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

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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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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

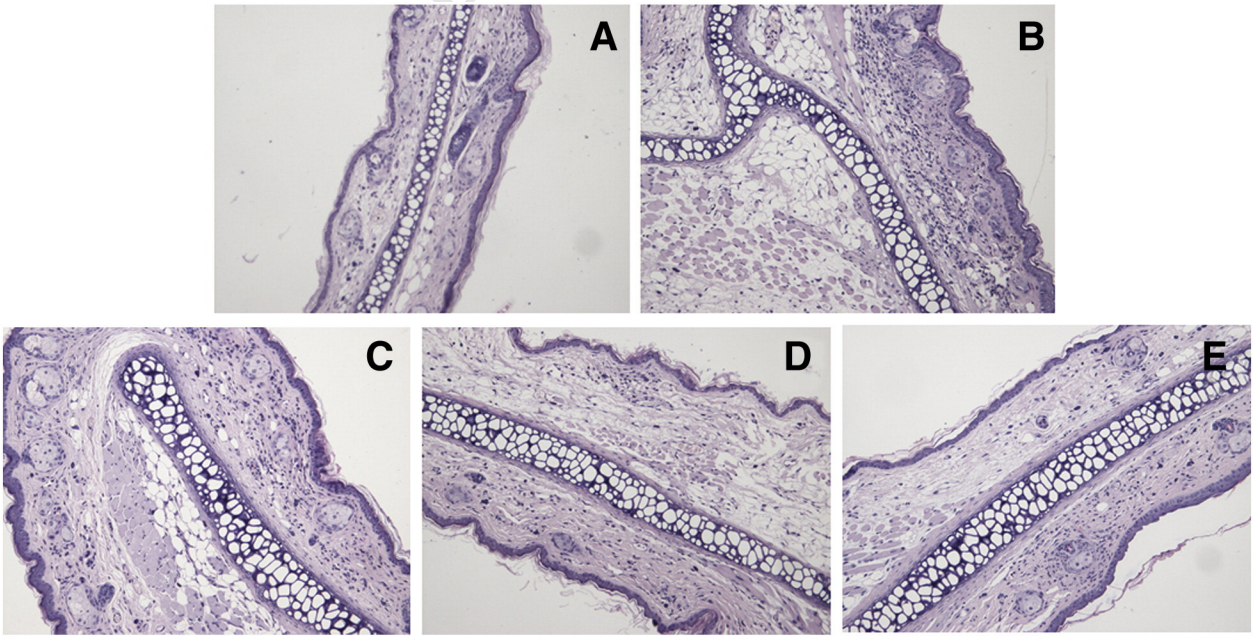

Fig. 1. Histological characteristics of mouse ears 6 h after the induction of croton oil dermatitis. Mouse ears were untreated (A), or croton oil was applied topically to induce dermatitis (B to E). Application of 1 μmol/cm^2 of casticin (C); 1 μmol/cm^2 of chrysosplenol D (D); or 1 μmol/cm^2 of indomethacin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

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