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Antinociceptive, Anti-inflammatory Effects and Safety of *Ziziphus mistol* Fruits

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ABSTRACT

Ziziphus mistol Griseb. (Rhamnaceae), popularly known as "mistol," is widely distributed throughout Perú, Bolivia, Paraguay and Argentina. Its fruit is consumed in different forms in several argentinean communities and used against biliary colic, dysentery, cold stomach and diseases of the respiratory system characterized by pain and inflammation. The present study was carried out to investigate the medicinal properties and safety of *Ziziphus mistol* (mistol) fruits ethanol and aqueous extracts and arrope. Antinociceptive activity was assessed using the formalin, acetic acid-induced writhing and tail-flick tests in rats. Anti-inflammatory effects were determined through carrageenan induced edema test and cotton pellet-induced granuloma formation, in rats. The safety was evaluated with test of acute toxicity (48 hours) and sub-chronic toxicity (91 days). All extracts (1,000 mg/kg b.w.) showed significant inhibition (P <0.05) in the three model of pain experimentally induced in comparison to control. In a combination test using naloxone, diminished analgesic activity of aqueous extract and arrope were observed, indicating that their antinociceptive activity is connected with the opioid receptors. At dose 1000 mg/kg bw, the aqueous extract and arrope showed higher anti-inflammatory activity than the ethanol extract, in carrageenan and cotton pellet granuloma model used. In the acute toxicity study, a single dose of 4000 and 8000 mg/kg b.w., produced no mortality and no clinical signs of disease were observed after 48 hours. In the sub-chronic toxicity study the extracts no caused significant visible signs of toxicity, nor mortality for 91 consecutive days of treatment. Extracts and arrope of *Z. mistol* fruits could be good source of antinociceptive and anti-inflammatory agents because of its good activity and safety.

Keywords: Antinociceptive, anti-inflammation, *Ziziphus mistol*, toxicity, rats.

INTRODUCTION

Ziziphus mistol Griseb., Rhamnaceae, is a plant widely distributed throughout Perú, Bolivia, Paraguay and Argentina. Its fruits have long been used in folk medicine in many preparations like "mistol tea" (infusion prepared with the fruit) used against biliary colic, dysentery, cold stomach, indigestion, coughing and as an antidote for the bites of poisonous snakes and insects. [1-2] The fruits can be eaten directly and can also be used to manufacture a sweet called arrope. Furthermore arrope has also employed to promote human health. The fruit and the derivate products have

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multiple traditional uses for the low. [3] This ancestral use continues today. Cardozo *et al*., demonstrated its antioxidant and anti-inflammatory properties *in vitro* and absence of genotoxic activity. [2]

The current study was designed to provide a scientific background for the traditional characteristics assigned to mistol and to check if activity was maintained in time in the main product, the arrope. The objective the present investigation was to validate the traditional indications in the folkloric medicine assigned to mistol, when administered orally to animal models. This work constitutes the first validation study of the antinociceptive and anti-inflammatory activities of *Ziziphus mistol* (mistol) *in vivo*. The acute toxicity of the aqueous extract and arrope was evaluated with a single high dose. In addition, long term studies are essential to determine a range of bioactivities for a no observed adverse effect level (NOAEL). [4]

Since subchronic toxicity studies can provide more information on the possible health hazards of test substances due to repeated exposure over a prolonged period of time, a 13-weeks subchronic oral toxicity test on *Ziziphus mistol* fruits aqueous extract and arrope was conducted in rats in this study.

MATERIALS AND METHODS

Plant material

The plant material used in this study consisted of fruits of *Ziziphus mistol* (mistol) collected during season of maturation of December-January (2013-2014) in Icaño, in the province of Santiago del Estero, Argentina. The specimen was identified by using morphological, anatomical and histochemical techniques, by Lic. Nora Muruaga. A voucher specimen LIL N°: 612552, was deposited in the herbarium of the Fundación Miguel Lillo, Tucumán, Argentina.

Preparation of ethanol and aqueous extracts of *Z. mistol* fruits

The first extraction of the fruits was performed with ethanol 96º after of 5 contact days (maceration) at room temperature under constant shaking and filtered with Whatman No 1 filter paper, for obtaining ethanol extract (EE). Then, the residue was extracted with boiled distilled water during 20 minutes and filtered, for obtaining aqueous extract (AE). The filtrates obtained from each extraction were concentrated under vacuum. The yields were 6.13% w/w and 13.09% w/w for the ethanol and aqueous extracts respectively. Dry extracts were stored at 4°C until used.

Preparation of arrope (Ar)

The mistol fruits were washed and boiled in water over medium heat, they were stirred with a wooden spoon from the time that the pulp begins to fall apart. They were boiled until a thick and creamy liquid syrup was formed. It was later filtered through a fine mesh. Through data obtained from literature, one can estimate the yield of 14%.

Animals

Wistar male rats were used (220-240 g). All animals were kept under normal laboratory conditions of humidity, temperature (25 ± 1ºC) and light (12 h dark/light cycle), and allowed free access to food and water *ad libitum*. The studies were conducted in accordance with the internationally accepted principles for laboratory animal use and care (EEC Directive of 1986; 86/609/EEC). All the experimental protocols were duly approved by the CICUAL (Comité Institucional para el Cuidado y Uso de Animales de Laboratorio) de la Universidad Nacional de Tucumán, under the current research project.

Antinociceptive assays

Formalin-induced nociception

The formalin test was carried similar to that described by Gorzalczany *et al*. [5] Rats were injected with 20µl of 2.5% formalin solution, into the sub-plantar region of the right hind paw 30 min after treatment with sterile water (control, *p.o.*), extracts (250, 500 and 1000 mg/kg b.w.) and reference drugs ibuprofen syrup (100 mg/kg b.w.) and morphine syrup (1 mg/kg b.w.). Licking time of the injected paw, was recorded as nociceptive response at 0-5 min (neurogenic phase) and 15-30 min (inflammatory phase) after formalin injection.

Involvement of opioid receptors

Four groups of animals received naloxone (1 mg/kg, *i.p.*, a non selective opioid receptor antagonist) to evaluate the participation of the opioid system. [6] These animals received morphine (1 mg/kg, *p.o.*), ethanol extract, aqueous extract and arrope (1000 mg/kg, *p.o.*) 15 min after naloxone administration. The formalin test was subsequently performed.

Acetic acid-induced writhing method

The acetic acid method was carried out as described by Koster *et al*. [7] Thirty minutes before to acetic acid injection, rats (n = 6 per group) were treated with ethanol extract, aqueous extract and arrope (250, 500 and 1000 mg/kg b.w., *p.o.*), sterile water (control, *p.o.*), morphine syrup (1 mg/kg b.w., *p.o.*) and ibuprofen syrup (100 mg/kg b.w., *p.o.*). Each group was administered 10 ml/kg b.w., *i.p.*, of an aqueous solution of acetic acid (1.0%). After five minutes the rats were observed and the number of writhing was counted for 30 min.

Tail immersion test

To evaluate the central analgesic property the tail immersion test was performed. [8] One to two cm of tail of the rats pretreated with EE, AE and Ar (250, 500 and 1000 mg/kg b.w., *p.o.*), morphine syrup (1 mg/kg b.w., *p.o.*), ibuprofen syrup (100 mg/kg b.w., *p.o.*) and sterile water (*p.o.*) were immersed in warm water kept constant at 54 ± 0.5°C. The latency between tail immersion and deflection of tail was recorded. A latency period of 20 s was maintained to avoid tail tissue damage in rat. The latency period of the tail withdrawal response was taken as the index of antinociception and was determined at 30, 60, 90, 120 and 150 min after the administration of the drug and

extracts. To avoid tissue injury, the cut-off time was set at 20 s. [9]

Anti-inflammatory study

Carrageenan-induced hind paw edema

The anti-inflammatory activity of the extract was evaluated according to the method of Winter *et al*. [10] Groups of six rats each were treated with the EE, AE and Ar (250, 500 and 1000 mg/kg b.w., *p.o.*), ibuprofen (100 mg/kg b.w., *p.o.*) and sterile water (2 ml/kg). Thirty minutes after the administration of the various agents, edema was induced by carrageenan injection (0.1 ml, 1%, w/v in saline solution) into the subplantar tissue of the right hind paw. The paw volume was measured before administering carrageenan (Vo) and 1, 2, 3, 4 and 6 h after (Vt).

Cotton pellet-induced granuloma formation

Male rats weighing 180-200 g were randomly divided into seven groups of six rats each. Two sterilized cotton pellets (20 mg) were implanted subcutaneously, one on each side of the abdomen in all groups, under light ether anesthesia. Rats in groups I (control group) received vehicle. Rats in groups II and III received ibuprofen and meprednisone, at the dose of 100 y 5 mg/kg/day, respectively. Rats in groups IV to IX received ethanol extract, aqueous extract and arrope at the dose of 500 and 1000 mg/kg/day respectively. Each test substance was administered for 7 d. On the eighth day, each rat was anesthetized. The rats were then sacrificed and the implanted pellets as well as the thymus were dissected out and determined for their wet and dry weights (dried at 60 ± 1°C for 18 h). [11]

Acute oral toxicity study

The animals were divided into five groups, with six animals each. They were treated orally with a single dose of the AE and Ar of mistol dissolved in distilled water and at doses of 4000 and 8000 mg/kg in 10 ml/kg volume. The control group received distilled water as a single dose. All animals were observed after treatment. The parameters evaluated were: death, alertness, sedation, ptosis, dyspnea, urination, diarrhea, convulsions, spontaneous motor activity, postural reflex, piloerection, response to touch, among others. The total number of deaths in each group was quantified by the end of the period of 48 hours. [12]

Sub chronic toxicity study

Aqueous extract and arrope were administered orally at doses of 1000 and 2000 mg/kg b.w., daily during 13 weeks. [12] The control group received sterile water. At the end of the 13 weeks experiment, all the animals were anaesthetized and blood samples were collected via cardiac puncture both biochemical and hematological analyses, respectively. The sacrificed rats were then dissected. Lung, spleen, heart, liver, pancreas and kidneys, were observed macroscopically in situ, based on the position, color, shape, size, weight and consistency of the organs.

Hematological and biochemical parameters

The parameters determined included: red blood cell count (RBC), white blood cell count (WBC), platelets count, hemoglobin and hematocrit. The blood chemistry tests were determined: alanine aminotransferase (ALT), gamma glutamyltransferase (GGT), bilirubin (BIL), urea, creatinine (CREA), albumin (ALB), total protein (PROT).

Statistical analysis

All experimental values are expressed as the mean ± the standard deviation of at least two independent experiments. Statistically significant differences from the vehicle group were identified by Student's test or ANOVA followed by Tukey test for paired data. The level of $p \leq 0.05$ was used to determine statistical significance.

RESULTS

Formalin-induced pain

Overall, the ethanol extract, aqueous extract and arrope showed a significant ($P < 0.05$) antinociceptive activity in both phases of the formalin-induced paw licking test (Figures 1A and 1B) with the dose of 1000 mg/kg b.w. Morphine was used as positive control (1 mg/kg b.w., *p.o.*) and the response time of the animals decreased significantly when compared to negative control in both phases, while the other positive control, ibuprofen (100 mg/kg b.w., *p.o.*), was effective only in the second phase (Fig 1B). Treatment with naloxone (1 mg/kg b.w., *i.p.*) greatly reversed the antinociceptive activity of AE (1000 mg/kg b.w., *p.o.*). So did morphine (1 mg/kg b.w., *p.o.*) in both neurogenic and inflammatory phases of formalin induced nociception (Figures 1A and 1B). Ar (1000 mg/kg b.w., *p.o.*), only reversed the antinociceptive activity in the first phase. As criterion for reversal of activity, was taken the loss of over 50% of the initial activity.

Acetic acid-induced writhing method

The oral antinociceptive doses of the extracts (EE and AE) and arrope (1000 mg/kg b.w.) produced a significant inhibition of acetic acid *i.p.* induced abdominal contrition in rats (Fig. 2).

The calculated inhibition for the EE, AE and arrope were 46.30%, 55.96% and 53.21% respectively, significantly lower compared with dose morphine (89.90%) and ibuprofen (94.50%).

Tail immersion test

A significant reduction of the painful sensation due to tail immersion in warm water was observed following oral administration of the ethanol extract, aqueous extract and arrope at doses of 500 and 1000 mg/kg b.w. (Table 1). The inhibitory effects of the aqueous extract and arrope became pronounced at 90 min, 106.79 % and 83.55% respectively, post dosing 1000 mg/kg b.w. The inhibitory effect of the ethanol extract became pronounced at 120 min (66.40%) at the same dose. The antinociceptive properties of the aqueous extract at this dose were similar to those of morphine (104.75%) at 60 min. Ibuprofen had no effect in this test.

Carrageenan-induced rat paw edema

In the carrageenan induced edema test, the average right back paw volumes by the extracts and standard

drug are shown in Table 2. Rats pre-treated with the ethanol extract, aqueous extract and arrope showed a significant reduction of the edema 3.0 h post dosing of 1000 mg/kg b.w. (70.10, 90.00 and 100.00% respectively). This behaviour is similar to the standard ibuprofen (100%) dose of 100 mg/kg b.w., p.o.

Cotton pellet-induced granuloma formation

Ibuprofen and meprednisone, ethanol extract, aqueous extract and arrope (1000 mg/kg/d), significantly reduced transudative and granuloma weights as shown by their granuloma inhibition of 45.56%, 57.10%, 29.64%, 40.59% and 33.85% respectively (Table 3). It was also found that the dry thymus weight were not significantly different among groups (control, ibuprofen, ethanol extract, aqueous extract and arrope), except in the meprednisone group which revealed a significant decrease from those of control group.

Toxicity studies

Studies of acute and sub-chronic toxicity were performed with aqueous extract and arrope, who had the highest pharmacological activity and is the most common form of consumption in rural communities.

Acute toxicity

No deaths or toxic symptoms were observed in any of the animals after oral administration of the different doses of the aqueous extract and arrope. There were no changes in body weight or food and water intake between the control and the treated groups. The treated rats did not present any behavioral alterations during the assessment period (48 h).

Table 1: Effect *Ziziphus mistol* fruits aqueous extract and arrope on pain with the tail immersion test

Treatment	Dose (mg/Kg, *p.o.*)	Interval following treatment (h)					
		0.0	0.5	1.0	1.5	2.0	2.5
		Reaction time (seg)					
Control	SW	2.10 ± 0.10	2.25 ± 0.20	2.30 ± 0.15	2.35 ± 0.15	2.30 ± 0.20	2.25 ± 0.09
Ibuprofen	100	2.10 ± 0.15	2.35 ± 0.25	2.35 ± 0.12	2.32 ± 0.20	2.29 ± 0.15	2.12 ± 0.10
Morphine	1	2.10 ± 0.05	3.85 ± 0.25 *	4.40 ± 0.19 *	4.00 ± 0.30 *	3.40 ± 0.15 *	2.95 ± 0.17 *
Ethanol Extract	250	2.20 ± 0.05	2.30 ± 0.15	2.35 ± 0.25	2.40 ± 0.10	2.65 ± 0.20	2.50 ± 0.25
	500	2.15 ± 0.03	2.30 ± 0.10	2.45 ± 0.22 *	2.55 ± 0.09 *	2.72 ± 0.25 *	2.49 ± 0.27
	1000	2.10 ± 0.15	2.35 ± 0.30	2.69 ± 0.09 *	2.82 ± 0.21 *	3.50 ± 0.25 *	3.15 ± 0.23 *
Aqueous Extract	250	2.10 ± 0.09	2.25 ± 0.22	2.60 ± 0.15	2.90 ± 0.20 *	2.75 ± 0.23 *	2.65 ± 0.30
	500	2.15 ± 0.09	2.39 ± 0.22	2.82 ± 0.15	3.18 ± 0.20 *	2.85 ± 0.22 *	2.72 ± 0.15 *
	1000	2.10 ± 0.05	2.94 ± 0.16 *	3.21 ± 0.15 *	4.34 ± 0.27 *	3.19 ± 0.15 *	2.88 ± 0.13 *
Arrope	250	2.20 ± 0.05	2.35 ± 0.25	2.50 ± 0.30	2.65 ± 0.35	2.50 ± 0.25	2.20 ± 0.22
	500	2.20 ± 0.09	2.55 ± 0.22 *	2.78 ± 0.15 *	2.95 ± 0.27 *	2.54 ± 0.19 *	2.17 ± 0.20
	1000	2.15 ± 0.15	3.15 ± 0.20 *	3.57 ± 0.26 *	3.85 ± 0.18 *	3.19 ± 0.21 *	2.42 ± 0.25

Values represent the mean ± SEM and are in seconds (n=6). * The asterisks denote the significance levels compared with the control group, $p < 0.05$ (one-way ANOVA, followed by Tukey's test). SW (sterile water).

Table 2: Effect of *Ziziphus mistol* fruits aqueous extract and arrope on carrageenan induced rat paw edema

Group (n=6)	Dose (mg/kg *p.o*)	Paw edema vol in ml (Mean ± S.E.)					
		0 H[a]	1 H[a]	2 H[a]	3 H[a]	4 H[a]	6 H[a]
Control	SW	1.40 ± 0.10	1.70 ± 0.05	1.85 ± 0.10	1.90 ± 0.05	2.20 ± 0.10	1.90 ± 0.20
Ibuprofen	100	1.40 ± 0.05	1.45 ± 0.15 *	1.40 ± 0.10 *	1.40 ± 0.10 *	1.45 ± 0.15 *	1.45 ± 0.05 *
Ethanol Extract	250	1.40 ± 0.10	1.70 ± 0.20	1.75 ± 0.20	1.85 ± 0.20	1.95 ± 0.25	1.85 ± 0.10
	500	1.40 ± 0.15	1.65 ± 0.20	1.75 ± 0.15	1.80 ± 0.15 *	1.90 ± 0.10 *	1.83 ± 0.15
	1000	1.40 ± 0.10	1.60 ± 0.15	1.63 ± 0.05 *	1.55 ± 0.10 *	1.80 ± 0.10 *	1.80 ± 0.25
Aqueous Extract	250	1.40 ± 0.05	1.65 ± 0.15	1.70 ± 0.05 *	1.65 ± 0.10 *	1.90 ± 0.15 *	1.85 ± 0.05
	500	1.40 ± 0.10	1.60 ± 0.20	1.60 ± 0.10 *	1.55 ± 0.15 *	1.80 ± 0.15 *	1.80 ± 0.20
	1000	1.40 ± 0.10	1.58 ± 0.05 *	1.50 ± 0.10 *	1.45 ± 0.00 *	1.65 ± 0.13 *	1.65 ± 0.15 *
Arrope	250	1.40 ± 0.05	1.65 ± 0.15	1.70 ± 0.05 *	1.75 ± 0.15 *	1.95 ± 0.10 *	1.80 ± 0.25
	500	1.40 ± 0.05	1.60 ± 0.25	1.62 ± 0.10 *	1.52 ± 0.15 *	1.83 ± 0.20 *	1.80 ± 0.20
	1000	1.40 ± 0.10	1.50 ± 0.10 *	1.52 ± 0.10 *	1.40 ± 0.20 *	1.60 ± 0.10 *	1.60 ± 0.15 *

Values are expressed in mean ± SEM (n = 6). [a]Time after carrageenan injection (h). The asterisks denote the significance levels compared with the control group, $p < 0.05$ (one-way ANOVA, followed by Tukey's test). SW (sterile water).

Table 3: Effect of *Ziziphus mistol* fruits aqueous extract and arrope on cotton pellet-induced granuloma formation in rats

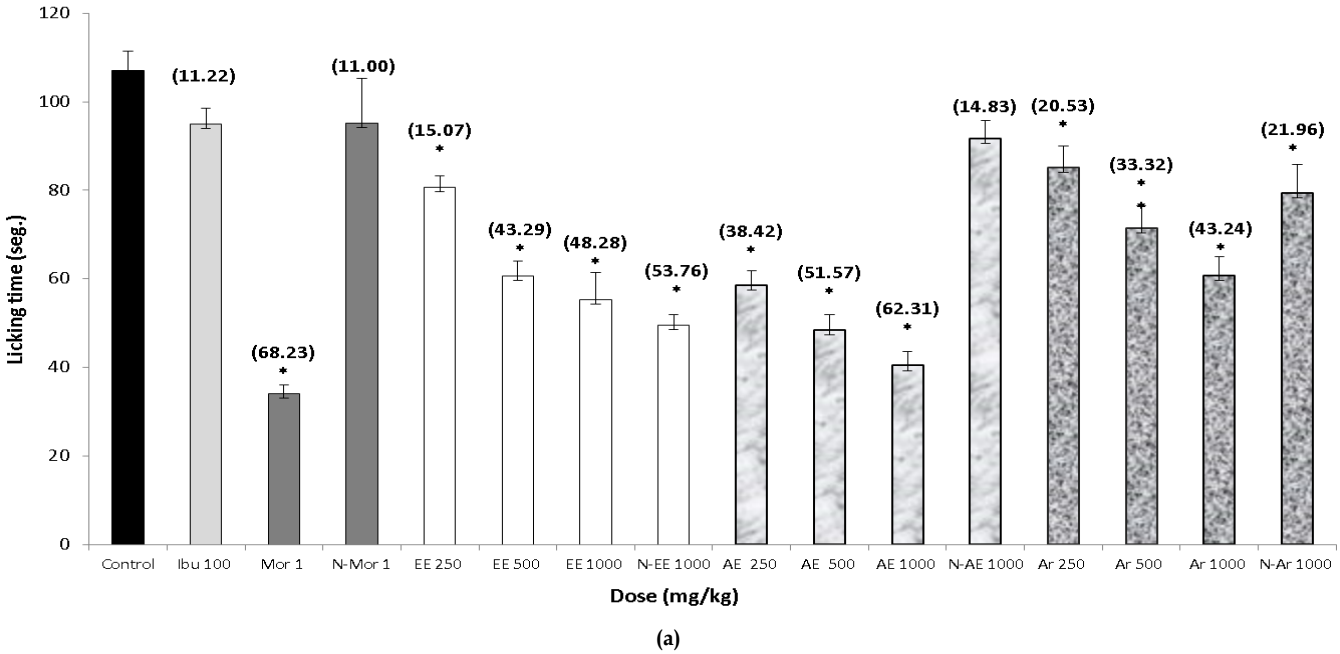
		Cotton pellet-induced granuloma formation			
Groups (n=6)	Dose (mg/kg/d)	Transudative weight (mg)	Granuloma weight (mg)	Granuloma inhibition (%)	dry Thymus weight (mg/100 g BW)
Control	SW	594.35 ± 25.60	156.70 ± 3.30	--	30.49 ± 2.06
Ibuprofen	100	178.90 ± 15.50 (*)	76.20 ± 2.80 (*)	45.56	33. 24 ± 5.40
Meprednisone	5	165.10 ± 14.85 (*)	55.80 ± 9.00 (*)	57.10	22.61 ± 3.95 (*)
Ethanol Extract	500	581.50 ± 20.15	144.50 ± 13.25 (*)	7.78	31.50 ± 8.05
	1000	470.75 ± 15.05	110.25 ± 10.40 (*)	29.64	32.90 ± 5.00
Aqueous extract	500	550.65 ± 16.15	130.45 ± 16.45 (*)	16.75	32.50 ± 6.50
	1000	406.00 ± 11.60 (*)	93.10 ± 4.10 (*)	40.59	33.43 ± 4.30
Arrope	500	565.15 ± 14.00	139.50 ± 12.50	10.97	34.15 ± 3.50
	1000	416.95 ± 10.60 (*)	103.65 ± 10.65 (*)	33.85	33.81 ± 3.80

Values are expressed as mean ± S.E.M. (n=6). TrW: Transudative weight, GrW: Granuloma weight, GI: Granuloma inhibition, BW: Body weight, TW: Thymus weight. * Significantly different from the control group, $p < 0.05$. SW (sterile water)

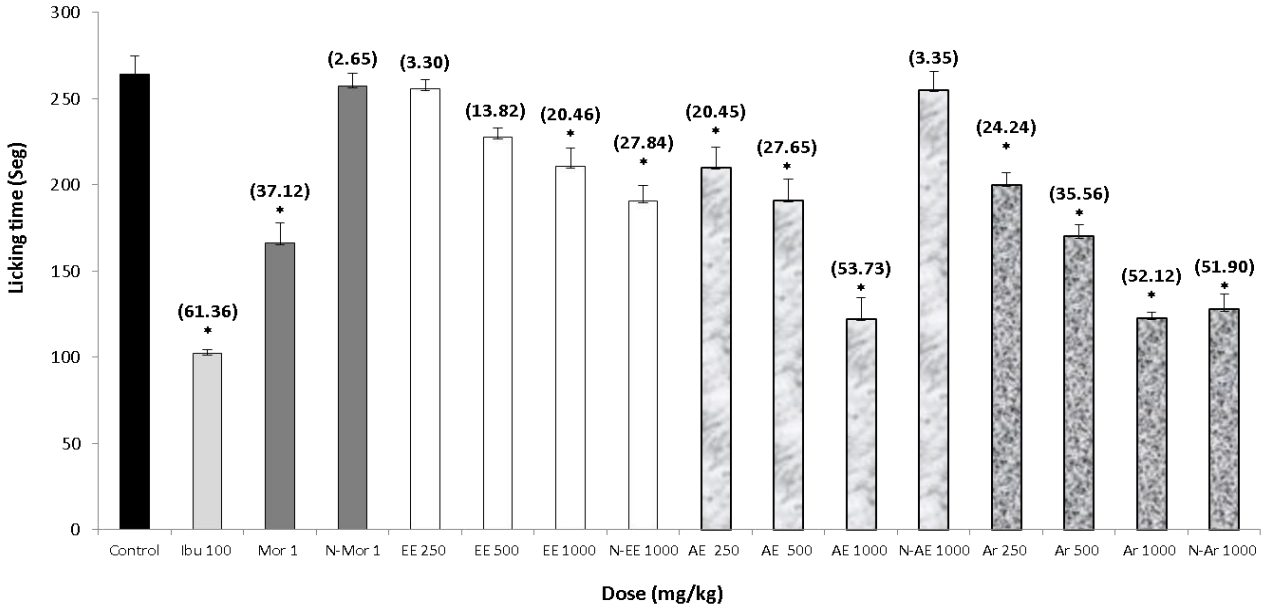
Table 4: Effects of *Ziziphus mistol* fruits aqueous extract (AE) and arrope (AR) in hematological and biochemical blood parameters of rats

Haematological and biochemical parameters	Dose group (expressed in mean±S.D., n=6)				
	Control	AE 1 g/Kg	AE 2 g/Kg	Ar 1 gr/Kg	Ar 2 gr/Kg
RBC (10^6 mm3)	8.35 ± 0.13	7.77 ± 0.17	8.41 ± 0.15	7.70 ± 0.07	8.07 ± 0.22
WBC (10^3 mm3)	5.40 ± 0.35	5.00 ± 0.36	5.75 ± 0.40	5.20 ± 0.36	6.00 ± 0.10
Haematocrit (%)	51.86 ± 4.86	45.40 ± 0.40	51.57 ± 1.77	46.50 ± 1.00	47.10 ± 1.90
Platelet (10^6 mm3)	1.03 ± 0.03	0.95 ± 0.04	1.01 ± 0.05	0.95 ± 0.04	1.05 ± 0.14
Hemoglobin (gr %)	16.70 ± 1.30	14.50 ± 0.05	16.15 ± 0.55	14.50 ± 0.05	15.25 ± 0.75
Urea (gr/l)	0.47 ± 0.03	0.44 ± 0.04	0.50 ± 0.06	0.45 ± 0.05	0.48 ± 0.05
Creatinine (mg/l)	6.53 ± 0.77	6.60 ± 1.20	7.55 ± 0.50	7.60 ± 0.10	8.10 ± 1.10
GGT (mU/ml)	13.00 ± 1.50	11.90 ± 2.50	12.50 ± 2.50	10.90 ± 1.50	11.30 ± 1.70
ALT (UI/l)	49.50 ± 1.45	50.50 ± 7.50	49.67 ± 5.60	53.50 ± 7.50	54.50 ± 3.50
Bilirrubin (mg %)	0.57 ± 0.45	0.44 ± 0.04	0.80 ± 0.05	0.54 ± 0.04	0.62 ± 0.10
Protein (gr/dl)	6.70 ± 0.50	6.00 ± 0.25	6.50 ± 0.40	5.90 ± 0.50	6.10 ± 0.10
Albumin (gr/dl)	4.15 ± 0.15	3.75 ± 0.25	4.05 ± 0.23	3.70 ± 0.30	3.87 ± 0.22

Groups of animals were pre-treated with aqueous extract (AE 250-500-1000 mg/kg b.w.) and arrope (AR 250-500-1000 mg/kg b.w.). Values are expressed in mean ± SEM (n = 6). No significant differences compared with the control group.



(a)



(b)

Fig. 1: Effect of *Ziziphus mistol* fruits extracts and arrope on the nociceptive response of the formalin test in first phase (a) and second phase (b). Control, ibuprofen (Ibu 100 mg/kg b.w.), morphine (Mor 1 mg/kg b.w.), ethanol extract (EE 250-500-1000 mg/kg b.w.), aqueous extract (AE 250-500-1000 mg/kg b.w.), arrope (Ar 250-500-1000 mg/kg b.w.), naloxone-morphine (N-Mor 1mg/kg b.w.), naloxone-ethanol extract (N-EE 1mg/kg-1000 mg/kg b.w.), naloxone-aqueous extract (N-AE 1mg/kg-1000 mg/kg b.w.) and naloxone-arrope (N-Ar 1mg/kg-1000 mg/kg b.w.). Values in parentheses are percentage of inhibition. * The asterisks denote the significance levels compared with the control group, $p < 0.05$ (one-way ANOVA, followed by Tukey's test). Values represent the mean ± SEM (n=6)

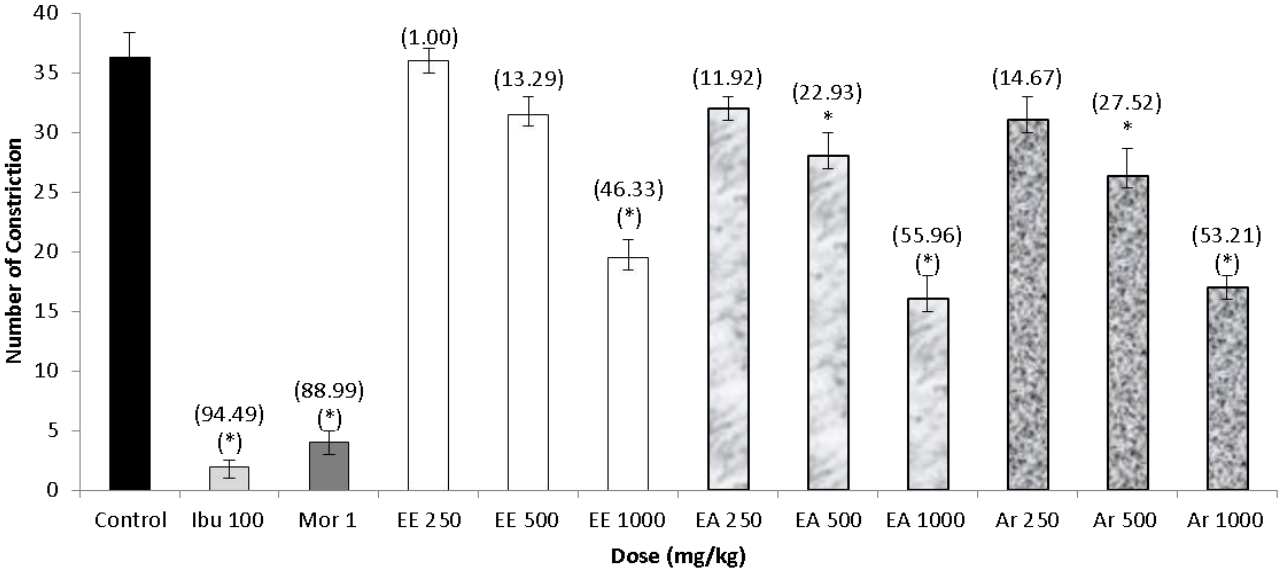


Fig. 2: Effect of oral administration on acetic acid induced writing in rats. The intensity of nociception behavior was cuantified by counting the total number or writhes occurring 20 min following the stimulus injection. Rats were orally treated with control, ibuprofen (Ibu 100 mg/kg b.w.), morphine (Mor 1 mg/kg b.w.), ethanol extract (EE 250-500-1000 mg/kg b.w.), aqueous extract (AE 250-500-1000 mg/kg b.w.) and arrope (Ar 250-500-1000 mg/kg b.w.). Values in parentheses are percentage of inhibition. * The asterisks denote the significance levels compared with the control group, $p < 0.05$ (one-way ANOVA, followed by Tukey's test). Values represent the mean ± SEM (n=6)

These results suggest that single oral doses of 4000 and 8000 mg/kg b.w. are safe to use in rats.

Subchronic toxicity study

Daily oral administration of *Z. mistol* aqueous extract and arrope for 91 consecutive days did not induce any obvious symptom of toxicity in rats even with the highest dose tested of 2000 mg/kg b.w. daily. There were no differences or significant changes in general behavior, body weight, food intake between the treated rats and those of the control group. Both the control and treated rats appeared uniformly healthy at the end and throughout the period of study. No deaths occurred at any of the doses administered. There was no significant effect on relative weights of liver, heart, spleen, kidneys, lung and pancreas between the treated and control rats. No treatment related gross pathology was observed. No significant changes were detected in the body weight ratio of the animals.

Hematological and biochemical parameters

The hematological and biochemical parameters data at end of the study are presented in Table 4. No significant changes between the treated and control groups measured at the dose of 1000 and 2000 mg/kg b.w. All the hematological and biochemical parameters tested were within normal clinical values throughout the period of treatment.

DISCUSSION

The results of this study showed that the *Ziziphus mistol* extracts and arrope had a significantly antinociceptive effect on three classical nociception models in rats: the formalin, the acetic acid induced writhing and the tail immersion tests, all of which are useful methods for screening prospective antinociceptive compounds. In relation to the anti-inflammatory effect, using the carrageenan test and the cotton pellet assay, both extracts and arrope showed anti-inflammatory activity. These two pharmacological activities support the traditional use of this fruits.

The formalin test represents a model of persistent pain. This test can also be used to determine the ability of new compounds to affect peripheral or central nociceptive pathways due to its biphasic nociceptive characteristics, known as the early and late phases that result from formalin administration. [13] The early phase, classified as a neurogenic pain, is an acute response observed immediately after the formalin injection and persists for 5 min (0–5 min) as a result of a its direct action on nociceptors. The late phase, classified as an inflammatory pain, is a tonic response resulting from the inflammatory processes generated by the release of inflammatory mediators. Centrally acting drugs (opioids) inhibit both phases, while peripherally acting drugs (NSAIDs) inhibit only the late phase. Based on the results obtained, the EE, AE and Ar of *Z. mistol* possess central and peripheral antinociceptive actions and additional anti-inflammatory activity. [14] It is also interesting to note that the pretreatment with a non selective opioid receptor antagonist, naloxone, reverses the antinociceptive effect of aqueous extract, arrope and morphine in the formalin induced paw licking test. Together, these results strongly suggest that the opioid system and central antinociception effect were involved in the induced antinociception for the aqueous extract and arrope. However, the ethanol extract does not exhibit opioid-mediated antinociceptive activity at the peripheral and central levels, which could suggest the presence of other chemical constituents with action at central level but with no opioid mechanisms.

The acetic acid induced abdominal constriction test, described as a typical model for inflammatory pain [15] and, in most cases, as a model to study the peripheral antinociceptive effect of extracts/compounds. Its algesic effect is due to the liberation of an increased level of several mediators such as histamine, serotonin, bradykinin, cytokines and eicosanoids in the peritoneal fluid. These mediators increase vascular permeability and eventually stimulate local peritoneal nociceptors. [16] In this study, EE, EA and arrope at the dose of 1000 mg/kg b.w. significantly reduced the number of writhing episodes in rats indicating the inhibition of acetic acid induced visceral nociception.

The tail immersion test evaluates a possible central action in which opioid agents exert their analgesic effects via supraspinal and spinal receptors. [8] The aqueous extract and arrope reached their maximum analgesic level 90 min and ethanol extract at 120 min after administration, similar to morphine. Ibuprofen did not show any activity in this test.

The carrageenan test was used because of its sensitivity in detecting orally active anti-inflammatory agents particularly in the acute phase of inflammation. [17] Its first phase (0 – 3 h after injection) results from the concomitant release of histamine, serotonin and kinin mediators on vascular permeability. The second phase is correlated with the high production of prostaglandins, oxygen-derived free radicals, and inducible cyclooxigenase. [18] The cotton pellet granuloma on the other hand, is a model of chronic inflammation. The dry weight of the pellet correlated well with the amount granulomatous tisuue. [11] Oral administration of *Z. mistol* fruit extracts (EE and AE) and arrope exhibited significant anti-inflammatory activity in the two models. Based on the results, the anti-inflammatory effects of extracts and arrope, may be mediated by the inhibition of prostaglandin biosynthesis. [2]

The current investigation, suggest that *Z mistol* fruits contain potential molecules with antinociceptive and anti-inflammatory activities. Phenolic compounds are very important for their biological activities. Cardoso et al. relates antioxidant and anti-inflammatory activities in vitro with the phenolic content of *Z. mistol*. [2] Other authors demonstrated beneficial properties of medicinal plants establishing relations between anti-inflammatory, analgesic and phenol/flavonoid content. [19-21] Additionally, the manufacturing process of arrope of *Z. mistol* not alter their antinociceptive and anti-inflammatory properties, unlike for arrope of chañar (*Geoffroea decorticans*) whose antinociceptive action in second phase (inflammatory pain) drops significantly, probably due to modifications caused by cooking. [22] It could be suggested that the antinociceptive and anti-inflammatory effects for *Z. mistol* extracts and arrope in this study, may be caused by the polyphenolic constituents and / or other thermo stable constituents present in the plant.

Our studies provide additional evidence of the safety of *Z. mistol* fruit aqueous extract and arrope at higher doses than those that produce a measurable anti-inflammatory and anti-nociceptive effect in animal models. The extract and arrope did not produce any mortality or alter the behavioral patterns of the rats during the acute toxicity testing; similar results were observed with other plant using the same toxicological method. [22] In the subchronic toxicity study, the aqueous extract and arrope at doses of 1000 or 2000 mg/kg b.w./day for 91 days did not produce any sign of toxicity in the treated rats, and no deaths were recorded. Treated rats body weight did not show significant variations when compared with that of control rats and no significant alterations were recorded in absolute or relative organ weight. Both control and treated groups appeared uniformly healthy at the end of the experiment. An important index to diagnose whether an organ has been exposed to injury is the organ to body weight ratio. [23] In the present study, the aqueous extract and arrope did not induce changes in rat's organ-to-body weight ratios or organ morphology. In addition, no significant differences between the treated and control groups were observed in gross anatomy, weight, size or color by macroscopic examination of internal organs. Therefore, histopathological studies were unnecessary. [24] Hematological and biochemical parameters were estimated. The statistical analysis of the results obtained did not show significant differences between the rats fed with aqueous extract and arrope and the control group in any of the selected parameters. This fact strongly indicates that the AE and arrope are non toxic for hepatocytes and kidney cells. The mutagenicity evaluation of mistol, evidenced the absence of a genotoxic response by the extracts against Salmonella. [2]

This study provides evidence that the extracts and arrope of *Z. mistol* have a significantly antinociceptive effect (1000 mg/kg b.w.) that may be mediated through its anti-inflammatory action and activation of the opioid system. At the oral doses tested, the aqueous extract and arrope can be considered safe without any observable adverse effect. Further bioassay directed fractionation studies are required to identify the active compound(s) and its/their exact mode(s) of action.

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