Antinociceptive Activity of Methanol Extract of Tabebuia hypoleuca (C. Wright ex Sauvalle) Urb. Stems

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Significance of the Study
• In this study Tabebuia hypoleuca was shown to have antinociceptive effects mediated by the participation of both peripheral and central antinociceptive mechanisms. The T. hypoleuca species could become a new therapeutic option for the treatment of pain.

Keywords
Tabebuia hypoleuca · Antinociceptive effect · Writhing test · Formalin test · Tail flick test · Hot plate test

Abstract
Objective: The aim of this study was to evaluate the antinociceptive activity of the methanol extract of Tabebuia hypoleuca stems (THME). Materials and Methods: The animals were divided into 5 groups of 8 mice for each test (negative controls, positive controls, and 3 groups treated with THME at doses of 150, 300, and 500 mg/kg, p.o.). The antinociceptive effect of THME was evaluated using the writhing, formalin, tail flick, and hot plate models in mice. Results: In the writhing test, THME (150, 300, and 500 mg/kg) produced significantly ($p < 0.001$) fewer writhes induced by acetic acid than in the control group. In the formalin test, the licking time for THME at doses of 300 and 500 mg/kg was significantly shorter ($p < 0.001$) compared to the control group in the first phase of the formalin test, whereas in the second phase only the dose of 500 mg/kg showed an antinociceptive effect. In addition, THME at doses of 300 and 500 mg/kg significantly increased the latency time in the tail flick test ($p < 0.05$ and $p < 0.001$, respectively) and in the hot plate test ($p < 0.01$ and $p < 0.001$, respectively) compared to the control group. Conclusions: These results show that THME had antinociceptive activity using several models of nociception, and they suggest that the effect is mediated by the participation of both peripheral and central antinociceptive mechanisms.

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Introduction

Pain is a subjective experience resulting from the perception of an injurious stimulus and it includes an emotional component that requires the individual to be conscious when this is happening [1]. In 1986, the International Association for the Study of Pain (IASP) defined pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage [2]. Pain also has a physiological component which is called nociception, i.e., the process by which intense thermal, mechanical, or chemical stimuli are detected by a subpopulation of peripheral nerve fibers called nociceptors [3].

In relief of pain, classical analgesic drugs, notably opiates and nonsteroidal anti-inflammatory drugs, are used [1]. However, long-term use of these agents may produce significant side effects, including gastric ulcers, renal damage, bronchospasm, cardiac abnormalities, dependence, and others, thus limiting their use [4]. Despite recent advances in the development of pain management therapies, there is still a need for effective painkillers. Over the years, natural products have been shown to be an unparalleled source of molecular diversity leading to drug discovery currently used in modern medicine, especially in the treatment of pain [5].

Tabebuia spp. (Bignoniaceae) includes approximately 100 species, known as strictly woody, found in tropical rain forest areas throughout Central and South America [6]. Species of the genus Tabebuia have been traditionally used to treat syphilis, malaria, cutaneous infections, stomach disorders, cancer, inflammation, pain, bacterial and fungal infections, anxiety, poor memory, irritability, depression, and others [7–11].

Tabebuia hypoleuca (C. Wright ex Sauvalle) Urb., commonly known as “Roble macho”, is an endemic species in Cuba, native to the Sierra Maestra and Guantanamo. We have previously reported the anti-inflammatory activity of the methanol extract of T. hypoleuca stems (THME) using carrageenin-induced paw edema models and croton oil-induced auricular edema in mice [12]. The present study was conducted to evaluate the antinociceptive activity of THME administered orally in animal models of pain.

Material and Methods

Plant Material and Extraction
T. hypoleuca stems were collected at the National Botanical Garden (JBN), Havana Province, Cuba. The identification of the plant was confirmed by Dr. Eldis R. Becquer and a sample was deposited in the herbarium of the experimental station with the number HFC-88204. Solid-liquid extraction in Soxhlet with methanol (Merck®) was used for the extraction of T. hypoleuca stems. The methanol extract was filtered and concentrated using rotary evaporation.

Drugs and Chemicals
The drugs and chemicals used were: indomethacin (SOLMED, Havana, Cuba), diclofenac (SOLMED), methanol (Merck, Germany), acetic acid (Merck), and formalin (Merck). The extract and all of the drugs were diluted in 0.9% saline solution (NaCl diluted in distilled water).

Animals
Male and female Balb/c mice (20–25 g) and female Sprague-Dawley rats (180–200 g) were supplied by the National Center for Laboratory Animal Production (CENPALAB, Santiago de Las Vegas, Havana, Cuba). The animals were kept under standard conditions of 23 ± 2 °C, 40–60% relative humidity, and a 12/12 h light-dark cycle, and they were given food and water ad libitum for 7 days. All experimental procedures were performed in accordance with the International Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Ethical Committee of the National Center for Animal and Plant Health (CENSA, Havana, Cuba) (protocol No. 03/FT/15).

Acute Oral Toxicity
The acute oral toxicity study was performed as per the guidelines of Organization for Economic Cooperation and Development (OECD; guideline 423). Nulliparous healthy female rats were used for this study. The rats were divided into 2 groups, with 3 animals in each group. From 12 h before until 3 h after the oral administration, the animals were kept without access to food and water. The control group received normal saline at 1 mL/kg by gavage while the exposed group received 2,000 mg/kg of THME. The safety of the 2,000 mg/kg dose was subsequently confirmed in another 3 animals as recommended in the OECD guidelines. Immediately after administration, all of the animals were observed for a total of 14 days based on established criteria, with special attention given during the first 4 h; clinical signs or mortality were noted. On day 15, all of the animals were euthanized by cervical dislocation, followed by necropsy and macroscopic observation of the organs [13].

Writhing Test
The writhing test was carried out as described by Koster et al. [14] with few modifications [15]. Male mice were divided into 5 groups of 8 mice each. The animals were treated orally with indomethacin (20 mg/kg), distilled water (10 mL/kg), and THME (150, 300, and 500 mg/kg). Writhing was induced by intraperitoneal injection of 0.8% acetic acid solution (0.01 mL/g body weight) 1 h after treatment. The writhes (abdominal constrictions and hind limbs stretching) were counted for 15 min after injection of the acetic acid solution. The percentage of analgesic activity was calculated as follows:

Percent inhibition = \( \frac{\text{number of writhes (control)} - \text{number of writhes (treated)}}{\text{number of writhes (control)}} \times 100. \)

Formalin Test
The formalin test was done as described by Santos and Calixto [16]. Formalin-induced pain behavior was biphasic; the initial acute phase (neurogenic pain) lasted 0–5 min and was followed by...
a relatively short quiescent period, after which came a prolonged tonic response (inflammatory pain) that lasted 15–30 min. Male mice were divided into 5 groups of 8 mice each. The animals were treated orally with diclofenac (10 mg/kg), distilled water (10 mL/kg), and THME (150, 300, and 500 mg/kg). After 30 min, the animals were injected with 20 μL of a 2.5% formalin solution (37% formaldehyde) on the plantar surface of the right hind paw. The index of nociception, i.e., the total time spent by each animal licking or biting the injected paw, was recorded for 30 min.

Tail Flick Test
The tail flick test was performed according to D’Amour and Smith [17]. Male mice were divided into 5 groups of 8 mice each. One to 2 cm of the mouse tail were immersed in warm water kept constant at 55 ± 0.5°C. The latency between tail submersion and deflection of the tail was recorded. Mice that showed a latency period between 1.5 and 3.5 s were selected for this study 24 h prior to the experiment and the pretreatment latency was recorded. The animals were treated orally with indomethacin (20 mg/kg), distilled water (10 mL/kg), and THME (150, 300, and 500 mg/kg). Sixty minutes after oral administration, the reaction time was again recorded. A cutoff time of 10 s was used to avoid tail tissue damage in mice. The percentage of analgesic activity was calculated as follows:

\[
\text{Percent inhibition} = \frac{\text{reaction time (control)} - \text{reaction time (treated)}}{\text{reaction time (control)}} \times 100.
\]

Hot Plate Test
The hot plate test was performed as described by Asongalem et al. [18]. Female mice were divided into 5 groups of 8 mice each. The device consisted of a water bath in which a metallic cylinder (diameter 20 cm and height 10 cm) was placed. The temperature of the cylinder was set at 55 ± 0.5°C. The mice that showed forepaw licking, withdrawal of the paw(s), or a jumping response within 15 s on the hot plate were selected for this study 24 h prior to the experiment, and the pretreatment latency was recorded. The animals were treated orally with indomethacin (20 mg/kg), distilled water (10 mL/kg), and THME (150, 300, and 500 mg/kg). Sixty minutes after oral administration, the reaction time was again recorded. A cutoff time of 15 s was used to avoid damage to the paw. The percentage analgesic activity was calculated using the same formula as in the tail flick test.

Statistical Analysis
Statistical analysis was performed using the statistical software package SPSS, version 21.0 for Windows (IBM Corp., Armonk, NY, USA). Data are expressed as means ± SEM. One-way ANOVA followed by the Dunnett post hoc test was used to determine the significant differences between the control and treatment groups. p < 0.05 was considered statistically significant.

Results

Acute Oral Toxicity Study
An acute oral toxicity study showed that THME at up to 2,000 mg/kg body weight did not produce any mortality or signs of behavioral or neurological toxicity in the animals after 14 days of observation. A normal body weight gain was observed and there was no difference in the organ weights of the control and treated rats (Table 1).

Table 1. Effect of oral administration of THME on various parameters evaluated in acute oral toxicity study

| Parameter | Treatment group | THME at 2,000 mg/kg |
|-----------|----------------|--------------------|
| control (vehicle) | 175.25 ± 0.33 | 180.00 ± 0.00 |
| Day 7  | 192.00 ± 0.88 | 194.75 ± 3.28 |
| Day 14 | 205.75 ± 4.48 | 210.00 ± 5.03 |
| Body weight gain, g  | Day 0  | – | – |
| Day 7  | 16.75 ± 0.88 | 14.75 ± 3.28 |
| Day 14 | 30.50 ± 4.48 | 30.00 ± 5.03 |
| Organ weight, g  | Liver | 9.60 ± 0.80 | 9.10 ± 0.25 |
| Kidney  | 1.86 ± 0.03 | 1.80 ± 0.10 |
| Lung | 1.33 ± 0.03 | 1.36 ± 0.12 |
| Heart  | 0.80 ± 0.00 | 0.80 ± 0.05 |
| Spleen | 0.53 ± 0.03 | 0.53 ± 0.03 |
| Thymus  | 0.46 ± 0.03 | 0.50 ± 0.05 |

Values are expressed as means ± SEM (n = 3). THME, methanol extract of Tabebuia hypoleuca stems.
Writhing Test

Oral administration of THME at 150, 300, and 500 mg/kg and indomethacin (20 mg/kg) caused a significant ($F[4, 35] = 168.63, p < 0.001$) decrease in the number of writhing episodes induced by acetic acid in a dose-dependent manner compared to the control group (Fig. 1). The calculated percentage inhibition of constrictions of indomethacin was 80%, for THME at 150 mg/kg it was 53%, for THME at 300 mg/kg it was 67%, and for THME at 500 mg/kg it was 87%.

Formalin Test

In this model, the time of licking for THME (300 and 500 mg/kg, p.o.) and diclofenac sodium (10 mg/kg, p.o.) was significantly ($F[4, 35] = 43.86, p < 0.001$) lower than that of the control group (Fig. 2a). In the first phase (0–5 min), the time of licking for THME at doses of 300 and 500 mg/kg, p.o., and diclofenac sodium (10 mg/kg, p.o.) was significantly shorter ($F[4, 35] = 50.08, p < 0.001$) than in the control group, with 82% (diclofenac sodium), 69% (THME at 300 mg/kg), and 86% (THME at 500 mg/kg) inhibition. THME at doses of 150 mg/kg, p.o., did not show a significant analgesic effect (Fig. 2b). In the second
phase (15–30 min), THME showed a significant ($F[4, 35] = 26.67$, $p < 0.001$) antinociceptive effect only at a dose of 500 mg/kg, p.o. Also, the licking time of the positive control group treated with diclofenac sodium (10 mg/kg, p.o.) was significantly shorter ($p < 0.001$) compared to the control group, with 91% (diclofenac sodium) and 79% (THME 500 mg/kg) inhibition. THME at doses of 150 and 300 mg/kg, p.o., did not show a significant analgesic effect in this phase (Fig. 2b).

**Tail Flick Test**

Oral administration of 300 and 500 mg/kg THME and 20 mg/kg indomethacin caused a significant ($F[1, 14] = 13.63$, $p < 0.002$ [indomethacin]; $F[1, 14] = 5.49$, $p < 0.034$ [THME at 300 mg/kg]; and $F[1, 14] = 26.86$, $p < 0.001$ [THME at 500 mg/kg]) increase in the latency time response compared to the pretreatment latency (indomethacin, 13%; THME at 300 mg/kg), 7%; and THME at 500 mg/kg, 16% inhibition). THME at doses of 150 mg/kg, p.o., did not induce a significant analgesic effect (Fig. 3).

**Hot Plate Test**

Oral administration of 300 and 500 mg/kg THME significantly ($F[1, 14] = 14.24$, $p < 0.002$ [THME at 300 mg/kg]; $F[1, 14] = 25.32$, $p < 0.001$ [THME at 500 mg/kg]) increased the latency time response compared to the pretreatment latency. The pain threshold was also significantly ($F[1, 14] = 6.76$, $p < 0.021$) reduced in the positive control.
group treated with indomethacin (20 mg/kg) compared to the pretreatment latency. The inhibition was for 51% for indomethacin, 51% for THME at 300 mg/kg, and 61% for THME at 500 mg/kg. The nociceptive responses were not significantly affected by THME at 150 mg/kg (Fig. 4).

**Discussion**

The present study showed that THME up to 2,000 mg/kg body weight (acute oral toxicity study) did not have any toxic effects. In addition, oral administration of THME in mice caused antinociceptive effects against the chemicals (writhing and formalin) and thermal (tail flick and hot plate) stimuli of nociception.

The writhing test describes a typical model of inflammatory pain. Acetic acid is an inducer of abdominal contractions and twisting of abdominal muscles by increasing the level of proinflammatory agents in the peripheral tissue fluid [19]. In this test, the number of writhes in mice treated with THME was lower than in the controls, indicating inhibition of the acetic acid-induced visceral nociception. This finding confirmed the previous report [12] that THME showed anti-inflammatory activity in 2 models of acute inflammation in mice, so the antinociceptive effect seen could have been due in part to inhibition of the release of inflammatory mediators or blockade of the peripheral cyclooxygenase activity. It is probable that the pain associated with this assay could be generated indirectly via stimulation of the peripheral nociceptive neurons by endogenous mediators like serotonin, histamine, bradykinin, and prostaglandins [20]. However, this chemical method has good sensitivity but poor specificity, allowing misinterpretation of the results, because this is an unspecific stimulus for nociception, sensitive to drugs with different mechanisms. This problem can be avoided by complementation with other nociception models [21].

The formalin test is considered a model of persistent pain produced in 2 phases. The first phase (0–5 min) is characterized by neurogenic pain and the second one (15–30 min) by inflammatory pain [22]. In this test, the licking time for THME at doses of 300 and 500 mg/kg was significantly shorter than in the control group in the first phase, whereas in the second one THME showed an antinociceptive effect only at a dose of 500 mg/kg. The antinociceptive effect in the second phase is related to previous studies in which THME showed anti-inflammatory activity only at a dose of 500 mg/kg. Pain in the early phase was predominantly caused by the activation of C fibers, while in the late phase a combination of an inflammatory reaction in peripheral tissue and functional changes in the dorsal horn of the spinal cord were involved [23]. Centrally acting drugs inhibit both phases of pain, while peripherally acting drugs inhibit mainly the second phase [24]. These results suggest that the antinociceptive activity of THME in the formalin test could be attributed to the action of both neurogenic and anti-inflammatory mediators.

In the tail flick test, the thermal stimulation activated peripheral nociceptors, leading to reflexive removal of the tail. An increase in the reaction time is generally considered to be an important parameter for evaluating central antinociceptive activity as reported previously [25]. The finding of the flick of the tail could be due to a reflex arc in the spinal cord which was modulated through a descending pathway mechanism [26]. The observed antinociceptive effect of THME (300 and 500 mg/kg, p.o.) in the tail flick test confirmed its central activity. In the hot plate test a predominantly supraspinal reflex revealed a centrally acting antinociceptive effect of THME. The plate, heated to a constant temperature, produced 2 behavioral components, i.e., paw licking and jumping, measured by reaction times, which could be due to supraspinally integrated responses [27]. These findings reveal that THME (300 and 500 mg/kg, p.o.) induced central antinociceptive effects because it significantly increased the latency time in this model, thereby confirming its central activity.

Several phytochemical studies had revealed that extracts from *Tabebuia* species contain a wide diversity of secondary metabolites such as tannins, flavonoids, quinones, alkaloids, naphthoquinones, and iridoids [11, 28]. Regarding THME, a preliminary phytochemical analysis revealed the presence of tannins, alkaloids, and phenolic compounds [12] that have been shown to have various biological actions, including antinociceptive and anti-inflammatory activities [29, 30], and hence the antinociceptive effects observed with THME could be attributable to the presence of those compounds. However, further investigations are required to identify the bioactive components and to determine the mechanism of action by which these compounds exert their antinociceptive properties.

**Conclusions**

This study demonstrated the antinociceptive activity of methanol extract of stems of *T. hypoleuca* using several models (chemical and thermal) of nociception in mice, thereby indicating that this species has centrally and peripherally mediated antinociceptive effects.
Acknowledgements

The authors are grateful for the technical support offered by Rafael Lorenzo and Damileysi Castro from the Division of Biopharmaceutical Development, CENSA. The authors would also like to thank Dr. Eduardo Sistachs for his assistance in the revision of the language.

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Disclosure Statement

The authors have no conflicts of interest to declare.