In vivo Analgesic Effect of Aqueous Extract of *Tamarindus indica* L. Fruits

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Key Words

Tamarindus indica • Analgesic • Opioid system

Abstract

Objective: To study the effects of *Tamarindus indica* L. aqueous fruit extract on the antinociceptive activities in rodent models. **Methods:** The analgesic effect was evaluated using acetic acid-induced writhing, hot plate and formalin tests. **Results:** The extract (60–600 mg/kg) significantly ($p < 0.05$) inhibited the writhing test in a dose-dependent manner with the percentage of analgesia recorded between 51.8 and 74.1%. In addition, the extract also significantly ($p < 0.05$) increased the latency time in the hot plate test in a dose-dependent manner. Further study showed that the extract elicited inhibitory activity in both the early and late phases of the formalin test. Moreover, pretreatment with 5 mg/kg naloxone, a nonselective opioid receptor antagonist, significantly ($p < 0.05$) modified the antinociceptive effect of the extract in all tests. **Conclusion:** The aqueous extract of *T. indica* possesses potential antinociceptive activity at both the peripheral and central levels, which are mediated via activation of the opioidergic mechanism.

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Introduction

Studies on plants that traditionally have been used as pain killers should still be seen as a fruitful and logical research area in the search for new analgesic drugs with low and possibly no side effects [1]. One of the plants being studied in our laboratory is *Tamarindus indica* L., which belongs to the family Caesalpiniaceae; the study focused on the pharmacological evaluation of its fruits to confirm the efficacy of its traditional uses in Malay culture. Popularly known as 'Asam Jawa' to the Malays, its fruit is known to have the ability to cure many disease conditions including stomach pain, fever, cough with throat pain, allergy, rheumatism and oral ulceration. The fruit pulp of *T. indica* is used to treat constipation, dysentery, loss of appetite, alcohol intoxication, worm infection, jaundice, morning sickness in pregnant women and asthma. It is also used for seasoning, and as a component in snacks and juices. In addition, juice prepared by squeezing the pulp into warm water is consumed to relieve sore throat [2, 3]. In traditional African medicine, *T. indica* is also used for the treatment of cold, fever, stomach disorders, diarrhea, jaundice and as a skin cleanser [4], while in ayurvedic medicine, the plant is used in the management of gastric ulcers, inflammation and digestion prob-

lems. The leaves and flowers of *T. indica* are also used as poultices for treatment of boils, swollen joints and sprains [5]. Previous pharmacological studies have shown the beneficial effects of *T. indica*, which include antioxidant [6] and antiobesity [3] properties. The extracts of the stem, bark and leaves of *T. indica* have been shown to possess broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria [4, 7]. Other pharmacological activities of *T. indica* are antidiabetic and antiulcerogenic [5, 8]. Phytochemical investigations have indicated the presence of flavonoids, saponins, tannins, alkaloids, sesquiterpenes and phlobatamins [4, 7]. Considering its use in Malay folk medicine, particularly in the management of pain-related ailments as described above and because there is no report on the analgesic activity of *T. indica*, we decided to investigate the analgesic activity of the aqueous extract of *T. indica* fruit pulp.

Materials and Methods

Preparation of the Aqueous Extract

The milled fruits of *T. indica* were diluted with 1,000 ml of distilled water, boiled for 20 min and filtered through a 2-layer mesh. The aqueous extract was then concentrated in a boiling bath to 500 ml, cooled, frozen at -70°C and lyophilized to give the crude *T. indica* aqueous extract (TIAE). The yield of the extract was approximately 20% (w/w).

Experimental Animals

Male imprinting control region albino mice (4 weeks old, 20–30 g) and Sprague-Dawley rats (4 weeks old, 180–200 g) were used throughout the experiments. The animals were maintained on standard conditions of food and water by being provided with pellet diet and tap water ad libitum and exposed to light (light period: 7.00–19.00 h) at a constant temperature ($27 \pm 2^{\circ}\text{C}$) cycle for at least 3 days before the experiment to allow acclimatization. Each animal was used once. All experiments were performed according to the ethical guidelines for investigation of experimental pain in conscious animals and approved by the Ethics Committee on Animal Experimentation of the Faculty of Medicine, Universiti Putra Malaysia.

Acetic Acid-Induced Writhing Test

The acetic acid-induced writhing test was used in mice to establish the antinociceptive property of TIAE as described previously [9]. The mice were divided into 6 groups ($n = 7$), which were treated orally with saline (negative control), acetylsalicylic acid (ASA; 5 mg/kg) or TIAE (60, 100, 300 and 600 mg/kg), respectively. The mice were injected intraperitoneally with 0.6% acetic acid solution 30 min after treatment with the test solutions to induce abdominal constriction or writhing effects. The numbers of abdominal constrictions (constriction of abdominal part together with full stretching of both hind legs) were observed for 30 min. The data were collected and computed according to the following formula:

$$\text{Percentage of inhibition (\%)} = \frac{\text{mean of control group} - \text{mean of test group}}{\text{mean of control group}} \times 100.$$

Hot Plate Test

The hot plate test was carried out in mice to determine the central antinociceptive property of TIAE according to the previously described method [10]. Briefly, the mice were divided into 6 groups ($n = 7$), which were treated orally with saline (negative control), morphine (5 mg/kg) or TIAE (60, 100, 300 and 600 mg/kg). They were placed on a hot plate maintained at $52 \pm 0.1^{\circ}\text{C}$, and the elapsed time until the occurrence of either a hind paw licking or a jump off the plate surface was recorded as the hot plate latency. Mice with baseline latencies of 6–9 s were used in the study. After the baseline determination of response latencies, hot plate latencies were measured at 30-, 90-, 150- and 210-min intervals after oral administration of test solutions. A latency period of 20 s was defined as complete analgesia.

Formalin Test

The formalin-induced paw licking test was carried out in a transparent plastic chamber on rats to determine the TIAE ability to affect the non-inflammation-mediated and inflammation-mediated nociceptive mechanisms according to the method described by Shaik Mossadeq et al. [11]: 50 μl of 2.5% formalin was injected into the subplantar region of the left hind paw of the rats 30 min after oral administration of test solutions. The time that the animals spent licking and/or biting the injected paw was measured as an index of nociception. The initial nociceptive response, measured between 0 and 5 min after formalin injection, indicates a neurogenic type of pain response and is termed first phase, while the nociceptive response measured between 15 and 30 min indicates an inflammatory type of pain response and is termed second phase. The data were collected and computed according to the following formula:

$$\text{Percentage of inhibition (\%)} = \frac{\text{mean of control group} - \text{mean of test group}}{\text{mean of control group}} \times 100.$$

Involvement of Opioid Receptors in the Antinociceptive Activity of TIAE

A separate study was carried out to determine the involvement of opioid receptors in the modulation of the antinociceptive activity of TIAE. Three groups of mice ($n = 6$) were pretreated subcutaneously with a nonselective opioid antagonist, naloxone (5 mg/kg) for 10 min, followed by the oral administration of 600 mg/kg TIAE and subcutaneous administration of morphine. Thirty minutes later, the animals were subjected to the hot plate and the formalin tests.

Statistical Analysis

The one-way analysis of variance followed by Dunnett's test was used for statistical evaluation, and the results in tables and figures are expressed as means $\pm$ SEM; $p < 0.05$ was used as the limit of significance.

Table 1. Antinociceptive profile of TIAE assessed by the writhing test in mice

Treatment	Dose, mg/kg	Number of writhings	Inhibition, %
Control		85.00 ± 8.49	–
TIAE	60	41.00 ± 1.41 ^a	51.76
	100	31.50 ± 1.61 ^a	62.94
	300	25.53 ± 1.41 ^b	70.58
	600	22.67 ± 1.38 ^b	73.33
TIAE + naloxone	600+5	38.25 ± 2.1 ^{a, c}	55.00
Morphine	5	12.8 ± 3.17 ^a	84.94
Morphine + naloxone	5+5	68.9 ± 4.56 ^{a, c}	18.94
ASA	100	18.83 ± 1.30 ^b	77.85

Each value represents the mean ± SEM; n = 7; ^a p < 0.05, ^b p < 0.01, significantly different from control; ^c p < 0.05, significantly different compared to the group receiving the appropriate drug/extract at the same dose without naloxone.

Table 3. Antinociceptive profile of TIAE assessed by the formalin-induced paw licking test in rats

Group	Dose, mg/kg	Licking time, s	
		first phase	second phase
Control		74.57 ± 2.35	126.35 ± 8.91
TIAE	60	45.83 ± 1.55 ^a	43.66 ± 4.21 ^a
	100	32.97 ± 1.66 ^a	34.39 ± 6.46 ^b
	300	23.72 ± 1.33 ^a	33.78 ± 6.01 ^b
	600	12.14 ± 1.33 ^b	11.88 ± 1.40 ^b
TIAE + naloxone	600+5	25.60 ± 1.84 ^{a, c}	48.27 ± 2.68 ^{a, c}
Morphine	5	15.7 ± 1.75 ^b	1.11 ± 1.11 ^b
Morphine + naloxone	5+5	68.31 ± 1.70 ^{a, c}	98.72 ± 2.86 ^{a, c}
ASA	100	74.11 ± 1.08	35.59 ± 1.91 ^b

Each value represents the mean ± SEM; n = 7; ^a p < 0.05, ^b p < 0.001, significantly different from control; ^c p < 0.05, significantly different compared to the group receiving the appropriate drug/extract at the same dose without naloxone.

Table 2. Antinociceptive profile of TIAE on the latency of discomfort assessed by the hot plate test in mice

Treatment	Dose, mg/kg	Reaction time, s			
		30 min	90 min	150 min	210 min
Control	–	7.22 ± 0.29	7.68 ± 0.26	7.57 ± 0.31	8.33 ± 0.34
TIAE	60	10.99 ± 0.47 ^a	12.13 ± 0.72 ^a	12.98 ± 0.44 ^a	14.56 ± 0.25 ^a
	100	11.55 ± 0.44 ^a	14.28 ± 0.55 ^a	14.90 ± 0.53 ^a	15.38 ± 0.26 ^a
	300	14.60 ± 0.56 ^a	16.35 ± 0.85 ^a	17.94 ± 0.49 ^a	18.17 ± 0.38 ^a
	600	15.84 ± 0.72 ^a	17.94 ± 1.03 ^a	19.94 ± 0.26 ^b	19.90 ± 0.08 ^b
TIAE + naloxone	600+5	10.55 ± 0.43 ^{a, c}	12.62 ± 0.21 ^{a, c}	13.59 ± 0.30 ^{a, c}	14.42 ± 0.40 ^{a, c}
Morphine	5	10.30 ± 0.81 ^a	11.92 ± 0.64 ^a	11.27 ± 0.67 ^a	10.37 ± 0.72 ^a
Morphine + naloxone	5+5	8.24 ± 1.23 ^{a, c}	9.98 ± 2.20 ^{a, c}	9.54 ± 3.12 ^{a, c}	9.31 ± 0.87 ^{a, c}

Each value represents the mean ± SEM; n = 7; ^a p < 0.05, ^b p < 0.01, significantly different from control; ^c p < 0.05, significantly different compared to the group receiving the appropriate drug/extract at the same dose without naloxone.

Results

Acetic Acid-Induced Writhing Test

Oral administration of TIAE (60–600 mg/kg) exhibited a significant dose-dependent reduction in the number of writhing responses induced by acetic acid compared to the control as shown in table 1 (p < 0.05). The dose of 600 mg/kg TIAE was found to produce an activity equivalent to that of 100 mg/kg ASA.

Hot Plate Test

As presented in table 2, TIAE administered at the dose of 60–600 mg/kg exerted a significant increase in the re-

sponse latency against the thermal stimulus-induced nociception compared to the control (p < 0.05).

Formalin Test

The formalin-induced nociception method in rats showed a similar pattern of dose-dependent antinociceptive effects. TIAE exhibited a significant dose-dependent reduction in response to the noxious stimulus during both early and late phases at all the doses tested (6–600 mg/kg) as shown in table 3. The extract at the dose of 100 mg/kg was found to produce approximately 50% analgesia in both phases when compared to the control group, and this activity was as effective as 100 mg/kg ASA. How-

ever, treatment of animals with ASA (100 mg/kg) caused significant analgesia of the late phase, but not the early phase.

Involvement of Opioid Receptors in the Antinociceptive Activity of TIAE

The results presented in tables 1–3 show that pretreatment of mice with naloxone (5 mg/kg s.c.) significantly reversed the antinociceptive effects of TIAE and morphine (5 mg/kg) in the acetic acid-induced writhing test, the hot plate and the formalin tests, respectively.

Discussion

The results of the present study demonstrated that oral administration of mice with TIAE caused significant antinociception against the chemicals (acetic acid and formalin) and thermal stimuli of nociception. The acetic acid-induced writhing test described as a typical model for inflammatory pain has long been widely used as a model for the evaluation of analgesic and anti-inflammatory properties of new agents [12]. Intraperitoneal injection of acetic acid produced peritoneal inflammation associated with increased levels of prostaglandins, such as PGE₂ and PGF_{2α} in the peritoneal fluids [13], and thus leads to an increase in the capillary permeability, which is thought to contribute to the enhancement of inflammatory pain [14]. In addition, it was also suggested that pain associated with this assay is generated indirectly via stimulation of the peripheral nociceptive neurons by endogenous mediators like serotonin, histamine, bradykinin and prostaglandins [15, 16]. Based on the previous reports [13–16] mentioned above and our present findings, several mechanisms of action could be suggested to explain the observed antinociceptive activity of TIAE, particularly, the activity assessed with the writhing test. The antinociceptive activity seen could be due in part to inhibition of the release of inflammatory mediators or blockade of the peripheral cyclooxygenase activity [17]. Despite being claimed as a highly sensitive and useful model for analgesic drug development, this model of visceral pain is not a selective pain test [18, 19]. The disadvantage of this model is that drugs such as muscle relaxants and sedatives can produce similar effects, leading to possible false-positive results [18]. Furthermore, according to Zakaria et al. [17], the writhing test did not specify the involvement of peripheral and/or central antinociceptive mechanism(s) of the extract. Thus, several other methods were used to clarify the mechanism(s) involved;

in the present study the hot plate test and formalin-induced paw licking test were also employed.

The hot plate test is a selective model for studying the central but not peripheral analgesic properties of extracts/compounds because heat stimulation sensitizes peripheral nerve endings and the impulses generated transmit by way of the spinal cord to the brain. This assay is widely used to screen potential substances or centrally acting opiate analgesic drugs that inhibit pain of central origin [12, 18]. The present findings confirmed the central antinociceptive effect of the TIAE. The mechanism of action involved in the TIAE's central antinociception included partly the opioid-mediated system, as indicated by the ability of naloxone to reverse the 600 mg/kg TIAE-induced antinociceptive activity.

The formalin test has been accepted as a reliable model of persistent nociception and has the advantage of discriminating between peripheral and central pain components [20, 21]. This test has a characteristic biphasic event, known as early and late phases. The early phase, which is generated in the periphery through the activation of nociceptive neurons by the direct action of formalin, is associated with a neurogenic pain, while the late phase, which occurs through the activation of the ventral horn neurons at the spinal cord level, is associated with an inflammatory pain [21, 22]. According to Shibata et al. [23], centrally acting drugs like morphine block both phases of the formalin test while the peripherally acting drugs like nonsteroidal anti-inflammatory drugs inhibit only the late phase. Furthermore, Martindale et al. [24] have reported that nonsteroidal anti-inflammatory drugs, by acting supraspinally, can reduce the pain in both phases. This is supported by the statement of Verma et al. [25] that the late phase is due to inflammation caused by the release of serotonin, histamine, bradykinin and prostaglandins, which at least to some degree can cause the sensitization of the central nociceptive neurons.

Our study showed that administration of TIAE produced a significant dose-dependent inhibition in both the early and late phases of licking responses at all tested doses (table 3). Similarly, morphine produced marked inhibition of both the early and late phases of the formalin test. In contrast, the treatment of animals with ASA caused significant inhibition of the late phase, but not the early phase. It is also interesting to note that pretreatment with naloxone significantly reversed the antinociceptive effect of TIAE and morphine in both phases of the formalin test compared to extract and morphine alone. Based on the findings of both the hot plate and the formalin tests, TIAE seems to possess significant central

and peripheral antinociceptive mechanisms that are partly mediated by the activation of opioid systems. The activity of TIAE is suggested to involve inhibition of both the inflammatory- and non-inflammatory-effect-mediated nociception [19].

Previous phytochemical screening carried out on these species showed the presence of numerous constituent classes, such as flavonoids, saponins, alkaloids, tannins and terpenes [4, 7]. Since several flavonoids, saponins, alkaloids, tannins and terpenes isolated from medicinal plants have been shown to have significant antinociceptive and/or anti-inflammatory activities [26–29], it is possible that the antinociceptive effects observed with the TIAE in the present study may be attributable to the presence of those components.

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Conclusion

The results of the present study demonstrated that TIAE exerts significant peripheral and central analgesic effects that are probably mediated by activation of the opioidergic mechanism. Further investigations are now in progress to identify the active compound(s) responsible for the observed analgesic activity of *T. indica* as well as to further explore its precise mechanism of action.

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