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Contributed Paper

Antinociceptive and Anti-inflammatory Effects of the Ethanolic Extract of *Curcuma* aff. *amada*

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ABSTRACT

Curcuma aff. *amada* is used for treatment of poisoning, detoxification and anti-inflammation in Thai folk medicine. However, there is no scientific evidence supporting the potential antinociceptive and anti-inflammatory activities of this plant. To investigate the antinociceptive and anti-inflammatory effects of the ethanolic extract of *Curcuma* aff. *amada* rhizome (CAE) and to examine the mechanisms of actions underlying these effects, the antinociceptive effect of CAE was assessed in mice using hot-plate, acetic-acid induced writhing and formalin tests. The anti-inflammatory effect of CAE was investigated by evaluation of carrageenan-induced paw edema, prostaglandin E_2 (PGE_2)-induced paw edema, arachidonic acid-induced paw edema, and cotton pellet-induced granuloma formation. To investigate the mechanism of anti-inflammatory action, paw tissues were examined histologically. Oral administration of CAE (100 and 200 mg/kg) significantly increased hot-plate latencies and this effect was reversed by naloxone, indicating involvement of opioid receptors. CAE (12.5, 25, 50, 100 and 200 mg/kg) significantly reduced acetic acid-induced writhing and caused significant inhibition of formalin-induced paw licking in both phases. In anti-inflammatory tests, CAE at doses of 12.5, 25, 50, 100 and 200 mg/kg significantly suppressed carrageenan-induced paw edema at 4, 5 and 6 h after carrageenan injection and showed significant activity against PGE_2-induced paw edema. CAE failed to inhibit paw edema induced by arachidonic acid. Histological studies showed that all doses of CAE decreased infiltration of neutrophils induced by carrageenan. CAE at 200 mg/kg also inhibited cotton pellet-induced granuloma formation in mice. Taken together, these results show that CAE possesses both central and peripheral antinociceptive activities and has anti-inflammatory effects against acute and chronic inflammation with no obvious acute toxic effects. These data support the ethnopharmacological use of this extract for treatment of pain and inflammatory disorders. However, further evaluation of the safety profile of the extract is needed.

Keywords: *Curcuma* aff. *amada*, Zingiberaceae, antinociceptive activity, anti-inflammatory activity, histological examination

1. INTRODUCTION

Pain and inflammation are major health problems in the general population and lead to extensive medical consultation. Pain is a common symptom of various inflammatory diseases that affect the quality of life, while inflammation is involved in the pathogenesis of diseases such as gout, rheumatoid arthritis, pulmonary emphysema, diabetes, cancer, and neurodegenerative, cardiovascular, and other life-threatening diseases.

Analgesic and anti-inflammatory drugs are used for pain and inflammation, but these drugs can have serious adverse effects. For example, acetaminophen causes mild to moderate hepatotoxicity, renal tubular necrosis, hypoglycemic coma and thrombocytopenia [1] and non-steroidal anti-inflammatory drugs (NSAIDs) are associated with upper gastrointestinal bleeding, ulcers, small intestinal perforation and hepatotoxicity [2]. Therefore, there is a need to find analgesic and anti-inflammatory agents with efficacy and fewer adverse effects, and such agents may be found in traditional plant-derived medicines.

Curcuma aff. *amada* (family Zingiberaceae) is known in Thai as "Wan Rang Chuet". A specific species name for the *Curcuma* aff. *amada* plant has yet to be assigned; however, it appears to have an affinity with *Curcuma amada*. The rhizome of *Curcuma* aff. *amada* is used for treatment of poisoning, detoxification and inflammation in Thai traditional medicine. The decoction of its rhizome is used to treat abscess and poisoning from insecticides and ethyl alcohol [3]. Aqueous and ethanolic extracts of *Curcuma* aff. *amada* were shown to have antioxidant activity in the Ferric reducing antioxidant power (FRAP) assay and to have natural phenolic components [4]. Several studies have indicated that reactive oxygen species scavenging and anti-inflammatory activities of medicinal plants are influenced by natural phenolic components with antioxidant effects [5].

Many different pharmacological actions of plants in the genus *Curcuma* have been shown in *in vitro* and *in vivo* models. For example, *Curcuma longa* (Turmeric) has been reported to have antioxidant, hepatoprotective, anticarcinogenic and cardioprotective effects [6]; and *Curcuma amada* Roxb. (Mango ginger) has antioxidant, antibacterial, antifungal, anti-inflammatory, platelet aggregation inhibitory, cytotoxicity, antiallergic, biopesticide, hypotriglyceridemic, enterokinase inhibitory and antitubercular, CNS depressant and analgesic activity, aphrodisiac, and antihypercholesterolemic properties [7]. Amadaldehyde, a pure compound isolated from chloroform extract of Mango ginger, has bactericidal, antioxidant, cytotoxicity and platelet aggregation inhibitory activities [8]. Most activities result from the presence of curcumin and its derivatives in the plant extract. Many previous studies demonstrate antinociceptive effects of curcumin such as the hyperalgesia activity of curcumin in the formalin-induced orofacial pain model in rats and its synergistic interaction with diclofenac; and curcumin treatment exerts modality-specific analgesic effects on mechanical allodynia and thermal hyperalgesia in neuropathic mice [9, 10]. However, the active compounds responsible for the biological activities of *Curcuma* aff. *amada* rhizome extract (CAE) have not been determined and there is no scientific evidence

supporting the anti-inflammatory activity and other pharmacological properties of *Curcuma* aff. *amada*.

The objective of this study was to investigate the antinociceptive and anti-inflammatory effects of CAE and to determine the possible mechanisms of action.

2. MATERIALS AND METHODS

2.1 Plant Material

Rhizomes of *Curcuma* aff. *amada* were collected from Chachoengsao province and identified by Dr. Chaiyo Chaichantipyuth, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The voucher specimen (No. SS-0809601) was deposited in the Museum of Natural Medicines of the Faculty of Pharmaceutical Sciences, Chulalongkorn University. CAE was provided Dr. Suchada Sukrong, Department of Pharmacognosy and Pharmaceutical Botany, Pharmaceutical Sciences, Chulalongkorn University.

2.2 Preparation of Plant Extract

Extraction procedures for CAE are described in Suwanchaikasem et al. (2013). Briefly, the plant was ground into powder with an electric blender, and the fine powder (20 g) was extracted with 200 mL of ethanol. The ethanolic extract was macerated at room temperature for 72 h and filtered with Whatman No.1 filter paper. The filtrate obtained from the extract was dried using a vacuum evaporator at 50□C. The resulting dry ethanol extract was weighed and the percentage yield calculated as 22.61 % (w/w). The extract was then stored at 4□C.

2.3 Thin-layer Chromatographic Identification

The ethanolic extract of *Curcuma* aff. *amada* rhizome (0.2 mg) was applied to a silica gel thin-layer chromatographic (TLC) plate as 8-mm-wide bands. The plates were developed in chloroform-methanol-formic acid (7:3:0.5). The chromatograms were evaluated under UV light at 365 nm to detect the target compounds. To detect flavonoid compounds, the TLC plate was sprayed with a 1% $AlCl_3$ solution and monitored under UV light at 254 and 365 nm. The TLC bioautography assay of free scavenging activity with the 2, 2-diphenyl-l-picrylhydrazyl (DPPH) radical was also employed. The chromatogram was sprayed with a 0.5 nM methanolic solution of DPPH to detect antioxidant compound [4].

2.4 Experimental 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 Animal facility of the Faculty of Pharmaceutical Sciences, Chulalongkorn University under conditions of 25±2 °C, 50-60% humidity, and a 12 h light/12 h dark cycle, with standard pellet diet and water provided *ad libitum*. The mice were acclimatized for 7 days before use in experiments. At the end of each experiment, mice were sacrificed by carbon dioxide asphyxiation. Animal experiments in this study were carried out in accordance with the Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes of the National Research Council of Thailand. The animal use protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand (Protocol Approval No. 14-33-004).

2.5 Drugs and Chemicals

Morphine sulfate (MO; Thai FDA), formaldehyde (Merck Chemical, Germany), acetic acid (Merck, Darmstadt, Germany), naloxone (NAL; Sigma, USA), λ-carrageenan (Sigma, USA), prostaglandin E_2 (PGE_2; Sigma, USA) and pentobarbitone sodium (Ceva Sante Animale, France) were dissolved in 0.9% sodium chloride solution (NSS; General Hospital Products Public, Thailand). Ethanol extract and indomethacin (IND; Sigma, USA) were suspended in 2% (w/v) Tween 80 (Srichansahaosoth, Thailand). Caffeic acid (Sigma, USA) was dissolved in 10% dimethyl sulfoxide (DMSO; Sigma, USA). Arachidonic acid (AA; Sigma, USA) was dissolved in 0.2 M sodium bicarbonate buffer (pH 8.43-8.56). MO and IND were used as standard analgesic drugs. Caffeic acid and IND were used as standard anti-inflammatory agents.

2.6 Hot-plate Test

A hot-plate test was conducted as described by Woolfe and Macdonald [11]. Mice were placed on a hot-plate (Harvard Apparatus, USA) maintained at 55±1 °C and confined by a clear Plexiglas wall cylinder. The hot-plate latency, the time from contact of the mouse with the hot plate to licking of a hind paw or jumping up from the surface, was recorded. Only animals with a pretreatment hot-plate latency time <45 s were utilized. If this behavior was not observed within 45 s, the animal was removed from the hot-plate to avoid burns and the latency was truncated as 45 s. Mice received oral administration of NSS (10 mL/kg), 2% Tween 80 (10 mL/kg), MO (2 mg/kg) or various doses of CAE (12.5, 25, 50, 100 and 200 mg/kg). The post-drug latency was determined at 15, 30, 45, 60, 90, 120 and 240 min after drug administration.

The time-course of hot-plate latency was expressed as the mean percent maximum possible effect (% MPE) using the following formula:

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

where cut-off time = 45 s.

Possible involvement of opioid receptors was also investigated using the hot-plate test. Mice were pretreated intraperitoneally with NAL 10 min before oral administration of CAE (200 mg/kg) and hot-plate latencies were measured.

2.7 Acetic Acid-induced Writhing Test

The acetic acid-induced writhing method in mice was conducted as described by Koster et al. [12]. Mice were pretreated orally with 2% Tween 80 (10 mL/kg) IND (10 mg/kg) or various doses of CAE (3.125, 6.25, 12.5, 25, 50, 100 and 200 mg/kg) 1 h before intraperitoneal injection of 0.6% acetic acid solution (10 mL/kg). The animals were then placed individually in a glass cylinder for observation of writhe response, manifesting as abdominal constriction, pelvic rotation and subsequent stretching of at least one hind limb. The number of writhes were counted in 5-min periods for 30 min after acetic acid administration. Antinociceptive activity was expressed as the percentage inhibition of the writhing response calculated using the following formula:

$$\%\text{Inhibition of writhes} = \frac{\text{MW(control)-MW(test)}}{\text{MW(control)}} \times 100$$

where MW = the mean number of writhes.

2.8 Formalin Test

The formalin test was conducted as described by Hunskaar and Hole [13]. Mice were orally administered with NSS (10 mL/kg), 2% Tween 80 (10 mL/kg), MO (2 mg/kg), IND (10 mg/kg) or various doses of CAE (3.125, 6.25, 12.5, 25, 50, 100 and 200

mg/kg) 1 h before subcutaneous injection of 2.5% formalin solution (20 µl) into the plantar surface of the left hind paw. Following formalin injection, the mouse was immediately placed individually in a glass cylinder for observation. The times spent for licking and biting the injected paw were recorded at 0-5 min (early phase) and 15-30 min (late phase) after formalin injection. The percentage inhibition of licking time was calculated using the following formula:

$$\%\text{Inhibition of licking time} = \frac{ML(control)-ML(test)}{ML(control)} \times 100$$

where ML = the mean of licking time (s)

2.9 Carrageenan-induced Mice Paw Edema

The experiment was conducted using the procedures described by Levy [14]. The mouse paw was marked with black ink at the level of the lateral malleolus. Mice were orally administered with 2% Tween 80 (10 mL/kg), IND (10 mg/kg) or various doses of CAE (12.5, 25, 50, 100 and 200 mg/kg) 1 h before subcutaneous injection of 1% carrageenan solution (50 .00l) into the plantar surface of the left hind paw. Paw volume was measured using a plethysmometer (Ugo Basile, Italy) before and 1, 2, 3, 4, 5 and 6 h after carrageenan injection. Edema was expressed as the mean increase in paw volume relative to the control. The percentage inhibition of paw edema was calculated using the following formula:

$$\%\text{Inhibition of paw edema} = \left(\frac{(Vt-Vb)control - (Vt-Vb)treated}{(Vt-Vb)control}\right) \times 100$$

where Vt = paw volume after carrageenan injection at each time point (mL); and Vb = paw volume before carrageenan injection (mL)

The mechanism of anti-inflammatory activity of CAE was examined using the PGE_2-induced mouse paw edema model described by Akkol et al. [15]. Mice were orally administered 2%Tween 80 (10 mL/kg), IND (10 mg/kg) or CAE (50 mg/kg) 1 h before subcutaneous injection of 0.01% PGE_2 solution (50 µl) into the plantar surface of the left hind paw. Paw volume was measured before and 0.5, 1, 1.5, 2, 3 and 4 h after PGE_2 injection.

2.10 Arachidonic Acid-induced Mice Paw Edema

The method used was first described by Di Martino et al. [16]. Mice were pretreated with 2%Tween 80 (10 mL/kg), IND (10 mg/kg) or various doses of CAE (12.5, 25, 50, 100 and 200 mg/kg) orally or 10% DMSO (10 mL/kg) or caffeic acid (100 mL/kg) intraperitoneally. One hour later, 0.5% AA solution (50 µl) was injected subcutaneously into the plantar surface of the left hind paw. Paw volume was measured before and 0.5, 1, 1.5, 2, 3 and 4 h after AA injection.

2.11 Histological Changes Associated with Carrageenan-induced Mice Paw Edema

After induction of carrageenan-induced paw edema, mice were sacrificed and the left hind paw of each mouse was removed and fixed in 10% neutral buffered formalin solution for 1 week at room temperature. Each sample was decalcified in ferric acid, dehydrated in graded ethanol, embedded in paraffin and cut into 3 mm thick sections, which were then stained with hematoxylin and eosin (H&E). Five representative tissue sections were randomly chosen from each group and examined histologically with a light microscope (Olympus, Japan). In each tissue section, the number of neutrophils was counted at a 400× magnification using the CellSense Standard program (Olympus, Japan). The average value from five fields

was used in statistical analysis [17].

2.12 Cotton Pellet-induced Granuloma Formation

The method was conducted as described by Meier et al. [18]. Mice were anesthetized with sodium pentobarbital (40 mg/kg). The back skin was shaved and disinfected with 70% ethanol. An incision was then made and pre-weighed sterile cotton pellets (10±0.5 mg) were implanted subcutaneously into the back area. Mice were treated orally with 2% Tween 80 (10 mL/kg), IND (10 mg/kg) or various doses of CAE (12.5, 25, 50, 100 and 200 mg/kg) once daily for 7 consecutive days. On day 8, the mice were sacrificed and pellets surrounded with granulomatous tissue were carefully removed and dried to a constant weight at 60°C for 16 h. The weight of granuloma was determined and the percentage inhibition of granuloma was calculated using the following formula:

$$\%\text{Inhibition of weight of granulowa} = \left(\frac{\text{Mg(control)} - \text{Mg(test)}}{\text{Mg(control)}}\right) \times 100$$

where Mg = the mean weight of granuloma (mg)

2.13 Safety Evaluation

To rule out a motor impairment or sedation effect of CAE, a rota-rod rest was performed as described by Dunham and Miya [19]. Mice were placed on a horizontal rod rotating at a speed of 16 rpm (Ugo Basile, Italy). Mice capable of remaining on the rotating rod for 60 s or more, in three successive trials were used in the study. Each mouse was treated orally with 2% Tween 80 (10 mL/kg) or CAE (200 mg/kg) and placed on the rotating rod at 30, 60, 90, 120 and 240 min after drug administration. The results are expressed as the time (s) for which the animal remained on the rota-rod. Animals used in the study were observed for any behavioral changes (such as diarrhea, salivation, respiratory stress) for 72 h. Morbidity or mortality was recorded for each group at the end of the observation period.

2.14 Statistical Analysis

Results are expressed as the mean ± standard error of mean (S.E.M.). Data were analyzed by one-way analysis of variance (ANOVA) and Student t-test followed by a *post hoc* Tukey test for multiple comparisons using SPSS for Windows ver. 17.0, with $p < 0.05$ indicating significance.

3. RESULTS

3.1 Thin-layer Chromatographic Identification

The chemical profiles of the ethanolic extract of *Curcuma* aff. *amada* rhizome is shown in Figure 1. Flavonoids, which turn yellow under UV light at 365 nm after being sprayed with an $AlCl_3$ solution, were not found in the ethanolic extract of *Curcuma* aff. *amada* rhizome.

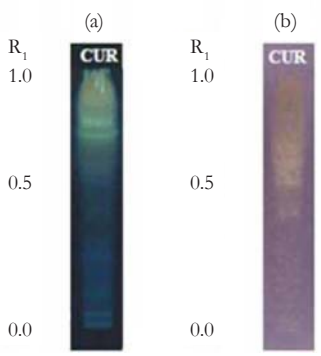


Figure 1. Thin-layer chromatograms of the ethanolic extract of *Curcuma* aff. *amada* rhizome. The plate of the ethanolic extract was developed with chloroform-methanol-formic acid (7:3:0.5) and then viewed (a) under UV at 365 nM after being sprayed with 1% $AlCl_3$ solution or (b) under visible light after being sprayed with a 0.5 nM DPPH solution.

3.2 Hot-plate Test

In the hot-plate test, MO, a centrally-acting analgesic drug, markedly increased hot-plate latency compared to controls. CAE at doses of 100 and 200 mg/kg significantly ($p < 0.01$ and $p < 0.001$, respectively) increased the hot-plate latencies compared to 2% Tween 80 (Figure 2). Animals pretreated with NAL, a mu opioid receptor antagonist, had a significantly ($p < 0.001$) attenuated analgesic response to CAE (200 mg/kg), indicating involvement of opioid receptors (Figure 3).

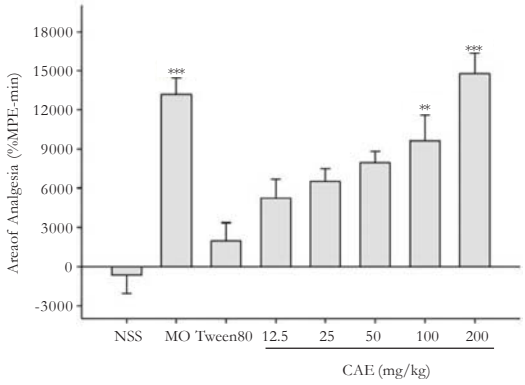


Figure 2. Area of analgesia (%MPE-min) from 0-240 min after oral administration of NSS (10 mL/kg), MO (2 mg/kg), 2% Tween 80 (10 mL/kg) and various doses of CAE (12.5-200 mg/kg). N=10 for all groups. Data are shown as the mean ± S.E.M. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group.

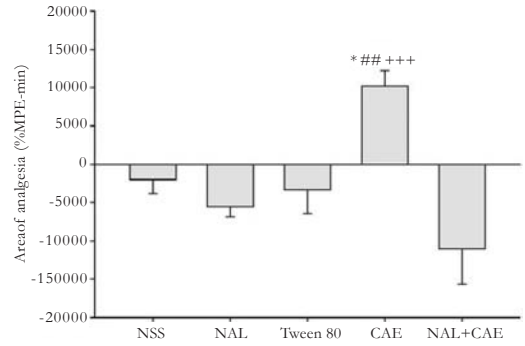


Figure 3. Area of analgesia (%MPE-min) from 0-240 min after oral administration of NSS (10 mL/kg, i.p.), NAL (5 mg/kg, i.p.), 2% Tween 80 (10 mL/kg, p.o.), CAE (200 mg/kg, p.o.) and NAL+CAE (5/200 mg/kg). N=10 for all groups. Data are shown as the mean ± S.E.M., $^{*}p < 0.05$ vs. control group, $^{\#\#}p < 0.01$ vs. NAL, $^{+++}p < 0.001$ vs. NAL+CAE.

3.3 Acetic Acid-induced Writhing Test

CAE at doses of 12.5, 25, 50, 100 and 200 mg/kg significantly decreased the number of writhes induced by acetic acid ($p < 0.01$) (Figure 4). The % inhibition of writhe with CAE at doses of 12.5, 25, 50, 100 and 200 mg/kg were 68%, 65%, 63%, 62% and 58%, respectively. CAE at doses of 12.5, 25, 50, 100, 200 mg/kg showed antinociceptive activities comparable to that of IND (76%, $p < 0.001$).

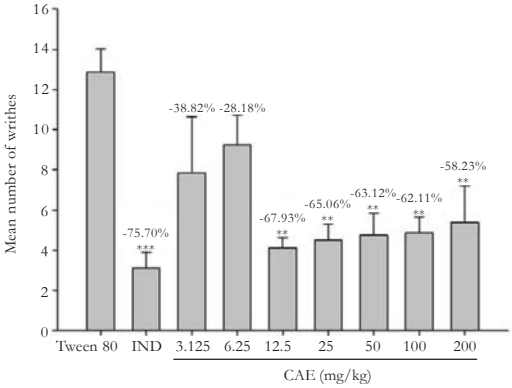


Figure 4. Mean number of writhes after oral administration of 2% Tween 80 (10 mL/kg), IND (10 mg/kg) and various doses of CAE (3.125-200 mg/kg). N=8 for all groups. Data are shown the as mean ± S.E.M. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group.

3.4 Formalin Test

MO (2 mg/kg) significantly decreased the licking time in the early (0-5 min after formalin injection) and late (15-30 min after formalin injection) phases of the pain response by 59% and 68%, respectively, compared with NSS ($p < 0.001$ and $p < 0.001$, respectively)

(Figure 5). The reference drug, IND (10 mg/kg), significantly ($p < 0.001$) reduced the time of licking and biting of injected paws during the late phase by 73%. However, the pain response during the early phase appeared to be unaffected by IND. CAE at doses of 12.5, 25, 50, 100 and 200 mg/kg caused significant inhibition of both phases of formalin-induced nociception by 45%, 50%, 40%, 41% and 56% ($p < 0.01$, $p < 0.001$, $p < 0.01$, $p < 0.01$, $p < 0.001$), respectively, in the early phase, and by 77%, 79%, 64%, 58% and 88% ($p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.01$, $p < 0.001$), respectively, in the late phase, compared to controls. The inhibitory effects of CAE at the doses tested (12.5, 25, 50, 100 and 200 mg/kg) on formalin-induced nociception in both phases were comparable to those of MO.

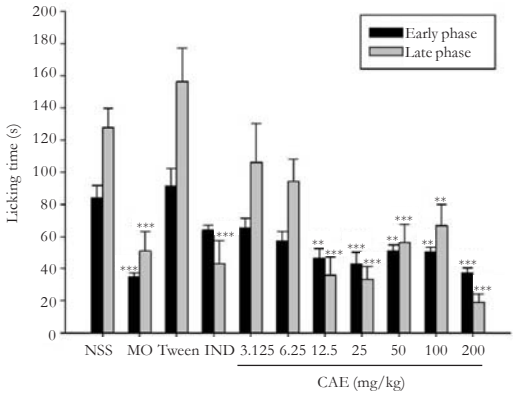


Figure 5. Time spent on paw licking after oral administration of NSS (10 mL/kg), MO (2 mg/kg), 2% Tween 80 (10 mL/kg), IND (10 mg/kg) and various doses of CAE (3.125-200 mg/kg). N=8 for all groups. Data are shown as the mean ± S.E.M. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group.

3.5 Carrageenan-induced Mice Paw Edema

Carrageenan injection in the control group induced localized paw edema that was evident 1 h after injection and increased progressively in severity up to 6 h after injection (Table 1). All doses of CAE (12.5, 25, 50, 100 and 200 mg/kg) significantly decreased paw edema induced by carrageenan at 4, 5 and 6 h (CAE 12.5 mg/kg: $p < 0.05$, $p < 0.01$, $p < 0.01$; CAE 25 mg/kg: $p < 0.05$, $p < 0.001$, $p < 0.001$; CAE 50 mg/kg: $p < 0.01$, $p < 0.001$, $p < 0.001$; CAE 100 mg/kg: $p < 0.01$, $p < 0.01$, $p < 0.01$; CAE 200 mg/kg: $p < 0.01$, $p < 0.001$, $p < 0.001$, respectively). The maximum inhibition of paw was 71% at 5 h, 76% at 4 and 5 h, 82% at 5 h, 69% at 4 h, and 82% at 6 h for doses of CAE of 12.5, 25, 50, 100 and 200 mg/kg, respectively. Thus, the inhibition by CAE at all doses tested is sustainable for 4-6 h after carrageenan injection. No significant effects of all doses of CAE on carrageenan-induced paw edema were observed at 1, 2 and 3 h. Compared to the control group, IND at 10 mg/kg caused a significant reduction of hind paw edema at 4, 5 and 6 h and a maximum inhibition of 82% at 5 and 6 h after carrageenan injection. In general, CAE at all doses tested appeared to have a similar inhibitory activity on carrageenan-induced paw edema to that of IND.

As shown in Table 2, CAE at 50 mg/kg significantly ($p < 0.001$) decreased paw edema at all times tested (0.5, 1, 1.5, 2, 3 and 4 h) after PGE_2 administration compared to the control group and showed a maximum inhibition of paw edema of 69% at 4 h. Compared to the control group, IND at 10 mg/kg also caused significant ($p < 0.001$) reduction of hind paw edema at all times tested, and showed a maximum inhibition of paw edema of 75% at 4 h.

Table 1. Changes in edema volume (mL) from 1 to 6 h after carrageenan administration following oral administration of 2% Tween 80 (10 mL/kg), IND (10 mg/kg), and various doses of CAE (12.5-200 mg/kg).

Treatment	Paw edema (mL) ± S.E.M. (% Inhibition)					
	1 h	2 h	3 h	4 h	5 h	6 h
Tween 80	0.09 ± 0.01	0.10 ± 0.02	0.13 ± 0.02	0.16 ± 0.02	0.17 ± 0.02	0.17 ± 0.02
IND 10 mg/kg	0.05 ± 0.02 (-44%)	0.08 ± 0.02 (-20%)	0.08 ± 0.02 (-38%)	0.05 ± 0.02** (-69%)	0.03 ± 0.01*** (-82%)	0.03 ± 0.01*** (-82%)
CAE 12.5 mg/kg	0.07 ± 0.02 (-22%)	0.08 ± 0.01 (-20%)	0.07 ± 0.02 (-46%)	0.06 ± 0.02* (-63%)	0.05 ± 0.01** (-71%)	0.06 ± 0.02** (-64%)
CAE 25 mg/kg	0.07 ± 0.02 (-22%)	0.08 ± 0.01 (-30%)	0.13 ± 0.01 (-0%)	0.06 ± 0.02* (-63%)	0.04 ± 0.02*** (-76%)	0.04 ± 0.01*** (-76%)
CAE 50 mg/kg	0.06 ± 0.02 (-33%)	0.08 ± 0.03 (-20%)	0.09 ± 0.03 (-31%)	0.03 ± 0.02** (-81%)	0.03 ± 0.02*** (-82%)	0.04 ± 0.02*** (-76%)
CAE 100 mg/kg	0.08 ± 0.01 (-11%)	0.08 ± 0.02 (-20%)	0.08 ± 0.03 (-38%)	0.05 ± 0.02** (-69%)	0.06 ± 0.02** (-65%)	0.06 ± 0.02** (-64%)
CAE 200 mg/kg	0.06 ± 0.02 (-33%)	0.07 ± 0.02 (-30%)	0.08 ± 0.02 (-38%)	0.06 ± 0.02** (-62%)	0.04 ± 0.01*** (-76%)	0.03 ± 0.01*** (-82%)

N=8 for all groups. Data are shown as the mean ± S.E.M. % inhibition compared to the vehicle control is shown in parentheses. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group.

Table 2. Changes in edema volume (mL) from 0.5 to 4 h after PGE_2 administration following oral administration of 2% Tween 80 (10 mL/kg), IND (10 mg/kg), and CAE (50 mg/kg).

Treatment	Paw edema (mL) ± S.E.M. (% Inhibition)					
	0.5 h	1 h	1.5 h	2 h	3 h	4 h
Tween 80	0.18 ± 0.01	0.17 ± 0.07	0.17 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.16 ± 0.01
IND 10 mg/kg	0.07 ± 0.01*** (-61%)	0.07 ± 0.01*** (-59%)	0.06 ± 0.01*** (-65%)	0.05 ± 0.01*** (-69%)	0.05 ± 0.01*** (-69%)	0.04 ± 0.01*** (-75%)
CAE 50 mg/kg	0.09 ± 0.01*** (-50%)	0.08 ± 0.01*** (-53%)	0.07 ± 0.01*** (-59%)	0.07 ± 0.01*** (-56%)	0.06 ± 0.01*** (-63%)	0.05 ± 0.01*** (-69%)

N=8 for all groups. Data are shown as the mean ± S.E.M. % inhibition compared to the vehicle control is shown in parentheses. $^{***}p < 0.001$ vs. control group.

3.6 Arachidonic Acid-induced Mouse Paw Edema

No significant effects of all doses tested of CAE and IND on AA-induced mouse paw edema were observed at all times tested (0.5, 1, 1.5, 2, 3 and 4 h) (Table 2). In contrast, caffeic acid, a positive control, caused significant ($p < 0.05$), reduction of mouse hind paw edema at all times tested and showed a maximum inhibition of paw edema of 76% at 4 h.

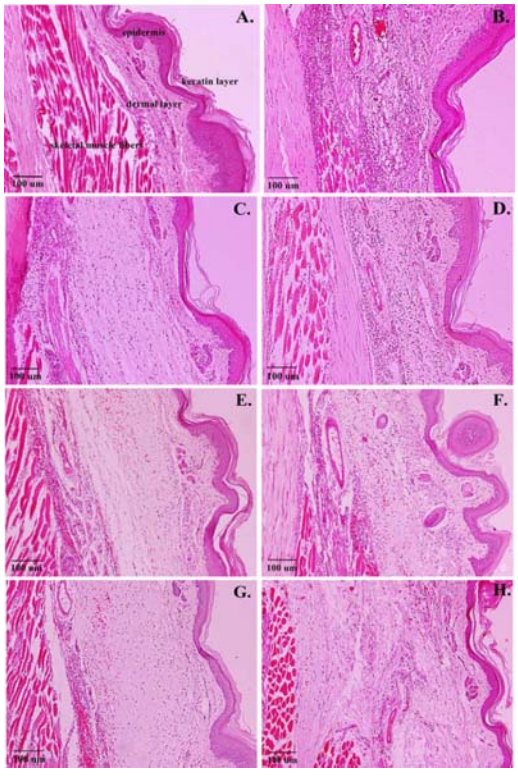
Table 3. Changes in edema volume (mL) from 0.5 to 4 h after AA administration following administration of DMSO (10 mL/kg, i.p.), Caffeic acid (100 mg/kg, i.p.), 2% Tween 80 (10 mL/kg, p.o.), IND (10 mg/kg, p.o.), and various doses of CAE (12.5-200 mg/kg, p.o.).

Treatment	Paw edema (mL) ± S.E.M. (% Inhibition)					
	0.5 h	1 h	1.5 h	2 h	3 h	4 h
DMSO	0.17 ± 0.04	0.15 ± 0.02	0.15 ± 0.02	0.13 ± 0.02	0.12 ± 0.02	0.12 ± 0.01
Caffeic acid 100 mg/kg	0.10 ± 0.02 (-44%)	0.08 ± 0.01 (-53%)	0.07 ± 0.01* (-61%)	0.07 ± 0.01 (-56%)	0.05 ± 0.01* (-69%)	0.04 ± 0.01** (-76%)
Tween 80	0.18 ± 0.04	0.17 ± 0.03	0.18 ± 0.04	0.16 ± 0.03	0.16 ± 0.04	0.17 ± 0.05
IND 10 mg/kg	0.11 ± 0.01 (-39%)	0.11 ± 0.01 (-36%)	0.11 ± 0.01 (-39%)	0.11 ± 0.01 (-31%)	0.09 ± 0.01 (-44%)	0.09 ± 0.01 (-47%)
CAE 12.5 mg/kg	0.13 ± 0.02 (-27%)	0.15 ± 0.02 (-12%)	0.12 ± 0.02 (-33%)	0.10 ± 0.02 (-38%)	0.12 ± 0.02 (-25%)	0.08 ± 0.01 (-53%)
CAE 25 mg/kg	0.18 ± 0.03 (-0%)	0.15 ± 0.03 (-12%)	0.16 ± 0.03 (-11%)	0.12 ± 0.03 (-25%)	0.12 ± 0.03 (-25%)	0.08 ± 0.02 (-53%)
CAE 50 mg/kg	0.16 ± 0.02 (-11%)	0.14 ± 0.02 (-18%)	0.14 ± 0.01 (-22%)	0.12 ± 0.01 (-25%)	0.10 ± 0.02 (-38%)	0.09 ± 0.02 (-47%)
CAE 100 mg/kg	0.14 ± 0.02 (-22%)	0.10 ± 0.02 (-41%)	0.13 ± 0.02 (-28%)	0.11 ± 0.02 (-31%)	0.09 ± 0.02 (-44%)	0.09 ± 0.02 (-47%)
CAE 200 mg/kg	0.13 ± 0.01 (-28%)	0.10 ± 0.02 (-41%)	0.09 ± 0.02 (-50%)	0.08 ± 0.02 (-38%)	0.09 ± 0.03 (-44%)	0.07 ± 0.02 (-59%)

N=8 for all groups. Data are shown as the mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. vehicle control group.

3.7 Histological Examination

Paw biopsies of carrageenan-treated mice showed massive infiltration of neutrophils in connective tissue. Paw biopsies of animals pretreated with CAE (12.5-200 mg/kg) showed reductions in carrageenan-induced inflammatory response. Pretreatment with IND showed less neutrophil infiltration compared to controls (Figure 6). IND and all doses of CAE (12.5, 25, 50, 100 and 200 mg/kg) significantly decreased the neutrophil count, compared to the carrageenan-treated group ($p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.01$, $p < 0.001$, respectively). All doses of CAE showed a similar neutrophil count to that with IND, with CAE 50 mg/kg producing the lowest number of neutrophils in paw tissues (Figure 7).



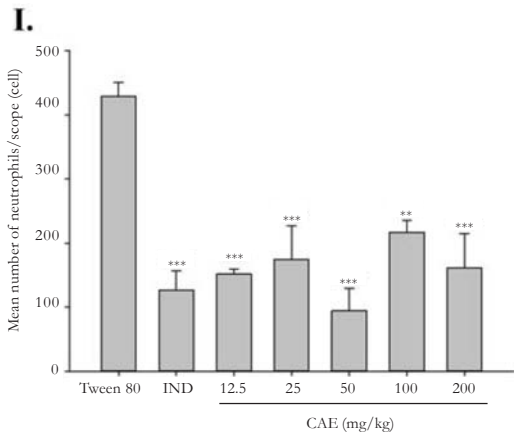


Figure 6. Representative light micrographs of H&E stained samples of mouse hind paw tissue. A: Histological structure of the epidermis and dermis of normal mouse hind paw with no obvious neutrophils infiltration, B: Carrageenan-treated mice with massive infiltration of neutrophils, C: Mice administered CAE 12.5 mg/kg before carrageenan, D: Mice administered CAE 25 mg/kg before carrageenan, E: Mice administered CAE 50 mg/kg before carrageenan, F: Mice administered CAE 100 mg/kg before carrageenan, G: Mice administered CAE 200 mg/kg before carrageenan, H: Mice administered IND 10 mg/kg before carrageenan. Scale bar = 100 .00m, I: The number of neutrophils in each scope (400×). Data are shown as the mean ± S.E.M. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control group.

3.8 Cotton Pellet-induced Granuloma Formation

Only CAE at 200 mg/kg significantly ($p < 0.05$) inhibited cotton pellet-induced granulomatous tissue formation, compared to 2% Tween 80 (Table 4), and had an inhibitory action similar to that of the standard anti-inflammatory agent, IND.

Table 4. Effects of oral administration of 2% Tween 80 (10 mL/kg), IND (10 mg/kg), and various doses of CAE (12.5-200 mg/kg) (for 7 consecutive days) on the weight of granuloma in mice.

Treatment	Weight of granuloma (mg) ± S.E.M	%Inhibition
2% Tween 80	45.69 ± 6.17	-
IND 10 mg/kg	16.86 ± 4.29**	63.10
CAE 12.5 mg/kg	36.31 ± 6.59	20.53
CAE 25 mg/kg	25.49 ± 3.34	44.21
CAE 50 mg/kg	21.95 ± 3.12	51.96
CAE 100 mg/kg	26.03 ± 2.96	43.03
CAE 200 mg/kg	19.81 ± 2.45*	56.64

N=7-8 for all groups. Data are shown as the mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group.

3.9 Safety Evaluation

Oral administration of CAE at 200 mg/kg did not affect the motor responses of animals in the rota-rod test at 30, 60, 90, 120 and 240 min after treatment (data not shown).

An acute toxicity study performed to evaluate the safety of CAE resulted in no morbidity or mortality with oral administration of all doses of CAE over an observation period of 72 h.

4. DISCUSSION

In this study, the antinociceptive and anti-inflammatory effects of CAE were investigated in several tests and animal models. The antinociceptive effects of CAE were studied using three laboratory models that allow assessment of behavioral responses to thermal and chemically-induced pain stimuli

[20]. The hot-plate test was established for evaluation of centrally-acting analgesic drugs [11]. This test measures complex responses to thermal stimuli induced hyperalgesia specific to centrally-mediated nociception [19]. Behavioral responses including jumping or hind paw licking are considered to be supraspinal sensory integration responses and are thought to involve opioids [11, 20]. MO is widely used as a reference drug in this test, and we found that MO had potent analgesic activity in the hot-plate test. CAE at doses of 100 and 200 mg/kg had significant effects on hot-plate latency compared to controls, with the highest antinociceptive effect at 200 mg/kg, indicating that CAE has a central analgesic effect. The antinociceptive action of CAE was inhibited by the specific opioid receptor antagonist, naloxone. These results suggest that CAE has opioid-like activity.

The acetic acid-induced writhing test is usually selected to evaluate the peripheral antinociceptive effects of drugs or natural compounds [22]. This method is sensitive to NSAIDs [23]. Intraperitoneal injection of acetic acid produces a painful reaction and acute inflammation, which is considered to be visceral pain model [22, 23]. Prostaglandin biosynthesis plays an important role in the nociceptive mechanism [24]. Acetic acid is thought to increase levels of lipoxygenase (LOX) and cyclooxygenase (COX) products (especially PGE_2) in peritoneal fluids [25], and to liberate endogenous mediators such as histamine, serotonin, bradykinin and cytokines, which stimulates the peripheral chemosensitive nociceptive receptors and manifests as abdominal constrictions that are accompanied by pelvic rotation and subsequent stretching of at least one hind limb [23, 24]. CAE at doses of 12.5-200 mg/kg showed significant activity against pain induced by acetic acid, compared to the control group. Thus, these data indicate that CAE has an antinociceptive effect on inflammatory pain. This activity of CAE may be related to blockage of the PGE_2 effect or reduction of the release of PGE_2 that excites nociceptors. Our results are consistent with the study of Mujumdar *et al.* [26], in which a significant reduction in acetic acid-induced-writhing was produced by the ethanolic extract of *Curcuma amada* Roxb. at doses of 50 and 100 mg/kg in mice. However, due to the lack of specificity of the writhing test, several other classes of drugs, including adrenergic blockers, antihistamines, muscle relaxants, monoamine oxidase inhibitors, and neuroleptics, can also inhibit writhing [20]. Therefore, other models are required for confirmation of the positive results in the writhing test. The formalin test was used in the current study.

The formalin test is a valid and reliable model in pain research. Formalin causes a behavioral syndrome most widely applicable to the study of acute and tonic pain states and the underlying physiology [27]. Subcutaneous injections of formalin produce a distinct biphasic nociceptive response of intensive licking and biting activities. The first phase (neurogenic pain: 0-5 min after injection) is due to direct chemical stimulation of nociceptors. Nociceptive mediators including substance P, bradykinin and glutamate are thought to participate in this phase [13]. The second phase (inflammatory pain: 20-30 min after injection) is thought to be an inflammatory response and several inflammatory mediators including histamine, serotonin, prostaglandins, NO and bradykinin, are involved in this phase. Centrally acting drugs such as morphine and codeine inhibit both phases equally, whereas peripherally acting drugs such as indomethacin and dexamethasone inhibit the second phase selectively [13, 28]. Therefore, this test was

used to determine the mechanism of action and mediators involved in the antinociceptive activity of the extract. In the current study, MO and IND were used as reference drugs. MO, a centrally acting drug, produced a significant analgesic effect in early and late phases, while IND, a peripherally acting drug, suppressed nociceptive responses only in the late phase. CAE at doses of 12.5-200 mg/kg significantly reduced the time spent for paw licking in both phases, indicating a centrally acting mechanism. Thus, this analgesic activity may be due to inhibition of production or action of pain mediators. These results from the formalin test were in good agreement with those obtained from the hot-plate and writhing tests, thereby indicating that CAE exerts antinociceptive activity both centrally and peripherally.

To determine the effect of CAE on motor response, a rota-rod test was performed. This test is designed to evaluate the motor coordination, skeletal muscle relaxation, convulsions and depression of the central nervous system produced by a test substance [19]. The results for the most effective dose of CAE (200 mg/kg) indicated no detectable relaxant or sedative effects. Therefore, the behavioral responses observed in the hot-plate, writhing and formalin tests were not due to motor impairment, but rather reflected a true antinociceptive effect.

To assess potential anti-inflammatory action, the effects of CAE on the acute and chronic phases of inflammation were evaluated in mouse models of carrageenan-induced paw edema, arachidonic acid (AA)-induced paw edema and cotton pellet-induced granuloma formation. The carrageenan-induced rat paw edema test is a well-established animal model of inflammation for evaluating the acute anti-inammatory effects of natural products [29]. The carrageenan-induced mouse paw edema test was later established by Levy (1969). Carrageenan-induced paw edema test is highly sensitive to NSAIDs and has long been accepted as a useful phlogistic tool for investigating new anti-inflammatory drugs [30]. The effect produced by carrageenan has been described as a biphasic event with involvement of multiple inflammatory mediators. The initial phase (0-2 h after injection of carrageenan) results from rapid production of pro-inflammatory agents such as histamine, serotonin and bradykinin [31]. The second phase of swelling (3-6 h after injection of carrageenan) is associated with release of prostaglandins and nitric oxide, produced by inducible isoforms of COX-2 and nitric oxide synthase (iNOS), respectively [32]. More recently, the second phase has also been attributed to involvement of free radicals, nitric oxide and COXs in hind paw exudates [33]. TNF-α is a mediator of carrageenan induced inflammatory incapacitation, and is able to induce further release of kinins and leukotrienes, which play an important role in the maintenance of a long lasting nociceptive response [34]. The carrageenan-induced inflammatory response has been linked to neutrophil infiltration and production of neutrophil-derived free radicals such as superoxide, hydrogen peroxide and hydroxyl radicals, as well as release of other neutrophil-derived mediators [35]. The inflammatory effect induced by carrageenan is associated with release of free radicals, prostaglandin and nitric oxide for 1-6 h after administration of carrageenan. The edema effect reaches a maximum in the third hour [36, 37]. Free radicals can induce biological damage and pathological events including aging, inflammation and carcinogenesis [38]. The infiltration of PMNs cells into site of inflammation induces secretion of several pro-inflammatory mediators such as NO,

prostaglandins and cytokines [39, 40].

CAE at all doses tested (12.5-200 mg/kg) significantly decreased mouse paw edema at 4, 5 and 6 h after carrageenan injection. These results indicate that CAE exerted anti-inflammatory action during the second phase of inflammation. The mechanism underlying this activity of CAE may be mediated through inhibition of prostaglandin synthesis. The ethanol extract of *Curcuma amada* Roxb at 200 mg/kg showed significant anti-inflammatory activity in carrageenan-induced rat paw edema at 1-4 h, and this activity was dose-dependent [41].

The effect of CAE on PGE_2-induced mouse paw edema was examined to determine if mechanisms other than COX inhibition were present. CAE significantly decreased paw edema induced by PGE_2 from 0.5-4 h after PGE_2 injection, indicating that the effect of CAE was due to degradation and/or inactivation of prostaglandins. The edematogenic response of PGE_2 in mouse paws is mediated by E-prostanoid $(EP)_3$ receptor activation, which involves stimulation of the protein kinase C (PKC), phospholipase C (PLC), mitogen protein-activated kinases (MAPKs) pathways, participation of neurokinin (NK1) and vanilloid receptor (TRPV1) receptors; and is largely prevented by a selective EP_3 receptor antagonist [42]. Our results in this model complement those obtained from other models involving inflammatory mediators, including the acetic acid-induced writhing and formalin licking responses. The reduction of PGE_2-induced paw edema could explain, at least in part, the inhibitory effects of CAE in the writhing and formalin tests.

The AA-induced mouse paw edema model [43] was also used to assess the acute anti-inflammatory activity of CAE. This model is highly sensitive to inhibition of the lipoxygenase pathway and is resistant to COX inhibitors. The involvement of lipoxygenase products and mast cell mediators in the edematous response to AA render this model potentially useful for studying anti-inflammatory agents with a mechanism of action that differs from that of COX inhibitors [16]. As expected, IND, a COX inhibitor, could not inhibit paw edema induced by AA. All doses of CAE tested were also found to be ineffective in this model. In contrast, caffeic acid, a known lipoxygenase inhibitors [44], exhibited an anti-inflammatory effect on AA-induced paw edema. The anti-inflammatory effect of CAE is thus suggested to be exerted through inhibition of COX pathway, rather than the LOX pathway. The mechanism of anti-inflammatory action of CAE is proposed to be similar to that of NSAIDs, which involves a decrease in prostaglandin production.

Histopathological evaluation of mouse paw tissues showed that CAE at all doses tested significantly suppressed massive neutrophil infiltration found in control animals following carrageenan injection. CAE exhibited an anti-inflammatory effect in the late phase of carrageenan-induced paw edema by inhibition of infiltration of PMN cells and production of neutrophil-derived free radicals.

The cotton pellet induced-granuloma formation test is common method used to investigate the transudative, exudative and proliferative components of chronic inflammation. The dried pellet weights correlate well with the amount of granulomatous tissue [45]. The subcutaneous implantation of a cotton pellet directly triggers acute inflammation, but is insufficient to eliminate pro-inflammatory mediators, leading to a chronic inflammatory reaction that includes infiltration of neutrophils and mononuclear cells [46]. Only the highest dose of CAE (200 mg/kg) effectively inhibited

cotton pellet-induced granuloma formation, which indicates that CAE has anti-inflammatory activity in the chronic phase of inflammation. The ethanol extract of *Curcuma amada* Roxb. at doses of 40 and 80 mg/kg showed dose-dependent reductions in granular tissue formation in a cotton pellet induced-granuloma model in rat [41].

In conclusion, CAE had antinociceptive properties in central and peripheral models of nociception in mice, and showed anti-inflammatory properties in acute and chronic inflammation models, which supports its ethnopharmacological use. Curcuminoids may be responsible for the antinociceptive and anti-inflammatory effects of CAE since curcuminoids is a significant bioactive compound of several species of plants in genus *Curcuma*.

Further studies are required to understand the mechanisms underlying these effects. Separation and isolation of active compounds in CAE are also needed. However, the results clarify the pharmacological actions of CAE and suggest the potential use of this extract for treatment of pain and inflammatory disorders.

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