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Analgesic properties of the ethanol extract of leaves of *Triumfetta cordifolia* A. Rich

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Abstract

Triumfetta cordifolia is used ethnomedicinally in the management of pain, inflammatory and mental disorders. No study has been carried out to scientifically confirm its ethnomedicinal use in the management of pain. Hence, this study was design to evaluate the antinociceptive property of ethanol extract of leaves of *T. cordifolia* (EETC) in experimental models of pain in mice. The antinociceptive property of EETC (8.8, 17.5 and 35 mg/kg) administered intraperitoneally (i.p.) was assessed on acetic acid-induced abdominal writhing, formalin-induced paw licking, hot water tail immersion and hot plate-induced nociception tests. In addition, naloxone (1 mg/kg, i.p.) was used to elucidate the involvement of opioidergic system in the antinociceptive activity of EETC in hot plate test. EETC (8.8, 17.5 and 35 mg/kg, i.p.) significantly ($p < 0.05$) reduced acetic acid-induced writhing when compared with control. Also, EETC (8.8, 17.5 and 35 mg/kg, i.p.) dose-dependently attenuated formalin-induced paw licking; as it significantly ($p < 0.05$) inhibited the inflammatory phase, although only EETC (35 mg/kg, i.p.) was found to suppressed the neurogenic phase. Moreover, EETC significantly ($p < 0.05$) increased the reaction time to pain in mice exposed to hot plate test, with no significant effect in the hot water tail immersion test when compared with control. Naloxone significantly ($p < 0.05$) reversed the effect of EETC on hot plate-induced pain. In conclusion, the results of this study provide evidence, showing that EETC possesses phytochemical compounds with analgesic activity and may be related to peripheral nociception mediation and central opioidergic pain pathway system.

Keywords: *Triumfetta cordifolia;* Analgesia; Writhing; Paw-licking

INTRODUCTION

Pain is a common and distressing feature of many diseases such as tumor, surgical procedures, physical trauma, noxious chemical stimulation [1, 2]. According to International Association for Study of Pain (IASP) [3], pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage. It is mostly a warning signal and primarily protective; however, excessive pain may lead to serious discomfort and behavioral stress such as sweating, apprehension, nausea and palpitation as well as other tissue damages [4]. Several epidemiological reports has shown increased prevalence rates of chronic pain approximately ranging 12-80% of patients in most hospital setting and this has become common with terminal illness such as cancer [5].

Treatments of pain have always included a non-specific inhibition of inflammatory mediators such as cyclooxygenases enzymes, prostaglandins, 5-hydroxytryptamine among others [6]. However, large numbers of drugs used in the

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management of pain have been shown to be associated with therapeutic failure. Indeed, a sizable proportion of patients with pain do not show significant improvement [7, 8] and the paucity of effective treatment in some serious terminal diseases with severe pain given their bodily discomfort has stimulated the continuous search for better therapies for pain management [8].

Triumfetta cordifolia A. Rich (*Tiliaceae*) is an erect perennial shrub [9] that grows on moister areas of Tropical Africa. It is commonly distributed locally on marshy grassland, secondary forest and riverine forest [9, 10]. The decoction of leaf of *Triumfetta cordifolia* is commonly used in locally for the managements of gastrointestinal disturbances such as dysentery, diarrhoeal, ulcerogenic conditions, indigestion and diabetes [10]. The leaf has also been indicated for the treatment of asthenia, marasmus, rhinitis, hepatitis, lumbago, muscle pain, backache, fever, inflammation and mental disorders [9, 10]. Moreover, ethnomedicinal survey revealed that *T. cordifolia* leaf extract is applied locally in the treatment of pain [10]. In addition, extracts of the flowers and fruits of *Triumfetta cordifolia* have been reported to show anti-malarial property [10] and to induce labor during childbirth [9]. Extracts of the roots of *Triumfetta cordifolia* has been used for the management of liver and kidney diseases [9]. Ethnopharmacological studies have revealed that *T. cordifolia* possesses antidiabetic, antidiarrhoeal, antihyperlipidemia, antiobesity, antiulcerogenic, antibacterial, anti-inflammatory, antimalarial and anti-HIV activity activities [11, 12, 13, 14]. Moreover, preclinical studies have also shown that *T. cordifolia* inhibits free radical scavenging activity, which suggests antioxidant activity [15]. The ethnomedicinal and ethno-pharmacological properties of *T. cordifolia* have been attributed to the presence of diverse phytochemical compounds (maslinic acid, betulinic acid, alkaloids, tannins, saponins, steroids, terpenes, stigmasterol, tormentic acid, oleanolic acid, cardiac glycosides and quercetin) present in the leaves, flowers and roots [14, 15]. However, literature review showed that no study has been carried to demonstrate the pharmacological property of *T. cordifolia* against pain. Hence, this study was design to evaluate the antinociceptive property of ethanol extract of leaves of *T. cordifolia* (EETC) in experimental models of pain in mice.

EXPERIMENTAL

Plant material. The leaves of *Triumfetta cordifolia* was collected and taxonomically identified by Mr O.A. Ugbogu and Mr O.S. Shasanya at the Forestry Reserve Institute of Nigeria (FRIN), Ibadan, Oyo State, Nigeria with an FHI No.109530.

Preparation of extract. Air-dried leaves (300g) were pulverized and soaked in 750 mL of 50% ethanol for 48 h. The filtrate was concentrated with a rotary evaporator to semi-solid residue at 38°C and evaporated to dryness to produce a solid residue, which was kept in the desiccator. However, the yield of the extract was 10.8 g with reference to the powdered leaves. The dried extract was subsequently dispensed in distilled water at different concentrations for various experiments.

Drug and chemicals. Acetic acid (May & Baker Ltd., Dagenham, England), formalin (BDH, England), acetylsalicylic acid (aspirin) (Sigma Chemicals Co. St. Louis, Missouri, USA) and morphine (Sigma Chemicals Co. St. Louis, Missouri, USA) were used in the study.

Drug preparation. The ethanol extract of leaves of *Triumfetta cordifolia* was dissolved in 5% dimethyl sulfoxide (DMSO). Acetylsalicylic acid and morphine, used as reference drugs were also dissolved 5% concentration of DMSO. All drugs including vehicle (5% DMSO, 10 mL/kg) were

administered intraperitoneally (i.p.). The doses of EETC used in this study were based on results obtained from preliminary investigation.

Experimental animals. Male Swiss mice (20-25 g; 6 weeks old) of either sex were obtained from the Central Animal House, University of Ibadan. The animals were housed five per plastic cage (42 x 30 x 27 cm) at a room temperature (25 ± 1°C) and relative humidity of 60 ± 5% with a 12-hr light/dark cycle. They were fed with standard rodent pellet food and water *ad libitum* throughout the experimental period. They were acclimatized for at least 1 week prior to commencement of the experiments. The experimental procedures were performed in accordance with the National institutes of Health (NIH) Guideline for the Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Experimental design

Evaluation of the effect of EETC on acetic acid-induced writhing. The effect of EETC on acetic acid-induced writhing behavior was assessed in a Plexiglas cage (45cm x 25cm x 25cm). Animals were randomly divided into 5 groups, (n=5). Group 1 received vehicle (10 mL/kg 5% DMSO, i.p) and served as negative control. Groups 2-4 received EETC (8.8, 17.5 and 35 mg/kg i.p) and group 5 was treated with the reference drug, aspirin (150 mg/kg i.p). Thirty minutes after single i.p. injection of extract, mice were administered with acetic acid (10 mL/kg of 0.6%, i.p.) and kept inside the Plexiglas cage. Thereafter (5 min), writhing behavior was assessed for 15 min inside the Plexiglas cage and the number of writhing exhibited by each mouse was counted and recorded. The reduction in writhing reflexes was taken as an indication of analgesic activity [16].

Effect of EETC on formalin-induced paw licking test. Formalin-induced tonic pain was carried out as previously described by Janssen *et al*., [17] based on frequency of paw licking behavior. Mice were randomly grouped into five treatment groups, (n=5); group 1 received vehicle (10 mL/kg 5% DMSO i.p) and served as negative control. Groups 2-4 was treated with EETC (8.8, 17.5 and 35 mg/kg i.p) and group 5 received reference drug, morphine (5 mg/kg, i.p). Thirty minutes later after i.p. injection of all drugs, mice from groups 1-5 received i.p. injection of 20 µL of 2.5% formalin into the intraplantar space of the right-hind paw of each mouse. The duration of paw licking between 0-5 minutes (1st phase, neurogenic) and 20-30 minutes (2nd phase, inflammatory) was recorded and the duration of paw licking behavior was used as an index of pain response [16].

Assessment of the effect of EETC on hot water tail immersion test. The effect of EETC on hot water-induced tail withdrawal reflex, as an index of nociception was also used to screen for the antinociceptive activity of EETC in mice [17]. Animals were randomly divided into 5 groups, (n=5). Group 1 received vehicle (10 mL/kg 5% DMSO i.p) and served as negative control. Groups 2-4 received EETC (8.8, 17.5 and 35 mg/kg i.p) and group 5 received morphine (5 mg/kg, i.p). Thereafter (30 min), the tail of each mouse was dipped (up to 5 cm) into hot water (55.0 ± 0.5°C) in a water bath. The latency to complete withdrawal of tail from the hot water by mice is taken as the reaction time to pain. However, a cut-off time of 15 s was used to avoid tissue damage.

Effect of EETC on hot plate test. Mice were randomly grouped into five treatment groups, (n=5); group 1 received vehicle (10 mL/kg 5% DMSO i.p) and served as negative control. Groups 2-4 was treated with EETC (8.8, 17.5 and 35 mg/kg i.p) and group 5 received reference drug, morphine (5 mg/kg, i.p). Thirty minutes after treatments, animals were singly placed on the hot plate set at 55±1 °C. The pain reaction time of the animals was recorded as indicated by the time it takes mice

to flick, lick the hind paw or jump about on the hot plate withdrawal time of mouse using stopwatch. However, a cut off time of 20 seconds was used to avoid tissue damage and mice that showed initial nociceptive responses within 1 s were selected for the experiment [18]. In order to elucidate possible involvement of opioid receptor activity in the analgesic property of EETC, the effects of EETC (35 mg/kg, i.p.) and morphine (5 mg/kg, i.p.) were interacted with naloxone (1 mg/kg, i.p.), an opioid receptor blocker. Accordingly, another set of three groups of mice (n=5) were randomly selected. Group 1 received naloxone (1 mg/kg, i.p.) alone while groups 2 and 3 received EETC (35 mg/kg, i.p.) and morphine (5 mg/kg, i.p.) 15 min prior to administration of EETC (35 mg/kg, i.p.) and morphine (5 mg/kg, i.p.). Thirty minutes later, mice were subjected to the test as earlier described.

Statistical analysis. All data are presented as Mean ± SEM. The results were analyzed by using One-way Analysis Of Variance (ANOVA) and Post-hoc test (Newman-Keuls) were carried out to determine the source of significant main effect using GraphPad InStat® Biostatistics software (Graphpad Software, Inc., La Jolla, USA version 4.0). The level of significance for all tests were set at $p \leq 0.05$.

RESULTS

Ethanol extract of leaves of *Triumfetta cordifolia* reduces acetic acid-induced abdominal writhing in mice. The effect of EETC on acetic acid-induced abdominal writhing is shown in Table 1. Intraperitoneal injection of acetic acid (10 mL/kg of 0.6%, i.p.) produced marked abdominal writhing in mice. However, administration of EETC (8.8, 17.5 and 35 mg/kg, i.p.) and aspirin (150 mg/kg, i.p.) significantly ($p < 0.05$) [$F (4, 20) = 56.54$, $P < 0.0001$] reduced acetic acid-induced abdominal writhes in dose dependent manner when compared with vehicle control in mice.

Ethanol extract of leaves of *Triumfetta cordifolia* decreases formalin-induced paw licking in mice. The effect of EETC on formalin-induced paw licking is shown in Table 2. Hind-paw intraplantar injection of formalin (20 µL, 2.5%) significantly ($p < 0.05$) increased paw licking behavior in mice. However, treatment with EETC (8.8, 17.5 and 35 mg/kg, i.p.) and morphine (5 mg/kg, i.p.) significantly ($p < 0.05$) [$F (4, 20) = 24.14$, $P < 0.0001$] decreased formalin-induced paw licking relative to vehicle-formalin control in the second phase (inflammatory stage). Meanwhile, only EETC (35 mg/kg, i.p.) and morphine (5 mg/kg, i.p.) significantly inhibited formalin-induced paw licking behavior in the first phase (neurogenic stage) when compared with vehicle-formalin control (Table 2).

Effect of Ethanol extract of leaves of *Triumfetta cordifolia* on hot water tail immersion test in mice. Administration of EETC (8.8, 17.5 and 35 mg/kg, i.p.) did not show any significant difference ($p > 0.05$) in reaction time to pain in the 30^{th}, 60^{th}, 90^{th} and 120^{th} min when compared with the vehicle group. However, treatment with the reference drug, morphine (5 mg/kg, i.p.) significantly ($p < 0.05$) [$F (4, 20) = 30.71$, $P < 0.0001$] increased the reaction time to pain when compared with the vehicle (Table 3).

Ethanol extract of leaves of *Triumfetta cordifolia* inhibits hot plate-induced pain reaction in mice. The effect of EETC on hot plate-induced pain reaction in mice is shown in Table 4. One-way ANOVA showed that there is significant difference at the different time interval between treatment group: 30^{th} min [$F (7, 32) = 13.56$, $P < 0.0001$], 60^{th} min [$F (7, 32) = 8.468$, $P < 0.0001$], 90^{th} min [$F (7, 32) = 174.1$, $P < 0.0001$] and 120^{th} min [$F (7, 32) = 11.0$, $P < 0.0001$]. Post hoc-analysis with Newman-Keuls test showed that

treatment with EETC (8.8, 17.5 and 35 mg/kg, i.p.) significantly ($p < 0.05$) increased the tolerance or reaction time to pain at 30th , 60th, 90th and 120th min when compared with vehicle group respectively; however, no effect was observed with EETC (8.8 mg/kg) at the 90th min. In addition, morphine (5 mg/kg) significantly ($p < 0.05$) inhibited hot plate-induced pain sensation, as evidenced by increased reaction time to pain when in comparison with vehicle control (Table 4). Intraperitoneal injection of naloxone (1 mg/kg, i.p.) did not show any significant difference relative to vehicle group (Table 4). However, pretreatment with naloxone significantly ($p < 0.05$) reversed the anti-nociceptic effects of EECT (35 mg/kg, i.p.) and morphine (5 mg/kg, i.p.) against hot plate-induced pain reaction when compared with EECT (35 mg/kg, i.p.)-treated group respectively (Table 4).

DISCUSSION

The study showed that ethanol extract of the leaves of *Triumfetta cordifolia* demonstrated significant antinociceptive property in mice. EETC significantly decreased abdominal writhing (vicerosomatic pain) induced by acetic acid in a dose-dependent manner. In addition, the increased paw licking due to intraplantar injection of formalin was significantly reduced by EETC.

Table 1. Ethanol extract of leaves of *Triumfetta cordifolia* reduces acetic acid-induced abdominal writhing in mice

Treatment	Number of writhes	Percentage analgesia
Vehicle (10 mL/kg)	57.20 ± 2.43	-
EETC (8.8 mg/kg)	46.60± 2.09*	18.53
EETC (17.5 mg/kg)	31.20 ± 2.73*	45.45
EETC (35 mg/kg)	13.40 ± 2.98*	76.57
Aspirin (150 mg/kg)	21.00 ± 1.45*	63.29

Value represents the mean of 6 animals / group. *$p < 0.05$ compared to vehicle group (one-way ANOVA followed by Newman-Keuls *post-hoc* test). EETC - ethanol extract of leaves of *Triumfetta cordifolia*

Table 2. Ethanol extract of leaves of *Triumfetta cordifolia* decreases formalin-induced paw licking in mice

Treatment	Licking time (in seconds)		Percentage inhibition	
	1st phase	2nd phase	1st phase	2nd phase
Vehicle (10 mL/kg)	77.00 ± 2.43	108.20 ± 5.85	-	-
EETC (8.8 mg/kg)	77.60 ± 1.97	50.00 ± 2.26*	-	53.79
EETC (17.5 mg/kg)	80.00 ± 2.15	17.20 ± 3.54*	-	84.10
EETC (35 mg/kg)	68.40 ± 3.27*	5.00 ± 1.00 *	11.17	95.38
Morphine (5 mg/kg)	14.20 ± 1.07*	14.20 ± 0.86*	81.56	86.88

Value represents the mean of 6 animals / group. *$p < 0.05$ compared to vehicle group (one-way ANOVA followed by Newman-Keuls *post-hoc* test). EETC - ethanol extract of leaves of *Triumfetta cordifolia*

Table 3. Effect of Ethanol extract of leaves of *Triumfetta cordifolia* on hot water tail immersion test in mice

Treatment	Reaction time (in seconds)			
	30 min	60 min	90 min	120 min
Vehicle (10 mL/kg)	0.39 ± 0.04	0.59 ± 0.03	0.57 ± 0.04	0.59 ± 0.03
EETC (8.8 mg/kg)	0.58 ± 0.07	0.87 ± 0.19	0.70 ± 0.11	0.72 ± 0.11
EETC (17.5 mg/kg)	0.62 ± 0.02	0.68 ± 0.05	0.76 ± 0.05	0.56 ± 0.03
EETC (35 mg/kg)	0.57 ± 0.05	0.68 ± 0.06	0.64 ± 0.08	0.68 ± 0.11
Morphine (5 mg/kg)	3.36 ± 0.15*	3.25 ± 0.10*	2.86 ± 0.14*	2.26 ± 0.03*

Value represents the mean of 6 animals / group. *$p < 0.05$ compared to vehicle group (one-way ANOVA followed by Newman-Keuls *post-hoc* test). EETC - ethanol extract of leaves of *Triumfetta cordifolia*

Table 4. Effect of Ethanol extract of leaves of *Triumfetta cordifolia* on the hot plate test in mice

Treatment (N=5 mice per group)	Reaction time (in seconds)			
	30 min	60 min	90 min	120 min
Vehicle (10 mL/kg)	0.55 ± 0.04	0.46 ± 0.03	0.47 ± 0.04	0.45 ± 0.04
EETC (8.8 mg/kg, i.p)	3.36 ± 0.49*	2.96 ± 0.54*	2.25 ± 0.08*	1.82 ± 0.34
EETC (17.5 mg/kg, i.p)	2.61 ± 0.45*	2.94 ± 0.34*	2.59 ± 0.06*	2.88 ± 0.52*
EETC (35 mg/kg, i.p)	2.18 ± 0.53*	2.26 ± 0.39*	2.43 ± 0.11*	3.12 ± 0.91*
Morphine (5 mg/kg, i.p)	2.13 ± 0.15*	2.95 ± 0.13*	2.94 ± 0.08*	3.05 ± 0.22*
Naloxone (1 mg/kg, i.p)	0.63 ± 0.02	0.52 ± 0.02	0.42 ± 0.02	0.58 ± 0.05
Naloxone (1 mg/kg, i.p) + EETC (35 mg/kg)	0.64 ± 0.07[a]	0.95 ± 0.11[a]	0.91±0.15[a]	0.88 ± 0.12[a]
Naloxone (1 mg/kg) + Morphine (5 mg/kg,)	0.49± 0.02[b]	0.53±0.03[b]	0.48±0.03[b]	0.50± 0.02[b]

Value represents the mean of 6 animals / group. *$p < 0.05$ compared to vehicle group; a$p < 0.05$ compared to EETC (35 mg/kg) group; b$p < 0.05$ compared to morphine group (one-way ANOVA followed by Newman-Keuls *post-hoc* test). EETC - ethanol extract of leaves of *Triumfetta cordifolia*

Moreover, the increased pain response induced by hot plate was also significantly suppressed by EETC as evidenced by prolonged reaction time to pain in the hot plate test. However, EETC did not show any significant effect against nociception induced by hot water tail immersion relative to control. The putative analgesic activity of EETC was evaluated to clarify its claim in management of pain [10] using experimental (chemical and thermal) animal models predictive of pain.

Acetic acid-induced abdominal writhing is a popular pain method used in the screening of test compounds for peripheral analgesic activity [19]. Acetic acid-induced abdominal writhing has been previously linked to increased release of pain mediators including prostaglandins (PGE), leukotrienes, 5-hydroxytryptamine, kinins and histamine from peripheral abdominal site, which is commonly manifested as abdominal writhing [6]. Abdominal writhing induced by acetic acid have been shown to be mediated by abdominal satellite cells such as acid sensing ion channels, peritoneal mast cells and prostaglandin chemosensitive nociceptors [20, 21, 22]. Specifically, acetic acid has been reported to cause an increased release of peritoneal fluids such as PGE_2 and PGF_2, which are particularly involved in PG-mediated nociception [23, 24]. Indeed, acetylsalicylic acid induced analgesia has been reported to be partially due to inhibition of PGE_2 and PGF_2 synthesis. Thus, the ability of EETC to reduced acetic acid induced abdominal writhing suggests analgesic property and may be partly due to the inhibition of synthesis of arachidonic acid metabolites.

Although acetic acid induced abdominal writhing has been used routinely in the screening of novel compounds for analgesic property, it lacks the potentials of inducing nociception centrally, which therefore requires the use of animal model capable of inducing pain centrally [25]. Accordingly, studies have shown that the formalin test comprises of two separate phases: an early phase and late phase. The early phase, also known as neurogenic phase has been interrelated to a direct effect on nociceptors and a consequent of C-fiber activation, which can be suppressed by centrally acting analgesics like morphine [25]. Whereas, the late phase, which is the inflammatory phase is assumed to be dependent on a combination of inflammatory reaction in the peripheral tissue and functional changes in the dorsal horn of the spinal cord, which is initiated by C-fiber barrage during the early phase [26]. Studies have shown that substance P and bradykinin participates in the early phase while histamine, serotonin, prostaglandins, nitric oxide and bradykinin are involved in the late phase of formalin test [27]. However, because both phases also helps to classify analgesic drugs into centrally

and peripherally acting agents, centrally acting drugs such as morphine have been shown to inhibit both the neurogenic and inflammatory phases of formalin test, whereas peripherally acting drugs including aspirin only attenuates the inflammatory phase peripherally [25]. Moreover, according to Trongsakul *et al.* [28], inflammatory pains from formalin-induced nociception are usually attenuated by non-steroidal anti-inflammatory drugs and corticosteroids. In this study, high dose of EETC was found to inhibit formalin-induced neurogenic pain from phase 1, as evidenced by decreased paw licking response relative to control group. In addition, EETC significantly inhibited inflammatory pain associated with the second phase of formalin-induced nociception in a dose-dependent manner; which suggests the presence of phytochemical compounds with both peripheral and central analgesic activities.

Previous studies have confirmed that hot plate and hot water tail immersion tests induced nociception associated with central nervous system in laboratory animals through thermal activation of central nociceptors like μ and κ-opioid receptors as well as C-fibers activation [29]. Also, central pain response induced by thermal models have been linked with increased supraspinal integrated response such as tail flick, paw licking and jumping, via increased influx of impulse from the dorsal horn of the spinal cord to cortical brain centers [30]. Thus, hot plate- and hot water-induced central pain is popularly used as an animal paradigm for assessing effects of novel compounds on central pain. It has been reported the usefulness of hot plate test in the assessment of central pain is based on natural preference and reaction time to pain of rodents to flick, lick the hind paw or jump about on the hot plate [17,18,31]. However, central analgesic activity in this test is normally judged by increased reaction time to pain, which is assumed as decreased sensitive to central pain and the ability of test compounds to prolong the tolerance to time suggests central analgesic property [31, 32]. Thus, the finding that EETC attenuates nociception induced by hot plate, as evidenced by increased reaction time to pain, further confirms its central analgesic activity and suggests a beneficial effect in conditions associated with central pain. However, EETC showed no effect on hot water tail immersion test. This is far from our expectation at the moment and may suggest a selective neurogenic effect of EETC against pain sensation and it is worthy of further investigation. Although receptor interaction study with naloxone, an opioid receptor antagonist did not demonstrate antinociceptive activity, it significantly reversed the central analgesic activity of EETC and morphine in the hot plate test. Thus, suggesting the involvement of opioidergic system in the central analgesic activity of EECT in mice.

In conclusion, the results of this study provide evidence, which suggest that ethanol extract of the leaves of *Triumfetta cordifolia* attenuated chemical and thermal nociception induced by acetic acid, formalin, hot plate and hot water immersion tests. These antinociceptive effects of EETC may be due to the present of phytochemical compounds with mechanisms related to inhibition of peripheral nociceptive mediators and central opioidergic system.

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