RESEARCH PAPER

Isoacteoside, a dihydroxyphenylethyl glycoside, exhibits anti-inflammatory effects through blocking toll-like receptor 4 dimerization

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BACKGROUND AND PURPOSE

Isoacteoside (is a phenylethanoid isolated from *Monochasma savatieri* Franch. ex Maxim., which is an anti-inflammatory herb widely used in traditional Chinese medicine. However, the exact mechanism of the anti-inflammatory activity of isoacteoside is not completely understood. In this study, its anti-inflammatory mechanism was elucidated in mouse macrophages.

EXPERIMENTAL APPROACH

The expression of the NF- κ B pathway, MAPK pathway, iNOS, TNF- α , IL-6 and IL-1 β was evaluated using Western blotting, quantitative real-time PCR or ELISA. TLR4 dimerization was determined by transfecting HEK293T cells with TLR4 plasmids. The *in vivo* anti-inflammatory effect of isoacteoside was determined using mouse models of xylene-induced ear oedema, LPS-induced endotoxic shock and LPS-induced endotoxaemia-associated acute kidney injury (AKI).

KEY RESULTS

Isoacteoside suppressed COX-2, iNOS, TNF- α , IL-6 and IL-1 β expression. Furthermore, isoacteoside attenuated the LPS-induced transcriptional activity of NF- κ B by decreasing the levels of phosphorylated I κ B- α and IKK and NF- κ B/p65 nuclear translocation. In addition, isoacteoside inhibited LPS-induced transcriptional activity of AP-1 by reducing the levels of phosphorylated JNK1/2 and p38MAPK. Isoacteoside blocked LPS-induced TLR4 dimerization, resulting in a reduction in the recruitment of MyD88 and TRIF and the phosphorylation of TGF- β -activated kinase-1 (TAK1). Pretreatment of mice with isoacteoside effectively inhibited xylene-induced ear oedema and LPS-induced endotoxic death and protected against LPS-induced AKI.

CONCLUSIONS AND IMPLICATIONS

Isoacteoside blocked TLR4 dimerization, which activates the MyD88–TAK1–NF- κ B/MAPK signalling cascades and TRIF pathway. Our data indicate that isoacteoside is a potential lead compound for the treatment of inflammatory diseases.

Abbreviations

AP-1, activator protein-1; IKK, I κ B kinase; iNOS, inducible NOS; TAK1, TGF- β activated kinase-1; TLR, toll-like receptor

Introduction

Inflammation is a self-protective response to invading pathogens, damaged cells and irritants. However, sustained inflammation generally leads to the aetiology of a panel of inflammatory-related diseases, such as rheumatoid arthritis (Shin *et al.*, 2012), endotoxaemia (Fichtner-Feigl *et al.*, 2005) and inflammatory bowel disease (Staniforth *et al.*, 2004). The transcriptional factor NF- κ B, one of the most important regulators of pro-inflammatory gene expression, is well known for playing a pivotal role in the initiation and amplification of the inflammatory process (Surh *et al.*, 2001). The cytoplasmic form of NF- κ B is a complex comprising I κ Bs, p65 and p50, whereas the main active form of NF- κ B is a heterodimer comprising p65 and p50 (Liu *et al.*, 2001). When the NF- κ B pathway is activated, NF- κ B/p65 is released and translocated from the cytoplasm to the nucleus, initiating the transcription of pro-inflammatory mediators such as **TNF- α** , **iNOS**, **COX-2**, **IL-1 β** and **IL-6** (Liu *et al.*, 2001; Ha *et al.*, 2014). Thus, suppressing the NF- κ B pathway to block the secretion of these cytokines is an important strategy in anti-inflammatory drug discovery. The transcriptional factor AP-1, which is activated by MAPK family members such as **p38MAPK**, **JNK1/2** and **ERK1/2**, also plays an important role in the inflammatory process (Chang and Karin, 2001; Xagorari *et al.*, 2002). Inhibiting the MAPK pathways is a well-established and effective approach for treating inflammatory diseases.

Currently, 11 toll-like receptor (TLR) family members have been identified. Among these TLRs, **TLR4** is a critical signalling receptor for **LPS** that mediates innate and acquired immunity (Saitoh *et al.*, 2004). When activated by LPS, TLR4 dimerizes and subsequently induces MAPK signalling transduction and NF- κ B translocation by interacting with its downstream 'bridging adaptor' molecules *via* both the MyD88-dependent and MyD88-independent pathways (Xu *et al.*, 2014). The MyD88-dependent signalling pathway activates **TAK1**, subsequently effecting signalling through the NF- κ B and/or MAPK pathways and eventually triggering the release of pro-inflammatory mediators (Yu *et al.*, 2008; Gao *et al.*, 2016). Therefore, inhibiting the homodimerization of TLR4 is proposed as an alternative strategy for treating inflammatory disorders.

Endotoxaemia, a clinical syndrome caused by injury or infection, is characterized by a whole-body inflammatory response (Cohen, 2002). Acute kidney injury (AKI), a devastating condition of endotoxaemia (Zarjou and Agarwal, 2011), is a worldwide health problem without an effective drug therapy. LPS has been identified as one of the most important factors that lead to AKI (Rivers *et al.*, 2008). LPS can up-regulate the production of the pro-inflammatory cytokines TNF- α , IL-6 and IL-1 β , which lead to the development of AKI (Chvojka *et al.*, 2010; Chen *et al.*, 2015a).

Isoacteoside is a compound isolated from *Monochasma savatieri* Franch. ex Maxim., a traditional Chinese medicine that is a component of Yanning particles. Yanning particles have been approved by the China Food and Drug Administration for the treatment of inflammatory diseases (Liu *et al.*, 2013). However, the material basis of Yanning particles that exerts its anti-inflammatory effects has not been clearly elucidated. In this study, four dihydroxyphenylethyl glycosides,

namely, acteoside (ACT), isoacteoside, torenoside B (TNB) and savaside A (SVA) (Figure 1A), were isolated and identified. Their anti-inflammatory activities were screened, and isoacteoside was investigated in detail.

Methods

Cell culture

RAW264.7 macrophages obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and L929 and HEK293T cells purchased from American Type Culture Collection (Manassas, VA, USA) were cultured in DMEM supplemented with 10% FBS. Cells were maintained at 37°C under a humidified atmosphere of 5% CO₂ in an incubator.

The isolation of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDMs) from 6- to 9-week-old female BALB/c mice were produced as reported previously (Troupin *et al.*, 2013). Briefly, mouse bone marrow cells were differentiated in DMEM supplemented with 10% FBS and 20% M-CSF-conditioned medium from L929 cells for 7 days. The BMDMs were then cultured overnight in six-well plates and 96-well plates at densities of 8×10^5 cells per well and 2×10^4 cells per well respectively.

MTT assay

RAW264.7 cells were seeded into 96-well plates at a density of 10^5 cells per well overnight. Then, the cells were treated with isoacteoside (20, 40 or 80 μ M) for 24 h, and the MTT assay was used to determine the cytotoxicity according to our previous report (Gao *et al.*, 2015).

Determination of nitrite and NO

RAW264.7 cells were plated in 24-well plates at a density of 5×10^5 per well overnight. After the cells were pretreated with isoacteoside (20, 40 or 80 μ M) for 1 h, LPS ($1 \mu\text{g}\cdot\text{mL}^{-1}$) was added to the isoacteoside containing medium and cultured for 24 h. The nitrite levels in the culture media were determined using the Griess reagent according to the protocol from the manufacturer.

Treated cells were collected and stained with DAF-FM ($1 \mu\text{M}$) for an additional 1 h. The fluorescence signal was determined by FACScan flow cytometry using the FITC channel.

ELISA

After being pretreated with isoacteoside (20, 40 or 80 μ M) for 1 h, BMDMs and RAW264.7 cells were then incubated with LPS ($1 \mu\text{g}\cdot\text{mL}^{-1}$) for 24 h. The medium was collected, and the secretion levels of TNF- α , IL-6 and IL-1 β were determined using ELISA kits according to the manufacturer's instructions.

Immunofluorescence

The immunofluorescence analysis of NF- κ B/p65 was conducted as previously described (Gao *et al.*, 2016). Briefly, 2×10^5 RAW264.7 cells were seeded into a 35-mm glass

Figure 1

Isoacteoside was chosen as an anti-inflammatory agent for further mechanistic study in LPS-stimulated RAW264.7 cells. (A) Chemical structures of ACT, isoacteoside, TNB and SVA. (B) Cells were pretreated with TNB, SVA, ACT or isoacteoside at the indicated concentrations for 1 h before being stimulated with LPS (1 $\mu\text{g}\cdot\text{mL}^{-1}$) for another 24 h. The effects of the four compounds on nitrite production were determined by the Griess assay ($n = 6$). The nitrite values of the control and LPS group were 0.99 and 20.67 μM respectively. (C, D) Cells were pretreated with TNB (80 μM), SVA (80 μM), ACT (80 μM) or isoacteoside (80 μM) for 1 h before being stimulated with LPS (1 $\mu\text{g}\cdot\text{mL}^{-1}$) for another 6 h. NO was measured using flow cytometry with DAF-FM (1 μM) ($n = 5$). (E) Cells were pretreated with the TNB (80 μM), SVA (80 μM), ACT (80 μM) or isoacteoside (80 μM) for 1 h before being stimulated with LPS (1 $\mu\text{g}\cdot\text{mL}^{-1}$) for another 24 h. The expression levels of iNOS and COX-2 were determined by immunoblotting ($n = 5$). * $P < 0.05$ compared with the LPS alone group.

bottom SPL confocal dish overnight. After the cells were pretreated with isoacteoside (80 μM) for 1 h and stimulated with LPS (1 $\mu\text{g}\cdot\text{mL}^{-1}$) for another 2 h, the primary antibody anti-NF- κB /p65 (1:50) and secondary antibody goat anti-rabbit Alexa Fluor 568 (1:200) were applied. Cells were fixed and stained with Hoechst 33 342 (1 μM) for imaging. The imaging was performed under a Leica TCS SP8 laser confocal

microscope at 60 $\times$ magnification using excitation/emission wavelengths of 588/615–690 nm.

Transient transfection and luciferase assay

HEK293T cells were seeded into a dish (10 cm, i.d.), at a density of 10^6 cells, and left overnight. TLR4-HA and TLR4-Flag plasmids obtained from Addgene were co-transfected using

TurboFect transfection reagents (Thermo Fisher Scientific) for 24 h. The transfected cells were seeded into a dish (5 cm, i.d.) at a density of 5×10^5 cells, and left overnight. The cells were pretreated with isoacteoside (80 μM) for 1 h and stimulated with LPS (1 $\mu\text{g}\cdot\text{mL}^{-1}$) for 24 h before harvesting.

RAW264.7 cells cultured in 96-well plates overnight were transiently co-transfected with piNOS-luc, pNF- κB -luc or pAP-1-luc plasmids and pRL-TK plasmid to detect iNOS, NF- κB or AP-1 transcriptional activity, respectively, according to the manufacturer's instructions. The pRL-TK plasmid was used as the reference control. After 48 h of transfection, the cells were pretreated with isoacteoside (80 μM) for 1 h and stimulated with LPS (1 $\mu\text{g}\cdot\text{mL}^{-1}$) for 16 h. The luciferase activity was determined using a Dual-Glo luciferase assay system kit according to the manufacturer's instructions.

Docking of isoacteoside to TLR4 complex

A docking simulation of isoacteoside with the TLR4 complex (PDB code: 3FXI) was performed using the molecular modelling packages in Ledock (<http://www.lephar.com>). TLR4 complex crystal structures were obtained from the RCSB Protein Data Bank (PDB code: 3FXI) (Park *et al.*, 2009).

Immunoblotting and immunoprecipitation

Protein samples were collected from treated cells, and the protein concentrations were examined using a Pierce Biotechnology BCA assay kit (Waltham, MA, USA). Then, 800 μg of proteins in IP lysis buffer were immunoprecipitated using anti-HA magnetic beads or protein A/G magnetic beads. The subsequent steps were performed according to the manufacturer's instructions. An equivalent amount of each protein sample was subjected to SDS-PAGE gel electrophoresis and transferred to PVDF membranes. After being blocked with 5% non-fat milk dissolved in Tris-buffered saline Tween 20 buffer for 1 h, the membranes were probed with primary antibodies (1:1000) overnight at 4°C and the corresponding secondary antibodies (1:5000) for another 1 h at 25°C. The protein band signals were detected with SuperSignal West Femto maximum sensitivity substrate (Pierce Biotechnology) under visualization in a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from each sample using a TRIzol assay kit (St. Louis, MO, USA). Then, 1 μg of RNA was employed in qRT-PCR using a qPCR master mix kit. PCR amplification was performed by incorporating SYBR green. The oligonucleotide primers derived from mice for iNOS were GGCAGCCTG TGAGACCTTTG (forward) and GCATTGGAAGTGAAGCGTT TC (reverse), for TNF- α were TTCTGTCTACTGAACCTCGGG GTGATCGGTCC (forward) and GTATGAGATAGCAAAT CGGCTGACGGTGTGGG (reverse), for IL-6 were TCCAGT TGCCTTCTTGGGAC (forward) and GTGTAATTAAGCCTCC GACTTG (reverse), for COX-2 were TGAGTACCGCAAACGC TTCTC (forward) and TGGACGAGGTTTTCCACCAG (reverse) for COX-2 and for IL-1 β were GAAAGACGGCACAC CCACCCT (forward) and GCTCTGCTTGTGAGGTGCTGA TGTA (reverse). The oligonucleotide primers derived from mice for GAPDH, which was used as a housekeeping gene, were CATGACCACAGTCCATGCCATCAC (forward) and TGAGGTCCACCACCCTGTTGCTGT (reverse). The steady-

state mRNA levels of iNOS, TNF- α , IL-6, COX-2, IL-1 β and GAPDH were determined using qRT-PCR on a Takara thermal cycler dice® (Takara Bio Inc., Shiga, Japan).

Animal experiments and ethical statement

BALB/c mice (male, 6–8 weeks-old and weighing 18–22 g) were obtained from the Experimental Animal Centre of Soochow University. All mice were reared in plastic cages (≤ 5 mice per cage) and given free access to food and water under standard conditions (specific-pathogen-free) with air filtration ($22 \pm 2^\circ\text{C}$, 12-h light/12-h dark). The study was performed in accordance with the Local Guide for the Care and Use of Laboratory Animals of Soochow University. These experiments are approved by the Ethics Committee of the Experimental Animal Centre of Soochow University (No. IACUC2016-13). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

For the xylene-induced mice ear oedema model (Ma *et al.*, 2013), isoacteoside (100 $\text{mg}\cdot\text{kg}^{-1}$) was administered i.p. After 2 h, 30 μL xylene ($\geq 98.5\%$) was applied to the posterior and anterior surfaces of the right ears. The left ears were untreated and served as controls. An hour later, the mice were killed by cervical dislocation under ether anaesthesia, and two ear punches (7 mm, i.d.) were collected and weighed. The oedema was evaluated by comparing the increase in the weight of the right ear punch with the increase in the weight of the left ear punch. The ear tissues were collected and fixed in 10% formaldehyde for at least 24 h at room temperature. After being dehydrated in different concentrations of alcohol, tissues were embedded in paraffin and sliced. The sections were stained with haematoxylin and eosin (H&E) and imaged under a light microscope.

For the endotoxic shock model (Chen *et al.*, 2015b), endotoxaemia was induced in mice by administering LPS (20 $\text{mg}\cdot\text{kg}^{-1}$, i.p.). The mice were pretreated with isoacteoside (100 $\text{mg}\cdot\text{kg}^{-1}$, i.p.) for 2 h before the LPS injection. Dexamethasone (DEX, 5 $\text{mg}\cdot\text{kg}^{-1}$, i.p.) was used as positive control because it has a superior anti-inflammatory effect (Zeng *et al.*, 2015; Qin *et al.*, 2016) and has been used in a clinical study to treat an endotoxic shock (Zhang *et al.*, 2003). The survival rate was monitored continuously for 132 h. After 132 h, the remaining mice were killed by cervical dislocation under ether anaesthesia.

For the AKI model (Zarjou and Agarwal, 2011), mice were randomly divided into six groups: a control group, isoacteoside (25, 50 and 100 $\text{mg}\cdot\text{kg}^{-1}$, i.p.) groups, an LPS group (10 $\text{mg}\cdot\text{kg}^{-1}$, i.p.) and a positive control **dexamethasone** (5 $\text{mg}\cdot\text{kg}^{-1}$, i.p.) group. At 2 h before the LPS injection, mice were pretreated with isoacteoside. After 12 h, serum samples were collected by retro-orbital bleeding under ether anaesthesia, and the cytokines (TNF- α , IL-6 and IL-1 β) were examined using ELISA kits according to the manufacturer's instructions. The mice were killed by cervical dislocation. The serum levels of urea nitrogen (BUN) and creatinine were determined on a Roche Module P800 (Roche, Shanghai, China). Furthermore, the kidney tissues were harvested and fixed in 10% formaldehyde. After being dehydrated in different concentrations of alcohol, the tissues were embedded in paraffin and sliced. The sections were stained with H&E and imaged under a light microscope.

Data analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). All results are presented as the means $\pm$ SEM. For statistical analysis, the significance of the intergroup differences was analysed by one-way ANOVA with Dunnett's multiple comparisons test using GraphPad Prism 6.0 software. The significant difference was defined as $P < 0.05$.

Materials

ACT (>98%), isoacteoside (>98%), TNB (>98%) and SVA (>98%) were isolated in our laboratory and determined by HPLC. LPS (*Escherichia coli*, serotype 0111: B4), GM-CSF, Griess reagent, and antibodies against HA and Flag were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM and FBS were purchased from Life Technologies/Gibco Laboratories (Grand Island, NY, USA). ELISA kits for IL-1 β , IL-6 and TNF- α were purchased from Neobioscience (Shenzhen, China). Antibodies against iNOS, COX-2, p65, p-p65, p-IKK- α/β , IKK- α , IKK- β , p-I κ B- α , I κ B- α , p-p38 MAPK, p38 MAPK, p-ERK1/2, ERK1/2, p-JNK1/2, JNK1/2, p-TAK1, TAK1, IL-1 β , TRIF and GAPDH were purchased from Cell Signaling Technologies (Beverly, MA, USA). Antibodies against TLR4 and MyD88 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Oligonucleotide primers for iNOS, COX-2, TNF- α , IL-6, IL-1 β and GAPDH were purchased from Nanjing KeyGEN Biotech. Co. Ltd. (Jiangsu, China). 4-Amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM), an HA-tag magnetic IP/Co-IP kit and a Protein A/G magnetic beads kit were purchased from Life Technologies/Thermo Fisher Scientific (Grand Island, NY, USA). Plasmids of pNF- κ B-luc, piNOS-luc, pAP-1-luc and pRL-TK were purchased from Addgene (Beijing, China). RT-PCR, quantitative PCR (qPCR) and Lipofectamine LTX™ kits were purchased from Promega (Madison, WI, USA).

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015a,b).

Results

Isoacteoside suppresses LPS-induced iNOS and COX-2 expression in RAW264.7 cells

The anti-inflammatory activities of the four phenylethanoids (Figure 1A) were first screened in LPS-stimulated RAW264.7 cells. As shown in Figure 1B, isoacteoside and ACT significantly decreased LPS-induced nitrite levels, whereas TNB and SVA had a much weaker inhibitory effect (Figure 1B). Similar trends were observed in the **NO** levels (Figure 1C, D). We also evaluated iNOS and COX-2 expression in RAW264.7 cells, and the results showed that isoacteoside was more potent in suppressing LPS-induced

iNOS and COX-2 expression than TNB, SVA and ACT (Figure 1E). Thus, isoacteoside was chosen for further mechanistic studies. The MTT assay showed that isoacteoside (20–80 μ M) had no significant cytotoxic effect on RAW264.7 cells after 24 h of treatment (data not shown). Figure 2A, B shows that isoacteoside suppressed LPS-induced NO production in a concentration-dependent manner. The LPS-stimulated protein expression of iNOS and COX-2 in RAW264.7 cells pretreated with isoacteoside was also inhibited in a concentration-dependent manner (Figure 2 C). Furthermore, isoacteoside significantly inhibited LPS-induced iNOS and COX-2 mRNA expression (Figure 2D, F) and suppressed LPS-induced iNOS promoter activity in a concentration-dependent manner, as determined by a reporter gene assay and luciferase activity assay (Figure 2E). Collectively, these findings indicate that isoacteoside suppresses LPS-induced iNOS and COX-2 expression and activity in RAW264.7 cells.

Isoacteoside inhibits LPS-induced cytokine release

LPS stimulates the production of pro-inflammatory mediators such as NO, TNF- α , IL-6 and IL-1 β in RAW264.7 cells *via* the NF- κ B pathway and/or MAPK pathway (Monica *et al.*, 2006; Lu *et al.*, 2007). The production of these cytokines was determined using ELISA. The TNF- α and IL-6 levels in isoacteoside-pretreated RAW264.7 cells were significantly decreased in a concentration-dependent manner (Figure 3A, D). After treatment of LPS-stimulated BMDMs with isoacteoside, cytokines such as TNF- α , IL-6 and IL-1 β were also concentration-dependently suppressed (Figure 3B, E, G). The inhibitory effect of isoacteoside on IL-1 β in both RAW264.7 and BMDMs was further confirmed by Western blotting (Supporting Information Figure S1). Furthermore, their mRNA expression levels were significantly decreased by isoacteoside (Figure 3C, F, H). In summary, isoacteoside effectively inhibited TNF- α , IL-6 and IL-1 β release and expression.

Isoacteoside inhibits LPS-induced NF- κ B and AP-1 activation in RAW264.7 cells

NF- κ B and AP-1 activation is known to play a pivotal role in the inflammatory process (Ha *et al.*, 2014). Reporter gene assays showed that isoacteoside significantly suppressed both NF- κ B-dependent luciferase activity and AP-1 luciferase activity in a concentration-dependent manner (Figure 4A, B). Furthermore, LPS significantly induced p65 phosphorylation, which was inhibited by isoacteoside (Figure 4C). In addition, immunofluorescence staining analysis revealed that isoacteoside inhibited the LPS-induced translocation of NF- κ B/p65 into the nucleus in RAW264.7 cells (Figure 4D).

Effects of isoacteoside on the degradation of I κ B- α and the activation of IKK- α/β in RAW264.7 cells

As an inhibitor protein of NF- κ B, I κ B- α keeps NF- κ B in an inactive state in the cytoplasm. However, in the presence of certain stimuli, such as LPS, I κ B- α is phosphorylated at specific sites, causing polyubiquitination and proteasomal degradation and thus allowing free NF- κ B to translocate from

Figure 2

Isoacteoside (ISO) had an inhibitory effect on iNOS and COX-2 expression in LPS-stimulated cells. (A, B) Cells were pretreated with the indicated concentrations of isoacteoside for 1 h before being stimulated with LPS ($1 \mu\text{g mL}^{-1}$) for another 6 h. NO was determined using flow cytometry with DAF-FM ($1 \mu\text{M}$) ($n = 5$). (C) Cells were pretreated with the indicated concentrations of isoacteoside for 1 h before being stimulated with LPS ($1 \mu\text{g mL}^{-1}$) for another 24 h. The iNOS and COX-2 expression was examined by Western blotting ($n = 5$). (D, F) Cells were pretreated with isoacteoside (20–80 μM) for 1 h before being stimulated with LPS for another 6 h. The iNOS and COX-2 mRNA levels were determined using the qRT-PCR assay described in the Methods section ($n = 6$). (E) RAW264.7 cells were transiently co-transfected with iNOS-luc and TK-luc for 48 h. The cells were pretreated with isoacteoside (80 μM) before being stimulated with LPS ($1 \mu\text{g mL}^{-1}$) for another 24 h. The luciferase activity was determined using the Dual-Glo luciferase assay described in the Methods section ($n = 6$). $P < 0.05$ compared with the LPS alone group.

the cytoplasm to the nucleus (Wen *et al.*, 2006). Figure 5A shows that LPS induced I κ B- α phosphorylation and degradation, which were significantly inhibited by isoacteoside pretreatment. Furthermore, the expression of **IKK- α** and

IKK- β , two upstream kinases of I κ B in the NF- κ B signal pathway, was determined. Isoacteoside clearly decreased LPS-induced IKK- α/β phosphorylation without affecting the total IKK- α/β expression.

Figure 3

Isoacteoside (ISO) suppressed LPS-induced pro-inflammatory cytokine release in RAW264.7 cells and BMDMs. (A, D) RAW264.7 cells were pretreated with the indicated concentrations of isoacteoside for 1 h before being stimulated with LPS for another 24 h. The pro-inflammatory cytokines, TNF- α and IL-6, were determined using ELISA ($n = 6$). (B, E, G) BMDMs were pretreated with the indicated concentrations of isoacteoside for 1 h before being stimulated with LPS (1 $\mu\text{g mL}^{-1}$) for another 24 h. The supernatants were collected. The pro-inflammatory cytokines, TNF- α , IL-6 and IL-1 β , were determined using ELISA ($n = 6$). (C, F, H) RAW264.7 cells were pretreated with the indicated concentrations of isoacteoside for 1 h before being stimulated with LPS for another 6 h. The TNF- α , IL-6 and IL-1 β mRNA levels were detected using the qRT-PCR assay described in the Methods section ($n = 6$). * $P < 0.05$ compared with the LPS alone group.

The MAPK signalling pathway contributes to the anti-inflammatory activity of isoacteoside

The activation of the MAPK (JNK1/2, ERK1/2 and p38 MAPK) signalling pathways is always a response to inflammatory stress (Chen *et al.*, 2015b). Furthermore, MAPK phosphorylation activates c-Jun, leading to its translocation into the nucleus and its binding with Jun or Fos family members to form the AP-1 transcriptional factor (Hossen *et al.*, 2015). To determine the role of MAPK signalling in the anti-inflammatory activity of isoacteoside, the expression of JNK1/2, ERK1/2 and p38 MAPK was determined using Western blotting. As shown in Figure 5B, isoacteoside pretreatment dramatically decreased p-JNK1/2 and p-p38 MAPK

expression in a concentration-dependent manner but did not alter total JNK1/2 and p38 MAPK expression. However, isoacteoside also did not obviously affect either p-ERK1/2 or total ERK1/2 levels. Thus, these findings show that JNK1/2 and p38 MAPK might participate in the anti-inflammatory activity of isoacteoside.

Isoacteoside disrupts LPS-induced TLR4 dimerization

TLR4, a transmembrane receptor situated on the surface of immune cells, plays a pivotal role in regulating innate and acquired immunity and the inflammatory process (Hennessy *et al.*, 2010). Upon stimulation with LPS, TLR4 forms a dimer

Figure 4

Effects of isoacteoside (ISO) on LPS-induced NF- κ B and AP-1 activation in RAW264.7 cells. (A, B) Cells were transiently co-transfected with NF- κ B-luc and TK-luc or AP-1-luc and TK-luc for 48 h. The cells were pretreated with isoacteoside (80 μM) before being stimulated with LPS ($1 \mu\text{g mL}^{-1}$) for another 24 h. The luciferase activity was determined using the Dual-Glo luciferase assay described in the Methods section ($n = 6$). (C) Cells were pretreated with the indicated concentrations of isoacteoside for 1 h before being stimulated with LPS for another 2 h. The expression of phosphorylated p65 was determined by Western blotting ($n = 5$). (D) RAW264.7 cells were pretreated with the indicated concentrations of isoacteoside for 1 h before being stimulated with LPS for another 2 h. The p65 translocation was determined using the immunofluorescence assay described in the Methods sections ($n = 3$). The primary antibody anti-NF- κ B/p65 (1:50) and secondary antibody goat anti-Rabbit Alexa Fluor 568 (1:200) were employed. * $P < 0.05$ compared with the LPS alone group.

and then activates the NF- κ B and/or MAPK signalling pathways, leading to a pathogen-specific innate immune response by releasing pro-inflammatory cytokines (Kawai and Akira, 2005). To explore whether LPS-induced TLR4

dimerization was affected by isoacteoside, HEK293T cells co-transfected with TLR4-HA and TLR4-Flag plasmids were employed. As shown in Figure 6A, compared with the LPS-treated group, the isoacteoside pretreatment group

Figure 5

Effects of isoacteoside (ISO) on the LPS-induced degradation of IκB-α and activation of IKK-α/β and the MAPK pathway. (A, B) Cells were pretreated with the indicated concentrations of isoacteoside for 1 h before being stimulated with LPS (1 μg mL⁻¹) for another 2 h. The expression of proteins such as IκB-α, IKK-α/β, IKK-α, IKK-β, p-JNK1/2, JNK1/2, p-ERK1/2, ERK1/2, p-p38 and p38 was determined by Western blotting (*n* = 5). **P* < 0.05 compared with the LPS alone group.

showed a decrease in TLR4-Flag in the TLR4-HA precipitation assay, suggesting that isoacteoside blocked LPS-induced TLR4 dimerization. Furthermore, molecular docking analysis showed that isoacteoside fitted into the hydrophobic pocket of TLR4, which overlaps part of the LPS binding sites on TLR4 (Figure 7), and effectively blocked TLR4 dimerization. Therefore, the anti-inflammatory effect of isoacteoside may initially be due to its ability to block TLR4 dimerization.

Isoacteoside blocks the LPS-induced MyD88-dependent and TRIF-dependent signalling cascades

TLR4 dimerization triggers two pathways during the pro-inflammatory process: the MyD88-dependent and MyD88-independent (TRIF) pathways (Kawai and Akira, 2005). The MyD88-dependent pathway is initiated by MyD88 being recruited to the domain of the TLR/IL-1 receptor and then binding with IRAK4 to enable IRAK1 to recruit TRAF6. The IRAK1-TRAF6 complex phosphorylates TAB2/TAB3 and TAK1 and thus activates the NF-κB and MAPK signalling pathway (Ajibade *et al.*, 2013). We explored whether isoacteoside exerted effects on the LPS-induced MyD88 and/or TRIF pathway. Figure 6B, C shows that RAW264.7 cells treated with LPS exhibited significantly increased the formation of the TLR4-MyD88 and TLR4-TRIF complexes. However, isoacteoside could

significantly decrease the LPS-induced formation of both complexes. In addition, isoacteoside inhibited TAK1 phosphorylation of LPS-stimulated RAW264.7 cells without affecting total TAK1 (Figure 6D). In summary, these findings indicate that isoacteoside exerts its anti-inflammatory effect mainly through the TLR4-MyD88-TAK1 and TLR4-TRIF pathways.

Isoacteoside inhibits inflammation in mice models

In a xylene-induced ear oedema mouse model, a significant inhibitory effect of isoacteoside (100 mg·kg⁻¹) on ear weight (Figure 8A) and ear section H&E staining was observed (Figure 8C-F). Furthermore, in an LPS-induced endotoxaemia model, all mice died within 72 h of LPS injection. However, 1-h pretreatment with isoacteoside (100 mg·kg⁻¹) significantly increased the survival rate. The survival rate was 45% at 132 h, which was higher than that of the mice treated with DEX (Figure 8B). In addition, in an LPS-induced AKI model, isoacteoside significantly reduced the levels of the LPS-induced inflammatory cytokines TNF-α, IL-6 and IL-1β (Figure 9A-C). In addition, the increase in creatinine and BUN caused by LPS was significantly suppressed by isoacteoside (Figure 9D, E). The H&E staining of renal tissues revealed normal kidney tubules in the control group (Figure 10A). In contrast, the LPS group exhibited oedema of the renal tubular epithelial cells, atrophy of the

Figure 6

Isoacteoside (ISO) blocked LPS-induced TLR4 dimerization, which is involved in the MyD88 and TRIF pathway. (A) HEK293T cells were co-transfected with TLR4-HA and TLR4-Flag for 24 h. Cells were pretreated with isoacteoside ($80 \mu\text{M}$) for 1 h before being stimulated with LPS ($1 \mu\text{g mL}^{-1}$) for another 24 h. The extent of TLR4 dimerization was determined using the co-immunoprecipitation assay described in the Methods section. Collected proteins were immunoprecipitated with anti-HA magnetic beads. Immunocomplexes were determined by Western blotting with anti-HA and anti-Flag antibodies ($n = 5$). (B, C) RAW264.7 cells were pretreated with isoacteoside ($80 \mu\text{M}$) for 1 h before being stimulated with LPS ($1 \mu\text{g mL}^{-1}$) for another 2 h. Collected proteins were immunoprecipitated with MyD88 or TRIF using magnetic beads. Immunocomplexes were determined by Western blotting with anti-MyD88, anti-TRIF and anti-TLR4 antibodies ($n = 5$). (D) RAW264.7 cells were pretreated with isoacteoside (20, 40 and $80 \mu\text{M}$) for 1 h before being stimulated with LPS ($1 \mu\text{g mL}^{-1}$) for another 2 h. The p-TAK1 and TAK1 expression was examined by Western blotting ($n = 5$). * $P < 0.05$ compared with the LPS alone group.

glomerulus, dilation of the renal capsule cavity, destruction of tubular structures, local focal necrosis and collapse of the epithelial cells and oedema of the renal interstitial epithelial cells (Figure 10B). Isoacteoside pretreatment significantly attenuated LPS-induced epithelial atrophy and necrosis and interstitial oedemas (Figure 10C–E). DEX was used as positive control (Figure 10F).

Discussions

To discover anti-inflammatory agents, we screened a series of dihydroxyphenylethyl glycoside derivatives that were isolated and identified from *M. savatieri* Franch. ex Maxim. in our laboratory. The anti-inflammatory activity of TNB, SVA and, in particular, ACT and isoacteoside has been reported.

Figure 7

The docking results of isoacteoside with the TLR4 complex. (A) The whole view of the TLR4 dimer and MD-2 depicting the small molecular binding pocket. (B) A close-up view of the small molecule binding pocket from the side. (C) Crystal structure of isoacteoside (green) showing TLR4 (yellow and pink) and MD-2 (grey) bound to the docking pocket. Hydrogen bonds are shown as blue lines.

Kim *et al.* (2009) reported that the anti-inflammatory activity of ACT was greater than that of isoacteoside in RBL 2H3 cells. Here, we found that isoacteoside and ACT had similar anti-inflammatory activities in RAW264.7 cells. However, ACT showed no significant effect on LPS-induced pro-inflammatory cytokines in animal models (data not shown). Furthermore, isoacteoside is a major constituent of Yanning particles, which have been used clinically for many years in China (SFDA Approval No. Z44020437). To the best of our knowledge, in this study we provide the first evidence that the anti-inflammatory mechanism of isoacteoside arises from its effect on TLR4-mediated signalling. Isoacteoside exhibited

a significant effect on three inflammatory mouse models: xylene-induced ear oedema, LPS-induced endotoxic shock and LPS-induced AKI.

TLR4, one of the first TLR family members to be identified in 1997 (Medzhitov *et al.*, 1997), is considered to be the gene encoding the LPS receptor (Di Lorenzo *et al.*, 2015). TLR4, in association with myeloid differentiation factor 2 (MD-2), is responsible for the physiological recognition of LPS in many different cells that express both proteins, such as macrophages, lymphoid cells and epithelial, endothelial and vascular smooth muscle cells (Hennessy *et al.*, 2010). TLR4 and MD-2 form a heterodimer that recognizes a common

Figure 8

Isoacteoside (ISO) decreased xylene-induced ear oedema and protected against LPS-induced endotoxic shock in mice. (A) Ear oedema was induced by the application of xylene. Mice were pretreated with isoacteoside (100 mg·kg⁻¹, i.p.) for 2 h before being administered xylene for 1 h, and then the ear weight was measured as described in the Methods section. Values are shown as the mean ± SEM ($n = 10$), * $P < 0.05$ compared with the xylene alone group. (B) Twenty mice per group were pretreated with vehicle or isoacteoside (100 mg·kg⁻¹, i.p.) for 2 h before the LPS (20 mg·kg⁻¹, i.p.) injection. DEX (5 mg·kg⁻¹, i.p.) was used as a positive control. The survival rates of these mice were observed for the next 132 h. (C) Histopathological examination of the ear sections. The left ear of mice not treated with isoacteoside was regarded as a vehicle control. (D) The right ear of mice treated with xylene (30 μL) but not with isoacteoside was regarded as the model control. (E) The left ear of a mouse pretreated with isoacteoside (100 mg·kg⁻¹, i.p.) for 2 h but not treated with xylene. (F) The right ear of a mouse pretreated with isoacteoside (100 mg·kg⁻¹, i.p.) for 2 h and then treated with xylene (30 μL) for another 1 h. The ear tissue samples were stained with H&E as described in the Methods section. (H&E, original magnification, 400×).

'pattern' in structurally diverse LPS molecules (Park *et al.*, 2009). Upon being released from the bacterial membrane, LPS is captured by the TLR4–MD-2 heterodimer, which has complex ligand specificity, *via* two accessory proteins: LPS binding protein and CD14 (Bryant *et al.*, 2010). Subsequently, the receptor multimer is formed, including two copies of the TLR4–MD-2–LPS complex displayed in a symmetrical manner to activate pro-inflammatory signalling pathways (Park *et al.*, 2009). Therefore, inhibiting TLR4 dimerization is a new strategy proposed to treat inflammatory disorders. Many previous studies have indicated that inhibiting LPS-induced

TLR4 expression is an alternative strategy for discovering new anti-inflammatory drugs with mechanisms involving TLR4 (Hennessy *et al.*, 2010). In contrast, we propose that blocking TLR4 dimerization is an alternative strategy for discovering new anti-inflammatory agents. In this study, we used two different plasmids, TLR4-HA and TLR4-Flag, to show that isoacteoside could significantly block the dimerization of TLR4 (Figure 6A). A docking simulation assay further confirmed that isoacteoside bound to the hydrophobic pocket of TLR4, which partially overlaps with the LPS binding sites on TLR4, and thus disrupted TLR4 dimerization (Figure 7).

Figure 9

Isoacteoside (ISO) suppressed inflammatory cytokine release and renal injury markers in BALB/c mice treated with LPS. Mice were pretreated with isoacteoside (25, 50 and 100 mg·kg⁻¹, i.p.) for 2 h before the LPS (10 mg·kg⁻¹, i.p.) injection. After 12 h, the blood samples were collected from mice under anaesthesia *via* the retro-orbital route. Inflammatory cytokines, such as TNF- α (A), IL-6 (B) and IL-1 β (C), and creatinine (D) and blood urea nitrogen (E) in serum were determined using ELISA kits. DEX (5 mg·kg⁻¹, i.p.) was used as a positive control. * $P < 0.05$ compared with the LPS alone group ($n = 10$).

The biological activity of TLR4 induced by ligand binding consists of the dimerization or oligomerization of receptor chains (Saitoh *et al.*, 2004). Activated TLR4 recruits two adaptor protein pairs: MAL–MyD88 and TRAM–TRIF. MAL and TRAM are considered to be ‘bridging adaptors’ that interact directly with TLR4 to recruit MyD88 and TRIF respectively (Hennessy *et al.*, 2010). MAL–MyD88, TRAM–TRIF or both are needed to activate the NF- κ B and MAPK pathways and the production of pro-inflammatory cytokines such as TNF- α , IL-1 β and IL-6. In the present study, we investigated the MyD88 and TRIF pathways using a co-immunoprecipitation assay between TLR4 and MyD88 or TRIF. Our data indicate

that isoacteoside can decrease the LPS-induced interactions between TLR4 and MyD88 and between TLR4 and TRIF in RAW264.7 cells (Figure 6B, C). However, the downstream processes of the TRIF pathway remain to be explored.

Activation of the transcription factors NF- κ B and AP-1 in the TLR4–MyD88 pathway involves TAK1 and two adaptor proteins: TAB1 and TAB2 (Kawai and Akira, 2005). Functionally, TAB1, an activator of TAK1, promotes the kinase activity of TAK1 (Kaisho *et al.*, 2001), whereas TAB2, an adaptor that connects TAK1 and TRAF6, facilitates TAK1 activation (Kaisho *et al.*, 2001). TAK1, one of MAPK kinase kinase (MAPKKK) family members, has been shown to be crucial

Figure 10

Histopathological examination of the kidney tissue of LPS-stimulated mice treated with isoacteoside (H&E, original magnification, 400 $\times$). The kidney tissues were collected from the mice from which the samples used to detect inflammatory cytokines were taken. (A) Normal group, (B) LPS (10 mg $\cdot$ kg $^{-1}$, i.p.) group, (C) isoacteoside (25 mg $\cdot$ kg $^{-1}$, i.p.) group, (D) isoacteoside (50 mg $\cdot$ kg $^{-1}$, i.p.) group, (E) isoacteoside (100 mg $\cdot$ kg $^{-1}$, i.p.) group and (F) DEX group (5 mg $\cdot$ kg $^{-1}$, i.p.).

for NF- κ B and AP-1 activation (Kawai and Akira, 2005). In our study, isoacteoside suppressed the LPS-induced phosphorylation of TAK1 while having no effect on the total TAK1 in RAW264.7 cells (Figure 6D). These results indicate that TAK1 is downstream of TLR4–MyD88 in LPS-stimulated RAW264.7 cells treated with isoacteoside.

The phosphorylation of the IKK complex by active TAK1 released from the TAK1–TAB1/2 complex in the membrane leads to the phosphorylation of I κ B for degradation (Adhikari *et al.*, 2007). Our data indicate that isoacteoside attenuates the LPS-induced phosphorylation of IKK- α/β and I κ B- α in RAW264.7 cells (Figure 5A). In addition, isoacteoside decreased LPS-induced I κ B- α degradation (Figure 5A). Decreasing LPS-induced phosphorylation of NF- κ B/p65 led to the suppression of the translocation of p65 from the cytosol to the nucleus (Figure 4C, D). Furthermore, the reporter gene

assay indicated that isoacteoside significantly inhibited LPS-induced p65-luc activity in RAW264.7 cells (Figure 4A). In addition, numerous reports have demonstrated that MAPKs are also activated by TAK1 phosphorylation (Adhikari *et al.*, 2007). Previous studies showed that some novel agents are effective in treating inflammatory diseases by acting *via* the JNK1/2 and p38 MAPK signalling pathways (Kumar *et al.*, 2003; Kaminska, 2005). AP-1 activity is modulated by different MAPK members. JNK phosphorylation activates the transcriptional potential of c-Jun, a critical part of AP-1 (Chang and Karin, 2001). We noted that JNK1/2, ERK1/2 and p38 MAPK contribute to the LPS-induced inflammatory process. Isoacteoside was found to inhibit the LPS-induced phosphorylation of JNK1/2 and p38 MAPK but did not affect ERK1/2 phosphorylation (Figure 5B), a result that was consistent with the suppression of AP-1 activity by isoacteoside in

LPS-stimulated RAW264.7 macrophages, as determined using the reporter gene assay (Figure 4B).

To evaluate the effect of isoacteoside on NF- κ B- and/or MAPK-regulated gene transcription, we determined that isoacteoside suppressed the LPS-induced expression of inflammatory mediators, such as iNOS, COX-2, TNF- α , IL-6 and IL-1 β . Isoacteoside was shown to suppress the induction of iNOS expression by down-regulating its promoter activities, which result in subsequent NO production and release. In addition, isoacteoside inhibited LPS-induced COX-2 expression in terms of transcription and protein levels in RAW264.7 cells. Our findings also indicate that isoacteoside inhibits the expression, transcription and protein levels of TNF- α , IL-6 and IL-1 β in a concentration-dependent manner. These results indicate that the suppression of NF- κ B activation by isoacteoside inhibits pro-inflammatory gene expression at the transcriptional level. Interestingly, IL-1 β release in RAW264.7 cells is controversial. Many studies have shown that RAW264.7 cells do not produce mature IL-1 β (Chujor *et al.*, 1996; Ndengele *et al.*, 2000). However, numerous other authors have reported that according to ELISA, LPS stimulation of RAW264.7 cells could lead to IL-1 β release (Shin *et al.*, 2012; Li *et al.*, 2016; Xiang *et al.*, 2016). Thus, we evaluated IL-1 β expression using Western blotting. The results showed that the mature IL-1 β was found in BMDMs but not in RAW264.7 cells (Supporting Information Figure S1). The Western blot results may be more reliable. However, to address this confusion, more data must be obtained.

To further confirm the anti-inflammatory effect of isoacteoside, three different mouse models of inflammation were used. The xylene-induced activation of immunity is dependent on TLR4, and the prolonged inflammatory response can trigger systemic inflammatory conditions such as oedema and endotoxaemia (Liu *et al.*, 2013). In our study, isoacteoside suppressed the xylene-induced ear oedema (Figure 8A, C–F), indicating that isoacteoside could attenuate acute inflammation by inhibiting the infiltration of inflammatory cells and the production of pro-inflammatory mediators. To examine the impact of isoacteoside on the systemic inflammatory response, the protective effect of isoacteoside on an LPS-induced endotoxaemia model was evaluated. This experiment revealed that isoacteoside can significantly protect mice and that it is more effective than DEX, a drug used clinically to treat inflammation (Figure 8B). A efficacy of a higher dose of DEX (7 or 10 mg·kg⁻¹) was found to be greater in mice with LPS-induced endotoxaemia (Rocksén *et al.*, 2000; Zeng *et al.*, 2015; Qin *et al.*, 2016). However, in terms of side effects, only those observed with the lower dosage of DEX (5 mg·kg⁻¹) are reasonable and still meet the clinical requirements (Cicarelli *et al.*, 2007). In addition, the dose of LPS used in many studies is 15, 16 or 18 mg·kg⁻¹ (Takahashi *et al.*, 2012). However, because our preliminary experiments showed that LPS at a dose of 15 mg·kg⁻¹ could not induce endotoxaemia death in 7 days, we employed a higher dose (20 mg·kg⁻¹) to establish the endotoxaemia model. Thus, the higher LPS dose and the lower DEX dose led to a survival rate of approximately 20% in the DEX group. Pretreatment with isoacteoside reduced the release of cytokines such as IL-1 β , IL-6 and TNF- α in serum in a dose-dependent manner and improve the survival rate (Figure 9A–C). In addition, endotoxic AKI caused by an aberrant inflammatory response

frequently leads to death (Chvojka *et al.*, 2010). In our study, isoacteoside pretreatment attenuated LPS-induced changes in BUN and serum creatinine (Figure 9D, E). Additionally, H&E staining indicated that isoacteoside ameliorated the LPS-induced AKI in a dose-dependent manner (Figure 10). Although the effect of isoacteoside on the AKI model was corroborated, further detailed mechanisms of the activity of isoacteoside in AKI remain to be explored in future research.

In summary, our data indicate that isoacteoside exerts anti-inflammatory effects *in vitro* and *in vivo*. The underlying mechanisms might be through modifications of the NF- κ B and MAPK pathways evoked by blocking TLR4 dimerization, which is involved in the MyD88 and TRIF pathways. Isoacteoside is a potent lead compound for the development of new therapeutics for inflammatory diseases.

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Author contributions

H.W.G., S.L.Y., X.R.L., Q.M.X. and X.P.C. designed the experiments; H.W.G., Y.K.C., N.X.K., X.L., Y.Z. and Z.Y.Z. performed the experiments; H.W.G. composed the draft; S.L.Y., X.R.L., Y.L.L., J.L. and X.P.C. revised the manuscript; and C.M.W. isolated and purified the BMDMs.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

<https://doi.org/10.1111/bph.13912>

Figure S1 Isoacteoside inhibits IL-1 β expression in RAW264.7 cells and BMDMs. (A) Isoacteoside inhibited LPS-induced IL-1 β expression in RAW264.7 cells. RAW264.7 cells were pretreated with isoacteoside (20, 40, and 80 μ M) for 1 h before LPS (1 μ g·mL⁻¹) stimulation for another 24 h. The IL-1 β expression was detected by Western blotting ($n = 5$). (B) Isoacteoside inhibited IL-1 β and mature IL-1 β expression in BMDMs. BMDMs were pretreated with isoacteoside (20, 40, and 80 μ M) for 1 h before LPS (1 μ g·mL⁻¹) stimulation for another 24 h. The IL-1 β and mature IL-1 β expressions were detected by Western blotting. * $P < 0.05$ compared to LPS alone group.

Figure S2 The chromatography of HPLC. The gradient elution conditions are as following: 0–5 min, 35% methanol; 5.01–20 min, 35–43% methanol, pure water contained 0.1% formic acid and the wavelength was at 330 nm. A: ACT, 98.8%; B: SAV, 99.1%; C: TNB, 98.1%; D: isoacteoside, 98.3%.