Pharmacology, Biochemistry and Behavior 95 (2010) 6–12

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Antinociceptive activity of *Annona diversifolia* Saff. leaf extracts and palmitone as a bioactive compound ☆

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ARTICLE INFO

Article history:
Received 31 July 2009
Received in revised form 28 October 2009
Accepted 30 November 2009
Available online 5 December 2009

Keywords:
Annona diversifolia Saff.
Annonaceae
Antinociception
Endogenous opioids
Palmitone
$5HT_{1A}$

ABSTRACT

Annonas are consumed as fresh fruits, but are also widely used in folk medicine for treating pain and other ailments. Antinociceptive properties of the *Annona diversifolia* ethanol crude extract were tested using the pain-induced functional impairment model in rat (PIFIR) and the writhing test in mice. The ethanol extract caused a 25% recovery of limb function in rats; this response was significant and dose-dependent. Furthermore, this extract produced a similar antinociceptive response (ED_{50} = 15.35 mg/kg) to that of the reference drug tramadol (ED_{50} = 12.42 mg/kg) when evaluated in the writhing test in mice. Bio-guided fractionation yielded hexane and acetone active fractions from which the presence of palmitone and flavonoids was respectively detected. Palmitone produced an antinociceptive response with an ED_{50} = 19.57 mg/kg in the writhing test. Antinociceptive responses from ethanol extract and tramadol were inhibited in the presence of either naloxone (1 mg/kg, s.c.)—an antagonist of endogenous opioids—or WAY100635 (0.8 mg/kg, s.c.)—a $5\text{-}HT_{1A}$ serotonin receptor antagonist. These results provide evidence that *A. diversifolia* possesses antinociceptive activity, giving support to their traditional use for treatment of spasmodic and arthritic pain. In addition, our results suggest the participation of endogenous opioids and $5\text{-}HT_{1A}$ receptors in this antinociceptive response.

1. Introduction

Annonaceae is a family of plants consisting of about 2300 to 2500 species and more than 130 genera. In fact, it is the largest family of the order Magnoliales. Only four genera—Annona, Rollinia, Uvaria and Asimina—produce edible fruits such as annona (Cañizares, 1966; Ruíz and Morett, 1997). Although annonas are generally consumed as fresh fruits, they are also widely used in folk medicine. Several reports have characterized the pharmacological activity of these plants because of their bioactive compounds (mainly acetogenins, flavonoids and alkaloids) found in roots, leaves, bark, seeds and fruit. Given their cytotoxic effect, some of these compounds are potential anti-cancer agents (Cassady, 1990; Rupprecht et al., 1990; Chang et al., 1993; Cortés et al., 1993). Other activities have been described for plants belonging to this genus, viz,: cardiotonic (Wagner et al., 1980), insecticidal (Sookvanichsilp et al., 1994), antiparasitic (Bories et al., 1991; Waechter et al., 1997), anticonvulsant (N´Gouemo et al, 1997; González-Trujano et al., 1998, 2001, 2006a) and anxiolytic-like activity (López-Rubalcava et al., 2005).

Annona diversifolia Saff. is a species belonging to the *Annona* genus; it is a tree commonly known in Mexico by many local names such as “ilama”, “ilama zapote”, “ilamazapotl” (in the Nahuatl language), “izlama”, “hilama” or “zapote de vieja” in the states of Colima, Guerrero and Mexico, whereas in the Tehuantepec region and Yucatan it is called “papausa”, “papauce” or “anona blanca” (Popenoe, 1920; Ruíz and Morett, 1997). The fruits of this plant are used as food, but its leaves are employed as anticonvulsant and analgesic, as well as an anti-inflammatory agent in traditional Mexican medicine (Estrada, 1985; 1994). Anticonvulsant properties of this species have been demonstrated in previous studies (González-Trujano et al., 1998, 2001, 2006a). However, there are no scientific reports supporting its antinociceptive and anti-inflammatory properties so that this plant can be validated in folk medicine for the treatment of pain. In the present study, these properties were analyzed by using experimental models of nociception such as the writhing test in mice and the pain-induced functional impairment model in rat (PIFIR model).

☆ All experimental procedures were carried out according to a protocol approved by the Local Animal Ethics Committee and in compliance with national (NOM-062-ZOO-1999) and international rules stated in the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

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doi:10.1016/j.pbb.2009.11.017

2. Materials and methods

2.1. Animals

Female Swiss mice (25–30 g) and male Wistar rats (180–200 g) (housed at the Instituto Nacional de Psiquiatria "Ramón de la Fuente Muñiz" and Cinvestav-Sede Sur) were used in this study. Animals were kept in a temperature- and light-controlled room under a 12 h light–dark cycle (lights on at 7:00 a.m.) with water and food *ad libitum*. Twelve hours before the experiments, food was discontinued, though animals had free access to tap drinking water. All experimental procedures followed the Guidelines on Ethical Standards for Investigations of Experimental Pain in Animals (Zimmermann, 1983) and were carried out according to a protocol approved by the local Animal Ethics Committee and in compliance with national (NOM-062-ZOO-1999) and international rules on care and use of laboratory animals (Publication No. 85-23, revised 1985). All tests were performed during the light phase. The number of experimental animals was kept to a minimum; they were used only once and sacrificed (CO_2 overdose) immediately after the experiment.

2.2. Plant material

Leaves of *A. diversifolia* Saff. were collected in Tejupilco, Guerrero in September 2005. Dr. Ernestina Cedillo-Portugal, a botanist from the Universidad Autónoma de Chapingo (UACH) certified the authenticity of the plant, a voucher specimen (AN9702) was deposited at the "Herbario de Plantas Utiles Efraím Hernández X" UACH, State of Mexico, México for future reference.

2.3. Drugs

Uric acid (nociceptive agent), naloxone (opioid antagonist) and WAY100635 (5-HT_{1A} receptor antagonist) were purchased from Sigma (St. Louis, MO, USA); acetic acid (nociceptive agent) was bought from Merck. Tramadol and acetylsalicylic acid (ASA), antinociceptive drugs, were acquired from Laboratories Grünental (Mexico City, Mexico) and Bayer (Mexico City, Mexico), respectively. Palmitone (16-hentriacontanone) was obtained as previously described (González-Trujano et al., 2001). Compounds were dissolved in saline solution (s.s.), or prepared by suspending them in 0.5% carboxymethylcellulose (ASA), mineral oil (uric acid) or 0.2–0.5% Tween 80 in s.s. (palmitone). All drugs were freshly prepared and administered in a volume of 0.1 or 0.2 mL/10 g in mice or 100 g of body weight in rats, respectively. Control animals received the same volume of the respective vehicle. Doses refer to the free base. ASA was used as a reference drug for p.o. administration, whereas tramadol was tested as positive control for the i.p. route. For each experimental procedure, animal groups consisted of either six mice or six rats. At least four doses were used in comparison to the control group to build a dose–response curve for *A. diversifolia* Saff. crude extract or reference drug.

2.4. Preparation of the extract and fractions or sub-fractions

The dried and powdered leaves of *A. diversifolia* (1.5 kg) were exhaustively extracted with hexane (28 L×3) and ethanol (28 L×3) through maceration at room temperature (22 °C) and subsequent evaporation in vacuum to give 42 g (2.8%) and 89 g (5.9%) of semisolid and syrupy hexane and ethanol crude extracts, respectively. The ethanol crude extract (32 g), which was the most active, was subjected to percolation over silica gel (1 kg) with one of the following solvents hexane (2 L), acetone (2 L) and methanol (2 L) to yield EtOH–FHexane (3.7 g, 11.7%), EtOH–FAcetone (6.3 g, 19.8%) and EtOH–FMeOH (11 g, 34.4%), respectively (Fig. 1). The most active fraction EtOH–FAcetone (5 g) was chromatographed on a silica gel column (30 g); it was initially eluted with hexane, followed by increasing amounts of ethyl acetate, then by a combination of ethyl acetate and acetone, and gradually enriched with methanol to yield 304 sub-fractions. Sub-fractions with similar thin-layer chromatography (TLC) profiles were combined to produce 24 pooled fractions as follows: P1 (7.9 mg F1–4, 0.15%), P2 (19.1 mg F5–16, 0.37%), P3 (1.6 mg F17–19, 0.31%), P4 (18.1 mg F20–27, 0.35%), P5 (8.1 mg F28–31, 0.16%), P6 (48.7 mg F32–37, 0.95%), P7 (28.2 mg F38–45, 0.55%), P8 (54 mg F46–53, 1.06%), P9 (32.3 mg F59–64, 0.63%), P10 (38.4 mg F65–79, 0.75%), P11 (18.9 mg F80–94, 0.37%), P12 (9.9 mg F95–116, 0.19%), P13 (610.2 mg F117–118, 11.97%), P14 (215.5 mg F119–120, 4.23%), P15 (120.4 mg F121–122, 2.36%), P16 (491.8 mg F123–136, 9.64%), P17 (26.2 mg F137–141, 0.51), P18 (113.4 mg F142–151, 2.22%), P19 (270.1 mg F152–192, 5.28%), P20 (1 420.5 mg F193–214, 27.9%), P21 (288.3 mg F215–260, 5.65%), P22 (23.9 mg F261–270, 0.47%), P23 (476.7 mg F271–277, 9.35%) and P24 (68.2 mg F278–304, 1.34%).

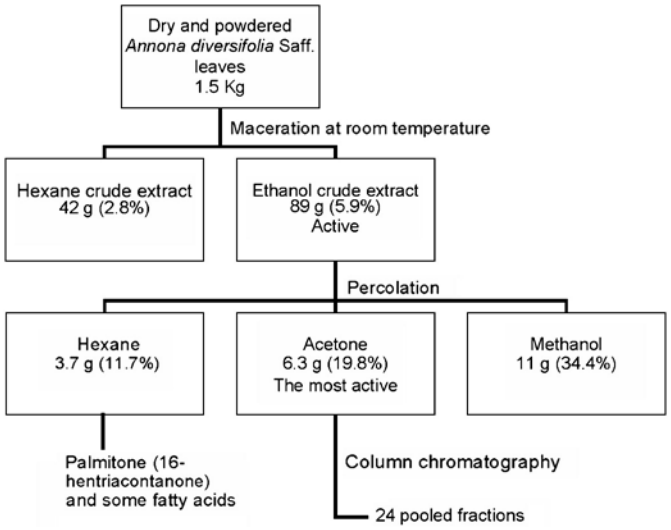

Fig. 1. Bioassay-guided fractionation of the *Annona diversifolia* Saff. leaves in the antinociceptive evaluation.

In summary, the ethanol crude extract was fractionated by percolation yielding hexane, acetone and methanol fractions which were tested in the writhing test and PIFIR model. The most active fraction evaluated in both models (acetone fractions) was in turn sub-fractioned using column chromatography. Ensuing sub-fractions with similar chromatography profiles were pooled and then evaluated in the writhing test to get the most active sub-fractions.

2.5. Antinociceptive activity evaluation

2.5.1. Writhing test

This test was performed as described by Koster et al. (1959). Antinociceptive activity was evaluated in mice by receiving hexane or ethanol crude extract at 100 mg/kg dosage via p.o or i.p. and 60 min later a 1% acetic acid injection (0.1 mL/10 g i.p.) was applied. The number of abdominal writhes was cumulatively counted in 5-min intervals over a 20-min period immediately after the acetic acid injection. Abdominal writhing was considered as a nociceptive behavior; it was defined as an exaggerated extension of the abdomen combined with the outstretching of the hind limbs. Control animals received vehicle and were similarly evaluated as above. Ethanol crude extract was the most active of the two; therefore, it was evaluated at 30, 100 and 300 mg/kg i.p. Active fractions were assessed at 100 mg/kg, whereas sub-fractions were tested at doses calculated depending on the yield obtained in 100 mg of the most active fraction.

2.5.2. PIFIR assay

Under light anesthesia with ether, nociception was induced by injection of 50 µL of 20% uric acid into the knee joint of the right hind

limb (intra-articular: i.art.) of each rat. After uric acid injection, animals developed a progressive dysfunction of the injured limb. Rats, with an attached electrode to the plantar surface of each hind paw, were allowed to recover from anesthesia and then placed on a stainless steel cylinder, 30 cm in diameter. The cylinder was rotated at 4 rpm forcing the rats to walk. When the electrode made contact with the cylinder floor, a circuit was closed and the time that the circuit remained closed was recorded. The variable measured in this model was the time of contact between each of the hind paws and the cylinder. The time of contact of the injured hind limb reached a zero value 2 to 2.5 h after uric acid injection. Rats were forced to walk on the rotating cylinder for 2-min intervals and allowed to rest between recording periods. Data are expressed as the percentage of the functionality index (FI%), i.e., the time of contact of injected foot divided by the time of contact of the control left paw multiplied by 100 (López-Muñoz et al., 1993).

Once the FI% reached the zero value, different groups of rats received the following treatments: Control groups: s.s. or 0.5% Tween 80 in s.s. via i.p., p.o., or s.c. Experimental groups: *A. diversifolia* ethanol extract (30, 100, 300 and 600 mg/kg, p.o.); naloxone (1 or 3.16 mg/kg, s.c.), or WAY 100635 (0.8 mg/kg, s.c.) alone or in combination with either *A. diversifolia* ethanol extract (300 and/or 600 mg/kg) or tramadol (30 mg/kg i.p.). Recordings were taken every 15 min for the first hour, and thereafter every 30 min until 4 h elapsed. Recovery of FI% was considered as an expression of the antinociceptive effect. A time–response curve of *A. diversifolia* was plotted to detect the onset and maximal antinociceptive effect. This methodology permitted determination of the time course of antinociceptive effects in the same animal. The area under the curve (AUC) as pharmacological parameter of antinociceptive efficacy was selected and determined in the temporal course. For the purpose of this study, inducing nociception in the experimental animals was unavoidable. However, care was taken to avoid unnecessary suffering.

An analysis to assess the possible mechanism of action was undertaken, in which either naloxone (1 or 3.16 mg/kg), an antagonist of endogenous opioids, or WAY100635 (0.8 mg/kg), a selective antagonist of 5-HT_{1A} serotonin receptors, was subcutaneously (s.c.) administrated 15 min before active compounds in both nociception models.

2.6. Phytochemical analysis

Conventional qualitative TLC analysis was performed to detect the presence of flavonoids, triterpenes and alkaloids in the active extracts. The chromogenic agent (1% diphenylboryloxyethylamine) NP-5% polyethylenglycol-400 (PEG) was employed for flavonoid detection (Wagner et al., 1996), whereas sulphuric acid/anisaldehyde or Dragendorff's reagents was used for the possible presence of triterpenes or alkaloids, respectively.

2.7. Acute toxicity (LD_{50})

The acute toxicity of the ethanol and hexane crude extracts in mice was estimated by i.p. and p.o. routes using the procedure reported by Lorke (1983). The method estimates the dose of a certain compound that will kill 50% of the animal population (LD_{50}) by a given route of administration. Extracts were administered at 10, 100 and 1000 mg/kg in the first stage. When 100% of mortality was induced at 10, 100 or 1000 mg/kg, a second dose stage was tried in which doses were reduced. Conversely, when no mortality was found at 10, 100 or 1000 mg/kg, the dose was increased according to the table described by Lorke. Mice were kept under observation for the following 14 days, their weights registered, and at the end of the study a macroscopic evaluation was carried out.

2.8. Data analysis

Data are expressed as the mean ± standard error of the mean (S.E.M.). The area under the curve (AUC) values were calculated from the respective temporal course curves obtained in the writhing and PIFIR assays, which were considered as expressions of the overall nociceptive or antinociceptive activity during 20 min or 4 h observation periods, respectively; maximal values were reached at 500 area units (au) and 387.5 au, respectively. AUC values were calculated using the trapezoidal rule (Rowland and Toser, 1989). All data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test or Student's *t*-test using SIGMA STAT® software, version 2.3. $P<0.05$ was considered statistically significant.

3. Results

3.1. Effect of A. diversifolia Saff in the writhing test

Fig. 2 shows that the ethanol crude extract administered either p.o. or i.p. produced a significant and dose-dependent antinociception on mouse abdominal constrictions induced by 1% acetic acid in mice. In this model, a stronger activity was obtained when this extract was tested i.p. [antinociceptive efficacy (%): 97.7 ± 1.86] rather than p.o. [antinociceptive efficacy (%): 67.2 ± 5.4]; its effect resembled that produced by tramadol as a positive control [antinociceptive efficacy (%): 99.18 ± 1.78]. Tramadol with an $ED_{50} = 12.42$ mg/kg, i.p. appeared to be the most potent of the assayed compounds. The i.p. administration of ethanol crude extract with an $ED_{50} = 15.35$ mg/kg was less potent than tramadol, but both compounds exhibited a higher efficacy than the ethanol crude extract administered p.o. A lower potency ($ED_{50} = 21.30$ mg/kg) was also observed for this last treatment. All fractions of the ethanol crude extract (100 mg/kg) obtained by percolation and tramadol (50 mg/kg) significantly decreased ($F_{5,30} = 85.55$, $P<0.001$) abdominal contractions induced by 1% acetic acid in mice. Antinociceptive activity of the acetone fraction was followed by the responses obtained with hexane and methanol fractions, in that order (Table 1). From the hexane fraction, as previously described by González-Trujano et al (2001), the presence of palmitone was detected. As a possible active principle, the antinociceptive response of this ketone was analyzed in a dose–response curve (10, 30, 100, 300 and 1000 mg/kg); its administration via p.o. served to obtain $ED_{50} = 19.57$ mg/kg (Fig. 2). Since the acetone fraction was the most active in producing antinociception in mice, as

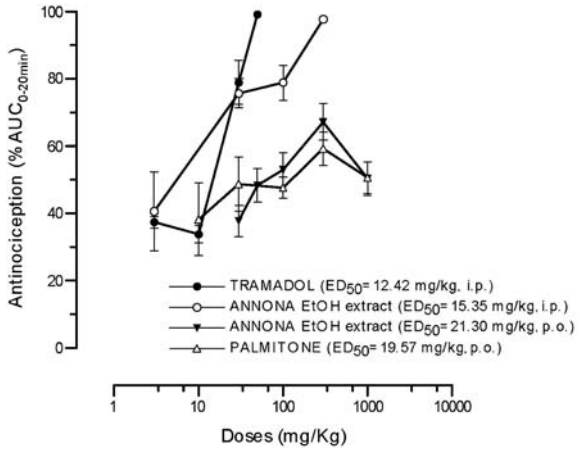

Fig. 2. Dose–response curves of the antinociceptive activity of the ethanol (EtOH) crude extract of *Annona diversifolia* Saff. leaves (3 to 300 and 1000 mg/kg) administered i.p. (○) or p.o. (▼) in comparison to tramadol (3 to 50 mg/kg, i.p.) (●) and palmitone (10 to 1000 mg/kg, p.o.) (△). Each point represents the average (mean ± SEM, $n = 6$) of antinociception (in percentage) taken as the area under the curve during a 20-min observation period after administration ($AUC_{0-20\ min}$).

Table 1
Antinociceptive effect of fractions obtained from the *Annona diversifolia* Saff. leaf ethanol extract on the abdominal contractions induced by acetic acid at 1% in mice ($n=6$ per group).

Treatment	Dose (mg/kg)	Latency to the first abdominal constriction (min)	Nociception ($AUC_{0-20\ min}$)	Antinociception (%)
Vehicle	–	3 ± 0.2	497.9 ± 37.6	–
Tramadol	50	18 ± 1.9	5.0 ± 4.1*,#	99
Ethanol crude extract	100	7 ± 0.3	107.1 ± 27.3*	78
Fraction hexane	100	7 ± 0.5	82.1 ± 19.6*	84
Fraction acetone	100	8 ± 1.4	72.1 ± 17.4*	86
Fraction methanol	100	4 ± 0.3	246.3 ± 30.4*,#	51

*,#$F_{5,30}=85.55$, $P<0.001$, one-way ANOVA followed by Dunnett's test vs vehicle and ethanol crude extract, respectively.

well as the one inducing the longest delay in the presentation of the first abdominal constriction (Table 1), it was processed by column chromatography and obtained enough yield to be tested in the writhing test (Table 2). Fifteen of the 24 pooled fractions were evaluated at doses which corresponded to their respective content found into the acetone fraction. Although none of these tested fractions produced the same degree of antinociception observed with the acetone fraction (81%), individually, each of them produced at least more than 50% of the antinociception, except for pooled fraction P11, which produced 43% (Table 2). The most active pooled fractions were P5, P16 and P24 showing 73%, 71% and 77% of antinociception, respectively (Table 2).

3.2. Effect of A. diversifolia Saff. in the PIFIR model

Controls receiving vehicle (s.s. or 0.5% Tween 80 in s.s.) or hexane crude extract (100 mg/kg) by i.p. or p.o. route of administration showed no recovery of the functionality index during the 4-h period experiments (Fig. 3A). The ethanol crude extract at 100 mg/kg p.o. produced the same antinociception—shown as the area under curve $AUC_{0-4\,h}$ (recovery of the loss of functionality induced by 20% of uric acid in a 4-h period)—than rats treated with this extract via p.o.

Table 2
Effect of pooled fractions obtained from the *Annona diversifolia* Saff. EtOH–FAcetone on the abdominal contractions induced by acetic acid at 1% in mice ($n=6$ per group).

Pooled fraction	Dose (mg/kg)	Nociception ($AUC_{0-20\ min}$)	Antinociception (%)
Vehicle	–	488.33 ± 37.30 au	–
Tramadol	50	5.00 ± 4.08	99
Acetone fraction	100	91.67 ± 19.45[a]	81
P5	5	131.25 ± 35.25[a,b]	73
P9	5	220.00 ± 10.35[a]	55
P10	0.75	215.42 ± 7.54[a]	56
P11	5	281.67 ± 18.27[a]	43
P13	12	192.50 ± 29.74[a]	61
P14	4.2	163.75 ± 19.96[a,b]	66
P15	2.4	170.00 ± 18.92[a,b]	65
P16	10	140.42 ± 17.47[a,b]	71
P17	2.2	187.08 ± 13.68[a]	62
P18	2.2	155.42 ± 30.12[a,b]	68
P19	5.3	259.20 ± 25.66[a]	47
P20	28	244.60 ± 15.23[a]	50
P21	5.6	198.33 ± 37.71[a]	59
P22	1	181.25 ± 34.79[a]	63
P24	1.3	109.58 ± 46.32[a,b]	77

[a] $F_{16,85}=9.19$, $P<0.001$, one-way ANOVA followed by Dunnett's test vs control.
[b] $F_{16,85}=9.19$, $P<0.001$, one-way ANOVA followed by Dunnett's test vs acetone fraction.

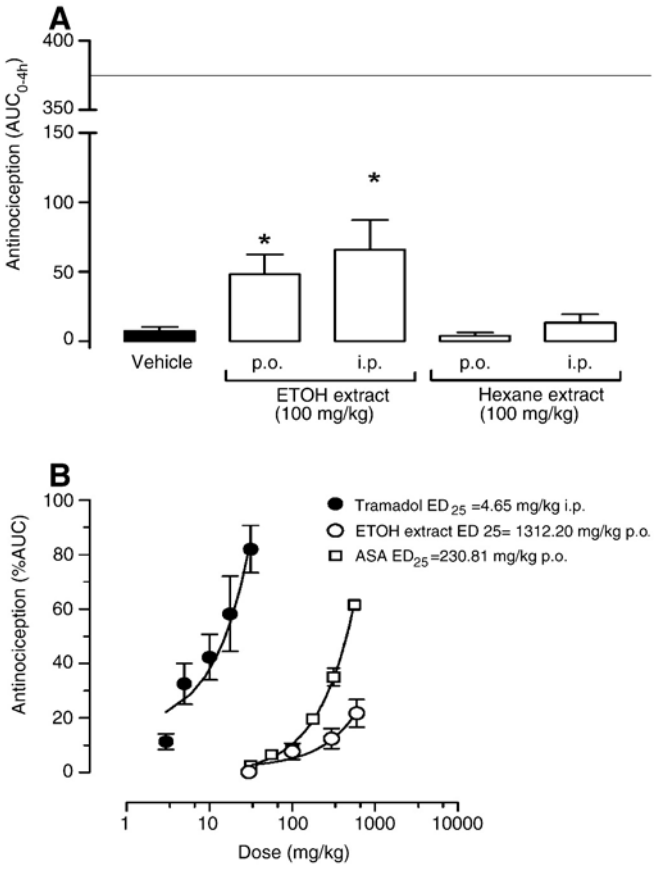

Fig. 3. Antinociceptive evaluation in the PIFIR model. A) Effect of hexane (100 mg/kg) and EtOH (100 mg/kg) crude extracts via i.p. and p.o. in comparison to vehicle (0.5% Tween 80 in s.s.) group. *$P<0.001$, one-way ANOVA followed by Dunnett's test. B) Dose–response curves of the ethanol (EtOH) crude extract (30 to 600 mg/kg, p.o.) (○) in comparison to tramadol (3 to 50 mg/kg, i.p.) (●). Each point represents the average (mean ± SEM, $n=6$) of antinociception (in percentage) taken as the area under the curve during a 4-h observation period after administration ($AUC_{0-4\,h}$).

(Fig. 3A). However, less variability was observed using the p.o. route, which produced a significant effect ($F_{4,25}=3.02$, $P<0.037$) in comparison to the vehicle group. A dose of ethanol crude extract of 30 mg/kg did not produce recovery (Fig. 3B), whereas 100, 300 and 600 mg/kg produced a significant ($F_{4,34}=10.12$, $P<0.001$) dose-dependent antinociceptive response (Fig. 3B). The dose–response curve of the ethanol crude extract showed that 25% of the maximal antinociceptive response was obtained with the highest dose tested (600 mg/kg, p.o.) when compared with the dose–response curve of tramadol in the PIFIR model. Tramadol ($ED_{25}=4.65$ mg/kg) was more potent than the ethanol crude extract ($ED_{25}=1\,312.20$ mg/kg, p.o.) in this model (Fig. 3B). Acetone and hexane fractions derived from the ethanol crude extract exhibited a significant ($F_{5,30}=5.88$, $P<0.001$) response in the PIFIR model tested at 300 mg/kg and 600 mg/kg, respectively (Fig. 4). The observed effect resembled that in rats treated with ASA at 300 mg/kg (Fig. 4). Minor activity was observed for hexane and methanol fractions at 300 mg/kg, which was significant and similar to those observed with ASA and palmitone, both tested at 100 mg/kg (Fig. 4).

The antinociceptive response produced by 300 mg/kg of the active ethanol crude extract in the writhing test was significantly prevented in the presence of naloxone (1 mg/kg) and in lesser proportion in the presence of WAY100635 (0.8 mg/kg) (Fig. 5A), whereas, the anti-nociceptive response produced by 300 or 600 mg/kg of this extract was partially or totally prevented in the PIFIR model, in the presence of the same doses of naloxone or WAY100635, respectively (Fig. 5B).

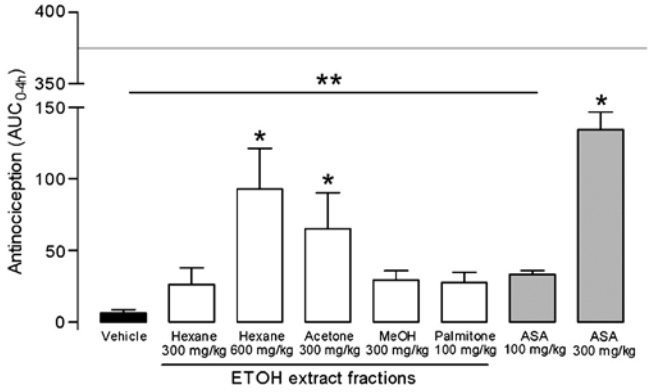

Fig. 4. Antinociception of hexane, acetone and methanol fractions obtained from the ethanol (EtOH) crude extract of *Annona diversifolia* Saff. leaves, as well as palmitone in comparison to vehicle (0.5% Tween 80 in s.s.) evaluated via p.o. in the PIFIR model. Acetylsalicylic acid (ASA) was used as a reference drug. Bars express the average (mean ± SEM, $n = 6$) of the area under the curve during a 4-h observation period after administration. *$P<0.001$, one-way ANOVA followed by Dunnett's test. **$P<0.001$, Student's *t*-test vs vehicle group.

For tramadol, a partial prevention of the antinociceptive response was observed in the presence of either WAY100635 (0.8 mg/kg) or naloxone (3.16 mg/kg) (Fig. 5B).

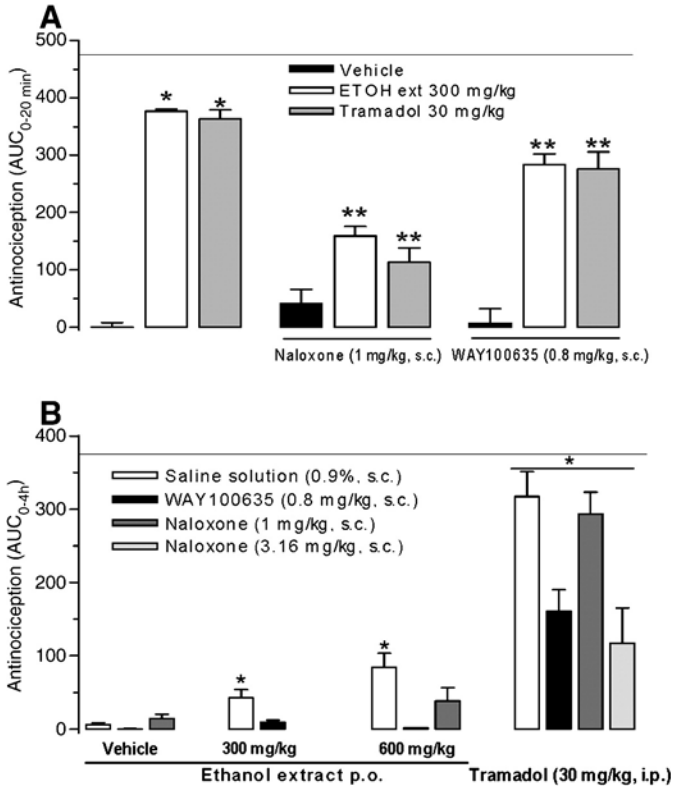

Fig. 5. A) Antinociceptive response of the active EtOH crude extract of *Annona diversifolia* Saff. leaves and tramadol via i.p. alone and in the presence of either naloxone (1 mg/kg, s.c.) or WAY100635 (0.8 mg/kg s.c.) in comparison to the respective control groups receiving vehicle (0.2% Tween 80 in s.s.) or s.s. alone or in the presence of their respective antagonists in the writhing test. Bars express the average of the area under the curve of at least 6 animals (mean ± SEM) during a 20-min observation period after administration. $P<0.001$, one-way ANOVA followed by Dunnett's test. *EtOH and tramadol alone vs vehicle. **Treatments in the presence of the antagonist vs treatment alone. B) Antinociceptive response of the active EtOH crude extract of *Annona diversifolia* Saff. leaves (300 and 600 mg/kg, p.o.) alone or in the presence of either naloxone (1 and 3.16 mg/kg, s.c.) or WAY100635 (0.8 mg/kg s.c.) in comparison to the respective control groups receiving vehicle (0.5% Tween 80 in s.s.) or s.s. alone and in the presence of their respective antagonists in the PIFIR model. Bars express the average (mean ± SEM, $n = 6$) during a 4-h observation period after administration. *$P<0.001$, one-way ANOVA followed by Dunnett's test.

3.3. Phytochemical screening

After percolation of the ethanol crude extract, the resulting fractions were chemically analyzed. The hexane fraction yielded triterpenes, as well as palmitone and other fatty acids such as palmitic, oleic and stearic acids. From the active acetone fraction and pooled sub-fractions, positive spots to flavonoids were detected by TLC analysis using the NP/PEG reagent. Finally, in the methanol fraction, the presence of alkaloids was also detected.

3.4. Acute LD_{50} study

No macroscopic tissue injury or weight loss was observed in mice surviving i.p. or p.o. administration of hexane or ethanol crude extract. The LD_{50} obtained in mice for hexane and ethanol extracts administered via i.p. was 141 mg/kg and 707 mg/kg, respectively. Whereas the LD_{50} calculated for tramadol, used as reference drug for i.p. administration, was 118 mg/kg (Table 3). On the other hand, with regard to the hexane crude extract administered p.o., the LD_{50} was 400 mg/kg (Table 3). Since a 33% mortality was observed at the highest dose tested for the ethanol crude extract (5000 mg/kg), it was not possible to determine its LD_{50} when using this route of administration (Table 3).

4. Discussion

In Mexican traditional medicine, maceration in ethanol and decoction of *A. diversifolia* leaves are commonly used preparations for topic and oral administration to cure spasmodic and arthritic pain (Estrada, 1985; González-Trujano et al., 1998). In spite of the analgesic properties attributed to this species, to our knowledge, there are no scientific studies corroborating this activity. In the present study, the antinociceptive activity of hexane and ethanol crude extracts of *A. diversifolia* Saff. was investigated, as well as the activity of some fractions and sub-fractions initially obtained from the most active fraction using bio-guided fractionation and assayed with the writhing test and/or the PIFIR model in mice and rats, respectively. Furthermore, acute toxicity was evaluated by LD_{50} determination of the crude extracts in mice.

Visceral pain is the response we feel when our internal organs are damaged or injured and it is by far the most common form of pain

Table 3
Effect of *Annona diversifolia* Saff. on the determination of acute lethal dose 50 (LD_{50}) via i.p. and/or p.o. in mice.

Treatment	1st stage		2nd stage		
	Dose (mg/kg)	Animals dying (%)	Dose (mg/kg)	Animals dying (%)	LD50 (mg/kg)
Via i.p.					
Hexane extract	10	0	140	33	
	100	**0**	**200**	**100**	141
	1000	100	600	100	
Ethanol extract	10	0	600	66	
	100	**0**	2900	66	707
	1000	66	**5000**	**100**	
Tramadol	10	0	**140**	**100**	
	100	**0**	200	100	118
	1000	100	600	100	
Via p.o.					
Hexane extract	10	0	**1600**	**100**	
	100	**0**	2900	100	400
	1000	33	5000	100	
Ethanol extract	10	0	1600	33	
	100	0	2900	33	>5000
	1000	0	5000	33	

Geometric mean on the doses for which 0% and 100% were found was estimated as LD_{50}.

(Cervero, 2000). In animal models, the writhing test is used to assess this kind of pain (Koster et al., 1959). In this study, the ethanol crude extract of *A. diversifolia* showed a differential antinociceptive efficacy when visceral nociception was induced by 1% acetic acid in mice either i.p. (97.7 ± 1.86%) or p.o. (67.2 ± 5.4%). In fact, the antinociceptive efficacy of the ethanol extract i.p. was similar to that observed for tramadol (99.18 ± 1.78%) when the same route of administration was utilized. As previously mentioned, palmitone was isolated as a bioactive chemical substance in *Annona* species to test its anxiolytic (López-Rubalcava et al., 2005; González-Trujano et al., 2006b) and anticonvulsant (González-Trujano et al., 2001, 2006a) effects. In this study, palmitone produced an antinociceptive response with an ED_{50} = 19.57 mg/kg, p.o. tested in the writhing test reinforcing its participation as a bioactive constituent in *Annona* species. It could be appreciated that using a test of visceral pain, in all cases, AUC increased in a dose-dependent manner. Tramadol was the most potent of the assayed drugs, followed by ethanol extract i.p. or palmitone p.o. The least potent was ethanol extract p.o., which also exhibited a limited efficacy.

On the other hand, the PIFIR model is a test validated to induce similar inflammatory and chronic nociception to that observed in clinical gout arthritis (López-Muñoz et al., 1993). Non-steroidal anti-inflammatory drugs (NSAIDs) are primarily used to treat inflammation and mild to moderate pain (Maclagan, 1876). In fact, specific uses for NSAIDs include the treatment for headaches, arthritis, sports injuries, and menstrual cramps. In our study, the *A. diversifolia* ethanol extract increased, in a dose-dependent and significant manner, the functionality index analyzed in rats with the PIFIR model. The antinociceptive response produced by this extract at 100 mg/kg in both i.p. or p.o. routes resembled the one produced by ASA, a NSAID drug, tested at the same dose and route of administration. When ethanol extract was increased to 600 mg/kg dosage, the antinociceptive response reached 25% efficacy of total antinociception. Fractionation of the ethanol crude extract produced an increase in the efficacy for both hexane (600 mg/kg) and acetone (300 mg/kg) fractions resembling the significant effect produced by ASA at 300 mg/kg. Palmitone was also evaluated at 100 mg/kg, p.o. demonstrating the same efficacy on the recovery of the functionality index as that of rats receiving ASA (100 mg/kg). These results indicate that palmitone is a bioactive compound that partially participates in the antinociceptive effect of *A. diversifolia* and its efficacy depends on the kind of nociceptive model tested.

Fractionation of the ethanol crude extract allowed the observation of various components in the hexane and acetone fractions that showed a partial participation in the antinociceptive effect. In the most active fraction (the acetone fraction) the presence of flavonoids was detected by qualitative analysis. Fifteen of the 24 pooled sub-fractions recovered after column chromatography produced different levels of antinociception in the same test suggesting participation of more than one constituent. Lizana and Reginato (1990) have reported the presence of caffeine in seeds of *Annona cherimolia*. It has been considered that caffeine might act as an analgesic adjuvant. In fact, our group has observed that a combination of NSAIDs with caffeine potentiates the antinociceptive response in the PIFIR model (Díaz-Reval et al., 2001; López et al., 2006). Moreover, quercetin, a natural anti-inflammatory flavonoid, has been isolated from the seeds of *Annona squamosa*. In a previous study, our group examined the antinociceptive properties of this flavonoid (Martínez et al., 2009). Since specific phytochemical studies concerning *A. diversifolia* are lacking we cannot discard the participation of these compounds in the antinociceptive activity of this species.

In *A. diversifolia* extracts, as well as in fractions and sub-fractions derived from these extracts, the presence of terpenes, flavonoids and alkaloids was detected by conventional qualitative TLC. These kinds of compounds are potentially useful natural products in medicine, together with unsaturated and saturated fatty acids, such as oleic, linoleic, palmitoleic, palmitic and stearic acids, previously identified in this species (González-Trujano et al., 2001). Some of these components were considered as possibly involved substances in the antinociceptive effect of *A. diversifolia* in this preliminary study. However, further investigation is needed to clarify the specific compounds involved in this effect.

Tramadol is a centrally-acting analgesic considered an effective drug in both experimental and clinical pain. The antinociceptive effect of tramadol in animals and its analgesic effect in humans are considered to be produced by the combined contribution of both opioid (Koga et al., 2005) and non-opioid analgesic mechanisms (Raffa et al., 1992; Kayser et al., 1992; Desmeules et al., 1996; Oliva et al., 2002; Barann et al., 2006; Berrocoso et al., 2006; Ide et al., 2006). These findings are in accordance with our results because the presence of either naloxone or WAY100635 partially reverted the antinociceptive effect of tramadol in both pain models. In fact, because of its antinociceptive efficacy in the two nociception models tested in this study, which involve different mechanisms of action, tramadol was chosen as a reference drug to be compared with the antinociceptive effects of *A. diversifolia*. The antinociceptive response produced by the ethanol crude extract was also differentially reverted in the presence of either antagonist in these two pain models. Previous studies have described the participation of a partial (buspirone) or total (8-OH-DPAT) 5-HT_{1A} receptor agonist and even some flavonoids in the antinociceptive effect using the PIFIR model (Martínez et al., 2009) and in models of abdominal nociception (Bardin et al. 2001; Sivarao et al., 2004). These results suggest and reinforce the involvement of serotoninergic neurotransmission mediated by 5-HT_{1A} receptors, as well as the participation of endogenous opioids in the antinociceptive effect of *A. diversifolia* and tramadol.

With regard to *A. diversifolia* toxicity, a sedative effect was shown in rats injected (i.p) with 600 mg/kg or higher doses of the ethanol extract; this effect was also observed at 1000 mg/kg p.o. A sedative effect was previously reported in mice treated with ethanol extract at doses higher than 200 mg/kg i.p. evaluated 30 min or 1 h after administration (González-Trujano et al., 1998). According to the Lorke method (1983), LD_{50} values in mice for hexane and ethanol crude extracts and tramadol administered i.p. were 141 mg/kg, 707 mg/kg and 118 mg/kg, respectively. For the oral administration, the LD_{50} calculated for the hexane extract was 400 mg/kg; it was not possible to calculate the LD_{50} for the ethanol extract by this route of administration, since a 33% mortality was observed with the highest dose (5000 mg/kg) tested. According to Victoria et al. (2006) administration of aqueous or ethanol extracts of *A. squamosa* by p.o. route produces no acute toxicity when tested in rats or mice at doses of 10 and 15.67 g/kg, respectively. However, Sookvanichsilp et al. (1994) described that *A. squamosa* seems to produce no toxicity to ear skin, but causes mild toxicity to rabbit eyes. In fact, toxicity from ethanol extracts obtained from other *Annona* species has been described as dependent on the part of the plant used—leaves against seeds, for example—and on the type of experimental model. In humans, atypical Parkinsonism has been associated with the consumption of infusions or decoctions prepared from leaves of *Annona muricata* L. in which acetogenins—mainly annonacin—have been detected. Annonacin is considered potentially toxic given its capacity for inducing brain lesions in rats (Champú et al., 2004); particularly, neurodegeneration of dopaminergic neurons in basal ganglia, as observed in human pathology (Champy et al., 2005; Lannuzel et al., 2006). Since an intravenous infusion was used in these last experiments and toxicity in our study was higher when i.p. administration was compared to the p.o. route, it appears that the form of administration is of key importance for the presence of adverse effects produced by Annonas. Therefore, more studies are necessary to establish the safety of the traditional use of decoction of Annona leaves to treat health problems, though the oral route is probably the most recommended.

In conclusion, these results provide evidence to support that plants of the *Annona* genus are a good source for the extraction of compounds with several pharmacological activities including those acting on the

CNS. Our study reinforces for the first time the utilization of the leaves of *A. diversifolia* to treat pain and inflammation. Our results demonstrate that although the ethanol extract of this species was partially efficacious in reducing arthritic nociception, it was able to produce total antinociception in visceral nociception at the dosages tested. Preliminary analyses indicate the presence of flavonoids in *A. diversifolia* and the participation of palmitone, in its antinociceptive activity. Our results also give some indications concerning the mechanism of action which might involve $5HT_{1A}$ receptors and endogenous opioids.

Acknowledgments

We thank Mr. Rubén Luviano, Mr. Luis Oliva and Mr. Froylán Sánchez for their technical support. We are also grateful to M. Sanchez-Álvarez for proof reading the English version of this manuscript and Dr. Benito Anton for allowing us access to the equipment facilities. This study was supported by INP3280A and CONACYT-80811 grants.

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