Ajudecumin A from Ajuga ovalifolia var. calantha exhibits anti-inflammatory activity in lipopolysaccharide-activated RAW264.7 murine macrophages and animal models of acute inflammation

Hai Zhanga, Qing-Cuo Rena, Yan Renb, Lin Zhaoa, Fan Yanga, Yi Zhanga, Wen-Ji Zhaod, Yu-Zhu Tanab and Xiao-Fei Shenb

aState Key Laboratory Breeding Base of Systematic Research Development and Utilization of Chinese Medicine Resources, Sichuan Province and Ministry of Science and Technology, College of Pharmacy and College of Ethnic Medicine, Chengdu University of Traditional Chinese Medicine, Chengdu, China; bDepartment of Traditional Chinese Medicine, College of Pharmacy, Southwest Minzu University, Chengdu, China; cKey Laboratory of Birth Defects and Related Diseases of Women and Children (Ministry of Education), West China Second University Hospital Sichuan University, Chengdu, China; dSichuan Academy of Grassland Sciences, Chengdu, China

ABSTRACT

Context: Ajuga ovalifolia Bur. et Franch. var. calantha (Diels) C. Y. Wu et C. Chen (Labiatae), a traditional Chinese medicine, has been used to treat several inflammatory diseases.

Objective: To assess the anti-inflammatory activity of ajudecumin A isolated from Ajuga ovalifolia var. calantha, and its possible mechanisms.

Materials and methods: Lipopolysaccharide (LPS, 0.5 μg/mL)-stimulated RAW264.7 macrophages were used to assess the anti-inflammatory activity of ajudecumin A (1–40 μM) in vitro. Nitric oxide levels were evaluated by Griess reagent. The mRNA levels of iNOS, COX-2, TNF-α, IL-1β and IL-6 were determined using qRT-PCR. Phosphorylation of ERK, JNK, p38 MAPK and IκBα were detected by western Blot. To further assess the anti-inflammatory activity of ajudecumin A in vivo, mice were oral treated with ajudecumin A (10 mg/kg) or dexamethasone (0.25 mg/kg, positive control) for 5 days before administration of carrageenan or xylene. Paw and ear edema were then measured, respectively.

Results: Ajudecumin A (10–40 μM) decreased LPS-induced nitric oxide production with an IC50 value of 16.19 μM. Ajudecumin A (20 and 40 μM) also attenuated cell spreading and formation of pseudopodia-like structures, and decreased the mRNA levels of iNOS (55.23–67.04%, p < 0.001), COX-2 (57.58–70.25%, p < 0.001), TNF-α (53.75–58.94%, p < 0.01–0.001), IL-1β (79.41–87.85%, p < 0.001) and IL-6 (54.26–80.52%, p < 0.01–0.001) in LPS-activated RAW264.7 cells. Furthermore, ajudecumin A suppressed LPS-induced phosphorylation of ERK, p38 MAPK, and IκBα, as well as IκBα degradation (p < 0.05–0.001). Finally, ajudecumin A (10 mg/kg) attenuated carrageenan- and xylene-induced inflammation in mice by about 28 and 24%, respectively.

Discussion and conclusions: Ajudecumin A exhibited a potent anti-inflammatory activity in vitro and in vivo through inhibition on NF-κB and ERK/p38 MAPK pathways, suggesting that ajudecumin A may be potentially developed as a lead compound in anti-inflammatory drug discovery.

Introduction

Inflammation, the most primitive protective response to a variety of stimuli, is induced and regulated by a series of immune cells (Tracey 2002). Macrophages, a highly plastic group of innate immune cells, play pivotal roles in immune responses and inflammation by producing many kinds of pro-inflammatory cytokines, inducible synthase and inflammatory mediators, including interleukin-1β (IL-1β), IL-6, tumour necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and nitric oxide (NO), etc. (Wynn et al. 2013). Furthermore, several signalling cascades such as nuclear factor κB (NF-κB) signalling pathway, and mitogen-activated protein kinases (MAPKs) signalling pathway, are activated and involved in macrophages-mediated inflammation (Huang et al. 2010; Hoesel and Schmid 2013). However, excessive activation of aforementioned pro-inflammatory signalling and overproduction of these pro-inflammatory factors in macrophages is responsible for many inflammatory diseases, such as rheumatoid arthritis, cancer, atherosclerosis, diabetes and Alzheimer disease (McNelis and Olefsky 2014; Heppner et al. 2015). Therefore, regulating the crucial proteins in these inflammatory signalling pathways or inhibiting the production of pro-inflammatory factors may serve to prevent or suppress a variety of inflammatory diseases (Pandurangan et al. 2016; Alvarez-Suarez et al. 2017; Xu et al. 2017).

CONTACT
Yu-Zhu Tan (yangzhu@cdutcm.edu.cn) State Key Laboratory Breeding Base of Systematic Research Development and Utilization of Chinese Medicine Resources, Sichuan Province and Ministry of Science and Technology, College of Pharmacy and College of Ethnic Medicine, Chengdu University of Traditional Chinese Medicine, Chengdu, China; Xiao-Fei Shen (szx379@126.com) Key Laboratory of Birth Defects and Related Diseases of Women and Children (Ministry of Education), West China Second University Hospital Sichuan University, Chengdu, China

Supplemental data for this article can be accessed here.

This article has been republished with minor changes. These changes do not impact the academic content of the article.

© 2018 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
The genus *Ajuga* (Labiatae) is distributed over the Eurasian continent. Several species of this genus have been reported to be rich sources of bioactive metabolites, including diterpenes, steroids and iridoids, which exhibit insect antifeedant, antimicrobial, cytotoxic and vasoconstrictor activities (Israili and Lyoussi 2009; Qing et al. 2017). The whole plant of *Ajuga ovalifolia* Bur. et Franch. var. *calantha* (Diels) C. Y. Wu et C. Chen (Labiatae) is used in folk medicine in China for the treatment of inflammation (Guo et al. 2011a, 2011b, 2012). Phytochemical studies showed that diterpenes are the main bioactive constituents in *Ajuga*. Recently, some new clerodane diterpenoids isolated from *Ajuga* have been regarded as neuroprotective agents against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPP⁺)-induced SH-SY5Y neuronal cell death and lipopolysaccharide (LPS)-induced inflammation in microglial BV-2 cells (Guo et al. 2011a, 2011b, 2012). However, pharmacological and mechanism studies on *Ajuga* and its bioactive components are limited. Previously, we isolated four diterpenes, including ajudecumin A (1), ajuforrestin B (2), (16S)-12,16-epoxy-11,14-dihydroxy-17(15→16)-abeo-abieta-8,11,13-trien-7-one (3) and 14,15-dihydroajugapitin (4)
Among these compounds, ajudecumin A exhibited moderate inhibitory activity on the proliferation of human breast cancer MCF-7 cells (Wang et al. 2012); 14,15-dihydroajugapitin showed an antibacterial activity against *Escherichia coli* (Ganaie et al. 2017). Diterpenes are known for their biological and pharmacological characteristics, such as antibacterial, anticancer and anti-inflammatory activities (Tran et al. 2017). In the present study, we further evaluated the anti-inflammatory activity and underlying mechanism of these four diterpenes in LPS-activated murine RAW264.7 macrophage cells, as well as carrageenan- and xylene-induced acute inflammation models.

**Materials and methods**

**Reagents**

The four diterpenes, including ajudecumin A (1), Ajuforrestin B (2), (16S)-12,16-epoxy-11,14-dihydroxy-17(15 →16)-abeo-abiet-8,11,13-trien-7-one (3), and 14,15-dihydroajugapitin (4) were isolated from *Ajuga ovalifolia var. calantha* (Chen et al. 2017b). Among these compounds, ajudecumin A exhibited moderate inhibitory activity on the proliferation of human breast cancer MCF-7 cells (Wang et al. 2012); 14,15-dihydroajugapitin showed an antibacterial activity against *Escherichia coli* (Ganaie et al. 2017). Diterpenes are known for their biological and pharmacological characteristics, such as antibacterial, anticancer and anti-inflammatory activities (Tran et al. 2017). In the present study, we further evaluated the anti-inflammatory activity and underlying mechanism of these four diterpenes in LPS-activated murine RAW264.7 macrophage cells, as well as carrageenan- and xylene-induced acute inflammation models.

**Cell culture**

Murine macrophage RAW264.7 cells were provided by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). RAW264.7 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (Hyclone, Beijing, China) in a humidified incubator with a 5% CO₂ atmosphere at 37 °C.

**Animal**

Male Kunming (KM) mice (about 6 weeks, and 22 g) were purchased from Chengdu Dashuo Biological Company (Chengdu, China). Animals were kept in plastic cages at 25 ± 1 °C with free access to pellet food and water and on a 12 h light/dark cycle. Animal welfare and experimental procedures were strictly
adhered to, in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The experimental scheme of animal study was approved by the ethics committee of Chengdu University of Traditional Chinese Medicine (No. 2018-05).

**Cell viability assay**

Cell viability was assessed by CCK8 assay. In brief, RAW264.7 cells were seeded into 96-well plates at a density of $2.5 \times 10^4$ cells/well, and incubated at 37°C overnight. Cells were treated with different concentrations of ajudecumin A (1, 2.5, 5, 10, 20 and 40 μM) for 24 h in presence of LPS (0.5 μg/mL). Next, cells were incubated with 10 μL of CCK-8 for 2 h at 37°C. Subsequently, absorbance at 562 nm was read using a scanning microtiter apparatus (Thermo Fisher Scientific, Waltham, USA). Relative cell viability was defined as the ratio of the absorbance in test wells compared to control wells.

**Determination of nitric oxide (NO)**

Briefly, RAW264.7 cells were seeded into a 24-well plate at a density of $2.5 \times 10^5$ cells/well, incubated overnight, and pre-treated with the four compounds (20 μM) mentioned above or different concentrations of ajudecumin A (2.5, 5, 10, 20 and 40 μM) for 2 h, followed by stimulation with LPS (0.5 μg/mL) for an additional 24 h. Levels of NO in cell culture medium were evaluated by Griess reaction.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

RAW264.7 cells ($2 \times 10^6$ cells/well) were plated in 6-well plate, incubated overnight, and treated with different concentrations of ajudecumin A and BAY 11-7082 (20 μM) for 2 h, followed by treatment with LPS for an additional 24 h. Total RNAs were extracted using a UNlQ-10 Column total RNA Purification Kit (Sangon Biotech, Shanghai, China), and then were reverse-transcribed to cDNAs by using All-in-One cDNA Synthesis SuperMix Kit (Bimake, Shanghai, China) according to the manufacturer’s protocol. qRT-PCR was performed on a CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) with SYBR Green (Bimake, Shanghai, China). Relative expression levels of the target genes were calculated based on $2^{-\Delta\Delta Ct}$ according to the manufacturer’s specifications by using the GAPDH gene as a reference gene. The primers were used as follows (Zhao et al. 2018). TNF-α forward primer: 5’-CAC CAC GCT CTT CTG TCT-3’, TNF-α reverse primer: 5’-GGC TAC AGG CCT TGC ACT C-3’, IL-1β forward primer: 5’-CAA CCA ACA AGT CAT ATT CTC CAT G-3’, IL-1β reverse primer: 5’-GAT CCA CAC TCT CCA GGT CCA-3’, IL-6 forward primer: 5’-TAG TCC TTC GTA CCC CAA TTT CC-3’, IL-6 reverse primer: 5’-TTG GTC CTG AGT AGA CCT TGG-3’, iNOS forward primer: 5’-CCT GTG AGA CCT TGG ACG TAC CAT G-3’, iNOS reverse primer: 5’-CCT ATA TTD AGA CCT TGG-3’, COX2 forward primer: 5’-CAC CTG AGC GGT TAC TCT G-3’, COX2 reverse primer: 5’-GTT CCA GGA GGA TGG AGT-3’, GAPDH forward primer: 5’-TGG ACC ACC AAC TGG TTA GC-3’, GAPDH reverse primer: 5’-GTC ATG GAC TGT GGT CAT GAG-3’.

**Figure 3.** Ajudecumin A suppressed the mRNA expression of pro-inflammatory cytokines in LPS-stimulated RAW264.7 cells. Cells were pre-treated with various concentrations of Ajudecumin A and BAY 11-7082 (5 μM) for 2 h, and then cultured with LPS (0.5 μg/mL) for 24 h. The mRNA levels of TNF-α (A), IL-1 β (B), and IL-6 (C) were measured by qRT-PCR with GAPDH used as an internal control. All data are represented as mean ± SD, n = 6. *p < 0.05, **p < 0.01, ***p < 0.001 vs. LPS control.
Photographs were taken. The sections were examined under a light microscope and paraffin, sectioned and stained with haematoxylin and eosin. Tissues were then dehydrated, processed, embedded in paraffin, sectioned and stained with haematoxylin and eosin. The left ear was considered as control. One hour after carrageenan injection, mice were pre-treated with dexamethasone and ajudecumin A for 2 h and then cultured with or without LPS (0.5 μg/mL) for additional 24 h. As shown in Figure 1(A), no significant cytotoxicity of ajudecumin A in RAW264.7 cells were observed at the tested concentrations up to 40 μM. Therefore, the concentrations ranging from 1 to 40 μM for ajudecumin A were used in the next assay.

Furthermore, we found that ajudecumin A obviously suppressed the NO production in a concentration-dependent manner in LPS-stimulated RAW 264.7 macrophages (Figure 1(B)), and the value of IC<sub>50</sub> is 16.19 μM. Additionally, the unstimulated macrophages generally display a smooth and smooth shaped forms. Upon exposing to various inflammatory stimuli, such as LPS, the macrophages display an activated phenotype, which mainly manifested as irregular and rough form with accelerated spreading and pseudopodia-like formations (Purushotham et al. 2017). As expected, LPS caused irregular and rough form with accelerated spreading in RAW264.7 cells, which can be obviously improved by ajudecumin A treatment (Figure 1(C)). Taken together, ajudecumin A suppressed the overproduction of NO and morphological changes in LPS-activated RAW264.7 macrophages, which suggesting the anti-inflammatory activity of ajudecumin A.
AJudecumin A decreased the mRNA and protein levels of iNOS and COX-2 in LPS-activated RAW264.7 macrophages

The production of NO is tightly regulated by iNOS, which is expressed predominantly in activated macrophages (Bogdan 2015). To determine whether the inhibition of NO production by ajudecumin A is attributed to its ability in inhibiting the expression of iNOS, we performed RT-qPCR and Western Blot assay to detect the mRNA and protein levels of iNOS, respectively. As a control, the 1xkB inhibitor BAY11-7082 remarkably decreased iNOS expression by 93.90 and 67.47% at both mRNA and protein levels (Figure 2(A,C)), consistent with the result that iNOS was tightly regulated by the NF-κB signalling. Ajudecumin A at doses of 10, 20 and 40 μM also suppressed the mRNA expression of iNOS by 21.57, 55.23 and 67.04% respectively (p < 0.05–0.001), respectively, compared with that in LPS-treated RAW264.7 cells (Figure 2(A)). Consistent with its effects on iNOS transcription, iNOS protein expression was decreased by treatment with ajudecumin A (20 and 40 μM) and ajudecumin A at 40 μM also diminished the TNF-α and IL-6 mRNA expression by 53.75 and 58.94%, 54.26 and 80.52%, respectively (p < 0.01–0.001). Likewise, compared to the vehicle control, treatment with 10, 20 and 40 μM of ajudecumin A also caused 47.28, 79.41 and 87.85% decrease in IL-1β gene expression in LPS-treated RAW264.7 cells, respectively (p < 0.01–0.001, Figure 2(C)). These results indicated that ajudecumin A inhibited NO production in LPS-activated RAW264.7 cells by suppression of iNOS expression.

COX-2 is another important inducible enzyme that amplifies inflammatory responses through catalyzing the rate-limiting step in the synthesis of prostaglandins (PGs) (Cha and DuBois 2007). Furthermore, the elevated expression and activity of COX-2 is always observed in a series of inflammatory cells, including macrophages (Dennis and Norris 2015). Therefore, COX-2 is considered as a promising target for the treatment of inflammatory diseases. We thus determined whether ajudecumin A affects COX-2 expression. As a potent inflammatory stimulator, LPS induced a remarkable elevation in mRNA and protein expression of COX-2 in RAW264.7 cells; and this abnormal elevation was attenuated by ajudecumin A treatment (40 μM), the inhibition rate on mRNA and protein levels were 70.25 and 46.07%, respectively, p < 0.001, Figure 2(B,D). This result indicated that ajudecumin A exerted an anti-inflammatory effect, in part, by inhibition of COX-2 expression.

AJudecumin A suppressed the mRNA level of pro-inflammatory cytokines in LPS-stimulated RAW264.7 cells

In addition to the inducible enzymes and inflammatory mediators, LPS expose also results in the production of several pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Mosser and Edwards 2008). To further address the anti-inflammatory activity of ajudecumin A, we thus evaluated whether it can alter the mRNA expression of TNF-α, IL-1β and IL-6 in LPS-stimulated RAW264.7 cells. As shown in Figure 3(A,C), treatment with ajudecumin A (20 and 40 μM) diminished the TNF-α and IL-6 mRNA expression by 53.75 and 58.94%, 54.26 and 80.52%, respectively (p < 0.01–0.001). Likewise, compared to the vehicle control, treatment with 10, 20 and 40 μM of ajudecumin A also caused 47.28, 79.41 and 87.85% decrease in IL-1β gene expression in LPS-treated RAW264.7 cells, respectively (p < 0.01–0.001, Figure 3(B)).

Synthesis and release of pro-inflammatory cytokines in macrophages by stimuli is a crucial step in the initiation and amplification of inflammation (Mosser and Edwards 2008). Among them, TNF-α and IL-6 can bind to their receptors, thereby triggering downstream activation of inflammatory gene expression, and are responsible for a series of inflammatory disorders, including rheumatoid arthritis and inflammatory bowel disease (Hodge et al. 2005; Billiet et al. 2014). Similarly, IL-1β, an early major pro-inflammatory cytokine mediating the inflammatory response at both the local and systemic levels, is also strongly involved in some autoimmune diseases, such as rheumatoid arthritis (Palomo et al. 2015). Thus, inhibition of these pro-inflammatory cytokines is conducive to the treatment of inflammatory diseases and has become a potential target for novel anti-inflammatory drugs. The present study has demonstrated that ajudecumin A can efficiently attenuate the production of pro-inflammatory cytokines at the transcriptional level.

AJudecumin A suppressed IkBα phosphorylation and degradation in LPS-activated RAW264.7 macrophages

NF-κB, a pleiotropic transcription factor, plays a crucial role in inflammation triggering and amplifying through up-regulating
the expression of multiple genes, such as pro-inflammatory cytokines, chemokines and inducible enzymes (Durand and Baldwin 2017). Thus, NF-κB has been considered as an attractive drug target for anti-inflammatory therapy (Killeen et al. 2014). Normally, NF-κB displays an inactive form in the cytoplasm by binding to its inhibitor protein of IκB. Upon stimulation by LPS or pro-inflammatory cytokines, the IκB protein can be rapidly phosphorylated by IκB kinase, thereby triggering a proteasome-mediated degradation, which is conducive to NF-κB activation. The activated NF-κB is then translocated into the nucleus, where it induces the expression of multiple inflammatory genes by binding to the specific sequences of DNA (Hoesel and Schmid 2013). Herein, we thus further determine whether the inhibitory action of ajudecumin A on pro-inflammatory cytokines and inducible enzymes was due to its inhibition of IκB phosphorylation and degradation.

As shown in Figure 4(A,B), in untreated cells, LPS stimulation caused a significant increase of IκB phosphorylation and reduction in IκB protein level, which was consistent with the previous finding that LPS stimulation can trigger IκB degradation (Akira and Takeda 2004). Treatment of ajudecumin A at doses of 20 and 40 μM decreased LPS-induced IκB phosphorylation in RAW264.7 cells by about 27.51% and 45.07% when compared with LPS control (p < 0.05–0.001, Figure 4(A,B)). Similarly, 20 and 40 μM of ajudecumin A also obviously elevated IκB protein level in LPS-stimulated RAW264.7 cells by about 23.30% and 39.57% when compared with vehicle control (p < 0.05–0.001, Figure 4(A,B)). These findings suggested that ajudecumin A exerted an anti-inflammatory action, in part, through suppression on the LPS-activated NF-κB signaling pathway.

Ajudecumin A attenuated the phosphorylation of ERK and p38 MAPK in LPS-stimulated RAW264.7 cells

The MAPK superfamily proteins, including ERK, p38 MAPK and JNK, plays a key role in regulating the secretion of pro-inflammatory cytokines, mediators, and inducible enzymes in activated macrophages and other cell types (Kim and Choi 2015). Previous work reported that activated MAPKs are found in several inflammatory diseases (Thalhamer et al. 2008). These findings emphasize MAPKs as potential therapeutic targets in some inflammatory diseases. Furthermore, several studies have indicated that some natural compounds including resveratrol and curcumin exert their anti-inflammatory activity through regulating the MAPKs signalling pathway (Koeberle and Werz 2014).

To explore whether MAPKs signalling is involved in ajudecumin A-mediated anti-inflammatory action in LPS-activated RAW264.7 cells, we assessed the phosphorylation levels of ERK, p38 MAPK, and JNK were via Western Blot. As displayed in Figure 5(A–C), stimulation of LPS significantly enhanced the phosphorylation of ERK, p38 MAPK, and JNK in RAW264.7 cells. In contrast, LPS-induced activation of ERK was attenuated by ajudecumin A treatment in a concentration-dependent manner (10, 20 and 40 μM, inhibition rate were 17.07, 30.50 and 53.26%, respectively, p < 0.05–0.001, Figure 5(A)). Likewise, compared with vehicle control, ajudecumin A also could inhibit the increased phosphorylation of p38 MAPK by 19.77, 25.50 and 29.08%, respectively (p < 0.05–0.01); but its inhibitory action was relatively moderate (Figure 5(B)). Unlike ERK and p38 MAPK, LPS-induced phosphorylated JNK could not be clearly altered by ajudecumin A (p > 0.05). This result indicated an important repressor effect of ajudecumin A on activation of ERK and p38...
MAPK in LPS-stimulated RAW264.7 cells, which may contribute
to its anti-inflammatory effect. Moreover, ERK and p38 MAPK
are known to regulate the NF-κB signalling pathway through
activating the mitogen- and stress-activated kinase (MSK) protein
(Saklatvala 2004; Vermeulen et al. 2009). Therefore, ajudecumin
A may repress the LPS-induced NF-κB pathway activation
through inhibition on the phosphorylation of ERK and
p38 MAPK.

**Ajudecumin A alleviated carrageenan-induced paw edema
and xylene-induced ear edema in mice**

To further evaluate the anti-inflammatory activity of ajudecumin
A in vivo, we established carrageenan-induced paw edema and
xylene-induced ear edema in mice. Carrageenan is a strong pro-
inflammatory agent that is used to stimulate the release of several
pro-inflammatory mediators, such as prostaglandins, leukotrienes,
histamine and TNF-α. Xylene-induced ear edema is mainly associ-
ated with the release of some pro-inflammatory mediators, includ-
ing substance P, prostaglandins, histamine. Furthermore, the carrageenan-
and xylene-induced acute inflammatory response is mainly charac-
terized by the exudation of fluid and plasma prote-
ins with a high degree of reproducibility (Vazquez et al. 2015).
Thus, these two models are considered suitable for evaluating the
effects of anti-inflammatory agents (Yoon et al. 2017; Chen et al.
2017a). As depicted in Figure 6(A), treatment with ajudecumin
A at 10 mg/kg or dexamethasone at 0.25 mg/kg for 5 days signifi-
cantly ameliorated carrageenan-induced acute paw edema in mice
by about 28% and 63% when compared with vehicle control
(p < 0.05–0.01, respectively). Furthermore, after 5 days treatment
of ajudecumin A (10 mg/kg) and dexamethasone (0.25 mg/kg)
decreased xylene-induced ear edema in mice by about 24% and
53% when compared with vehicle control (p < 0.05–0.001, Figure
6(C)), respectively. Meanwhile, histologic evaluation showed that
the carrageenan- and xylene-induced edema, hyperaemia, and
inflammatory cell infiltration could be lessened by treatment of
ajudecumin A or dexamethasone (Figure 6(B,D)). The inhibitory
action of ajudecumin A on carrageenan- and xylene-induced acute
inflammation may be related to the inhibition of pro-inflamma-
tory mediators.

**Conclusions**

Ajudecumin A from *Ajuga ovalifolia* var. *calantha* possesses anti-
inflammatory activity in LPS-activated RAW264.7 murine mac-
rophages, and carrageenan- and xylene-induced acute inflammation
in mice. All these actions may be attributed to its inhibition on NF-κB and ERK/p38 MAPK signalling. Furthermore, these
results may also explain the anti-inflammatory activity of *Ajuga*
reported previously, and suggesting that ajudecumin A may be
an important bioactive ingredient in *Ajuga*. Finally, these find-
ings provide additional pharmacological information and may
contribute for the further study and use of ajudecumin A as a
phytomedicine.

**Disclosure statement**

The authors report that they have no conflicts of interest.

**Acknowledgements**

This work was supported by the Discipline Talent Promotion Program of “Xinglin Scholars” (No.QNXZ2018005), Miaozi
Cultivation Project of Sichuan Science and Technology Innovation
(No. 2018137), the Key Fund Project for Education Department of
Sichuan (No. 18ZA0181), Education Department of Sichuan
Province (No. 17ZB0156), and Department of Science and Technology of Sichuan Province (No. 2016SZYZF0002).

**References**

Akira S, Takeda K. 2004. Toll-like receptor signalling. Nat Rev Immunol. 4:
499–511.

Alvarez-Suarez JM, Carrillo-Perdomo E, Aller A, Giampieri F, Gasparriini M,
González-Pérez L, Beltrán-Ayala P, Battino M. 2017. Anti-inflammatory
effect of Capsul cherry against LPS-induced cytotoxic damage in RAW
264.7 macrophages. Food Chem Toxicol. 102:46–52.

Billet T, Rutgeerts P, Ferrante M, Van Asche G, Vermeire S. 2014.
Targeting TNF-α for the treatment of inflammatory bowel disease. Expert
Opin Biol Ther. 14:75–101.

Bogdan C. 2001. Nitric oxide and the immune response. Nat Immunol. 2:
907–916.

Bogdan C. 2015. Nitric oxide synthase in innate and adaptive immunity: an
update. Trends Immunol. 36:161–178.

Cha YI, DuBois RN. 2007. NSAIDs and cancer prevention: targets down-
stream of COX-2. Ann Rev Med. 58:239–252.

Chen LZ, Sun WW, Bo L, Wang QJ, Xiu C, Tang WJ, Shi JB, Zhou HP, Liu
XH. 2017a. New arylpyrazoline-coumarins: Synthesis and anti-inflamma-
tory activity. Eur J Med Chem. 138:170–181.

Chen Y, Yang F, Ao H, Pan Y, Li HX, SA TH, Tang X, Li KL, Chen Z, Zhao
WJ, et al. 2017b. Chemical constituents of *Ajuga ovalifolia* var. *calantha*.
Chin Traditional Herbal Drugs. 48:3475–3479.

Dennis EA, Norris PC. 2015. Eicosanoid storm in infection and inflamma-
tion. Nat Rev Immunol. 15:511–523.

Durand JK, Baldwin AS. 2017. Targeting IKK and NF-κB for therapy. Adv
Protein Chem Struct Biol. 107:77–115.

Ganie HA, Ali MN, Ganai BA, Meraj M, Ahmad M. 2017. Antibacterial
activity of 14, 15-dihydroajugapitin and 8-
acetylharpagide isolated from *Ajuga bracteosa* Wall ex. Benth.
W. B. Chen. Microb Pathog. 103:114–118.

Guo P, Li YS, Xu J, Guo YQ, Jin DQ, Gao J, Hou W, Zhang T. 2011a. Neo-
Clerodane diterpenes from *Ajuga ciliata* Bunge and their neuroprotective
activities. Fitoterapia. 82:1123–1127.

Guo P, Li YS, Jin DQ, Xu J, He YS, Zhang L, Guo YQ. 2012. Neo-clerodane
diterpenes from *Ajuga ciliata* and their inhibitory activities on LPS-
induced NO production. Phytochem Lett. 5:563–566.

Guo P, Li YS, Xu J, Liu C, Ma Y, Guo YQ. 2011b. Bioactive neo-clerodane
diterpenoids from the whole plants of *Ajuga ciliata* Bunge. J Nat Prod. 74:
1575–1583.

Heppner FL, Ransohoff RM, Recher B. 2015. Immune attack: the role of
inflammation in Alzheimer disease. Nat Rev Neurosci. 16:358–372.

Hodge DR, Hurt EM, Farrar WL. 2008. The role of IL-6 and STAT3 in
inflammation and cancer. Eur J Cancer (Oxford, England : 1990). 44:
907–918.

Hoese B, Schmid JA. 2013. The complexity of NF-κB signaling in inflamma-
tion and cancer. Mol Cancer. 12:1–15.

Huang PY, Han J, Hui L. 2010. MAPK signaling in inflammation-associated
cancer development. Protein Cell. 1:218–226.

Israli ZH, Lyoussi B. 2009. Ethnopharmacology of the plants of genus *Ajuga*.
Pakistan J Pharmacut Sci. 22:425–462.

Killeen MJ, Linder M, Pontoniere P, Crea R. 2014. NF-κB signaling and
chronic inflammatory diseases: exploring the potential of natural prod-
cuts to drive new therapeutic opportunities. Drug Discov Today. 19:
373–378.

Kim EK, Choi EJ. 2015. Compromised MAPK signaling in human diseases:
an update. Arch Toxicol. 89:867–882.

Koeberle A, Werz O. 2014. Multi-target approach for natural products in
inflammation. Drug Discov Today. 19:1871–1882.

McNels JC, Ofelsky JM. 2014. Macrophages, immunity, and metabolic dis-
ease. Immunity. 41:36–48.

Musser DM, Edwards JP. 2008. Exploring the full spectrum of macrophage
activation. Nat Rev Immunol. 8:958–969.
Palomo J, Dietrich D, Martin P, Palmer G, Gabay C. 2015. The interleukin (IL)-1 cytokine family-Balance between agonists and antagonists in inflammatory diseases. Cytokine. 76:25–37.

Pandurangan AK, Mohebali N, Hasanpourghadi M, Looi CY, Mustafa MR, Mohd Esa N. 2016. Boldine suppresses dextran sulfate sodium-induced mouse experimental colitis: NF-kB and IL-6/STAT3 as potential targets. Biofactors (Oxford, England). 42:247–258.

Purushotham PM, Kim JM, Jo EK, Senthil K. 2017. Withanolides against TLR4-activated innate inflammatory signalling pathways: A comparative computational and experimental study. Phytother Res. 31:152–163.

Qing X, Yan HM, Ni ZY, Vavricka CJ, Zhang ML, Shi QW, Gu YC, Kiyota H. 2017. Chemical and pharmacological research on the plants from genus Ajuga. Heterocycl Comm. 23:245–268.

Saklatvala J. 2004. The p38 MAP kinase pathway as a therapeutic target in inflammatory disease. Curr Opin Pharmacol. 4:372–377.

Shen XF, Zeng Y, Li JC, Tang C, Zhang Y, Meng XL. 2017. The anti-arthritis activity of total glycosides from Pterocephalus hookeri, a traditional Tibetan herbal medicine. Pharmaceut Biol. 55:560–570.

Thalhamer T, McGrath MA, Harnett MM. 2008. MAPKs and their relevance to arthritis and inflammation. Rheumatology (Oxford, England). 47:409–414.

Tracey KJ. 2002. The inflammatory reflex. Nature. 420:853–859.

Tran QTN, Wong WSF, Chai CLL. 2017. Labdane diterpenoids as potential anti-inflammatory agents. Pharmacol Res. 124:43–63.

Vazquez E, Navarro M, Salazar Y, Crespo G, Bruges G, Osorio C, Tortorici V, Vanegas H, Lópe M. 2015. Systemic changes following carrageenan-induced paw inflammation in rats. Inflamm Res. 64:333–342.

Vermeulen L, Vanden Berghe W, Beck IM, De Bosscher K, Haegeman G. 2009. The versatile role of MSKs in transcriptional regulation. Trends Biochem Sci. 34:311–318.

Wang B, Wang XN, Shen T, Wang SQ, Guo DX, Lou BX. 2012. Rearranged abietane diterpenoid hydroquinones from aerial parts of Ajuga decumbens Thumb. Phytochem Lett. 5:271–275.

Wynn TA, Chawla A, Pollard JW. 2013. Macrophage biology in development, homeostasis and disease. Nature. 496:445–455.

Xu JJ, Zhao Y, Aisa HA. 2017. Anti-inflammatory effect of pomegranate flower in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. Pharmaceut Biol. 55:2095–2101.

Yoon JJ, Sohn EJ, Kim JH, Seo JW, Kim SH. 2017. Anti-rheumatoid arthritis effect of Kaejadan via analgesic and antiinflammatory activity in vivo and in vitro. Phytother Res. 31:418–424.

Zhao L, Wang L, Di SN, Xu Q, Ren QC, Chen SZ, Huang N, Jia D, Shen XF. 2018. Steroidal alkaloid solanine A from Solanum nigrum Linn. exhibits its anti-inflammatory activity in lipopolysaccharide/interferon γ-activated murine macrophages and animal models of inflammation. Biomed & Pharmacother. 105:606–615.