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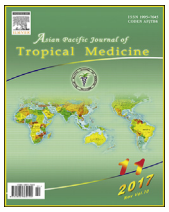

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Antioxidant, anti-inflammatory and antinociceptive potential of *Ternstroemia sylvatica* Schltdl. & Cham

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ABSTRACT

Objective: To evaluate the anti-inflammatory, analgesic, antioxidant and acute toxicity of extracts obtained from a successive extraction with solvents of ascending polarity [hexane, hex; chloroform, $CHCl_3$ and ethanol (EtOH)] of *Ternstroemia sylvatica* Schltdl. & Cham.

Methods: The antioxidant potential was evaluated by 2,2 diphenyl-1-picrylhydrazyl, the ferric reducing/antioxidant power assays and by determining the total phenolic content. The anti-inflammatory and antinociceptive effects were evaluated using the *in vivo* croton oil-induced ear edema, phorbol 12-myristate 13-acetate induced ear edema, carrageenan-induced paw edema, acetic acid-induced writhing and formalin murine models. The acute toxicity was tested using the Lorke's method in mice.

Results: The EtOH extract was the most active for the antioxidant potential tests diphenyl-1-picrylhydrazyl (68.70% inhibition), ferric reducing/antioxidant power [(2431.30 ± 102.10) mmol Fe^{2+} and total polyphenols content (215.80 ± 8.50) meqAG/g]. The anti-inflammatory activity was evaluated by topical application of croton oil (2 mg/ear dose) where the EtOH extract showed the strongest activity compared to the control group (45.13% inhibition), whereas in the phorbol 12-myristate 13-acetate model, at the same dose, the $CHCl_3$ extract showed the highest inhibition (42.88%). In the carrageenan induced edema model, the EtOH extract showed a stronger inhibition compared to indomethacin (56.34% and 50.70% at doses of 250 and 500 mg/kg of extract, respectively) during the first hour. Similarly, the same extract showed the highest analgesic activity (30.60% inhibition) in the acetic acid contortion assay, and in the formalin test it showed a greater effect with respect to the control group in both phases.

Conclusions: Our work confirms the value of *Ternstroemia sylvatica* as an important anti-inflammatory and analgesic plant, whose mechanism seems to be associated to its antioxidant effects, and supports its uses in the Mexican traditional medicine.

1. Introduction

Ternstroemia sylvatica (*T. sylvatica*) Schltdl. & Cham. (Theaceae), is a tree about 1.5 m–5.0 m high, which is very common in wooded areas of Mexico. It is commonly known as “flor de tila, “hierba del cura” or “trompillo”. This species is used in traditional Mexican medicine for treating sleep and anxiety disorders; in the State of Veracruz, the leaves are used in alcoholic cataplasms for the treatment of inflammatory and rheumatic processes [1]. Phytochemical studies on other species of the genus *Ternstroemia* such as *Ternstroemia gymnanthera*, *Ternstroemia japonica* and *Ternstroemia pringlie*, have reported the presence of triterpenes, saponins, carotenoids and the identification of jacaranone [2–6]. Pharmacological studies on *T. sylvatica* have demonstrated a sedative activity of the extracts of flowers and fruits in murine models [7,8]. Recently, the toxic effects of aqueous extracts from flowers and fruits in murine models have also been reported. This effect has been attributed to the presence of a triterpenoid glycoside identified as 28-*O*- [β-L-6-

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ramnopyranosyl]-R1-barrigenol [9]. However, there are no reports on the inflammatory and analgesic effects of *T. sylvatica* leaves.

Inflammation is a response of the cellular and humoral innate system, triggered against an infectious agent, irritation and other injuries in order to restore damaged tissue [10]. The presence of reactive oxygen species at different stages of the inflammatory process contributes to inducing a state of short-duration oxidative stress. They are important mediators that support the inflammation process, either by initiating or amplifying the responses through stimulating the release of cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin 1 (IL-1), and arachidonic acid-derived metabolites. This results in the recruitment of additional inflammatory cells at the injury site and the production of additional reactive oxygen species [11].

However, a continuous state of oxidative stress is related to multiple diseases in which the inflammatory process plays an important role, such as rheumatoid arthritis, asthma, neurodegenerative, cardiovascular, autoimmune diseases and cancer [12,13]. Inflammatory processes are part of several diseases and are directly or indirectly related to mechanisms of nociceptive stimuli generation; since inflammatory mediators such as prostaglandins (PG), TNF-α, IL-1 and interleukin-6 (IL-6) are released at the site of injury and interact with nociceptors, they facilitate the transmission of pain signals through the nervous system [14]. Pain is an uncomfortable sensation that alerts the organism of harmful situations to avoid major injuries. More than half of the patients suffering pain receive inadequate therapeutic management which severely affects their quality of life [15]. Hence, reducing the inflammatory response is an effective way of decreasing the pain process. Because inflammation involves the generation of various chemical and cellular mediators, it is difficult to identify a main component for its treatment, so there is a growing interest in the search for alternative medicinal plants that, because of their chemical composition, have a better ability to mitigate these mediators and their multiple mechanisms to treat symptoms related to a painful inflammatory process more effectively [16]; for this reason the present work evaluated the anti-inflammatory, analgesic, antioxidant and acute toxicity properties of *T. sylvatica* extracts in murine models.

2. Materials and methods

2.1. Chemicals

All chemicals used in this study were analytical grade. Methanol (MeOH), ferric chloride, acetone, sodium carbonate, sodium acetate, acetic acid, ethanol (EtOH), chloroform ($CHCl_3$), ethyl acetate (EtOAc), sodium picrate, acetic anhydride, Grignard reagent, sulfuric acid, potassium hydroxide, potassium iodine, basic bismuth salts and cobalt chloride were purchased from Merck Co (Germany). Diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu reagent, 2,4,6-tripyridyl-5-triazine, phorbol 12-myristate 13-acetate (TPA), indomethacin, carrageenan, ascorbic acid, gallic acid and thin-layer chromatography plates were purchased from Sigma–Aldrich Cod. 2193291 (St. Louis, MO, E.U.A.)

2.2. Plant collection

T. sylvatica Schltdl. & Cham (leaves) was collected in Acatlán municipality, Veracruz State, Mexico in March 2009. Plants were identified by Luis Hermann Bojorquez Galván and a voucher specimen (CIB-UV-8905) was deposited at the herbarium of the Centro de Investigaciones Biológicas, Universidad Veracruzana.

2.3. Preparation of plant extracts

The collected plant material was dried at room temperature, and then 853.0 g of dried aerial parts were extracted by maceration using solvents of ascending polarity at room temperature, 25 °C. The extraction was carried out successively using hexane (EHex, 10.0 g), chloroform ($ECHCl_3$, 12.0 g) and ethanol (EEtOH, 7.5 g) and kept in darkness at room temperature for further use. Solvents were removed using a rotary evaporator (Heidolph LABOROTA 4000) and extracts fully dried in a vacuum oven (ShelLab) at 25 °C.

2.4. Phytochemical and chemical analyses

Phytochemical analyses of the plant extracts were carried out by using standard qualitative methods (color test and/or thin layer chromatography) to detect the presence of sterols, terpenoids, coumarins, flavonoids, lignans and alkaloids [17,18]. Two mg of the EHex and $ECHCl_3$ extracts were dissolved in 5 mL $CHCl_3$, the systems of elution were Hex/AcOEt (9:1), the EEtOH was dissolved in MeOH and the elution system was Hex/MeOH (4:1).

2.5. Antioxidant activity

2.5.1. DPPH radical-scavenging activity

The radical-scavenging activity was determined according to the Brand-Williams method modified by Domínguez-Ortíz [19,20]. These determinations are spectrophotometric-based methods and are widely accepted for measuring antioxidant activity in plant extracts *in vitro* [20]. Briefly, 2.9 mL of freshly prepared DPPH solution (9×10^{-5}) M in MeOH was placed in an amber vial followed by the addition of 100 µL of plant extract solution dissolved in MeOH, all extracts were tested at three concentrations (33, 16.5 and 8.25 µg/mL) (for the blank, 100 µL of MeOH were added instead of the sample). After mixing, samples were incubated for 30 min at 37 °C in a water bath. Absorbance values of samples (A_E) and blank (A_B) were measured at 517 nm using a UV–Vis spectrophotometer (Varian Model Cary-100). Experiments were carried out in triplicate and the activity was calculated using the following formula:

$$\%\ inhibition = [(A_B - A_E)/A_B] \times 100$$

2.5.2. Total phenolic content

The total phenolic concentration was determined using the Folin–Ciocalteu reagent according to Cai and Luo [21] with some modifications. Fifty µL of each sample, 2.5 mL 1/10 dilution of Folin–Ciocalteu's reagent and 2 mL of Na_2CO_3 (7.5%, w/v) were mixed and incubated at 45 °C for 15 min. The absorbance of all samples was measured at 765 nm using a UV–Vis spectrophotometer (Varian, model Cary 100). The experiments were carried out in triplicates. Results were expressed as gallic acid equivalent GAE (mg/L) using a standard gallic acid graph (range 10–1 000 mg/L, $R^2 = 0.9965$).

Absorbance = 0.001 [GAE (mg/L)] + 0.075 4.

2.5.3. Ferric reducing/antioxidant power (FRAP)

The reductive power of samples was measured using the Benzie and Strain method [22]. FRAP reagent was prepared by mixing 100 mL of sodium acetate buffer solution (300 mM, pH 3.6), 10 mL of 2,4,6-tripyridyl-5-triazine (10 mM) in HCl 40 mM, 10 mL of $FeCl_3 \cdot 6H_2O$ (20 mM); then incubated at 37 °C for 4 min. About 2.7 mL of FRAP solution was transferred into an amber vial and added with 150 µL of methanol solution of plant extract (1 mg/mL) and 150 µL of distilled water. The absorbance was measured at 593 nm for triplicate. The blank of the experiment was prepared by adding 300 µL of distilled water to 2.7 mL of FRAP solution. The results were expressed in mmol Fe^{2+}/L, based on a calibration curve obtained from different concentrations of $FeSO_4$ (10–750 mmol/L, R^2 = 0.998 9).

Absorbance = 0.001 0 (mmol Fe^{2+}/L) + 0.166 0.

2.6. In vivo assays of animals

Male CD1 mice (20–25 g) were used for the anti-inflammatory and acute toxicity assays. All animals were maintained under standard laboratory conditions (25 °C, 12 h dark/12 h light, 50% relative humidity) according to the NOM-062-ZOO-1999 [23]. Food and water were provided *ad libitum*.

2.7. Anti-inflammatory activity

2.7.1. Croton oil (CO) induced ear edema in mice

CO, extracts $ECHCl_3$ and EEtOH of *T. sylvatica* and indomethacin were applied topically, and employed for each group (n = 6). The mice, 1 h before the induced edema, were previously treated in the inner and outer surfaces if the right ear with 50 µL $ECHCl_3$ and EEtOH of *T. sylvatica* and indomethacin to doses of 1–2 µg/ear (W_T). The ear edema was induced with 50 µg of CO 5% (v/v) in ethanol in the right ear and the left ear received only acetone (W_C). Mice were sacrificed by cervical dislocation 6 h after TPA treatment and 6 mm diameter sections of the right and left ears were cut and weighted. Anti-inflammatory inhibition activity was calculated according to the weight difference between the right and the left ear sections compared to the control group [24].

$$\%\ inh.\ edema = \frac{(W_T \times 100)}{W_C} - 100$$

2.7.2. TPA induced ear edema in mice

In this experiment, TPA and indomethacin were applied topically. In the test group, animals (n = 6) were initially treated with 2.5 µg of TPA dissolved in 25 µL of acetone in the right ear. The left ear received only acetone. After 30 min, the $ECHCl_3$, EEtOH or indomethacin (2 mg/ear dissolved in 50 µL of acetone) was applied. Mice were sacrificed by cervical dislocation 6 h after TPA treatment and 6 mm diameter sections of the right and left ears were cut and weighted. Anti-inflammatory inhibition activity was calculated according to the weight difference between the right and the left ear sections compared to the control group [25–27].

2.7.3. Carrageenan induced edema in mice

In this assay, edema was induced by subcutaneous injection of 20 µL of 1% carrageenan in saline solution. Following the administration of carrageenan, the size of the edema was measured at t = 1, 3, 5 and 7 h using a digital micrometer, t = 0 corresponded to a measurement before the administration of carrageenan [28,29]. The test groups (n = 6) were injected intraperitoneally indomethacin (4 mg/kg) or plant extract (250 mg/kg and 500 mg/kg) 30 min before administration of carrageenan. The control group only received the vehicle (tween 80-water 1:9). The percentage of inhibition was calculated comparing the measurement of the edema at different times to the measurement at t = 0. The results were analyzed according to Olajide [30].

2.7.4. Acetic acid-induced writhing test

This test was done using the method described by Collier [31]. Muscle contractions were induced in mice (n = 8) by intra peritoneal injection of 0.6% solution of acetic acid (0.1 mL/10 g). Immediately after administration of acetic acid, the animals were placed in glass cages, and the number of 'stretching' per animal was recorded during the following 20 min. $ECHCl_3$ and EEtOH (500 mg/kg and 1 000 mg/kg), ibuprofen (200 mg/kg) were administered by the intragastric route. The activity was expressed as the inhibition percent.

2.7.5. Formalin test

$ECHCl_3$ and EEtOH (500 and 1 000 mg/kg), ibuprofen (200 mg/kg) were administered by the intragastric route, 1 h before the formalin. Pain was induced with formalin via subcutaneous administration of 20 µL of 2.5% formalin into the right hind paw of the mouse (n = 8). The time of licking of the injected paw was defined as a nociceptive response, which was recorded during a 30 min period and measured every 5 min after analgesic injection. The formalin-induced licking behavior was biphasic. The initial acute phase (first phase, 0–10 min) was followed by a relatively short quiescent period that was then followed by a prolonged tonic response (second phase, 15–30 min) [32].

2.7.6. Acute toxicity (LD_{50})

Animals were fasted 12 h before oral administration via a gastric catheter and intraperitoneal route of the plant extracts (1 500–5 000 mg/kg) or vehicle (tween 80-water 1:9), n = 3. The control group only received the vehicle. Animals were observed daily for 14 d registering deaths and behavioral changes. At the end of the experiment, mice were sacrificed by clavicle dislocation, and selected organs (liver, lungs, heart, spleen and kidney) were excised, weighed and macroscopically examined [33,34].

2.8. Statistical analysis

Data are presented as means ± SEM. For statistical evaluation, comparison between experimental and control groups were performed by one-way analysis or variance followed by Tukey test. $P \leq 0.05$ were accepted as statistically significant difference.

3. Results

3.1. Antioxidant activity

DPPH and FRAP tests were carried out to evaluate the antioxidant activities in extracts of *T. sylvatica* (Table 1).

Table 1
Antioxidant activity of *T. sylvatica* extracts.

Extract	DPPH	FRAP	Total phenolics
	Inhibition (%)	(μmol Fe^{+2}/L)	(meqAG/g)
EHex	8.90 ± 0.80	206.90 ± 20.00	2.81 ± 0.40
$ECHCl_3$	7.30 ± 0.90	317.90 ± 6.00	27.80 ± 1.60
EEtOH	68.70 ± 3.20	2431.30 ± 102.00	215.80 ± 8.50
Ascorbic acid	98.00 ± 0.30	3276.50 ± 40.60	ND

ND: No determinated.

Additionally, the total polyphenol content was also measured. The EEtOH extract showed both the highest radical scavenging capacity of DPPH (68.70% at 33 μg/mL; IC_{50} 22.00 μg/mL) and the highest ferric reducing ability [(2431.30 ± 102.00) μmol Fe^{2+}/L]. The same extract showed the highest concentration of phenolic content [(215.80 ± 8.50) meqAG/g].

3.2. Anti-inflammatory effect

Table 2 showed the anti-inflammatory activity results of *T. sylvatica* extracts in the ear edema induced models with CO and with TPA. It was observed that EEtOH had a greater anti-edematous effect in the CO-induced model with a 45.13% edema inhibition, a statistically significant result compared to the control group and slightly higher than that obtained with indomethacin (39.65%). On the other hand, in the model of ear edema with TPA, $ECHCl_3$ showed the greatest effect (42.88%). However, in this model both extracts showed a lower effect compared to indomethacin (51.46% inhibition).

In the carrageenan model (Table 3), the group treated with $ECHCl_3$ at 250 mg/kg showed an edema inhibition activity after the first hour (57.74%). The best effect was then observed after 3 h (75.47%) and maintained nearly until the end of the experiment, only to decrease after 7 h (38.77%). The groups treated with EEtOH at doses of 250 mg/kg and 500 mg/kg, showed activity (56.34% and 50.70%, respectively) after the first hour of the experiments, this effect was maintained throughout the study at both doses. However, we observed that the effect was better at the lowest doses tested. It was important to note that the effect shown by the extracts was much better than that shown by our positive control, indomethacin.

3.3. Antinociceptive effect

Table 4 showed the results of the acetic acid-induced writhing test, as observed in our previous anti-inflammatory and antioxidant tests, the EEtOH at both doses (500 and 1 000 mg/kg) resulted in a significant decrease ($P < 0.05$) in the number of writhes compared with the control group. On the other hand, the $ECHCl_3$ only showed a significant activity at 1 000 mg/kg. However, none of the extracts showed a similar or higher effect in decreasing the number of writhes, compared with the control group, ibuprofen (71.70%, decrease).

The results of the formalin test in mice (Table 5) showed that in the first phase of the analgesic effect measurement, EEtOH was the most effective in inhibiting the number of licks in the study (48.70%) compared to $ECHCl_3$ and our reference compound, ibuprofen (24.90% and 37.60%, respectively). In the second phase of the experiment, only EEtOH and ibuprofen maintained an analgesic effect (25.67% and 65.40%, respectively).

Table 4
Antinociceptive effect of the *T. sylvatica* extracts on acetic acid-induced writhing in mice ($n = 8$).

Treatment	Dose (mg/kg)	Number of writhing	Inhibition (%)
Control	0	79.00 ± 4.10	0.00
$ECHCl_3$	500	65.60 ± 3.60	16.90
	1000	56.50 ± 4.70*	28.50
EEtOH	500	58.00 ± 1.70*	26.60
	1000	54.80 ± 1.80*	30.60
Ibuprofen	200	22.30 ± 2.30*	71.70

* $P < 0.05$, difference from the control group.

Table 2
Anti-inflammatory effect of *T. sylvatica* on CO and TPA induced ear acute edema in mice ($n = 6$).

Treatment	Doses (mg/ear)	5% CO induced edema		TPA induced edema	
		Weight (mg)	Inhibition (%)	Weight (mg)	Inhibition (%)
Control	0	9.66 ± 0.54	0.00	11.66 ± 0.46	0.00
$ECHCl_3$	2	7.16 ± 0.18*	25.88	6.66 ± 0.87*	42.88
EEtOH	2	5.33 ± 0.46*	45.13	8.83 ± 1.07*	24.27
Indomethacin	2	5.83 ± 0.33*	39.65	5.66 ± 0.61*	51.46

* $P < 0.05$, difference from the control group.

Table 3
Anti-inflammatory effect of *T. sylvatica* on carrageenan induced paw edema in mice ($n = 6$).

Treatment	Doses (mg/kg)	1 h		3 h		5 h		7 h	
		Length (mm)	Inhibition (%)	Length (mm)	Inhibition (%)	Length (mm)	Inhibition (%)	Length (mm)	Inhibition (%)
Control	0	0.71 ± 0.08	0.00	1.06 ± 0.13	0.00	0.72 ± 0.19	0.00	0.49 ± 0.14	0.00
$ECHCl_3$	250	0.30 ± 0.07*	57.74	0.26 ± 0.03*	75.47	0.19 ± 0.05*	73.61	0.30 ± 0.07*	38.77
	500	0.53 ± 0.11*	25.35	0.93 ± 0.15*	12.26	0.71 ± 0.23*	1.38	0.85 ± 0.15*	0.00
EEtOH	250	0.31 ± 0.10*	56.34	0.45 ± 0.08*	57.54	0.35 ± 0.11*	51.38	0.12 ± 0.07*	75.51
	500	0.35 ± 0.04*	50.70	0.23 ± 0.07*	78.30	0.16 ± 0.08*	77.77	0.26 ± 0.05*	46.93
Indomethacin	4	0.66 ± 0.20	7.04	0.73 ± 0.30	31.10	0.31 ± 0.07	56.94	0.39 ± 0.19	20.40

* $P < 0.05$, difference from the control group.

Table 5
Antinociceptive effect of *T. sylvatica* extracts on formalin test in mice (n = 8).

Treatment	Dose (mg/kg)	Licking (sec)		Inhibition (%)	
		First phase (0–10 min)	Second phase (15–30 min)	First phase (0–10 min)	Second phase (15–30 min)
Control	0	81.00 ± 2.98	59.60 ± 1.63	–	–
Ibuprofen	200	50.50 ± 1.12*	20.60 ± 1.87*	37.60	65.40
$ECHCl_3$	500	60.83 ± 5.98*	56.00 ± 1.94	24.90	6.10
EEtOH	500	41.50 ± 2.52*	44.30 ± 4.49*	48.70	25.67

* $P < 0.05$, difference from the control group.

3.4. Acute toxicity

In this study, we initially tested in the first phase of the study the acute toxicity of the extracts at 10, 100 and 1 000 mg/kg doses of both $ECHCl_3$ and EEtOH, the results showed that none of the animals administered died. In the second phase of the study, the doses 5 000 mg/kg/o.r and 3 000 mg/kg/ip.r of $ECHCl_3$ caused death of the mice during the first 24 h after administration of the extract. Additionally, the behavior of the animals was different from that of the control group, presenting a decrease in their locomotor activity, lethargy and piloerection. In the group administered with EEtOH, deaths of mice were observed when they were administered at doses of 2 000 mg/kg/ip.r and 5 000 mg/kg/o.r. The behavior in the animals administered with EEtOH was the same as that observed in $ECHCl_3$ group. LD_{50} (o.r.) for both the $ECHCl_3$ and EEtOH extracts was 3 872.98 mg/kg, when the extracts were administrated via intraperitoneally route, the LD_{50} was 2 449.48 mg/kg and 1 732.05 mg/kg for the $ECHCl_3$ and the EEtOH extracts, respectively.

4. Discussion

Previous phytochemical reports on other species of the genus *Ternstroemia* have shown the presence of main triterpenes such as oleanolic acid, primulagenina A, and A1-barrigenol and quinolic-type compounds such as jacaranona [2,5,9]. In our qualitative phytochemical analysis we were able to observe the presence of terpenoids compounds in EEtOH. It is known that terpenes are responsible for multiple ecological and physiological functions in plants, such as defense against environmental stress, insects and repair of lesions in plant tissue [35]. Physiologically, terpenoid compounds are reported to suppress the expression of nuclear factor kappa B, the main regulator in the pathogenesis of chronic inflammatory diseases such as atherosclerosis and rheumatoid arthritis. Once nuclear factor kappa B factor is activated, it migrates to the nucleus and stimulates the transcription of mediators of inflammatory processes. Studies on the anti-inflammatory effects of oleanolic acid have demonstrated the inhibitory effects on the activation of nuclear factor kappa B signaling, hence decreasing the expression of T2 cells (T_h2) and the production of cytokines such as IL-10, IL-13 and TNF-α [36].

The results observed in the antioxidant tests showed that *T. sylvatica* EEtOH offers better protection against oxidative stress in an inflammatory process. This extract showed a high content of phenolic compounds, which could be contributing to the antioxidant protection and the reduction of the expression of inflammatory and nociceptive mediators such as TNF-α and IL-1 [37]. The presence of reactive species and free radicals are known to result in adverse reactions in the organism such as tissue lesions, over stimulation of inflammatory mediators and damage to biomolecules that further increase the symptoms of the inflammation process [38].

Murine ear-induced edema models are widely accepted as pharmacological models for testing novel anti-inflammatory drugs [25]. CO induces an inflammatory response characterized by edema and leukocyte infiltration via phospholipase A_2 (PLA_2) activation. This results in an increase in cyclooxygenase-2 expression at the inflammation site [39]. On the other hand, in the TPA (a phorbol ester and main component of croton oil) model, an acute inflammatory response is observed, leading to vasodilation, platelet aggregation and leukocyte tissue infiltration [40]. All these events result from protein kinase C activation, leading to other enzymatic events, such as mitogen-activated protein kinases, increased PLA_2 activity. Hence an increase of arachidonic acid and its metabolites, such as PG and leukotrienes, thromboxanes and proinflammatory mediators such as NF-κB, TNF-α and IL-6 is observed [41,42]. Both extracts ($ECHCl_3$ and EEtOH), when tested *in vivo* in the two models showed significant edema inhibition effects compared to the control group, so the mechanisms of the anti-inflammatory effect of the compounds contained in these extracts could be associated with their capacity to regulate both PLA_2 and cyclooxygenase-2 and the metabolites derived from these enzymes [43].

It is known that an inflammatory reaction is divided into three phases. The initial phase, (0–1.5 h) is attributed to the release of histamine and serotonin. In our model this was induced in the sub-plantar edema using carrageenan. This is followed by a second phase (1.5–2.5 h), mediated by bradykinin and finally, in the third phase (2.5–6 h) PG, leukotrienes and other arachidonic acid derivatives are overproduced [44]. The results of this study showed that $ECHCl_3$ at a dose of 250 mg/kg and EEtOH at doses of 500 mg/kg significantly inhibited inflammation during phase three mainly, anti-inflammatory activities could be associated with an inhibitory effect of PG biosynthesis. However, EEtOH at 500 mg/kg doses showed a significative edema inhibition since the first hour (50% inhibition), this suggests that the extract influences other mediators such as histamine and serotonin.

The acetic acid-induced writhing test has been widely used as a tool for evaluating analgesic properties of extracts of plant species. The nociceptive properties of acetic acid are due to the release of cytokines, such as TNF-α, IL-1 and interleukin-8 by macrophages and mast cells [45]. Additionally, mediators such as histamine, serotonin and eicosanoids lead to increased levels of PG in peritoneal fluids [46]. In the present study there was a slightly significant reduction in the number of contortions for the EEtOH treated groups at the two doses used (500 and

1 000 mg/k) with a percentage inhibition of 26.50% and 30.60% respectively compared to the control group. In order to confirm this effect, the formalin test was also carried out. This model is useful not only for the evaluation of analgesic substances but also to elucidate the possible mechanism. The first phase (acute pain) begins immediately after the injection of formalin and lasts only a few minutes, and it is believed to be driven by afferent activity to a primary nociceptor. The second phase (inflammatory pain) lasts about 20–40 min. In this phase the production of PG, cyclooxygenase-2 and the release of nitric oxide are elevated [45]. Drugs that act principally on the central nervous system suppress both phases, whereas peripheral drugs only suppress the second phase [46]. The results of the formalin test show that $ECHCl_3$ does not have a remarkable inhibition percentage in any of the two phases, especially in the second (6.10%). However, EEtOH significantly suppressed the response in the first and second phases with percentage inhibition of the number of licks by 48.70% and 25.67% respectively, relative to the control group. Based on the whole set of experiments carried out in this study, the anti-inflammatory and antinociceptive assays, the pharmacological effects of *T. sylvatica* can be associated to the inhibition of cyclo-oxygen-ase and PLA_2 by terpenoid and phenolic compounds, both of them found in EEtOH, which show the highest effects in our assays. These pharmacological effects can also be associated to the antioxidant potential showed by the various types of components found in *T. sylvatica* extracts. It is important to note that this is the first study reporting on the effects of *T. sylvatica* leaves, a commonly used medicinal plant in Veracruz, Mexico, for the treatment of inflammatory and rheumatic processes. However, there is a lack of pharmacological studies that can add evidence to its traditional use. It is clear that *T. sylvatica* leaves has a significant anti-inflammatory, antinociceptive activities associated to the inhibition of cyclo-oxygen-ase and PLA_2 that seems to be related to the antioxidant mechanisms. Additionally, *T. sylvatica* shows no lethal effects at a single dose, when given orally. Finally, this study supports the therapeutic use of this species in the Mexican traditional medicine.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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