Toxicology and Applied Pharmacology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Flavonoids casticin and chrysosplenol D from *Artemisia annua* L. inhibit inflammation *in vitro* and *in vivo*

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ARTICLE INFO

Article history:
Received 18 December 2014
Revised 27 March 2015
Accepted 8 April 2015
Available online xxxx

Keywords:
Artemisia annua L.
Flavonoids
Casticin
Chrysosplenol D
Inflammation

ABSTRACT

Background: The aim of our experiments was to investigate the anti-inflammatory properties of casticin and chrysosplenol D, two flavonoids present in *Artemisia annua* L.
Methods: Topical inflammation was induced in ICR mice using croton oil. Mice were then treated with casticin or chrysosplenol D. Cutaneous histological changes and edema were assessed. ICR mice were intragastrically administrated with casticin or chrysosplenol D followed by intraperitoneal injection of lipopolysaccharide (LPS). Mouse Raw264.7 macrophage cells were incubated with casticin or chrysosplenol D. Intracellular phosphorylation was detected, and migration was assessed by trans-well assay. HT-29/NFκB-luc cells were incubated with casticin or chrysosplenol D in the presence or absence of LPS, and NF-κB activation was quantified.
Results: In mice, administration of casticin (0.5, 1 and 1.5 μmol/cm^2) and chrysosplenol D (1 and 1.5 μmol/cm^2) inhibited croton oil-induced ear edema (casticin: 29.39–64.95%; chrysosplenol D: 37.76–65.89%, all $P < 0.05$) in a manner similar to indomethacin (0.5, 1 and 1.5 μmol/cm^2; 55.63–84.58%). Casticin (0.07, 0.13 and 0.27 mmol/kg) and chrysosplenol D (0.07, 0.14 and 0.28 mmol/kg) protected against LPS-induced systemic inflammatory response syndrome (SIRS) in mice (all $P < 0.05$), in a manner similar to dexamethasone (0.03 mmol/kg). Casticin and chrysosplenol D suppressed LPS-induced release of IL-1 beta, IL-6 and MCP-1, inhibited cell migration, and reduced LPS-induced IκB and c-JUN phosphorylation in Raw264.7 cells. JNK inhibitor SP600125 blocked the inhibitory effect of chrysosplenol D on cytokine release.
Conclusions: The flavonoids casticin and chrysosplenol D from *A. annua* L. inhibited inflammation *in vitro* and *in vivo*.

Introduction

Artemisia annua L. (Qinghao) is an annual herb native to China and it grows naturally as a part of steppe vegetation at 1000–1500 m above sea level. It is also called wormwood, Chinese wormwood, sweet wormwood, annual mugwort, and sweet sagewort. Among the herbal extracts of *A. annua* L., artemisinin has been identified as having effects against parasitemia. A series of potent anti-malarial derivatives were developed from artemisinin including dihydroartemisinin, which is currently widely used as an anti-malarial drug (Krishna et al., 2008, 2010; Ding et al., 2011; Tu, 2011). Over the past decade, artemisinins from *A. annua* L. have been used in the treatment of not only malaria (Ho et al., 2014), but also cancers (Berger et al., 2005; Krishna et al., 2008; Ferreira et al., 2010; He et al., 2010; Aung et al., 2011), viruses (Deng et al., 1992; Romero et al., 2005; Rocha Martins et al., 2011) and other parasite-related infections (Shuhua et al., 2000; Tang et al., 2000; Galal et al., 2005; Seif el-Din et al., 2011). Artemisinins have been reported to alleviate the symptoms of autoimmune diseases (Jin et al., 2009; Shakir et al., 2011; Ho et al., 2012; Li et al., 2013), allergic disorders (Chen and Maibach, 1994; Mohapatra et al., 2009; Cheng et al., 2013) and septic inflammation (Li et al., 2008, 2010; Jiang et al., 2011). Our preliminary experiments indicated that Arteannuin B and the flavonoids casticin and chrysosplenol D suppressed the lipopolysaccharide (LPS)-induced production of nitric oxide (NO), prostaglandin E2 (PGE2) and proinflammatory cytokines like TNF-alpha, IL-1 beta and IL-6 in both rat peritoneal cells and human peripheral blood mononuclear cells (Zhu et al., 2013). The capacity to inhibit mediators of angiogenesis may explain the anticancer activity of *A. annua* L. (Zhu et al., 2013).

The flavonoids present in *A. annua* L. are also reported to have significant pharmacological activities including antitumor and antibacterial activities that contribute to the therapeutic effects of the herb (Zheng, 1994; Ferreira et al., 2010). Previous studies have reported that casticin and chrysosplenol D isolated from *A. annua* L. increased DPPH x scavenging (Luo et al., 2013). Casticin and chrysosplenol D isolated from

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http://dx.doi.org/10.1016/j.taap.2015.04.005
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Vitex negundo or *Achillea millefolium* have been reported to reduce the proliferation and growth of cancer cells and were recommended as promising anti-cancer agents (Li et al., 2005; Csupor-Loffler et al., 2009; Awale et al., 2011). Casticin isolated from *Fructus viticis* also inhibited acute inflammation in a mouse model (Lin et al., 2007) and could induce cancer cell apoptosis (Chen et al., 2011; Kikuchi et al., 2013; Zhou et al., 2013; Liu et al., 2014). Casticin from *Vitex agnus-castus* exhibited a potent lipoxygenase inhibition (Choudhary et al., 2009), and also inhibited monocyte oxidative burst and suppressed the chemotaxic activity of N-formyl-L-leucyl-L-phenylalanine-stimulated neutrophils as well as phytohemagglutinin stimulated peripheral blood mononuclear cells (Mesaik et al., 2009).

In this study, we sought to investigate the anti-inflammatory properties of casticin and chrysosplenol D isolated from *A. annua* L. in a mouse model of local cutaneous inflammation and systemic inflammatory response syndrome (SIRS).

We also tried to explore the mechanisms underlying the functions of these flavonoids using mouse Raw264.7 macrophage cells. This study underlines the potentially therapeutically important anti-inflammatory activities of casticin and chrysosplenol D.

Methods

Croton oil-induced ear dermatitis and edema in mice. Forty 4-week old male ICR mice weighing 20–24 g were supplied by the Laboratory Animal Center of the Academy of Military Medical Sciences. Topical inflammation was induced on the surface of the right ear (about 1 cm^2) by applying 80 μg of croton oil (Sigma) dissolved in 15 μL of acetone, as previously described (Baumgartner et al., 2011). Groups of mice (n = 10/group) received no treatment, casticin (1 μmol/cm^2), chrysosplenol D (1 μmol/cm^2) or the nonsteroidal anti-inflammatory drug (NSAID) indomethacin (1 μmol/cm^2). These compounds were dissolved in acetone at the indicated concentrations and applied to the same site as the croton oil. The left ear remained untreated. Mice were sacrificed after 6 or 12 h, and a 6-mm punch was taken from both ears. All animal experiments complied with the guidelines of the Peking University Health Science Center Animal Research Committee (Protocol: SYXK JUN 2007–004).

Table 1
Topical anti-inflammatory activity of casticin and chrysosplenol D from *Artemisia annua* L. (n = 10).

Test substance	Dose		Edema	Inhibition	ID_{50}
	(μmol/cm^2)	(μg/cm^2)	(mg)	(%)	(μmol/cm^2)
Control	–	–	15.89 ± 2.31		
Casticin	0.5	187	11.22 ± 4.03*	29.39	1.16
	1	374	10.44 ± 3.37*	34.30	
	1.5	561	5.50 ± 2.03**	64.95	
Chrysosplenol D	0.5	180	12.30 ± 1.82	22.59	1.12
	1	360	9.89 ± 2.82**	37.76	
	1.5	540	5.42 ± 2.15**	65.89	
Indomethacin	0.5	179	7.05 ± 1.84**	55.63	0.41
	1	358	3.56 ± 1.17**	77.60	
	1.5	716	2.45 ± 0.94**	84.58	

Note: *P < 0.05, **P < 0.01 *vs.* controls (ANOVA).

Evaluation of the edematous response. Edema was quantified by the difference in weight between punch samples taken from the treated and untreated ears. Anti-edema activity was expressed as percent inhibition of the edematous response in animals treated with the test substances compared with edema in model animals treated with irritant alone, as previously described (Gomig et al., 2008; Baumgartner et al., 2011). Development of edema over 12 h was quantified by calculating the areas under the curves (AUCs) and, subsequently, the ratio between the AUCs of these animals and the AUCs of controls.

Histological analysis. Ear biopsies were fixed in 10% formalin, dehydrated in ascending grades of ethanol, cleared in xylene, and embedded in paraffin. Sections (10 μm) were stained with hematoxylin–eosin and evaluated using a light microscope (Olympus).

Lipopolysaccharide (LPS)-induced systemic inflammatory response syndrome (SIRS) in mice. LPS was used to induce SIRS (Gosemann et al., 2012). Ninety 10–12-week old ICR mice were purchased from Peking University Medical Department (protocol: SCXK2006–0008) and received an intragastric gavage of 0.9% saline (10 mL/kg) containing casticin at 0.07, 0.13 or 0.27 mmol/kg, chrysosplenol D at 0.07, 0.14 or

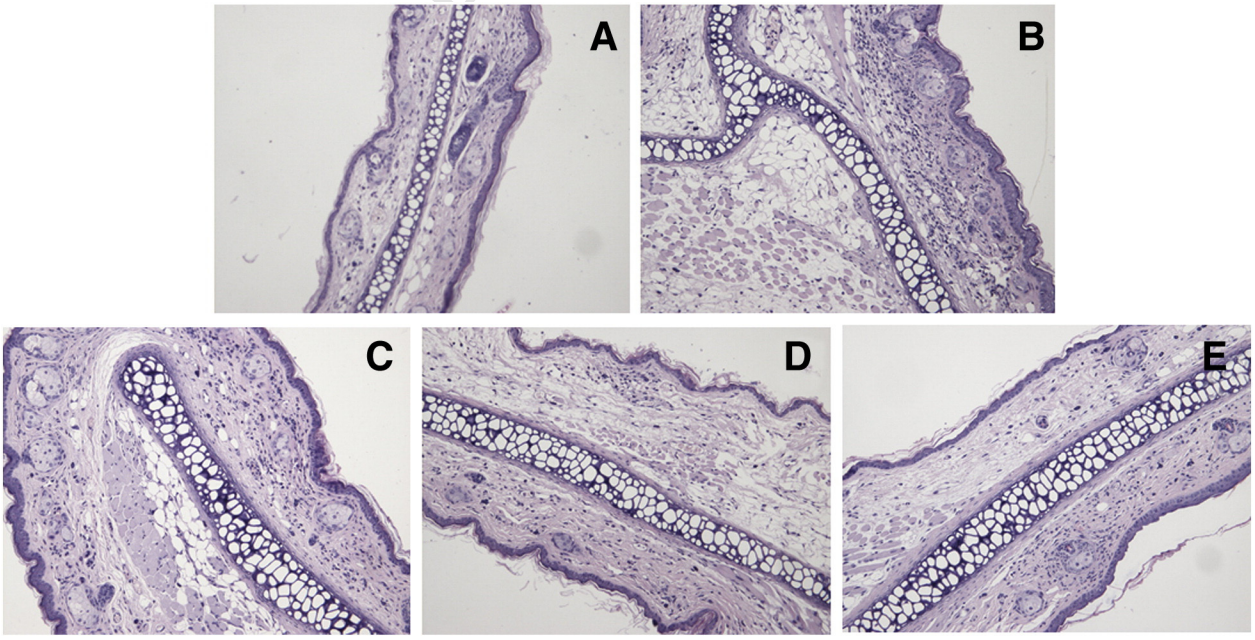

Fig. 1. Histological characteristics of mouse ears 6 h after the induction of croton oil dermatitis. Mouse ears were untreated (A), or croton oil was applied topically to induce dermatitis (B to E). Application of 1 μmol/cm^2 of casticin (C); 1 μmol/cm^2 of chrysosplenol D (D); or 1 μmol/cm^2 of indomethacin (E) improved croton-oil induced dermatitis. Hematoxylin and eosin staining, 200× magnification.

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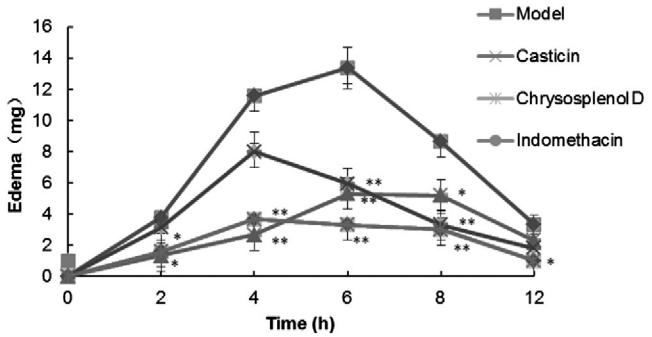

Fig. 2. Effect of casticin, chrysosplenol D and indomethacin on the time course of the edematous response up to 12 h (■model; ▲1 μmol/cm^2 of casticin; ♦1 μmol/cm^2 of chrysosplenol D; ●1 μmol/cm^2 of indomethacin). *P < 0.05, **P < 0.01 *vs.* models (ANOVA). Each point represents the mean of the results from 10 mice.

0.28 mmol/kg, dexamethasone (0.03 mmol/kg) daily continuously for 7 days, or no treatment (10 mice/group). One hour after the last intragastric gavage, mice were injected intraperitoneally with 10 mL/kg LPS (*Escherichia coli*, 0111:B4, Sigma, 6 mg/kg) (Gosemann et al., 2012) dissolved in 0.9% saline. Body temperature and respiratory rate were monitored for 4 h before and after LPS injection. Thereafter, mice were sacrificed, and serum levels of IL-1 beta and tumor necrosis factor (TNF)-alpha were measured by ELISA (Rapidbio, CA, USA).

Cell culture. HUVEC cells were purchased from Sciencell Inc. (CA, USA). Raw264 cells were purchased from the American Typical Collection Center (Maryland, USA). The human colorectal adenocarcinoma cell line HT-29, stably transfected with a NF-κB luciferase reporter (HT-29/NFκB-luc cells), was provided by Professor Zhuo-yu Li of the Institute of Biotechnology, Key Laboratory of Chemical Biology and Molecular Engineering of National Ministry of Education, Shanxi. All cells were cultured in RPMI1640 supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA), 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

MTT cell viability assay. Cell viability was determined using a MTT assay. Cells were plated in 96-well plates at 5000 cells/well with the indicated additives. After 24 h, 10 μL of MTT (Sigma) was added in each well and cell viability was determined after 4 h by the OD_{570} values for each well.

Transwell cell migration assay. Raw264.7 cells were labeled with a fluorescent dye, 3′-O-Acetyl-2′,7′-bis(carboxyethyl)-4 or 5-carboxyfluorescein, diacetoxymethyl ester (BCECF-AM, Dojindo Molecular, Japan), by incubating 2.0 × 10^6 cells in 4 mL of IMDM (containing 10% FCS and 40 μL of BCECF-AM to achieve a final BCECF-AM concentration of 10 μM) at 37 °C for 1 h. Dye loading was stopped by adding 4 mL of cold IMDM containing 10% FCS to the suspension and rinsing.

Table 2
Casticin and chrysosplenol ameliorate LPS-induced hypothermia and respiratory symptoms in a mouse model of SIRS.

Group	N	Dose mmol/kg	Body temperature change (°C)	Respiratory rate (breaths/min)
Control		–	−0.12 ± 0.43**	159 ± 14**
Model	10	–	−2.21 ± 1.94	122 ± 12
Casticin	10	0.07	−0.62 ± 0.70*	151 ± 13**
	10	0.13	−0.80 ± 0.54*	147 ± 7**
	10	0.27	−0.37 ± 0.92*	157 ± 10**
Chrysosplenol D	10	0.07	−0.38 ± 0.66*	149 ± 23**
	10	0.14	−0.62 ± 0.71*	150 ± 6**
	10	0.28	−0.57 ± 0.92*	152 ± 9**
Dexamethasone	10	0.03	−0.80 ± 1.02*	152 ± 7**

Note: **P < 0.01 *vs.* controls (ANOVA).

Table 3
The inhibitory effects of casticin and chrysosplenol D on TNF-alpha and IL-1 beta production by LPS on the systemic inflammatory response in mice.

Group	N	Dose (mmol/kg)	TNF-alpha (mmol/L)	IL-1 beta (mmol/L)
Control	10	–	0.11 ± 0.08**	10.44 ± 1.83*
Model	10	–	0.21 ± 0.04	17.61 ± 5.69
Casticin	10	0.07	0.19 ± 0.06	10.43 ± 2.34*
	10	0.13	0.19 ± 0.08	9.25 ± 2.31**
	10	0.27	0.18 ± 0.11	9.89 ± 1.84**
Chrysosplenol D	10	0.07	0.20 ± 0.06	13.01 ± 1.87
	10	0.14	0.22 ± 0.04	10.08 ± 2.27*
	10	0.28	0.22 ± 0.09	9.80 ± 2.31**
Dexamethasone	10	0.03	0.16 ± 0.08*	8.52 ± 4.07**

Note: *P < 0.05, **P < 0.01 *vs.* controls (ANOVA).

Fluorescence-labeled cells were resuspended in 4 mL of IMDM containing 10% FCS and prepared for use in migration experiments.

Transwell chambers (8 μm pore size) (BD Falcon, New Jersey, USA) were used for migration assay. Cells were cultured in FBS-free RPMI-1640 (Invitrogen, Carlsbad, CA, USA) for 24 h. BCECF-AM-labeled cells (1 × 10^5) were seeded onto the upper chamber and then inserted into a 24-well plate. The upper chamber contained serum-free medium and the lower chambers contained culture medium. After 24 h, the number of cells remaining in the upper chamber were collected by swabbing, rinsed with PBS, and counted by a fluorescence microscope (Olympus, Tokyo, Japan) using a fluorescence plate reader at excitation/emission wavelengths of 485/535 nm.

Cytokine assays. Raw264.7 cells were plated at 2 × 10^5 cells per well in 24-well plates and pretreated with casticin or chrysosplenol D (1, 5 and 10 μM) for 4 h before exposure to LPS (200 ng/mL). After 20 h, the concentrations of IL-1 beta, MCP-1 and IL-6 in the cell culture supernatant were measured by ELISA according to the manufacturer's instructions (Rapidbio, CA, USA).

Bio-Plex phosphoprotein assay. Raw264.7 cells (1.5 × 10^5/mL) were treated with LPS (200 ng/mL) for 2 h. Then, protein lysates were prepared using the Cell lysis kit (Bio-Rad). The presence of p-IκB, p-ERK1/2, p-p38 MAPK, p-Stat3, p-MEK and p-c-JUN was detected using the Bio-Plex 6-plex phosphoprotein assay kit (Bio-Rad Laboratories Inc., Hercules, USA) and the Phosphoprotein Testing Reagent kit (Bio-Rad), according to the manufacturer's protocol. Data from the reaction was then acquired and analyzed using the Bio-Plex suspension array system (Bio-Plex 200 reader).

To test whether activation of the c-JUN pathway was involved in the anti-inflammatory effects of chrysosplenol D, RAW264.7 cells pretreated with 20 nM of the JNK inhibitor SP600125 (Calbiochem, San Diego, CA), were subjected to the previously described experiments, and cytokine release was quantified after the addition of LPS.

NF-κB transactivation activity. HT-29/NFκB-luc cells were maintained at 37 °C and 5% CO_2 in DMEM with phenol red supplemented with 2 mM glutamine, 100 U/mL benzylpenicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum. HT-29/NFκB-luc cells were seeded in 96-well plates and incubated at 37 °C and 5% CO_2 overnight. On the next day, the medium was replaced with serum-free DMEM and the indicated concentrations of casticin or chrysosplenol D were added. One hour after treatment, the cells were stimulated with 20 ng/mL of LPS for 2 h. After lysis, firefly luciferase and ZSGreen fluorescence were quantified on a GeniosProplate reader (Tecan, Austria). The luciferase signal derived from the NF-κB reporter was normalized to the ZSGreen-derived fluorescence to account for differences in cell numbers or transfection efficiency.

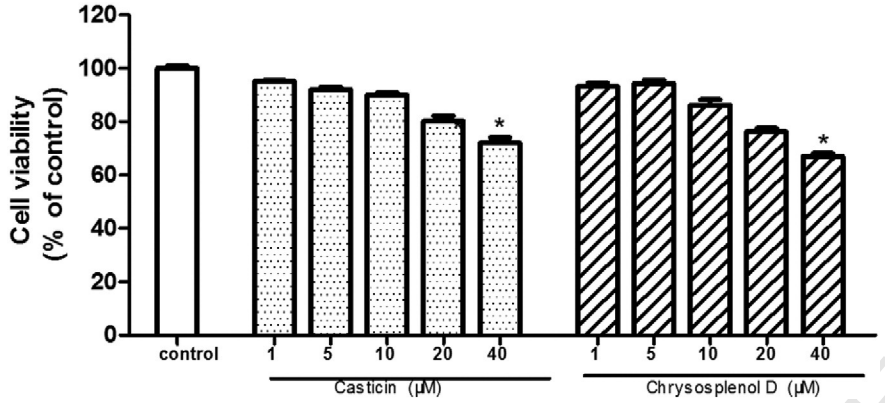

Fig. 3. The inhibitory effects of casticin and chrysosplenol D on cell viability measured by MTT assay. Raw264.7 cells were treated with casticin or chrysosplenol D. Data represents the mean ± SD from three separate experiments *P < 0.05, **P < 0.01, ***P < 0.001 *vs.* vehicle-treated control.

Statistical analysis. The results shown in each figure are expressed as arithmetic mean ± SD. Data analysis was performed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison post hoc test using GraphPad Prism. P-values < 0.05 were considered statistically significant. All experiments were repeated at least three times.

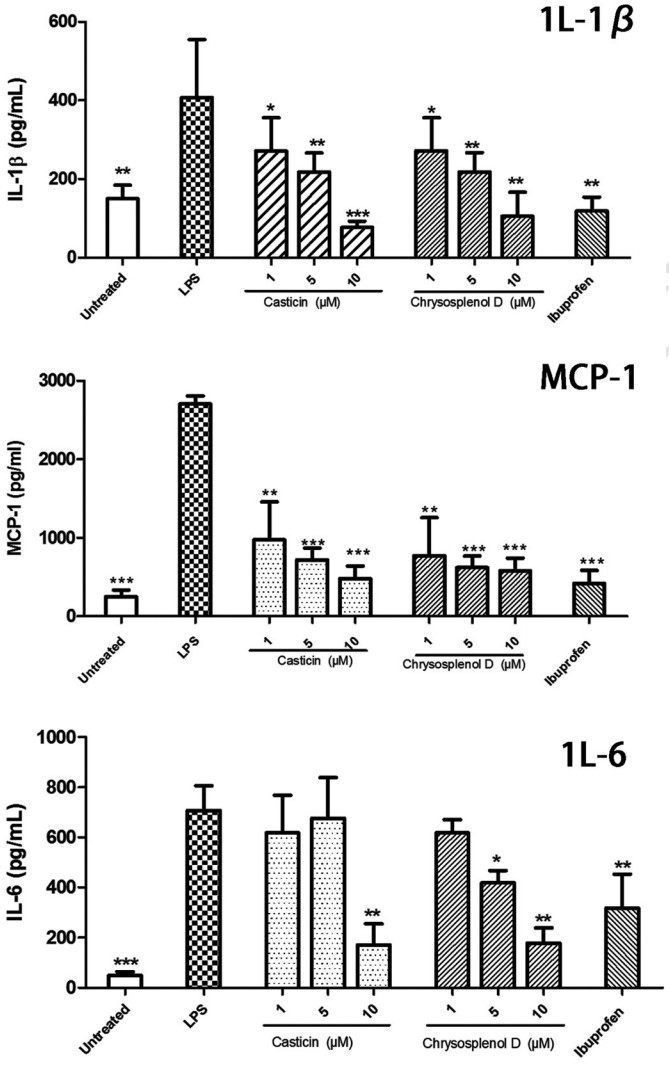

Fig. 4. Casticin and chrysosplenol D inhibits LPS-mediated IL-1 beta, IL-6 and MCP-1 production in Raw264.7 cells. RAW264.7 cells were pretreated for 4 h with casticin or chrysosplenol D at 1, 5 or 10 μM. Cytokine concentrations were evaluated after 20 h in the presence of LPS by ELISA. Data are presented as the mean values of six independent experiments; bars represent mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 *vs.* the LPS group.

Results

A. annua L. flavonoid casticin and chrysosplenol D reduce croton oil-induced dermatitis and edema

Croton oil induced dermatitis, and dilated blood vessels and dermal swelling were observed after 6 h (Fig. 1B). To assess the anti-inflammatory properties of casticin and chrysosplenol D, these compounds were applied topically to the previously described mouse model of local inflammation at 1 μmol/cm^2. After 6 h, reduced inflammation was observed in the ears of mice treated with casticin or chrysosplenol D (Figs. 1D to E). Similarly, ear tissues from mice treated with indomethacin (1 μmol/cm^2) revealed attenuation of all the vascular and cellular signs of inflammation (Fig. 1F).

The flavonoids and indomethacin significantly reduced the edematous response. To evaluate the anti-inflammatory potency of the isolated compounds, ID_{50} values were assessed. Casticin and chrysosplenol D showed ID_{50} values in the range 1.12–1.27 μmol/cm^2, which were higher than indomethacin (ID_{50} 0.41 μmol/cm^2) (Table 1).

The anti-inflammatory activities of casticin and chrysosplenol D at 1 μmol/cm^2, a dose leading to about 50% edema reduction at 6 h, were investigated with regard to edema development up to 12 h after dermatitis induction and were compared with indomethacin (Fig. 1). Local inflammation models developed an edematous response that was still measurable after 12 h, reaching a peak at 6 h after croton oil application, followed by a progressive decrease. Casticin and chrysosplenol D exerted a significant inhibitory activity at each observation time, showing reductions in the ranges of 30.72–77.10% and 16.76–65.16%, respectively. Interestingly, despite the similar activity profile, casticin achieved a long-lasting steady anti-inflammatory effect, which was observed from 2 h, peaked at 4 h (77.10%) and persisted until 12 h (30.72%). Chrysosplenol D exhibited anti-inflammatory effect from 4 h, with a maximum response being observed between 6–12 h (55.66–65.16%). Induction (1 μmol/cm^2) significantly reduced edema at all observed time points (Fig. 2).

The activity profile of casticin and chrysosplenol D on the whole edematous response up to 12 h was quantified by calculating the ratio between the AUCs for mice treated with these compounds and the AUCs of model animals of local inflammation. Casticin, chrysosplenol D and indomethacin reduced the global edematous response by the same extent (38.72%, 45.44% and 36.70% respectively), significantly lower compared with untreated mouse models of local inflammation (89.83%) (Fig. 2).

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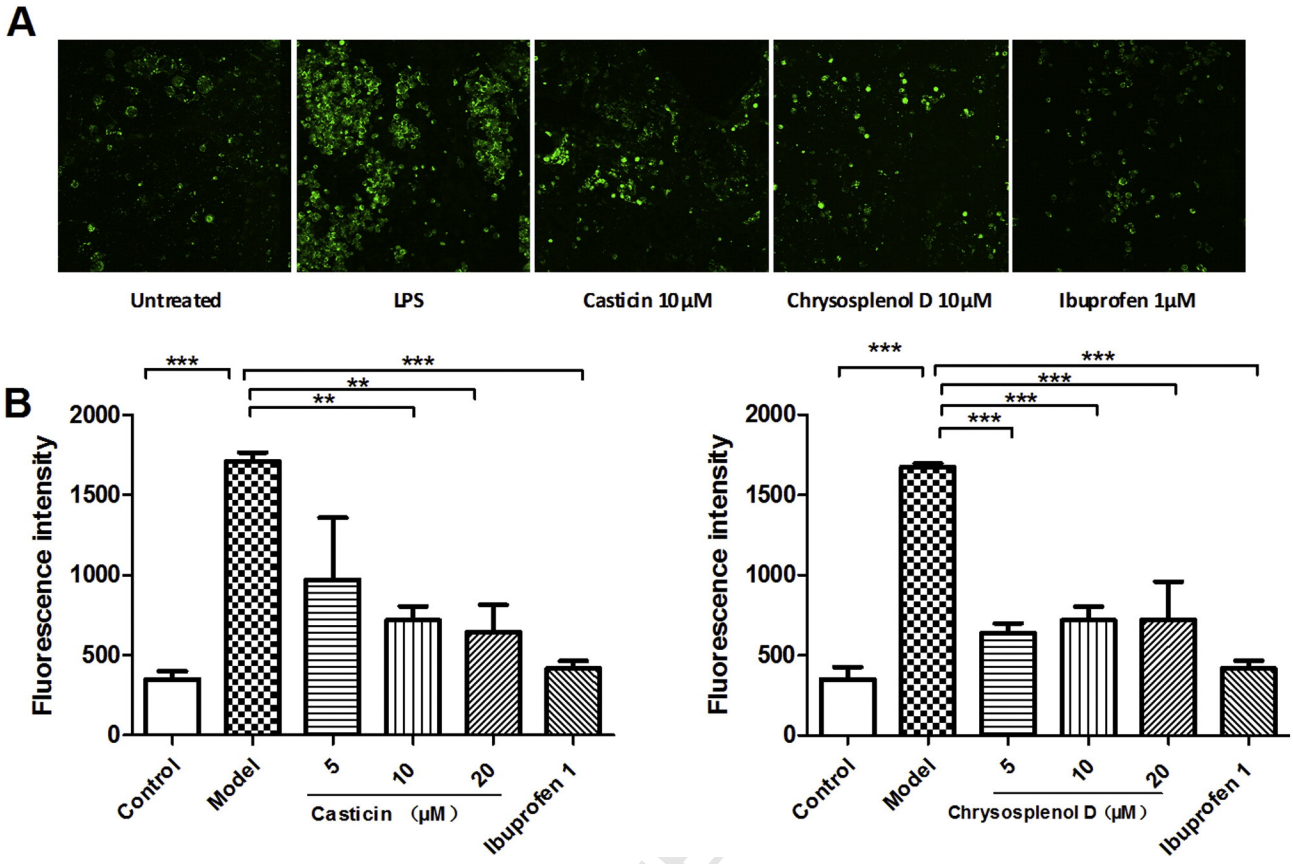

Fig. 5. En face fluorescence microscopic images of BCECF-AM-labeled Raw264.7 cells. Cells that migrated to the outside surface of upper chamber were seen after incubation in media containing LPS for 2 h at three different compounds. (A) Confocal microscopy imaging and (B) migrated cells were measured by a fluorescence plate reader at excitation/emission wavelengths of 485/535 nm. Data represents the mean ± SD from three separate experiments. *P < 0.05, **P < 0.01, ***P < 0.001 *vs.* the LPS group.

Murine model of SIRS

To establish a murine model of SIRS, mice were administered LPS by intraperitoneal injection. As shown in Table 2, the body temperature declined gradually after intraperitoneal injection of LPS and reached a maximal reduction to 34 °C in 4 h (Table 2). LPS also caused a rapid increase in respiratory rate compared with controls (Table 2), and increased serum levels of TNFα and IL-1 beta within 4 h (Table 3).

Casticin and chrysosplenol D reduce LPS-induced SIRS

Pretreatment with casticin (0.07–0.27 mmol/kg) or chrysosplenol D (0.07–0.28 mmol/kg) significantly reduced the drop in body temperature following LPS administration by 30.72 to 77.10% and 16.76 to 65.16%, respectively. Animals treated with casticin (0.27 mmol/kg) or chrysosplenol D (0.14 or 0.28 mmol/kg) had improved respiratory rate by 23.22% and 25.02%, respectively, roughly equivalent to the effect of dexamethasone (24.53%) (Table 2).

Four hours after LPS administration, serum TNF-alpha and IL-1 beta levels were increased compared with controls. Administration of casticin, (0.07, 0.13 or 0.27 mmol/kg) significantly reduced the production of IL-1 beta by 39.86%, 40.77% and 47.47%, respectively. Chrysosplenol D (0.14 or 0.28 mmol/kg) markedly suppressed the production of IL-1 beta by 42.75% and 44.35%, respectively. Dexamethasone also caused a significant reduction in the production of TNF-alpha and IL-1 beta by 23.81% and 51.62%, respectively. However, casticin and chrysosplenol D did not reduce the production of TNF-alpha in this model of systemic inflammation (Table 3).

In vitro casticin and chrysosplenol D suppress LPS-induced release of inflammatory mediators

The murine macrophage cell line Raw264.7 is commonly used to model inflammatory responses *in vitro*. To investigate the effect of casticin and chrysosplenol D on LPS-mediated secretion of proinflammatory mediators, we incubated Raw264.7 murine macrophages with casticin or chrysosplenol D at 1, 5, 10 μM for 18 h, after which LPS was added for a further 12 h. Pilot concentration response experiments (from 1 μM to 40 μM) established the optimal dosing of casticin or chrysosplenol D and excluded detrimental effects on cell viability (Fig. 3). In this cell model, the addition of LPS stimulated the release of IL-1 beta, IL-6 and MCP-1 by 2–14 folds. Casticin induced an increase in release of IL-1 beta by up to 81.08%; IL-6 by up to 60.82%; and MCP-1 by up to 82.32%. Chrysosplenol D induced an increase in release of TNF-alpha by up to 40.65%; IL-1 beta by up to 74.20%; IL-6 by up to 74.96%; and MCP-1 by up to 78.68% (Fig. 4). These observations suggest that the anti-inflammatory properties of these flavonoids may involve reducing LPS-inducible pro-inflammatory cytokine production.

Casticin and chrysosplenol D potently inhibit Raw264.7 cell migration

Using a Transwell chamber assay we investigated whether casticin or chrysosplenol D altered the chemotactic activity of Raw264.7 cells. Casticin (10 μM) reduced Raw264.7 macrophage migration by 62.29%, and 10 μM chrysosplenol D reduced macrophage migration by 57.97% (Fig. 5).

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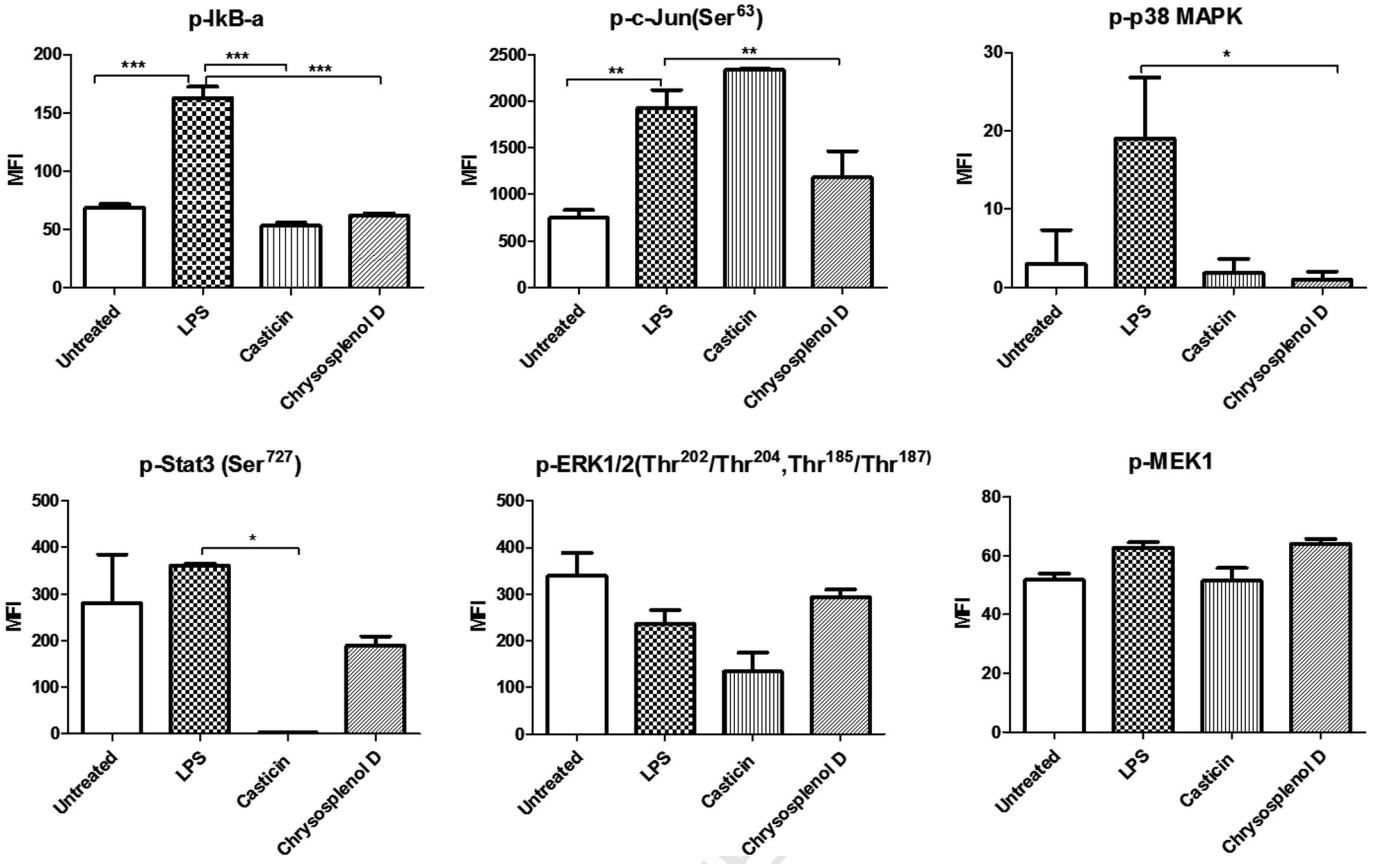

Fig. 6. Change in IκB, p38 MAPKs, ERK, 1/2, Stat3, MEK and c-JUN phosphorylation in response to treatment with casticin or chrysosplenol D (10 μmol) for 2 h. Data acquired using the Bio-Plex phosphoprotein and total protein assay kits. All values were expressed as mean ± SD of three individual experiments. *P < 0.05, **P < 0.01, ***P < 0.001 *vs.* LPS-treated controls.

Casticin and chrysosplenol D reduce LPS-induced IκB and c-JUN phosphorylation

To investigate the molecular mechanisms of anti-inflammatory properties of casticin and chrysosplenol D observed *in vivo* and *vitro*, phosphorylation of cell signaling proteins was assessed. Raw264.7 cells incubated with LPS for 2 h contained higher levels of phosphorylated IκB and c-JUN, but phosphorylation of ERK1/2, Stat3, MEK and p38 MAPK proteins was not altered by LPS (Fig. 6). IκB phosphorylation was reduced in the presence of casticin or chrysosplenol D, and c-JUN phosphorylation was reduced in the presence of chrysosplenol D (Fig. 6).

Casticin and chrysosplenol D act on the NF-κB transcription pathway

The NF-κB pathway is a key mediator of inflammation and result in increased production of cytokines and chemokines including IL-6, IL-1, TNF-alpha, and MCP-1. The capacity for chrysosplenol D to inhibit NF-κB transactivation was assessed in LPS-stimulated HT-29 cells stably transfected with a NF-κB-driven luciferase reporter gene. Pre-incubation with 10 μM casticin and chrysosplenol D significantly inhibited NF-κB activation by >90% and 50%, respectively (Fig. 7A).

Chrysosplenol D inhibition of cytokine release is mediated via JNK

To investigate whether activation of the JNK pathway was involved in the mechanism of chrysosplenol D anti-inflammatory activity, Raw264.7 cells were incubated with the JNK inhibitor SP600125 prior to incubation with chrysosplenol D and LPS, and IL-6 and MCP-1 release was measured by ELISA. Pre-treatment with JNK inhibitor improved the effect of chrysosplenol D on LPS-induced IL-6 and MCP-1 release (Fig. 7B).

Discussion

We previously reported that the anticancer activity of *A. annua* L. may be attributed to the inhibition of immune mediators including pro-inflammatory cytokines by arteannuin B, casticin and chrysosplenol D (Zhu et al., 2013). In this study we sought to further characterize the anti-inflammatory activity of casticin and chrysosplenol D *in vivo* and *vitro*.

A previous study has shown the anti-inflammatory effects of casticin in LPS-stimulated mouse macrophages (Liou et al., 2014), but the effects of chrysosplenol D on inflammation were unstudied. The present study showed the substantial anti-inflammatory effects of flavonoids present in *A. annua* L. in mouse models of local and systemic inflammation, as well as in cultured mouse macrophages. Administration of casticin or chrysosplenol D reduced croton oil-induced edema and improved LPS-induced systemic inflammatory responses. *In vitro*, incubation with casticin or chrysosplenol D decreased Raw 264.7 cell migration, reduced chemokine and cytokine production in response to LPS, suppressed LPS-induced Raw264.7 cell migration and release of inflammatory mediators in a NF-κB- and c-JUN-dependent manner. Each of these functions highlights the potential therapeutic role for these compounds in the treatment of inflammatory diseases.

Croton oil-induced ear edema is a useful model for testing topical anti-inflammatory activity of drugs (Tonelli et al., 1965; Tubaro et al., 1986). Application of croton oil induces the production of pro-inflammatory compounds and edema (Fernandez-Arche et al., 2010; Saraiva et al., 2011). This study showed that topically applied casticin

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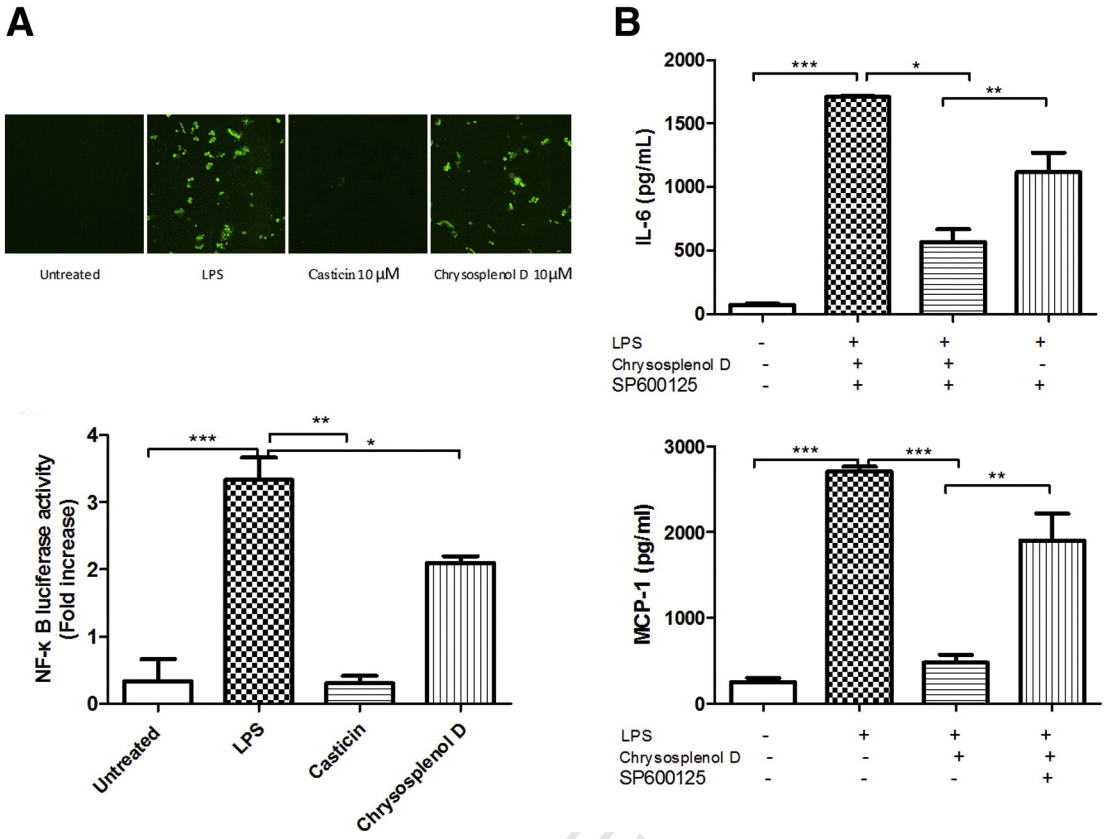

Fig. 7. IκB and c-JUN phosphorylation in response to treatment with casticin or chrysosplenol D (10 μmol) for 2 h. (A) HT-29 cells were transfected with plasmid constructs for NF-κB-Luc. The luciferase activity was measured with a luminometer. (B) IL-6 and MCP-1 content of the supernatants of LPS-treated Raw264.7 cells pretreated with a standard JNK inhibitor (SP600125) was measured by ELISA. Each sample was tested three times. All values are expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 *vs.* LPS-treated controls.

or chrysosplenol D reduced edema, indicating that casticin and chrysosplenol D are able of reducing the inflammatory local reaction induced by croton oil.

SIRS is a complex immune response often induced in response to severe trauma, hemorrhage, pancreatitis and septic shock (Botwinski, 2001). SIRS is characterized by excessive production of proinflammatory mediators, including TNF-alpha, IL-1 beta, IL-6, MMP-1, CCL2 and CXCLs. High levels of these proinflammatory mediators contribute to severe organ damage and multiple organ dysfunction syndrome (Mendes Sdos et al., 2009). LPS, the main trigger of SIRS, activates monocytes and macrophages, inducing release of proinflammatory cytokines and mediators (Botwinski, 2001). In this study, we reported that pretreatment of mice with casticin and chrysosplenol D reduced the systemic immune response to LPS.

Then, we tried to explore the mechanisms of casticin and chrysosplenol D anti-inflammatory activity using cell models of inflammation. Stimulation of macrophages with LPS elicits a variety of different signaling events, including the production of cytokines, chemokines and other signals important for the coordination of the inflammatory response (Joseph et al., 2003). These inflammatory responses promote the secretion of inflammatory cytokines (Bode et al., 2012). LPS promotes the production of inflammatory cytokines *via* IκB/NF-κB and mitogen activated protein kinase (MAPK)-dependent pathways (Bode et al., 2012). These proteins play critical roles in regulating pro-inflammatory gene expression. In the present study, we demonstrated *in vitro* that the capacity of casticin and chrysosplenol D to reduce the pro-inflammatory effect of LPS was dependent upon NF-κB and c-JUN. These results complement the results of a previous study that showed that casticin decreased the secretion of proinflammatory cytokines by activated macrophages through an inhibition of the nuclear NF-κB subunit of p65 as well as through decreased Akt and MAPK activation. However, further study is necessary to determine the exact mechanisms responsible for the effects of casticin and chrysosplenol D on inflammation. In addition, further preclinical study is still necessary before these compounds can be used as drugs in humans.

In the present study, casticin and chrysosplenol D decreased the migration of macrophages in response to LPS. These results are supported by a previous study of the effects of casticin on eosinophil migration in lung epithelial cells through decreased ICAM-1 expression (Koh et al., 2011). Further study focusing on cellular migration, adhesion, chemotactic molecules is necessary.

In summary, the flavonoids casticin and chrysosplenol D extracted from *A. annua* L. suppress the expression of inflammatory mediators through the regulation of NF-κB and c-JUN in a murine macrophage cell line. The biological effects of the casticin and chrysosplenol D confirmed in this study indicate that these components might be useful in the treatment of inflammatory disorders.

Conflict of interests

The authors have no conflicting financial interests.

Author contributions

Yu-Jie Li, Yan Guo, Qing Yang, Xiao-Gang Weng and Lan Yang carried out the studies, participated in collecting data, and drafted the manuscript. Dong Zhang, Qi Li, Xu-Cen Liu, Xiao-Xi Kan, Xi Chen, Ya-Jie Wang, and Ying Chen performed the statistical analysis and participated in its design. Xiao-Xin Zhu, Eva Kmoníèková and Zdeníìk Zídek helped to draft the manuscript. All authors read and approved the final manuscript.

Please cite this article as: Li, Y.-J., et al., Flavonoids casticin and chrysosplenol D from *Artemisia annua* L. inhibit inflammation *in vitro* and *in vivo*, Toxicol. Appl. Pharmacol. (2015), http://dx.doi.org/10.1016/j.taap.2015.04.005

Transparency document

The transparency document associated with this article can be found, in the online version.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (30973901), International Science and Technology Cooperation Program of the People's Republic of China (2011DFA30870) and Joint Research Project of The Twentieth Session of China–Thailand Joint Committee on Science and Technology Cooperation (20-602J). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Please cite this article as: Li, Y.-J., et al., Flavonoids casticin and chrysosplenol D from *Artemisia annua* L. inhibit inflammation *in vitro* and *in vivo*, Toxicol. Appl. Pharmacol. (2015), http://dx.doi.org/10.1016/j.taap.2015.04.005