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Antinociceptive and anti-inflammatory effects of rosmarinic acid isolated from *Thunbergia laurifolia* Lindl.

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ABSTRACT

Rosmarinic acid (RA) was isolated from an ethanolic extract of *Thunbergia laurifolia* leaves. The antinociceptive activity of RA was assessed in mice using hot-plate, acetic acid-induced writhing, and formalin tests. The anti-inflammatory effects of RA were determined in two mouse models of carrageenan-induced paw edema and cotton pellet-induced granuloma formation. Orally administered RA (50, 100, and 150 mg/kg) showed significant ($p < 0.001$) antinociceptive activity in the hot-plate test and this effect was reversed by naloxone. RA at doses of 50 and 100 mg/kg significantly reduced acetic acid-induced writhing by 52% ($p < 0.01$) and 85% ($p < 0.001$), respectively, and RA at 100 mg/kg also caused significant inhibition of formalin-induced pain in the early and late phases ($p < 0.01$ and $p < 0.001$, respectively). RA at 100 mg/kg significantly suppressed carrageenan-induced paw edema at 3, 4, 5 and 6 h after carrageenan injection ($p < 0.01$, $p < 0.05$ $p < 0.01$, and $p < 0.05$, respectively) and showed significant activity against PGE_2-induced paw edema. RA at 100 mg/kg also inhibited cotton pellet-induced granuloma formation in mice. Taken together, these results demonstrate that RA possesses both central and peripheral antinociceptive activities and has anti-inflammatory effects against acute and chronic inflammation. While further evaluation regarding the safety profile of RA is needed, these data may provide a basis for the rational use of RA and *T. laurifolia* for treatment of pain and inflammatory disorders.

1. Introduction

Pain and inflammation are major problems in the general population that affect lifestyle and health. Many analgesic and anti-inflammatory drugs are available for treating these symptoms, but these drugs can cause adverse effects when used for long term treatment. Therefore, there is considerable interest in the discovery and development of new analgesic and anti-inflammatory drugs from natural sources with high efficacy and low side effects.

Thunbergia laurifolia Lindl. belongs to the Acanthaceae family and is known in Thai as "Rang Chuet" (Chan and Lim, 2006). This plant is commonly used for relief of symptoms including pain, inflammation, edema, headache, and excessive thirst, and as an antidote for poisons in Thai traditional medicine. An aqueous extract preparation of *T. laurifolia* leaves has anticholinergic effects and decreases mortality in rats treated with folidol, an organophosphate insecticide (Tejasen and Thongthapp, 1980). This extract also has hepatoprotective activity against ethanol-induced liver injury *in vitro* and *in vivo* (Pramyothin et al., 2005). Topical application of alcohol and hexane extracts of *T. laurifolia* leaves also produces anti-inflammatory activity, with significant inhibition of carrageenan-induced paw edema in mice (Charumanee et al., 1998). Subcutaneous administration of an ethanol extract of *T. laurifolia* leaves has also been shown to have antinociceptive and anti-inflammatory effects in several animal models (Phosri et al., 2008). However, the active compounds responsible for these effects of *T. laurifolia* leaf extracts have not been determined.

An ethanolic extract of *T. laurifolia* leaves was shown to have antioxidant activity in a 2,2-diphenyl-L-picrylhydrazyl (DPPH) radical scavenging assay (Suwanchaikasem et al., 2014). TLC bioautography used for separation of the bioactive constituents indicated that rosmarinic acid (RA) was responsible for the antioxidant activity (Suwanchaikasem et al., 2014). The antioxidative effect of RA is also apparent in its reduction of liver injury induced by D-galactosamine (Won et al., 2003). Rosmarinic acid (RA) has diverse immunoregulatory functions including antimicrobial, antioxidant, and antiinflammatory activities (Van Kessel et al., 1986, Kelm et al., 2000, Sahu et al., 1999). Rosmarinic acid also has an additive effect in treating inflammatory diseases such as rheumatoid arthritis due to its free radical-scavenging capacity (Youn et al., 2003). The anti-inflammatory activity of RA is mainly attributable to inhibition of cyclooxygenase (COX) and lipoxygenase (LOX) activities and complement activation (Sahu et al., 1999). In animal models, RA significantly inhibited paw edema induced by *Bothrops jararacussu* snake venom (Ticli et al., 2005) and RA given intraperitoneally reduced the number of total

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exudate cells in a carrageenan-induced pleurisy model, but was ineffective on tail-flick latencies in a tail-flick assay in rats (Gamaro et al., 2011).

Previous studies have used RA isolated from plants of other families including Boraginaceae, Lamiaceae but not Acanthaceae for analgesic and anti-inflammatory activity testing. RA is found to be a major constituent of *T. laurifolia* belonging to the Acanthaceae family that has not been observed previously. Suwanchaikasem et al. (2014) was the first group to isolate *trans*-rosmarinic acid from the ethanolic extract of *T. laurifolia* leaves. RA used in this study was isolated from the ethanolic extract of *T. laurifolia* leaves by Suwanchaikasem group.

The aim of the present study was to investigate the effects of orally administered RA isolated from *T. laurifolia* leaves on nociception and inflammation in mice, and to examine the mechanisms of actions of RA underlying these effects. The findings indicate that RA is a potential lead for development of treatment options for pain and inflammation.

2. Materials and methods

2.1. Plant material

T. laurifolia leaves were collected from Nakhon Pathom Province, Thailand, and identified by Associate Professor Thatree Phadungcharoen, Department of Pharmacognosy and pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The voucher specimen (SS-0510105) was deposited at the Museum of Natural Medicines of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.2. Antioxidant-guided isolation of rosmarinic acid (RA)

The extraction and isolation procedures for RA were described by Suwanchaikasem et al. (2014). In brief, dried *T. laurifolia* leaves (900 g) were macerated in 95% ethanol (8 L) at room temperature for 72 h. The extract was filtered and evaporated to dryness at 50 °C in a rotary evaporator. Extraction of the residue was repeated using the same conditions and the two filtrates were combined. The ethanolic extract (64.75 g) was applied to a column of ion-exchange resin and eluted with a gradient mixture of water and acetone. All of the obtained fractions were examined for DPPH-scavenging properties via TLC bioautography. The DPPH-scavenging active fraction was identified as a yellowish spot on the purplish background of the TLC plate and was further separated on a silica gel column eluted with a chloroform–methanol–formic acid (8.5:1.5:0.5) mixture. The fraction containing the targeted compound was then separated using Sephadex LH-20 with methanol, resulting in four fractions. The DPPH-scavenging active fraction was purified on a silica gel column with a gradient system starting with dichloromethane–methanol (7:3) and increasing to 100% methanol to obtain the pure, yellow antioxidant compound (235.3 mg). The isolated compound was identified as RA and represented yield at least 0.36% of the dry extract. The molecular structure of RA (Fig. 1) was confirmed using spectral analysis, NMR, and mass spectrometry using an AB-SCIEX QTRAP 5500 spectrometer.

2.3. Animals

Male ICR mice weighing 18–25 g and 25–35 g were used in the experiments. The mice were obtained from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakhon Pathom, Thailand. The animals were housed in the Laboratory Animal Unit of the Faculty of Pharmaceutical Sciences, Chulalongkorn University at 25 ± 2 °C, 50–60% humidity, and under a 12/12 h light/dark cycle, with food and water provided *ad libitum*. The mice were kept for one week under laboratory conditions before use in experiments. At the end of each experiment, mice were sacrificed by carbon dioxide asphyxiation. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

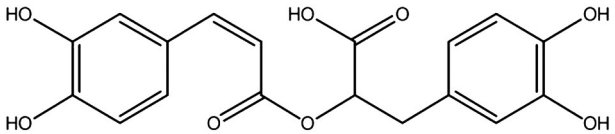

Fig. 1. Structure of rosmarinic acid.

2.4. Drugs and chemicals

Morphine sulfate (MO; Thai FDA), acetic acid (Merck, Darmstadt, Germany), formaldehyde (Merck), naloxone (NAL, Sigma, St. Louis, MO, USA), λ-carrageenan (Sigma), prostaglandin E_2 (PGE_2; Sigma), pentobarbitone sodium (Ceva Sante Animale, Libourne, France) were dissolved in 0.9% sodium chloride solution (NSS; General Hospital Products Public Co., Thailand). Indomethacin (IND; Sigma) was suspended in 2% (w/v) Tween 80 (Srichansahasoth Co., Thailand). RA was suspended in sterile water (SW; General Hospital Products Public Co.). MO and IND were used as standard analgesic drugs, and IND was also used as a standard anti-inflammatory agent. Control animals were administered an equivalent volume of vehicle via the same route as the test compound.

2.5. Hot-plate test

The hot-plate test was conducted as described by Woolfe and Macdonald (1944). Mice were placed on a hot-plate (Harvard Apparatus) maintained at 55 ± 0.5 °C and were confined by a Plexiglas wall cylinder. Only animals with a pretreatment hot-plate latency time <45 s were used. Animals were treated with NSS (10 mL/kg) or MO (10 mg/kg) intraperitoneally or SW (10 mL/kg) or various doses of RA (12.5, 25, 50, 100 and 150 mg/kg) orally. The latency to licking of a hind paw or jumping from the surface of the hot-plate was measured. If this behavior was not observed within 45 s, the animal was removed from the hot-plate to avoid tissue damage. The post-drug latency was measured in 7 trials at 15, 30, 45, 60, 90, 120 and 240 min after drug administration. The hot-plate latency was expressed as the mean % maximum possible effect (%MPE):

$$\%\text{MPE} = \frac{(\text{post-drug latency}) - (\text{pre-drug latency})}{(\text{cut-off time}) - (\text{pre-drug latency})} \times 100$$

where cut-off time = 45 s.

Possible involvement of the opioid system in the antinociceptive effect of RA was also analyzed. The animals were pretreated intraperitoneally with naloxone (5 mg/kg) 10 min before oral administration of RA (100 mg/kg) and the hot-plate latencies were measured.

2.6 . Acetic acid-induced writhing test

The acetic acid-induced writhing test in mice was conducted as described by Koster et al. (1959). Mice were pretreated orally with 2% Tween 80 (10 mL/kg), IND (10 mg/kg), SW (10 mL/kg) or various doses of RA (12.5, 25, 50, 100 and 150 mg/kg) 1 h before intraperitoneal injection of 0.6% acetic acid (10 mL/kg). The animals were then placed in an observation glass cylinder. The number of writhes (contraction of the abdominal muscles together with hind limb extension) were counted in 5-min periods for 30 min after acetic acid injection. Antinociceptive activity was expressed as the % inhibition of the writhing response compared with the vehicle control group:

$$\%\text{Inhibition of writhing response} = \frac{\text{Wr(control)} - \text{Wr(test)}}{\text{Wr(control)}} \times 100$$

where Wr = the mean number of writhing responses.

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