr705) (G) were observed by confocal microscopy (600×, LSM880, Carl Zeiss Co., Ltd., Oberkochen, Germany). NF-κB p65 and p-STAT3 were labelled in green, nuclei were stained blue with DAPI. Data were represented as mean ± SD from three independent experiments. *P < 0.05, **P < 0.01 vs. the control group, ***P < 0.001 vs. the control group, #P < 0.05, ##P < 0.01 vs. the model group. Ctrl, control group; Dex, dexamethasone group; LPS, lipopolysaccharide group.

Figure 5 Daidzin inhibited the NF-κB and STAT3 pathway by up-regulation of α7nAChR. (A) Daidzin up-regulated the protein expression of α7nAChR. The cells were pretreated with daidzin (25, 50 and 100 μM) for 12 h and then further co-incubated with LPS (100 ng/mL) for another 12 h. The protein expression level of α7nAChR was determined by Western blot analysis. (B) α7nAChR siRNA blocked the protein expression of α7nAChR. RAW264.7 cells were transfected with α7nAChR siRNA for 24 h and protein expression of α7nAChR was determined by Western blot analysis. The secretion levels of IL-6 (C) and TNF-α (D) in the culture supernatants were detected by ELISA kits. The nuclear protein levels of NF-κB p65, p-STAT3 (Tyr705) (E) and the total protein expressions of IκBα, p-IκBα (Ser32), STAT3 (F) were determined by Western blot analysis. Data were represented as mean ± SD from three independent experiments. ****P < 0.001 vs. the control group, ###P < 0.001 vs. the model group, ####P < 0.001 vs. the daidzin treatment group. LPS, lipopolysaccharide group.
In the current study, four compounds were firstly isolated from the crude soybean glycolipids and two of them were found to exert notable anti-inflammatory activities in zebrafish. These results provide a cheap and easily available strategy to obtain bioactive compounds from soybean glycolipids, which promotes the recycling and reutilization of this by-product.

NF-κB is a classical transcription factor that coordinates the expression of immune-related genes [39]. It is pertinent that STAT3 interacts closely with NF-κB. For example, some inflammatory cytokines encoded by genes related to NF-κB, particularly IL-6, are significant STAT3 activators [40]. In turn, STAT3 directly interacts with the NF-κB family member p65 by prolonging the stagnation time of active NF-κB in the nucleus and thus promoting the NF-κB activation [41]. Despite the component from soybean has been reported to repress the pro-inflammatory mediators release by suppressing the activation of NF-κB and STAT-1, there is currently no research on the inhibitory effects of daidzin in vivo and its possible mechanism of action [42]. Our study firstly elucidated that daidzin attenuates the secretion of IL-6 and TNF-α by inhibiting the NF-κB and STAT3 signaling pathways. Moreover, our results showed that α7nAChR regulates its downstream NF-κB and STAT3 signaling pathways. These results suggested that daidzin exhibited anti-inflammatory effects by partially activating the cholinergic anti-inflammatory pathway and then inhibiting the NF-κB and STAT3 pathways, indicating its potential to act as natural dietary anti-inflammatory agents.

Conclusion

In conclusion, four compounds were firstly isolated from the soybean glycolipids, among which verticilloide and daidzin significantly hampered the inflammation. Additionally, daidzin exerted the best anti-inflammatory effects, which are closely associates with activating the cholinergic anti-inflammatory pathway and further inhibition of NF-κB and STAT3 pathways. Taken together, our findings investigated that daidzin is a novel α7nAChR agonist, which supports it as a natural anti-inflammatory agent. Moreover, this new application of daidzin also promoted the recycling of the crude soybean glycolipids.

References
