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Antinociceptive and anti-inflammatory activities of Tree-Phon-Thad remedy

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Abstract

Tree-Phon-Thad remedy is used as an antipyretic and anti-inflammatory agent by traditional practitioners in Thailand, and includes roots of *Zingiber zerumbet* (Kra-Thue), *Zingiber montanum* (Plai) and *Cymbopogon nadus* (Ta-Krai-Hom). In this study, the antinociceptive activity of the root extract of Tree-Phon-Thad remedy (TPT; 75-300 mg/kg) was determined using hot-plate, formalin and acetic acid-induced writhing models in mice. The anti-inflammatory properties of orally administered TPT were determined using a carrageenan-induced mouse paw edema model. All doses of TPT (75, 150 and 300 mg/kg, p.o.) produced a significant analgesic response in the hot-plate test. TPT at doses of 150 and 300 mg/kg caused significant inhibition of formalin-induced pain in the late phase ($p<0.05$) with % inhibition of 57.91 % and 51.17 %, respectively. All doses of TPT (75, 150 and 300 mg/kg) significantly reduced acetic acid-induced writhing by 62.50 % ($p<0.01$), 62.50 % ($p<0.01$), and 61.81 % ($p<0.01$), respectively. Mice treated with indomethacin and TPT at a dose of 300 mg/kg showed a significant reduction in carrageenan-induced paw edema in the second phase. Taken together, these results demonstrate that TPT possesses both antinociceptive and anti-inflammatory activities, and suggest that the antinociceptive effects occur through both central and peripheral mechanisms.

Keywords: Tree-Phon-Thad, Hot-plate, Formalin test, Writhing Test, Carrageenan-induced paw edema

Introduction

Natural products from minerals, plants and animals have been the main sources of drugs for a long time. In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants [1]. Tree-Phon-Thad remedy is a Thai traditional remedy that has been used as an antipyretic and anti-inflammatory drug by traditional practitioners in Thailand. The revealed of this remedy has been in the Thai traditional medical text book called "Paad sard song khor", but there is no scientific evidence for its biological activity. Tree-Phon-Thad remedy consists of three herbal roots in equal parts by weight, including roots of *Zingiber zerumbet* (L.) Smith (Kra-Thue), *Zingiber montanum* (Koenig) Link ex Dietr. (Plai), and *Cymbopogon nardus* Rendle (Ta-Krai-Hom).

In contrast to TPT itself, many studies have shown biological activities of compounds from the three

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component roots. Methanolic extracts of *Z. zerumbet* (25, 50, and 100 mg/kg; s.c.) and essential oil of *Z. zerumbet* (30, 100, and 300 mg/kg; i.p.) exhibit significant antinociceptive activity at the peripheral and central levels when assessed using the writhing, hot plate, and formalin tests [2]. Intraperitoneally administered aqueous and ethanol extracts of *Z. zerumbet* at doses of 25-100 mg/kg have an anti-inflammatory profile in prostaglandin E_2-induced paw edema test [3]. Zerumbone, a major bioactive compound isolated from the rhizome of *Z. zerumbet*, has anti-inflammatory effects against the exudative and proliferative phases of inflammation in mice[4, 5]. Compound D ((E)-4-(3',4'-dimethoxyphenyl) but-3-en-2-ol), a compound found in the hexane extract of *Z. cassumunar*, elicited analgesic activity on the acetic acid-induced writhing response in mice [6]. Pongprayoon et al. [7] reported topical anti-inflammatory activity of (E)-1-(3,4-dimethoxyphenyl) butadiene (DMPBD), which is also isolated from *Z. cassumunar*, in a model of carrageenan-induced hind paw edema in rats. Based on this background, the current study was designed to investigate the antinociceptive and anti-inflammatory activities of the root extract of Tree-Phon-Thad remedy.

Materials and Methods

Plant materials and extraction: All three plants species of Tree-Phon-Thad remedy were collected from December 2011 to February 2012 from Chiang Rai and Nakhon Pathom provinces in Thailand. These plants materials were authenticated by one of the authors (N. Ruangrungsi). The voucher specimens were deposited at the College of Public Health Sciences, Chulalongkorn University. Roots were washed, shade-dried and ground to coarse powders. Each plant powder was macerated with ethanol and water, respectively, for 24 h at room temperature and filtered. The ethanolic extracts were evaporated under vacuum and the aqueous extracts were lyophilized to dryness. The percent yields of the ethanolic and aqueous extracts of each herbal root were recorded. The extracts were stored at -20°C to decrease the possibility of degradation of active compounds. The root extract of Tree-Phon-Thad remedy (TPT) was prepared by mixing each extract in the quantity (based on the yield of each root extract) equivalent to that in the remedy. A weighed amount of TPT suspended in 2 % aqueous Tween 80 solution was used in the study.

Animals: Experiments were conducted using adult male ICR mice weighing 18-25 g purchased from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakhon Prathom, Thailand. Animals were housed at 25 ± 2 °C under a 12-h light/12-h dark cycle, relative humidity (50-60 %) and with access to food and water *ad libitum*. The mice were kept under these laboratory conditions for one week prior to the start of the experiments. On the day of the experiment, animals were starved for 2-3 h prior to the administration of test compounds. At the end of each experiment, animals were sacrificed with carbon dioxide asphyxiation. The study protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Drugs and chemicals: Morphine sulfate (MO; Thai FDA), formaldehyde (Merck, Germany), acetic acid (Merck, Germany), and λ-carrageenan (Sigma Chemical Co., USA) were dissolved in 0.9 % sodium chloride solution (NSS; General Hospital Products Public Co., Thailand). Indomethacin (IND; Sigma Chemical Co., USA) and TPT were suspended in 2 % (w/v) Tween 80 solution. MO (10 mg/kg) and IND (10 mg/kg) were used as standard analgesic drugs. IND (10 mg/kg) was also used as a standard anti-inflammatory drug. Control animals were given an equivalent volume of vehicle via the same route.

Antinociceptive activity test:

Hot-plate Test: The hot-plate test was conducted as a thermal pain model using the method described by Woolfe and MacDonald [8]. In these experiments, a hot-plate (Harvard Apparatus, USA) measuring 28×28 cm with a surface temperature at 55±0.5°C was used for assessment of analgesia. The hot-plate was surrounded by a clear Plexiglas wall cylinder (20 cm in diameter and 30 cm in height) to confine the animal to the heated surface during testing. Prior to treatment, three pre-drug baseline trials were performed on the hot-plate spaced 5-10 min apart and the baseline latencies of each mouse were recorded as an indicator of hypo- or hyper-sensitive mice. The average of the last two trials served as the baseline pre-drug latency. Only those animals with a pretreatment hot-plate latency time of <45 s were utilized in the study. After the third pre-drug baseline latency, the animals were administered NSS (10 mL/kg) and MO (10 mg/kg) intraperitoneally or 2 % Tween 80 (10 mL/kg) and various doses of TPT (75,150 and 300 mg/kg) orally. Each mouse was placed on the hot-plate and the latency to licking of a hind paw or vigorous jumping up from the surface of the metal plate was recorded by a stop-watch and used as the end point. The post-drug latency was measured at 15, 30, 45, 60, 90, 120 and 240 min after drug administration. To avoid possible tissue injury, the cut-off time was set at 45 s. The time-course of hot-plate latency was expressed as the mean percent maximum possible effect (%MPE) using the following ratio: [(post-drug latency) - (pre-drug latency)] ×100/ [(cut-off time) - (pre-drug latency)]. The area of analgesia for the hot-plate assays was derived by computing the area under the corresponding 0-240 min time-course vs. %MPE curves, with areas calculated using the trapezoidal rule [9]. Ten mice were used in each group.

Formalin test: The formalin test was based on the method described by Hunskaar and Hole [10]. Using a minimum of restraint, 20 μL of 2.5 % formalin solution was injected subcutaneously into the left hind paw of each mouse 1 h after oral administration of 2 % Tween 80 (10 mL/kg), IND (10 mg/kg) and various doses of TPT (75,150 and 300 mg/kg). Following the formalin injection, animals were immediately placed in an observation cylinder. Two distinct periods of intensive licking activity were identified. The duration of paw licking was

measured during the first period (early phase: 0-5 min) and the second period (late phase: 15-30 min) after formalin injection with a stopwatch. The % inhibition of licking was calculated using the formula: $[(T_c - T_t)/T_c] \times 100$; where T_c and T_t are the values for the vehicle-treated control group and the treated group, respectively, for each phase. Eight mice were used in each group.

Acetic acid-induced writhing test: Mice were administered 2% Tween 80 (10 mL/kg), IND (10 mg/kg) and various doses of TPT (75,150 and 300 mg/kg) orally 1 h before intraperitoneal injection of 0.6% acetic acid (10 mL/kg). Each animal was then placed in a transparent observation cylinder. The number of writhing events (contraction of abdominal muscle together with hind limb extension) was counted at 5 min intervals for a period of 30 min after administration of acetic acid. The number of writhing events was recorded and the % inhibition was calculated using the formula: $(W_{control} - W_{treated}) \times 100/ W_{control}$; where W is the mean number of writhing events. Eight mice were used in each group.

Anti-inflammatory activity testing:

Mouse model of carrageenan-induced paw edema: Each mouse was pretreated with 2 % Tween 80 (10 mL/kg), IND (10 mg/kg) and various doses of TPT (75,150 and 300 mg/kg) orally. One hour later, 1 % carrageenan solution (50 µL) was injected subcutaneously into the plantar surface of the left hind paw of each mouse. The paw was marked with black ink at the level of the lateral malleolus and immersed in dipping solution up to this mark. The volume of the injected paw was measured before and after application of the irritant and the paw volume of the treated animals was compared to controls at 1, 2, 3, 4, 5 and 6 h using a plethysmometer (Ugo Basile7150, Italy). The % inhibition of edema was analyzed using the following formula: $[(V_t - V_o)$ control $- (V_t - V_o)$ treated$] \times 100/ (V_t - V_o)$ control; where V_o and V_t are the average volumes for each group before and after treatment, respectively. Eight mice were used in each group.

Rota-rod test: A rota-rod machine with automatic timers and falling sensors (Ugo Basile7600, Italy) was used in the study. Mice were placed on a horizontal rod (3.5 cm diameter) rotating at 16 rpm. Those capable of remaining on the rotating rod for ≥60 s in three successive trials were selected for the study. Each mouse was treated with 2 % Tween 80 (10 mL/kg) or TPT (75,150 and 300 mg/kg) orally and placed on the rotating rod at 30, 60, 90, 120 and 240 min after drug administration. Results are expressed as the time (s) for which the animal remained on the rota-rod during 60 s [11]. Eight mice were used in each group.

Acute toxicity: Animals used in the rota-rod test were observed for 72 h and morbidity or mortality was recorded for each group.

Statistical analysis: Results are expressed as the mean ± S.E.M. to show variation in groups. Differences in means between groups were analyzed by one-way analysis of variance (ANOVA) and Student's t-test followed by a *post hoc* Tukey test for multiple comparisons. Statistical significance was defined as $p<0.05$.

Results

Hot-plate test: All doses of TPT (75,150 and 300 mg/kg) significantly ($p<0.01$, $p<0.01$ and $p<0.001$, respectively) increased hot-plate latencies compared to the vehicle group (Figure 1). However, the antinociceptive effect of TPT was not dose-related.

Formalin test: The reference drug, IND (10 mg/kg), significantly reduced the time of licking and biting of injected paws during the late phase of formalin-induced nociception (61.52 % inhibition vs. vehicle controls, $p<0.05$; Figure. 2). Nociception during the early phase was unaffected by IND. TPT at doses of 150 and 300 mg/kg caused significant inhibition of late phase formalin-induced nociception (57.91 % and 51.17 % inhibition vs. controls, both $p<0.05$). The inhibitory effects of TPT at 150 and 300 mg/kg during the late phase were comparable to that of IND. Also similarly to IND, TPT had little effect on the early phase.

Acetic acid-induced writhing test: IND significantly ($p<0.001$) decreased the acetic acid-induced writhing response by 78.47 % compared to the vehicle control (Figure. 3). All doses of TPT (75, 150 and 300 mg/kg) also significantly reduced the writhing response compared with the vehicle group ($p<0.05$, $p<0.01$ and $p<0.001$, respectively), with % inhibitions of 62.50 %, 62.50 % and 61.81 %, respectively but were not dose-related. All doses of TPT had antinociceptive activities comparable to that of IND.

Carrageenan-induced paw edema: Carrageenan-induced paw edema was evident 1 h after injection and

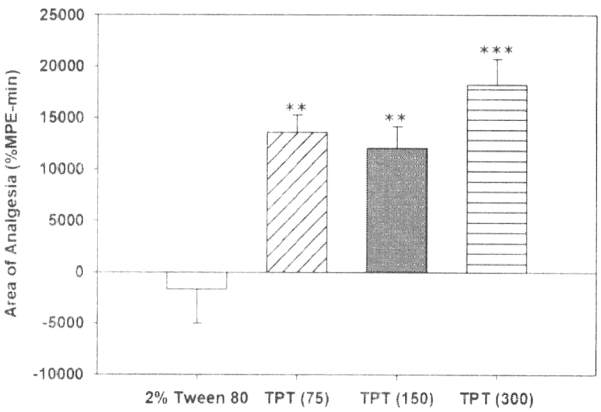

Figure 1 Area of analgesia (%MPE-min) from 0 to 240 min after oral administration of 2% Tween 80 (10 mL/kg) and various doses of TPT (75-150 mg/kg). N = 10 for all groups. Data are shown as the mean ± S.E.M.; $^{**}p<0.01$, $^{***}p<0.001$ vs. control animals

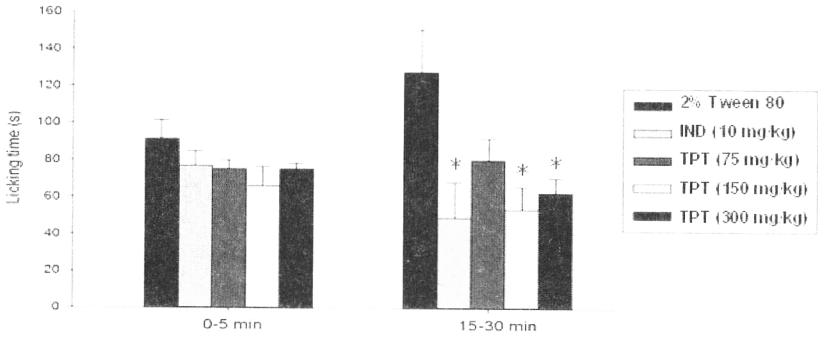

Figure 2 Time spent on paw licking after oral administration of 2% Tween 80 (10 mL/kg), IND (10 mg/kg) and various doses of TPT (75-150 mg/kg). N = 8 for all groups. Data are shown as the mean ± S.E.M. *p<0.05 vs. control animals

reached a maximum at 3 h. The swollen paw persisted up to 6 h (1). Only the highest dose of TPT (300 mg/kg) produced a significant (p<0.05) decrease in paw edema induced by carrageenan at 3, 4, 5 and 6 h, with % inhibition of 55.95 %, 72.15 %, 86.67 % and 90.14 %, respectively. The maximum inhibition of paw edema by TPT was reached at 6 h. Thus, the % inhibition effect of TPT at the highest dose appears to be increased and sustained for 3-6 h after carrageenan injection. No significant effects of TPT on carrageenan-induced paw edema were observed at 1 and 2 h for all doses tested. Compared to the 2 % Tween 80 (vehicle) group, IND at 10 mg/kg caused a significant reduction of hind paw edema at 2, 3, 4, 5 and 6 h after carrageenan injection with % inhibition of 76.67 %, 70.24 %, 81.01 %, 89.33 % and 95.77 %, respectively. The maximum inhibition of paw edema of IND was reached at 6 h. Thus, TPT had comparable inhibitory activity on carrageenan-induced paw edema compared to that of IND.

Rota-rod test: Oral administration of TPT at all doses tested (75-300 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.

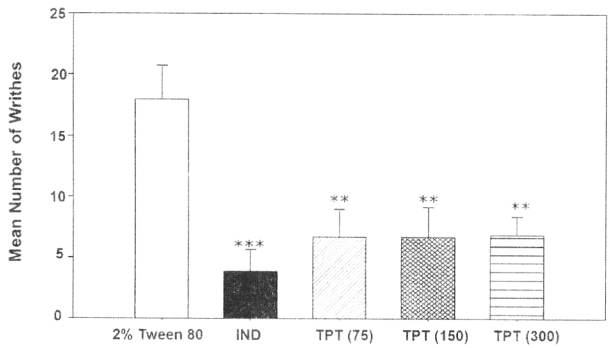

Figure 3 Mean number of writhes after oral administration of 2 % Tween 80 (10 mL/kg), IND (10 mg/kg) and various doses of TPT (75-150 mg/kg). N = 8 for all groups. Data are shown as the mean ± S.E.M. **p<0.01; ***p<0.001 vs. control animals

Acute toxicity: No acute toxicity or mortality was observed with oral administration of all doses of TPT tested over an observation period of 72 h, indicating the safety of TPT.

Discussion

The results of this study show that TPT has antinociceptive effects in thermally and chemically induced pain models in mice, and has anti-inflammatory activity in a carrageenan-induced mouse paw edema model.

The hot-plate test is a well-validated model for detection of centrally-acting analgesics, while peripherally-acting analgesics show little to no activity in this test. A plate heated to a constant temperature produces two behavioral components (hind paw licking and jumping with all four feet) in mice that can be measured in terms of reaction times. Both are considered to be supraspinally integrated responses [12]. This model uses morphine as a reference drug with a potent analgesic effect in this model that indicates the sensitivity of the test. Our results showed that TPT had significant analgesic activity in the hot-plate test. All animals treated with TPT at all doses showed analgesia greater than the control group, with the highest antinociceptive effect produced at 300 mg/kg. These data show that TPT has a central analgesic effect.

The properties of TPT were further investigated in a formalin-induced nociception model of chronic pain. The formalin test in mice is a valid and reliable model of nociception and is sensitive for various classes of analgesic drugs. The response to formalin has an early and a late phase. The early phase (0-5 min) seems to be caused predominantly by C-fiber activation due to the peripheral stimulus, while the late phase (15-30 min) is dependent on the combination of an inflammatory reaction in peripheral tissue and functional changes in the dorsal horn of the spinal cord [13]. Substance P and bradykinin participate in the early phase (acute pain), whereas histamine, serotonin, prostaglandins, nitric oxide and bradykinin are involved in the late phase (inflammatory pain) [10, 13]. Drugs that act predominantly on the central nervous system inhibit both phases, while peripherally-acting

Table 1 Changes in edema volume (mL) from 1 to 6 h after carrageenan injection following oral administration of 2% Tween 80 (10 mL/kg), IND (10 mg/kg) and various doses of TPT (75-150 mg/kg)

Treatments (mg/kg)	Paw edema (mL) ± S.E.M. (% Inhibition)		
	1 h	2 h	3 h
2 % Tween 80	0.0750 ± 0.0195	0.0938 ± 0.0285	0.1050 ± 0.0283
IND (10)	0.0538 ± 0.0750 (-28.33 %)	0.0238 ± 0.0938* (-74.67 %)	0.0313 ± 0.1050* (-70.24 %)
TPT (75)	0.0838 ± 0.0149 (11.67 %)	0.0875 ± 0.0183 (-6.67 %)	0.0963 ± 0.0235 (-8.33 %)
TPT (150)	0.0700 ± 0.0098 (-6.67 %)	0.0800 ± 0.0172 (-14.67 %)	0.0850 ± 0.0203 (-19.05 %)
TPT (300)	0.0750 ± 0.0091 (-0.00 %)	0.068 8± 0.0144 (-26.67 %)	0.0463 ± 0.0116* (-55.95 %)

Treatments (mg/kg)	Paw edema (mL) ± S.E.M. (% Inhibition)		
	4 h	5 h	6 h
2 % Tween 80	0.0988 ± 0.0259	0.0938 ± 0.0227	0.0888 ± 0.0230
IND (10)	0.0188 ± 0.0988* (-81.01 %)	0.0100 ± 0.0938* (-89.33 %)	0.0038 ± 0.0888* (-95.77 %)
TPT (75)	0.0800 ± 0.0196 (-18.99 %)	0.0675 ± 0.0175 (-28.00 %)	0.0600 ± 0.0145 (-32.39 %)
TPT (150)	0.0775 ± 0.0136 (-21.52 %)	0.0675 ± 0.0128 (-28.00%)	0.056 3± 0.0092 (-36.62 %)
TPT (300)	0.0275 ± 0.0094* (-72.15%)	0.0125 ± 0.0031* (-86.67 %)	0.0087 ± 0.0030* (-90.14 %)

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 vs. control animals

drugs inhibit only the late phase [10, 14, 15]. The late phase is also selectively attenuated by cyclooxygenase inhibitors [16]. IND, a peripherally acting reference drug, showed an analgesic response only in the late phase. TPT at doses of 150 and 300 mg/kg also suppressed the late phase and had no significant effects on the early phase. These results indicate that TPT has antinociceptive activity against inflammatory pain.

The acetic acid-induced writhing test is commonly used for measuring peripheral analgesic activity. Intraperitoneal injection of a noxious agent that irritates serous membranes provokes a stereotypical behavior in mice that is characterized by abdominal contractions, movements of the body as a whole (particularly of the hind paws), twisting of dorsoabdominal muscles, and a reduction in motor activity and motor incoordination. The test is sometimes called the abdominal contortion test, the abdominal constriction response, or the stretching test, but more commonly is known as the "writhing test" [12]. The writhing response is presumed to be induced by local peritoneal receptor activation as a result of prostanoid mediators. In mice, there is an increase in the peritoneal fluid levels of PGE_2 and PGF_2, as well as lipooxygenase products, and release of sympathetic nervous system mediators [17, 18]. The nociceptive properties of acetic acid may also be due to release of cytokines, including TNF-α, interleukin-1β, and interleukin-8 by resident peritoneal macrophages and mast cells [19]. In this study, there was a significant reduction in the number of writhes in animals treated with IND and TPT at all doses tested, and the efficacy of TPT at all doses was comparable to that of IND. The results obtained from the formalin and writhing tests indicate that TPT has peripheral analgesic effects. Our results were in accordance with previous studies that showed the antinociceptive activities of the methanol extract of *Zingiber zerumbet* (25, 50, and 100 mg/kg) given subcutaneously and essential oil of *Z. zerumbet* (30, 100, and 300 mg/kg) given intraperitoneally when assessed with hot-plate, acetic acid induced-writhing and formalin tests [2, 20].

The rota-rod test is widely used to evaluate the activity of drugs interfering with motor coordination and

determine the ability of a compound to produce skeletal muscle relaxation, convulsions and depression of the central nervous system. In this study, the results of the rota-rod test indicated a lack of detectable relaxant and sedative effects, even at the highest dose of TPT tested. Therefore, the behavioral responses observed in the hot-plate, formalin and writhing tests were unlikely to have been due to motor dysfunction, but rather were true antinociceptive effects.

Screening of anti-inflammatory drugs is commonly based on inhibition of edema in the hind paw of a mouse after injection of a phlogistic agent (an irritant) such as carrageenan [21]. In this model, multiple physical and behavioral responses can be followed to assess the extent of injury and its prevention or reversal by NSAIDs [22]. The carrageenan-induced paw edema test is characterized by a biphasic response. The first phase (1 to 2 h after injection of carrageenan) is mainly due to release of pro-inflammatory agents such as histamine from damaged surrounding tissues, whereas the second phase of swelling (3 to 6 h after injection) has been correlated with enhanced production of prostaglandins. Continuity between the two phases is believed to be mediated by kinins [23, 24]. The current study showed that pre-treatment of animals with the highest dose of TPT resulted in inhibition of the second phase of carrageenan-induced mouse paw edema at 3, 4, 5 and 6 h. This effect may be due to interference of TPT with liberation of prostaglandins or blockade of prostaglandin receptors. Our results were in agreement with the study of Zakaria et al., 2010 that showed the anti-inflammatory activity of the methanol extract of *Z. zerumbet* (25-100 mg/kg) given subcutaneously when assessed using the acute (carrageenan-induced paw edema test) and chronic (cotton pellet-induced granuloma test) models of inflammation [2].

Conclusion

In conclusion, this study showed that a root extract of Tree-Phon-Thad remedy had antinociceptive properties in central and peripheral models of nociception in mice, and anti-inflammatory properties in an acute inflammation model. Additional studies are required to understand the mechanism of actions underlying these effects. The current study clarifies the pharmacological action of the TPT herbal remedy and provides a scientific basis for the effects of Thai traditional medicine. An assessment of acute toxicity indicated that all doses of TPT used in the study were safe. Thus, the collective findings highlight the potential use of this remedy for alleviating pain and treating inflammatory disorders.

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