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Antinociceptive and anti-inflammatory activities of ethyl acetate fraction from *Zanthoxylum armatum* in mice

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ABSTRACT

Zanthoxylum armatum DC. is a traditional Chinese medicine that is prescribed to alleviate pain and treat inflammatory disorders. This species is distributed mainly in the southeast and southwest regions of China. In the present study, we found that ethyl acetate fraction of ethanolic extract of *Z. armatum* could significantly decrease acetic acid-induced writhing numbers, and suppress formalin induced licking times in the first phase at the highest dose and in the second phase at all tested doses. This observation revealed that *Z. armatum* extract possessed powerful antinociceptive activity. The mechanisms of the antinociceptive effect might be mainly involved in the periphery inflammatory analgesic. In addition, the ethyl acetate fraction also inhibited xylene-induced ear swelling in a dose-dependent manner in mice. Eight lignans [eudesmin, horsfieldin, fargesin, kobusin, sesamin, asarinin, planispine A, and pinoresinol-di-3,3-dimethylallyl] were identified as major components of the ethyl acetate fraction. Considering related studies reporting the anti-inflammatory activity for the identified lignans, lignan might be responsible for its anti-inflammatory activity. Our results confirm that the traditional use of *Z. armatum* in the treatment of inflammation and pain is warranted.

1. Introduction

The genus *Zanthoxylum* (Rutaceae) comprises of 250 species distributed in the tropical and subtropical zones of Asia, Africa, America and Oceania. There are 39 species and 14 varieties in China. *Zanthoxylum armatum* DC., a wild deciduous arbor (3–5 m high), is distributed mainly in southeast and southwest China [1]. It is widely used as a folk medicine for the prevention of stomach ache, tooth ache, treating cold in the chest and abdomen, preventing snake bites and expelling roundworms. Previous phytochemical investigations have revealed the presence of amides [2], alkaloids, flavonoids, coumarins [3], lignans [4–6],triterpene compounds [6].

Zhuyejiao tablets, are made up of 4/5 ethanolic extract and 1/5 raw herb powder of *Z. armatum*. This is a Chinese patented drug for the treatment of acute appendicitis, abdominal pain and stomach ache [7]. The results of animal experiments showed that Zhuyejiao tablets possessed antinociceptive and anti-inflammatory activities [8]. There has been no further report on these pharmacological effects for this plant so far.

Therefore, based on previous investigations, we studied the bioactivity of the petroleum ether, ethyl acetate, *n*-butanol and water fraction from the ethanolic extract, and found that the ethyl acetate fraction had powerful antinociceptive and anti-inflammatory activities. In present study, we also identified the major components of the ethyl acetate fraction by HPLC, and examined further antinociceptive and anti-inflammatory activities of the fraction to further elucidate the mechanisms for its bioactivity and to provide a scientific basis for the clinical use of *Z. armatum*.

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2. Materials and methods

2.1. Plant material

Stems and roots of the plant material were collected in Nanning (located at 22.8° N and 108.3° E), Guangxi Province in August 2008, and were identified by Pro. Yi Cai. A voucher specimen of the plant (#08081) was deposited at the Herbarium of the Department of Pharmacognosy, School of Pharmacy, Fudan University.

2.2. Extraction and partition procedures

Dried powdered stems and roots of *Z. armatum* DC. (9.5 kg) were percolated with 95% EtOH, and the solvent was concentrated under reduced pressure to give a crude extract (560 g) with a yield of 5.8%. The crude extract was subsequently suspended in distilled water and partitioned successively with petroleum, ethyl acetate and *n*-butanol. The ethyl acetate fraction of the extract was used in activity tests and in the phytochemical study.

2.3. Drugs

Acetic acid, formaldehyde, acetylsalicylic acid (ASA), xylene and Tween80 were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China), morphine hydrochloride from Shenyang First Pharmaceutical Company (Shenyang, China). All other drugs were of analytical grade.

The control group received vehicle (1.5% Tween80 suspension in saline). The ethyl acetate fraction from the ethanolic extract (EAF) was administered in 100, 200, 400, and 800 mg/kg doses after being suspended in vehicle. ASA (100 or 200 mg/kg, p.o.) in vehicle and morphine hydrochloride (10 mg/kg) in saline were used as reference drugs. All test drugs were orally administered, except for morphine hydrochloride, which was intraperitoneally administered in an equivalent volume of 10 ml/kg body weight of the animal.

2.4. Analysis of major components of ethyl acetate fraction by HPLC

HPLC analysis was performed on a liquid chromatograph system (LC-10AT, Shimadzu), equipped with an UV–Vis detector (SPD-10A, Shimadzu). Data analyses were carried out using ChemStation software (HW-2000, Dianpu Co., Ltd, China). Separation was conducted on a ODS column (250×4.6 mm, Shimadzu). The solvent system was composed of A (acetonitrile) and B (water), with an elution program performed as follows: 0–20 min, linear gradient from 30% A to 50% A; 20–35 min, 50% A; 35–50 min, linear gradient from 50% A to 90% A, and 50 min–57 min, 90% A. The total time of analysis was 57 min. The column was equilibrated for 10 min. The flow rate was 1 ml/min. Injection volume was 20 μl. The detection was conducted using the wavelength of 233 nm.

2.5. Animals

Mice were purchased from the Department of Experimental Animal Center of Fudan University. Adult KM mice were housed in plastic cages, with food and tap water available ad libitum. Mice were acclimatized to the laboratory for at least 5 days prior to the test procedure. The experiment procedures were conducted in compliance with the National Institutes of Health Guide for Care and Use of the laboratory animals, and were approved by the Local Bioethics Committee (School of Pharmacy, Fudan University, China; document number: SYXK2007-002).

2.6. Acetic acid-induced writhing test

The method was used according to previously described techniques [9]. Mice weighing 18–22 g ($n=10$) were treated orally one hour before injection of acetic acid with EAF at doses of 100, 200, 400 and 800 mg/kg. The control group received only vehicle (10 ml/kg), and the reference group received ASA (100 mg/kg). Writhing was induced by an intraperitoneal injection (10 ml/kg) of a 0.6% acetic acid solution. The numbers of writhing that occurred between 5 and 25 min were counted for each animal.

2.7. Formalin test

The method used was similar to that described by Hunskaar and Hole [10]. Mice weighing 18–22 g ($n=10$) were orally treated with 100, 200, 400, and 800 mg/kg of the EAF. The control group received only vehicle (10 ml/kg), and the reference group received morphine hydrochloride (10 mg/kg, i.p.). One hour after, they were injected under the dorsal skin surface of the right hind paw with 20 μl of 2% formalin (in 0.9% saline). Immediately after, each mouse was placed into a glass cylinder provided with mirrors to enable a total panorama of the nociceptive behaviour. The time spent in licking the injected paw (an index of nociception) was measured in the first (0–5 min) and second (15–45 min) phases after formalin injection.

2.8. Xylene-induced ear swelling in mice

The improved method was adopted as previously described [11]. Mice weighing 25–30 g ($n=10$) were pretreated with EAF (100, 200, 400 and 800 mg/kg; p.o.), ASA (200 mg/kg, p.o.), or the vehicle alone. One hour later, each animal received 20 μl of xylene on the anterior and posterior surfaces of the right ear lobe, the left ear serving as the control. Four hours later, the animals were sacrificed by cervical dislocation. The bilateral ears were removed, one round ear piece (two for each ear) were cut with a punch of 9 mm in diameter, and each round ear piece was weighed precisely .The degree of ear swelling was calculated based on the weight of the left ear without applying xylene.

2.9. Statistical analysis of data

The results were presented as the mean ± S.D. Statistical significance between groups was performed by the application of analysis of variance ANOVA followed by Bonferroni's test. p values less than 0.05 ($^{*}p<0.05$) were used as the significant level.

3. Results and discussion

The writhing test has long been used as a screening tool for the assessment of analgesic or anti-inflammatory properties of plant extracts and natural products. It has been suggested that acetic acid acts by releasing endogenous mediators that stimulate the nociceptive neurons [12]. It is postulated that the abdominal constriction response is induced by local peritoneal receptor activation [13] and involves prostanoids mediators. In mice there is an increase in the peritoneal fluid levels of PGE2 and PGF2, as well as lipooxygenase products [14], release of sympathetic nervous system mediators [15]. The nociceptive properties of acetic acid might also be due to the release of cytokines, such as TNF-α, interleukin-1β, and interleukin-8, by resident peritoneal macrophages and mast cells [16,17]. In the present writhing test, there was a significant reduction ($p<0.05$) in writhing for the groups treated with ASA (100 mg/kg) and EAF at all doses (100, 200, 400 and 800 mg/kg); 49.09%, 13.96%, 27.47%, 56.31% and 58.56%, respectively, in comparison to the control (Fig. 1). This datum suggested that EAF had powerful antinociceptive effect. However, writhing test shows poor specificity. The results alone didn't ascertain whether the antinociceptive effect was central or peripheral.

In order to confirm these effects, the formalin test was also carried out. The formalin model of nociception [10], is useful not only for assessing analgesic substances but also for elucidating the mechanism of analgesia [18]. The first phase (acute pain) initiates immediately after formalin injection and lasts only a few minutes, and is believed to be driven by primary afferent nociceptor activity [17]. The second phase (tonic pain) initiates 15 min after formalin injection and lasts about 20–40 min, and is thought to arise from nociceptive spinal neuron hyperactivity. In this second phase various mediators operate in a sequence to produce an inflammatory response and have been correlated with the elevated production of prostaglandin (PG), induction of cyclo-oxygenase (COX) and the release of nitric oxide (NO) [19,20]. Drugs that act primarily on the central nervous system suppress both phases equally, while peripherally acting drugs suppress the second phase [10]. There is evidence that suggests peripheral inflammatory processes are involved in the second phase, and are blocked by non-steroidal anti-inflammatory drugs (NSAIDs) while the first phase seems to be unaffected [21].

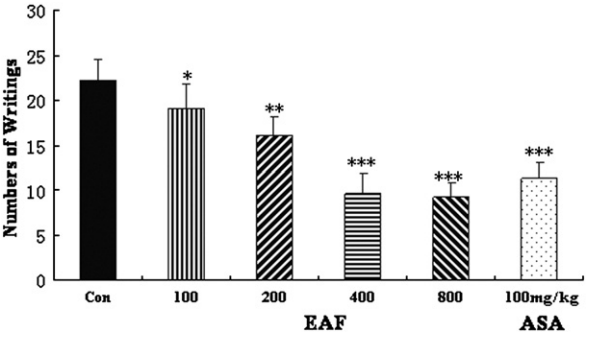

Fig. 1. Effects of EAF on acetic acid-induced writhing behaviour in mice. Results are expressed as mean ± S.D.($n=10$). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with control. EAF: ethyl acetate fraction of ethanolic extract from *Zanthoxylum armatum*. ASA: Acetylsalicylic acid.

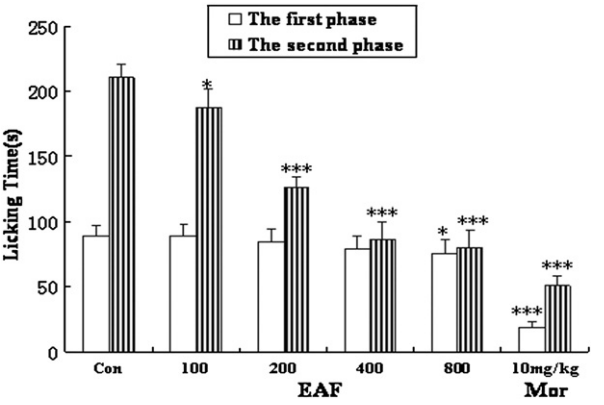

Fig. 2. Effects of EAF on formalin induced-pain in mice. Results are expressed as mean ± S.D.($n=10$). $^{*}P<0.05$ and $^{***}P<0.001$ compared with control. EAF: ethyl acetate fraction of ethanolic extract from *Zanthoxylum armatum*. Mor: Morphine.

The results of the formalin test are shown in Fig. 2, morphine hydrochloride (10 mg/kg), the reference drug, significantly suppressed the formalin response in the first and second phases by 82.03% and 75.30%, respectively. Pretreatment with EAF at doses of 800 mg/kg caused 18% and 60.42% inhibition of the licking times in both phases, while EAF at doses of 100, 200, and 400 mg/kg only caused 11.03%, 36.81%, and 59.82% reduction in licking times in the second phase respectively. This suggested that the mechanisms of antinociceptive activity might be mainly involved in the periphery inflammatory analgesic.

To complement the results obtained on the second phase of formalin induced licking response, we tested the effects of EAF on ear swelling in mice induced by xylene, this animal model is an acute inflammatory model. Xylene-induced neurogenous swelling is partially associated with substance P, and is a common inflammatory model for evaluating vascular permeability [22]. EAF at all tested doses, as well as ASA (200 mg/kg), significantly inhibited xylene-induced ear swelling in a dose-dependent manner in mice (Fig. 3), and suggests it might reduce the release of substance P or antagonize its action. Therefore, these results revealed that EAF displayed anti-inflammatory activity.

According to the HPLC conditions mentioned earlier, a chromatogram of the ethyl acetate fraction was obtained (Fig. 4). Eight lignans were identified as the major components of this fraction (Fig. 5). These are eudesmin (**1**),

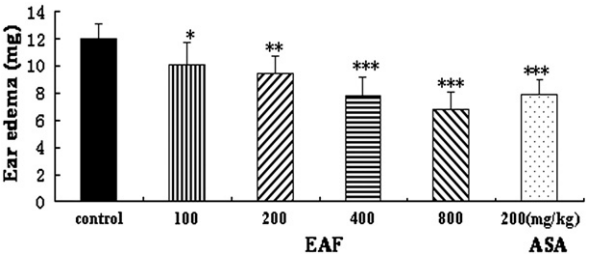

Fig. 3. Effects of EAF on xylene-induced ear edema in mice. Results are expressed as mean ± S.D.($n=10$). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with control. EAF: ethyl acetate fraction of ethanolic extract from *Zanthoxylum armatum*. ASA: Acetylsalicylic acid.

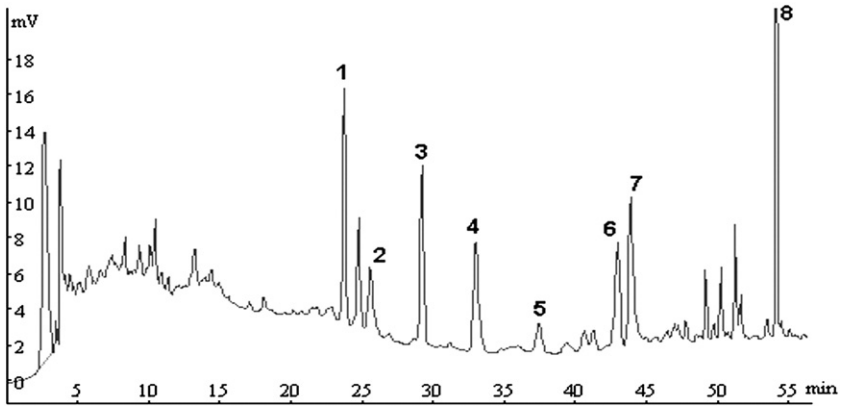

Fig. 4. HPLC chromatogram of ethyl acetate fraction of *Zanthoxylum armatum*. (compounds **12345678**). Peak numbers as in Fig. 5.

horsfieldin (**2**), fargesin (**3**), kobusin (**4**), sesamin (**5**), asarinin (**6**), planispine A (**7**), and pinoresinol-di-3,3-dimethylallyl (**8**). In our present phytochemical studies of the ethyl acetate fraction, 39 compounds were obtained, including large amounts of lignans, some alkaloids, small quantities of coumarins, triterpenoids and steroids. Among which, eudesmin, yangambin, Sesamin and pinoresinol [23] have been proved to possess anti-inflammatory properties. Sesamin has displayed a role of action in the inflammatory process by inhibiting primary activation pathways, such as mitogen-activated protein kinase and nuclear factor kappa B. Yet, it is not able to inhibit PGE2 in-vitro [24]. It has also been reported that eudesmin and yangambin also have anti-inflammatory effects by inhibiting in the production of NO in LPS-activated microglia [25]. In our studies, fargesin in a dose of 80 mg/mg and yangambin in 30 mg/mg significantly inhibited

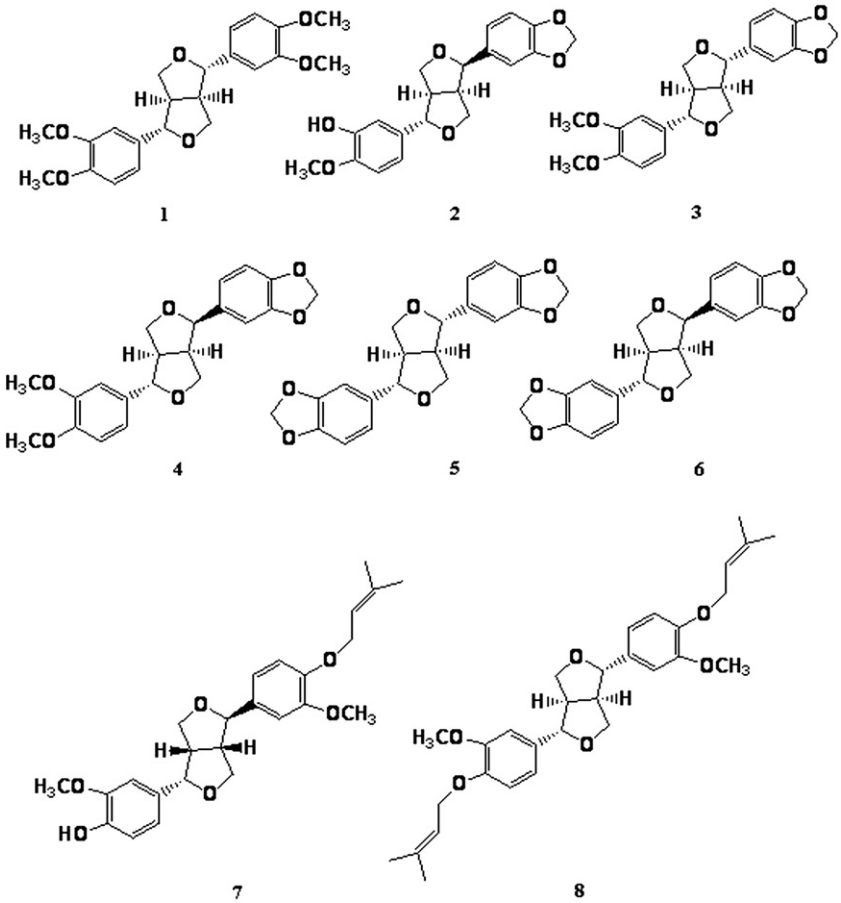

Fig. 5. Chemical structures of compounds **12345678**. Eudesmin (**1**), horsfieldin (**2**), fargesin (**3**), kobusin (**4**), sesamin (**5**), asarinin (**6**), planispine A (**7**), and pinoresinol-di-3,3-dimethylallyl (**8**).

xylene-induced ear swelling. According to these results, it is suggested that lignans seemed to be one of the constituents responsible for anti-inflammatory activity of this plant species.

In conclusion, the results of this study show that the ethyl acetate fraction of *Z. armatum* possessed antinociceptive and anti-inflammatory activities, and further supported its use in traditional medicine for alleviating pain and treating inflammatory disorders. It is likely that some of these effects are due to lignan constituents that are known to have anti-inflammatory properties. Due to the remarkable bioactive properties of the ethyl acetate fraction, further studies are required for the identification and isolation of active components.

Acknowledgements

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