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Identification of some bioactive metabolites and inhibitory receptors in the antinociceptive activity of *Tagetes lucida* Cav.

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ABSTRACT

Tagetes lucida Cav. is an ancient medicinal plant used to treat different ailments involving neurological diseases and pain. However, scientific studies to validate their medicinal properties as analgesic have not been described. The aim of this study was to evaluate the *T. lucida* antinociceptive response using pain models. Bioactive compounds and a possible mechanism of action were also explored. Dose-response effects of an ethanol crude extract were investigated in the writhing and formalin tests in mice and rats, respectively. The extract was fractionated to isolate active fractions and bioactive compounds (quercetagetin 7-O-β-D-glucoside and 6,7-dimethoxycoumarin) using the formalin test. The antinociceptive effects were compared to the reference drugs (tramadol 10 mg/kg, diclofenac 50 mg/kg, and/or ketorolac 1 mg/kg, i.p.). The ethanol extract was explored in the presence of naloxone (3 mg/kg, i.p. a non-selective opioid receptor antagonist) and WAY100635 (0.5 mg/kg, s.c., a selective 5-HT_{1A} receptor antagonist) to screen their participation as possible inhibitory mechanisms involved in the antinociceptive response of *T. lucida*. The ethanol crude extract, fractions, and pure compounds caused a significant antinociceptive response resembling the effect of the reference drugs. Both opioid and 5-HT_{1A} receptors participated in the analgesic –like activity of the extract, which did not produce gastric damage. On the contrary, the gastric damage produced as an adverse effect of the analgesic ketorolac was prevented when combined with the extract. In conclusion, these preliminary data provide evidence and give support to the properties attributed to *T. lucida* in the traditional medicine to alleviate pain.

1. Introduction

Pain, though usually perceived as an unpleasant feeling, is important for survival. In physiological circumstances, it has a protective function but there are pathologies in which pain is a major symptom that becomes the center of a disease [1]. Despite several analgesic drugs have demonstrated its efficacy to treat pain, adverse effects continuous being an obstacle for a better treatment or relief.

The use of medicinal plants to treat pain dates from the earliest times of mankind, aboriginal civilizations used parts of these to prepare their potions for healing purposes. At present, its use as a modality of Traditional and Natural Medicine is fundamental within the medical-pharmacological procedure since they constitute an arsenal of biologically active substances with pharmacological activity, in many cases with proven scientific evidence.

Tagetes lucida Cav. is a native and very ancient plant from Nahuatl ethnicity that was and remains one of the most important sacred plants used frequently in Mexico. Its common names include "yauhtli", "pericón", "San Miguel", "mangy", "flor de Santa María", "yerba anís", among others [2]. It belongs to the group of plants of God "Tláloc", and perhaps it was the main sacred plant because it occupied the main site since it was an epiphany of God. It is traditionally cultivated with an ornamental and ceremonial use for their showy flowers [3].

Mexican traditional healers recommend consuming the *T. lucida* aerial parts orally in infusion and hydroalcoholic extracts for analgesic and anti-inflammatory, diuretic, menstruation stimulant, abortive, antidiarrheic, antiulcer, and antihypertensive effects [2,4–6].

Pharmacological studies of their aerial parts or flowers alone have reported antibacterial [7–9], insecticidal [10], cytotoxic [11,12], and antioxidant [13] activities. Regarding the central nervous system

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(CNS), their antidepressant [4], anxiolytic, and sedative [6] actions have been reported. Phytochemical analysis of *T. lucida* aerial parts have reported the presence of bioactive compounds like flavonoids and coumarins [6]. However, the pharmacological antinociceptive and anti-inflammatory effects of *T. lucida*, responsible bioactive metabolites, and mechanisms of action have not been studied at all.

Our aim is to give evidence of the popular uses, recognizing ways of use and recommended traditional preparation forms and bioactive metabolites of this potential medicinal species. Our data contributes to the systematic analysis of Mexican traditional medicine by evaluating the dose-response antinociceptive activity of *T. lucida* prepared as an ethanol extract and tested in different doses in two experimental models of acute pain in mice. A chromatographic fractionation to look for bioactive compounds was included in the study. It is known that analgesic effects involve opioids and 5-HT_{1A} receptors participation in the CNS descending pathway [14]. The participation of opioidergic and 5-HT_{1A} serotonergic receptors as possible inhibitory neurotransmission involved in the antinociceptive activity of *T. lucida* extract was also explored.

2. Material and methods

2.1. Plant material

Aerial parts of *Tagetes lucida* Cav. (Asteraceae) are used mainly in flowering, the time of flowering might occur from July to December. People using this plant species commonly collect it from the field or buy it in the markets. For this study, the plant material was collected in San Cristóbal, Municipality of Ecatepec Mexico State, Mexico, in September 2016. The species identification was confirmed by the taxonomists M.Sc. Ana Rosa López. The voucher specimen was deposited at the Herbarium of the Biology Department of the UAM-Iztapalapa (No. 82038).

2.2. Preparation of the extract

The dried mature aerial parts of *T. lucida* were cut into small bits (500 g) and kept in a container; an extraction was carried out by three successive macerations in 5 L of hexane to degrease vegetal material at room temperature (22 ± 2 °C) for 72 h each time. Vegetal residue was discarded after filtration and then macerated in 3 L of absolute ethanol to give a final filtrated. Solvents were completely removed in a rotatory evaporator (Büchi R-210, Switzerland) under reduced pressure. The final product yielded 5.13 g (1.03%) of a dark green crude hexane extract and 40.49 g (8.14%) of a clear green crude ethanol extract (Fig. 1).

The extract was analyzed by thin chromatography in plates covered with silica gel 60 GF254 Merck to observe the presence of similar constituents. Thus, two compounds (one flavonoid and one coumarin) were isolated and purified as the most abundant constituents in the ethanol extract after chromatographic fractionation (Fig. 2). An open column chromatography and a subsequent percolation was done to get fractions containing metabolites. Insoluble fraction was washed with methanol solvent to obtain a yield of 0.37 g (2.47%) of a green taffy-like liquid and a yellow precipitate powder (0.13 g, 0.86%). An ultra-performance liquid chromatography analysis was also done to obtain the profiles of the identified compounds or a mixture of them (Fig. 2).

2.3. Fractionation of the ethanol crude extract

The ethanol crude extract (15 g) was separated using an open column chromatographic packed with 250 g of silica gel (60–200 mesh, Macherey-Nagel). The elution started with hexane (500 mL) followed by hexane-ethyl acetate mixture (50:50, 500 mL), ethyl acetate (500 mL), ethyl acetate–methanol mixture (50:50, 500 mL), and finally methanol (1200 mL). An unexpected preliminary fraction containing a mixture of flavonoids was got as a precipitated. This fraction was obtained just when the crude extract was applied into the column. Extraction of the coumarins mixture was done by using solvents hexane-ethyl acetate 50:50 (Figs. 1 and 2). The yielded of fractions obtained in this process is described also in Fig. 2.

2.4. Chromatographic analysis

2.4.1. Ultra-performance liquid chromatography (UPLC) analysis

Chromatographic analysis was performed using an Acquity UPLC-H class with PDA eλ detector (UPLC, Acquity Waters, Singapore) equipped with a Symmetry C18 column (100 Å, 150 mm × 4.6 mm, 5 mm, Waters, Ireland) with the thermostat at 43 °C. The mobile phase consisted of acidified water at 0.1% phosphoric acid in milli Q water (A) and methanol (B) (HPLC grade). The initial gradient elution was 80% A: 20% B to reach 100% B (17 min). The initial condition was regenerated during 3 min (80% A: 20% B). Constant flow rate was 1.0 mL/min with an elution curve of 6. The extract (4 mg/mL), fraction (4 mg/mL), mixture of compounds or a pure standard (2 mg/mL) were diluted in methanol and injected directly after filtration through 0.2 mm filter (GHP, Acrodisc 13, Waters) in a volume of 10 μL. Data acquisition, data handling and instrument control were performed by the Empower® 3 software (Waters, Milford, MA, USA).

2.5. Pharmacological study

2.5.1. Animals

Male Swiss Webster mice (25–30 g) and Female Wistar rats (180–200 g) were used in the pharmacological evaluation. Animals were provided by Instituto Nacional de Psiquiatría "Ramón de la Fuente Muñiz", they were kept at a controlled temperature of (22 ± 1 °C) with light/dark cycle of 12 h and fed *ad libitum* with standard water and food. Exploration was carried out in the Institute following the specifications issued by the Committee of Ethics and Research of that institution with the approval of the project number NC-123280.0 and NC-17073.0, as well as according to the Official Mexican Norm for the care and handling animal (NOM-062-ZOO-1999), and the international rules of care and use for laboratory animals.

2.5.2. Reagents and drugs

Naloxone, WAY100635, and Tween 80 were purchased at Sigma (St. Louis MO, USA). Sodium pentobarbital (SP) was acquired from PISA Farmacéutica (Mexico City, Mexico). The reference nonsteroidal anti-inflammatory drugs (NSAIDs) diclofenac and ketorolac were from AMSA and Liomont (Mexico City, Mexico), respectively. Tramadol was acquired by Grünenthal de México S.A. de C.V. (Mexico City, Mexico). Hexane, ethyl acetate and ethanol were analytic grade. Glacial acetic acid and formaldehyde at 37% solution were purchased by J.T.Baker, Mexico. The crude extract, fractions, the purified flavonoid (quercetagetin 7-O-β-D-glucoside) or the coumarin (6,7-dimethoxy coumarin) were used in fresh preparation and intraperitoneally (i.p.) administered. All the treatments were injected in a volume of 0.1 mL/10 g body weight. Vehicle consisted in 0.2 or 0.5% tween 80 in distilled water or saline solution alone (s.s. 0.9% NaCl) depending on their solubility. The isolated flavonoid and coumarin were used individually in the pharmacological evaluation as possible bioactive compounds.

2.5.3. Experimental design

2.5.3.1. Antinociceptive activity. Groups of at least 6 mice or rats received the vehicle or the *T. lucida* extract, fraction or pure compounds. After 30 min, the nociceptive agent was injected to induce writhing or shaking behavior, respectively, as follows:

Writhing test. - Abdominal nociception was induced by an i.p. injection of 1% acetic acid, this test is characterized by abdominal contractions known as writhes, described as an exaggerated extension of the abdomen combined with the outstretching of the hind limbs [15]. The crude ethanol extract (30, 100, 150, and 300 mg/kg, i.p.), ketorolac

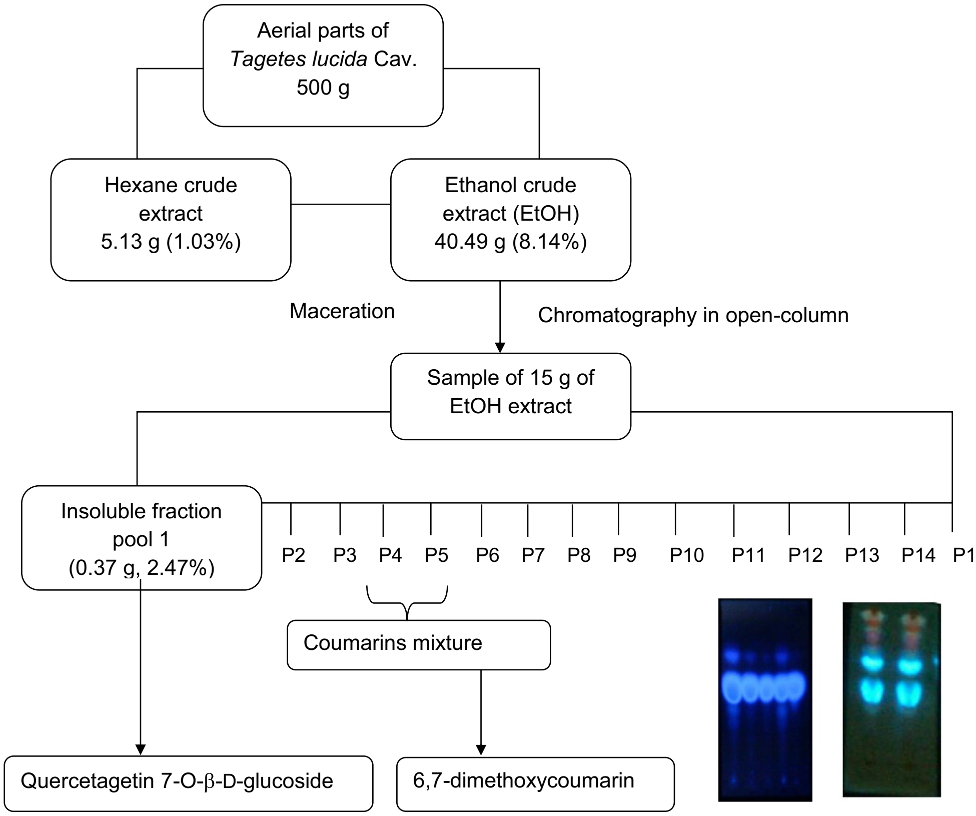

Fig. 1. Diagram resumes preparation of the crude extracts of the *Tagetes lucida* aerial parts and fractionation of the ethanol crude extract.

(reference drug, 1 mg/kg, i.p.), and purified compounds quercetagetin 7-O-β-D-glucoside (3, 10, 30, and 100 mg/kg, i.p.) and 6,7-dimethoxycoumarin (0.3, 1, 3, and 10 mg/kg, i.p.) were tested in this test. Immediately after acetic acid administration, the total number of writhes was recorded in the following periods: 0–5, 5–10, 10–15, 15–20, 20–25, and 25–30 min.

Data were obtained as temporal course curves of the writhes induced in mice. Then, a dose–response curve was plotted using the area under the curve (AUC) to determine the significant antinociceptive dose and to calculate an effective dose fifty (ED_{50}) of the extract. These doses were chosen after a preliminary exploration of toxicity according to a modified Lorke method [16] by performing OECD test guidelines for laboratory animals [17].

Formalin test. - After a habituation of 20 min, animals were administered with several doses of the ethanol crude extract (30, 100, 150, and 300 mg/kg, i.p.), fractions (pools, 100 mg/kg, i.p.), or the purified compounds quercetagetin 7-O-β-D-glucoside (3, 10, 30 and 100 mg/kg, i.p.) and 6,7-dimethoxycoumarin (0.3, 1, 3, and 10 mg/kg, i.p.), and compared to the reference drug diclofenac (DIC, 50 mg/kg, i.p.) or tramadol (TRM, 10 mg/kg, i.p.). The coumarin doses were selected due to previous studies of CNS activity of *T. lucida* (Pérez-Ortega et al., 2016).

Thirty min later, the chemical nociception was induced as follows: immediately after 50 μL injection in the subplantar area of the right hind paw with 1% formalin by using a 30-gauge needle, each rat was placed into a glass cylinder provided with mirrors to enable a total panorama of the nociceptive behavior. The number of shakings observed in the injected paw was taken as nociceptive response. Two periods of high shaking activity were considered: the first one was present immediately after injection and lasted 5 min; this was known as early phase (neurogenic phase). A second period was observed 5–30 min after formalin injection and denominated late phase (inflammatory phase). Control animals received vehicle by the same route and time of administration.

2.5.3.2. Gastric damage evaluation. Rats received vehicle or treatment with the reference drug ketorolac (1 mg/kg) and the *T. lucida* ethanol crude extract at the highest dosage tested (300 mg/kg, i.p.); as well as the combination of different doses of the crude extract (30, 100 and 300 mg/kg, i.p.) and ketorolac (1 mg/kg, i.p.). All the rats were euthanized inside of a CO_2 chamber and then the stomachs were dissected and filled with 10 mL of 10% formaldehyde for their fixation and observation for possible gastric damage. After 10 min, the stomachs were opened by the greater curvature and rinsed with distilled water to eliminate their content and proceed to their scanning for the subsequent observation and measurement of possible gastric lesions [18].

2.5.3.3. Mechanism of action analysis. Participation of the endogenous opioids and serotonin $5\text{-}HT_{1A}$ receptors were explored in the antinociceptive effect of the *T. lucida* ethanol extract. For this, independent groups of rats were administrated with naloxone (3 mg/kg, i.p., a non-selective opioid receptor antagonist) or WAY100635 (0.5 mg/kg, s.c., a $5\text{-}HT_{1A}$ receptor antagonist) 15 min before the dosage of treatments or the vehicle. Thirty minutes after treatments, mice or rats were submitted to the corresponding nociceptive test to be compared also with the effect of naloxone or WAY100635 *per se* alone or combined.

2.5.3.4. Statistical data analysis. Using the trapezoidal rule, the area under the curve (AUC) values (writhes or shakings per min) were calculated from the respective temporal course curves obtained in the nociceptive behavior assays and they were considered as an expression of both the nociceptive behavior in the writhing and formalin tests. Data are expressed as the mean ± standard error of the mean (S.E.M.) of at least 6 repetitions. Dose-response data were analyzed by one-way ANOVA followed by Dunnett's *post hoc* test, or even by a Student's *t*-test when comparing only two groups, using GraphPad Prism software, version 6.0. $P < 0.05$ was considered statistically significant.

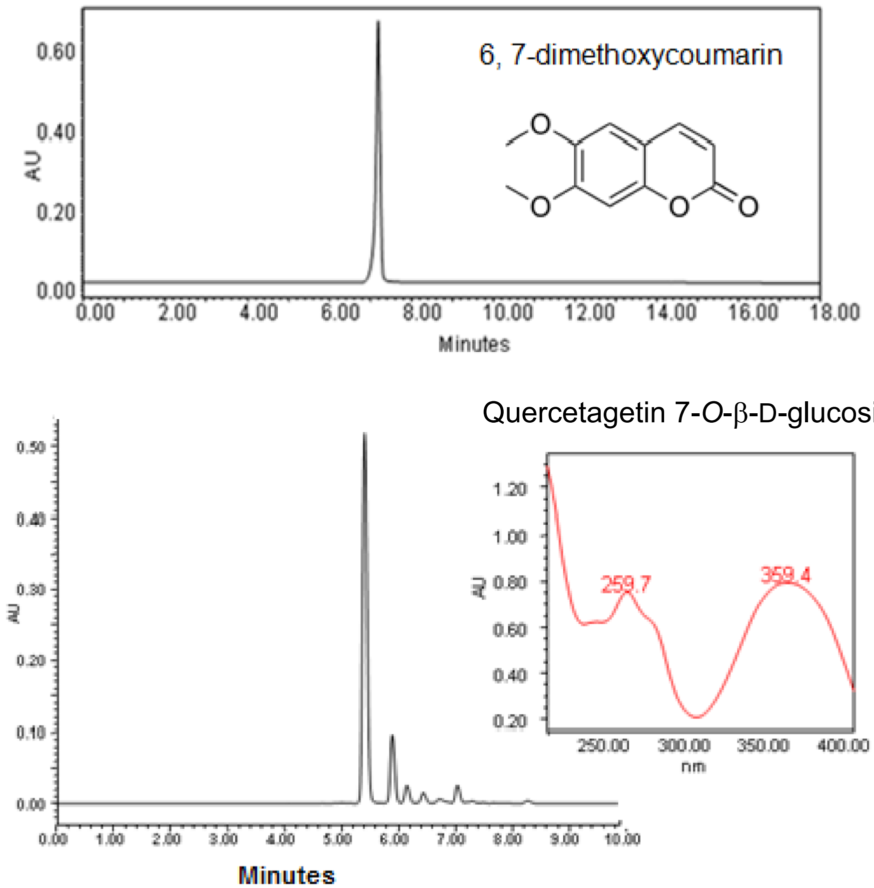

Fig. 2. The inserted table describes fractions and solvents used, as well as the yield obtained in the extraction of the ethanol crude extract. Chromatographic profile refers the presence of quercetagetin 7-O-β-D-glucoside [retention time (Rt) 5.65 min] and 6,7-dimethoxy coumarin (Rt 7.2 min).

3. Results

3.1. Antinociceptive activity in writhing test

Maximal nociception was obtained in mice receiving only 0.9% saline solution (s.s.) or 0.2% tween 80 in s.s. as vehicle. *T. lucida* crude extract produced antinociceptive activity in a dose-dependent manner by reducing the number of writhes observed in the vehicle groups. Data expressed as the percentage of the AUC in a period of 30 min allowed observing significant diminution in nociception from a dosage of 30 mg/kg resembling that obtained with the analgesic ketorolac (1 mg/kg, i.p.) ($F_{5,30} = 14.76$, $P < 0.0001$) (Fig. 3A). Since the antinociceptive effects showed a dose-dependent manner, an $ED_{50} = 67$ mg/kg, i.p. was calculated. Whereas acute toxicity of the extract according to the modified method of Lorke (1983) was determined as a $LD_{50} = 1250$ mg/kg, i.p. to give a therapeutic index of 19.

Quercetagetin-7-O-β-D-glucoside was obtained as pure compound in pool 1 from fractionation of the crude extract of *T. lucida* (Fig. 1). It significantly reduced writhing response in mice receiving the nociceptive agent and pretreated with doses of 10, 30, and 100 mg/kg, i.p. in comparison to the saline solution group ($F_{5,30} = 8.57$, $P < 0.0001$) (Fig. 3B).

The 6,7-dimethoxycoumarin was the other pure compound extracted mainly in pool 4 after fractionation of the crude extract (Figs. 1 and 2). This compound reduced writhing behavior in mice from a dosage of 0.3 mg/kg, but this effect was significant after a dosage of 1 mg/kg remaining at the same level in mice receiving also 3 or 10 mg/kg, i.p. ($F_{5,30} = 5.64$, $P = 0.0009$) in comparison to the vehicle (0.2% tween 80 in s.s.) (Fig. 3B).

3.2. Antinociceptive activity in the formalin test

The crude extract administrated at 100, 150, and 300 mg/kg, i.p., and tramadol 10 mg/kg, i.p. (a partial opioid agonist) produced significant antinociceptive response in the neurogenic phase ($F_{5,30} = 13.34$, $P < 0.0001$) (Fig. 4A). Pools 2, 3, 4, 6, and 8 reduced nociception but it was not significant ($F_{5,30} = 1.43$, $P = 0.24$) in this phase (Fig. 4A). Statistical significance in the response of diclofenac in the neurogenic phase was reached using Student's *t*-test analysis ($t = 3.30$, df = 10) (Fig. 4A).

In contrast, a significant antinociceptive response was observed in the inflammatory phase in groups receiving pools 2, 3, 4, and 8 resembling the effect of diclofenac and tramadol, less significance was obtained with pool 6 ($F_{8,47} = 15.40$, $P < 0.0001$) (Fig. 4B). These fractions were obtained from the fractionation of the crude extract using non-polar and medium polar solvents, all of them were evaluated at 100 mg/kg, i.p. and compared to the vehicle group receiving 0.2% tween 80 in s.s. The ethanol extract at doses of 100, 150, and 300 mg/kg, i.p. produced also a dose-dependent and significant response in the inflammatory phase ($F_{5,30} = 11.11$, $P < 0.0001$) (Fig. 4B).

Regarding to the pool 1, it revealed a mixture of flavonoids with the presence of quercetagetin 7-O-β-D-glucoside (supplementary material). This mixture and Pool 9 administrated at 100 mg/kg, i.p. produced significant reduction in the shaking nociceptive behavior in the neurogenic phase (Fig. 4C). This antinociceptive response was equivalent to that obtained with the crude extract at the same dosage and resembled that of the reference drugs tramadol (TR, 10 mg/kg, i.p.) and diclofenac (DIC, 50 mg/kg, i.p.) ($F_{7,42} = 2.907$, $P = 0.01$) (Fig. 4C). Pools 10 and 11 did not reach significance in the neurogenic phase as compared to the vehicle group (s.s.) (Fig. 4C).

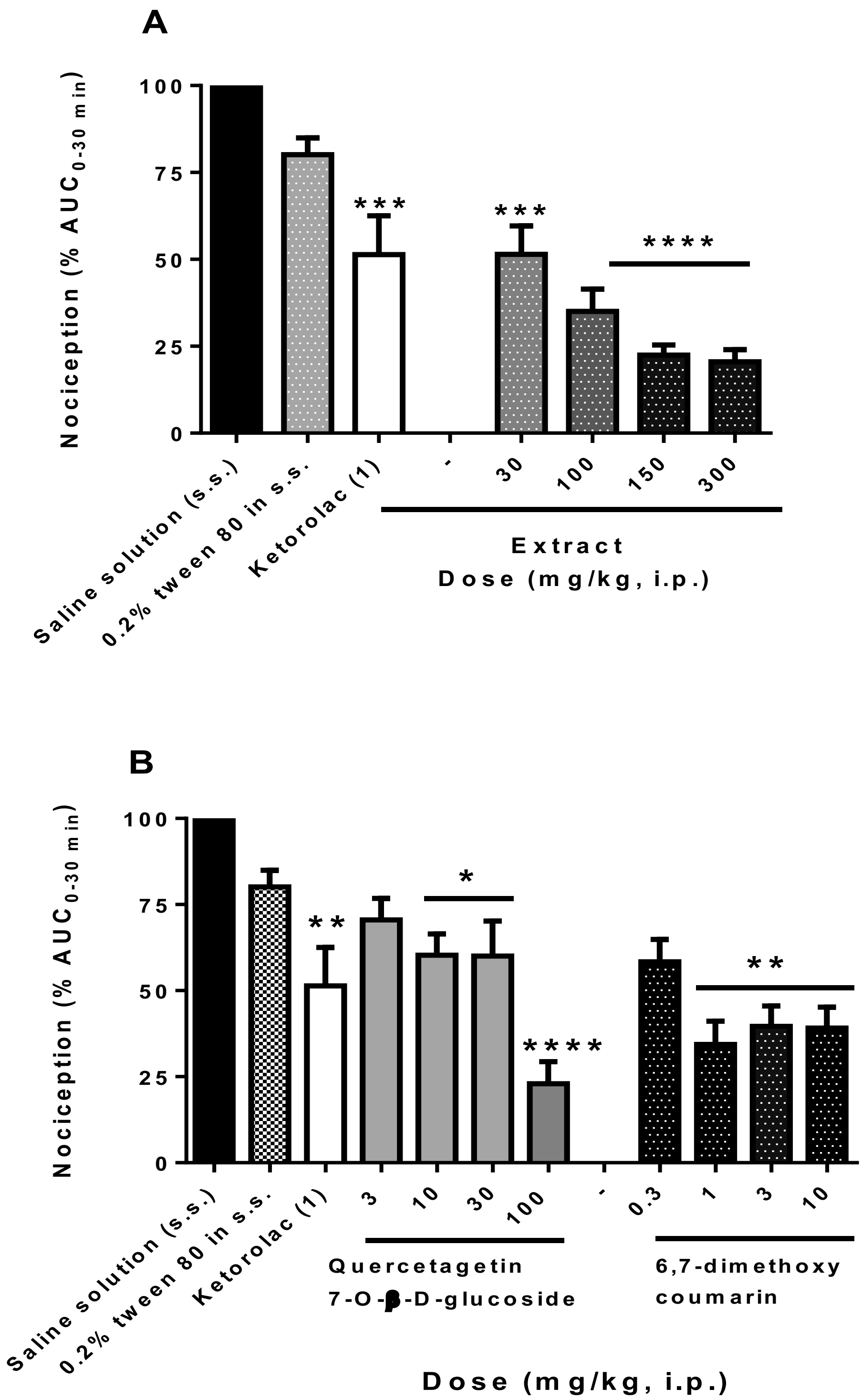

Fig. 3. Dose-response antinociceptive effects of *Tagetes lucida* ethanol crude extract (A), and the pure compounds quercetagetin 7-O-β-D-glucoside and 6,7-dimethoxy coumarin (B) were compared to the effect of the analgesic drug ketorolac (1 mg/kg, i.p.) and the corresponding vehicle group (0.9% saline solution, s.s. or 0.2% tween 80 in s.s.) in the writhing test in mice. One-way ANOVA followed by Dunnett's *post hoc* test, respectively: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ or ****$P < 0.0001$, $n = 6$ repetitions.

In contrast, all these pools diminished antinociceptive behavior in a significant manner in the inflammatory phase (Fig. 4D), all of them showed equivalent response than the crude extract and the reference drugs ($F_{7,42} = 16.40$, $P < 0.0001$) (Fig. 4D).

In the neurogenic phase of the formalin, significant and dose-dependent reduction in the nociceptive behavior was produced in rats receiving quercetagetin-7-O-β-D-glucoside at 30 and 100 mg/kg, i.p. ($F_{4,25} = 10.37$, $P < 0.0001$) (Fig. 4E). In this phase, no significant reduction was obtained in the case of rats receiving 6,7-dimethoxycoumarin (0.3, 1, and 3 mg/kg, i.p.) ($F_{3,20} = 3.86$, $P = 0.02$), but 10 mg/kg, i.p. reduced nociception reaching significance compared alone with the vehicle group ($t = 2.84$, df = 10, $P = 0.01$) (Fig. 4E).

On the contrary, significant and dose-dependent reduction in the nociceptive behavior was produced in rats receiving quercetagetin-7-O-β-D-glucoside (10, 30, and 100 mg/kg, i.p.) ($F_{4,25} = 8.42$, $P = 0.0002$) and 6,7-dimethoxycoumarin (3 and 10 mg/

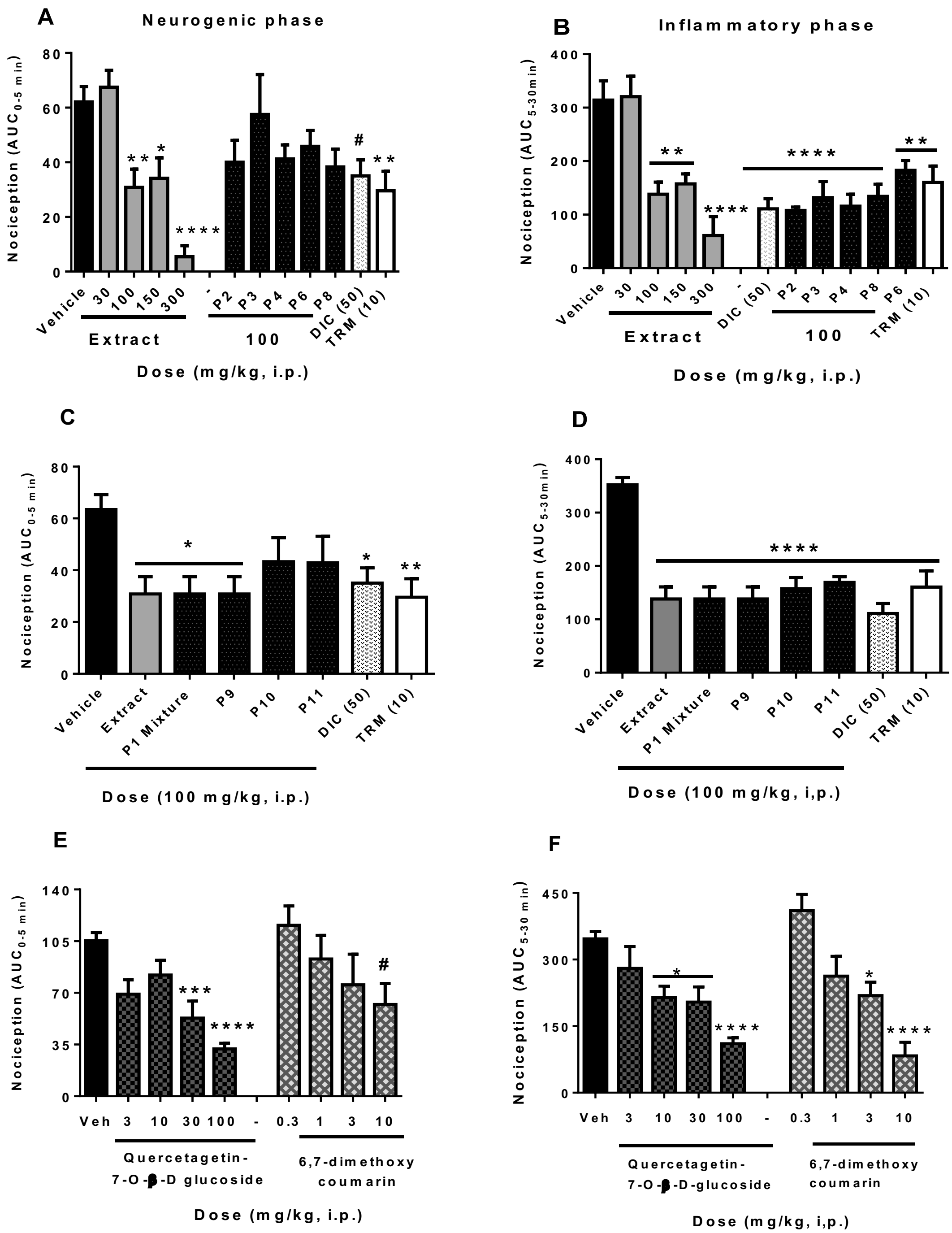

Fig. 4. Antinociceptive effects of several doses of the crude ethanol extract and a dosage of 100 mg/kg for pools 2, 3, 4, 6, and 8 were compared to those produced by analgesic drugs diclofenac (DIC, 50 mg/kg, i.p.) or tramadol (TRM, 10 mg/kg, i.p.) in the neurogenic (**A**) and inflammatory (**B**) phases of the formalin test in rats. Antinociceptive response of pools 1 (mixture of flavonoids), 9, 10, and 11 were also tested at 100 mg/kg, i.p., in the neurogenic (**C**) and inflammatory (**D**) phases of the formalin test in rats. Antinociceptive dose-response activity of the pure compounds quercetagetin-7-O-β-D-glucoside and 6,7-dimethoxycoumarin were also tested in the neurogenic (**E**) and inflammatory (**F**) phases of the formalin in rats in comparison to the vehicle group (Veh, saline solution). One-way ANOVA followed by Dunnett's *post hoc* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. $^{\#}$Student's *t*-test, $P < 0.05$, $n = 6$ repetitions. P: Pool.

kg, i.p.) ($F_{4,25} = 14.18$, $P < 0.0001$) in the inflammatory phase (Fig. 4F).

3.3. Gastric damage evaluation

The *T. lucida* extract administered alone at the highest dosage of 300 mg/kg, i.p. did not produce gastric damage, as it was observed with ketorolac alone (Fig. 5A). In addition, combined administration of *T. lucida* at 30, 100 or 300 mg/kg with ketorolac (1 mg/kg, i.p.) protected from this adverse effect observed in the reference analgesic drug alone (Fig. 5B).

3.4. Mechanism of action

Nociceptive behavior in the neurogenic (Fig. 6A) and inflammatory (Fig. 6B) phases of the formalin test was not modified after administration of an antagonist of opioid (naloxone, NX 3 mg/kg, i.p.) or 5-HT_{1A} serotonin receptors (WAY100635, WAY 0.5 mg/kg, s.c.) alone

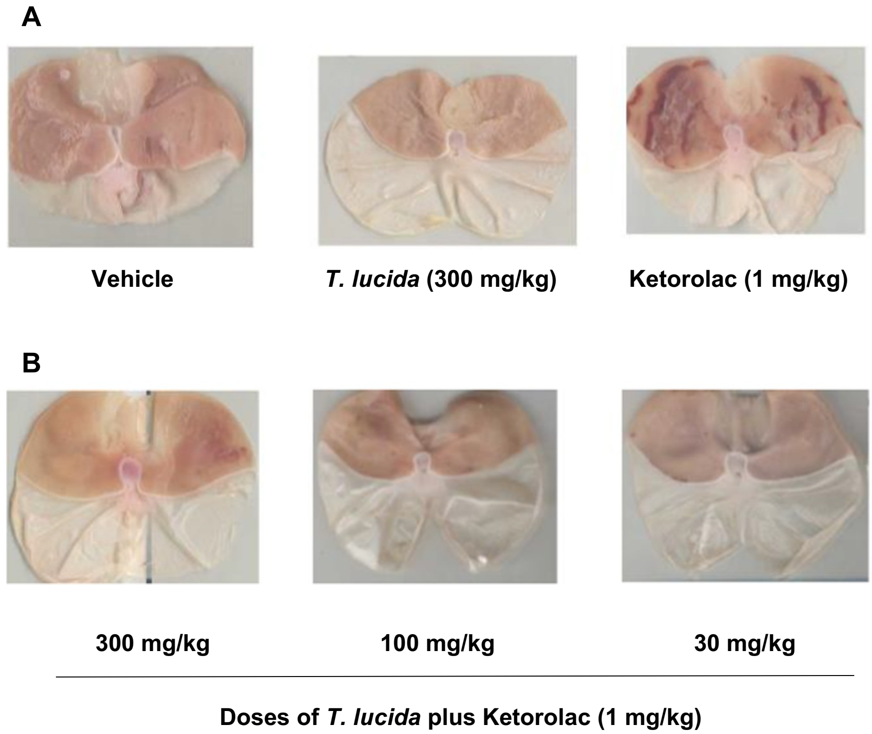

Fig. 5. Representative photographs exploring gastric damage in the presence of the highest dosage of the *Tagetes lucida* ethanol crude extract or the reference drug ketorolac in rats; as well as after combination of several doses of the crude extract plus ketorolac.

(Fig. 6A and B). In contrast, the significant effect of the extract ($F_{6,37} = 3.13$, $P = 0.014$ and $F_{6,37} = 5.05$, $P = 0.0007$, respectively) was avoided in the presence of these two antagonists in the neurogenic (Fig. 6A) and in the inflammatory (Fig. 6B) phases of the formalin test.

4. Discussion

Tagetes lucida Cav. belongs to the Asteraceae family. It is an ancient medicinal species that according to the literature and recent verbal reports of merchants and healers of Mexican regions [6] is used to treat several gastrointestinal affections involving colic and ulcer, as well as for alleviating musculoskeletal pain. It is also used in other countries, like Carib population of Guatemala [19] or Egypt [10] where transcultural beliefs are frequently used to maintain health. Nature of the bioactive compounds involved in the activity of plants is important to recognize as origin of new pharmaceutical products. Furthermore, it is known that there is no a unique principle active responsible for the total medicinal activity of plants. In this study, we describe scientific pharmacological evidence of the use of this *Tagetes* species in traditional medicine, specifically about its properties to alleviate pain. Additionally, we inform about some possible bioactive compounds participating in its antinociceptive activity and some inhibitory neurotransmission involved.

As it was mentioned, abdominal pain is the most frequent affection alleviated by using infusion of this medicinal plant, it includes digestive disorders, stomachache and menstrual colic (dysmenorrhea) or abdominal pain associated to inflammation [20], among other affections [21]. Our data support the analgesic efficacy of *T. lucida* to alleviate abdominal pain at doses might be far from possible toxicological or lethal effects since the therapeutic index calculated in mice was 19. According to the OECD, this difference might situate *T. lucida* in a category 5 suggesting minimal hazard or no toxic substance [17].

The antinociceptive response of the polar extract was reinforced in this study with the effects observed in the nociceptive behavior in both neurogenic (central) and inflammatory (peripheral) phases of the formalin test in rats. It is reported that centrally acting analgesics such as opioids inhibit both phases of the formalin test [22]; however, peripheral analgesics such as NSAIDs and corticosteroids inhibit only the second phase [23]. Interestingly, central and peripheral influence of *T. lucida* is involved in its analgesic effects. Our present study gives evidence for the first time of the antinociceptive and anti-inflammatory properties of *T. lucida* prepared as an alcoholic extract and evaluated in an *in vivo* assay. All these results together reinforce efficacy of this species not only for abdominal pain but also musculoskeletal pain including inflammatory process.

Fractionation of the crude extracts reveled that several fractions showed significant antinociceptive activity in the formalin test, these results indicated participation of several constituents from different polarity mainly in the inflammatory phase. The pharmacological properties of a plant could be limited when they depend on a single active principle, however if the species possesses several active components there will always be some that maintain the medicinal property of the species, even if conditions of collection of the vegetal material are varied.

A mixture of flavonoids was obtained as part of the antinociceptive fractions of *T. lucida* producing effect also in the neurogenic phase. This result reinforced CNS activity for this type of compounds as previous reported for rutin (glycoside of quercetin) [14] and others combined flavonoids [24], which depressant action on the CNS [25,26] has been useful for brain disorders [27]. In this study, quercetagetin 7-O-β-D-glucoside was purified as principal bioactive compound in an antinociceptive mixture of flavonoids of *T. lucida*. These compounds are part of the main constituents in *Tagetes* genus and this flavonoid has been found in a more specific manner in *T. lucida* [28], among other glycosides and mono-, di- and tri-substituted methyl derivatives of quercetagetin [29].

Other constituents isolated as bioactive analgesics in this species were coumarins, one of them identified as 6-7-dimethoxycoumarin. Coumarins have been associated to the anti-inflammatory effects including peripheral receptors like TRPV1 or pro-inflammatory cytokines

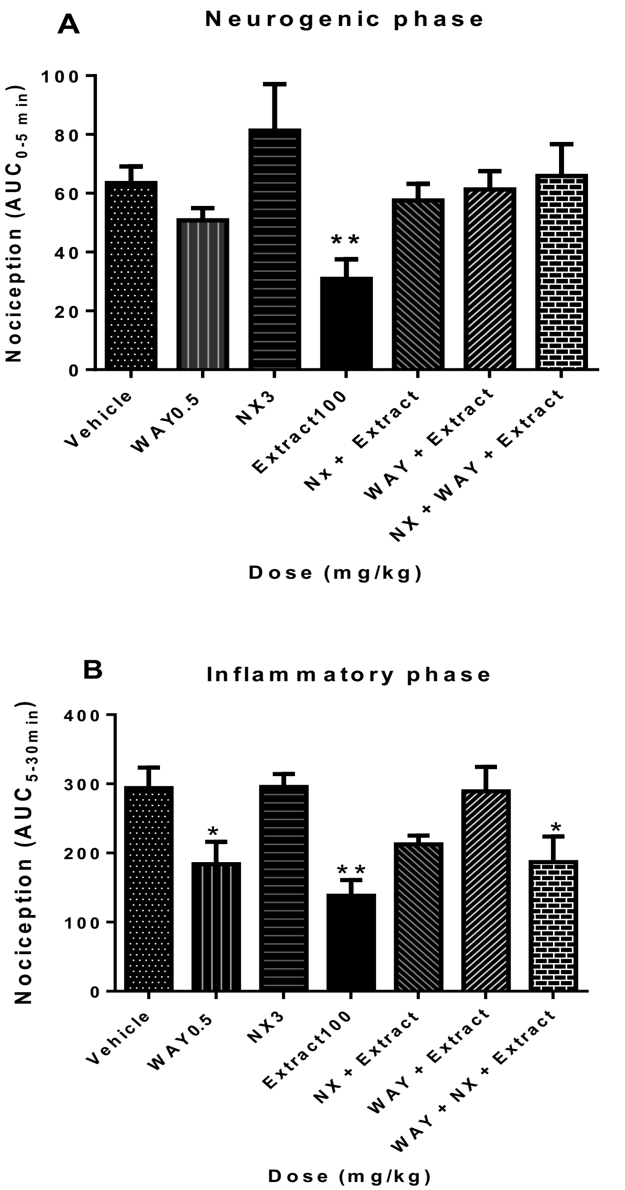

Fig. 6. Nociceptive response represented as the neurogenic ($AUC_{0–5min}$) and inflammatory ($AUC_{5–30min}$) phase in the formalin test in rats receiving vehicle (Veh, 0.2% tween 80 in saline solution) and the antagonists of the opioids (NX3: naloxone, 3 mg/kg, i.p.) and serotonin 5-HT_{1A} (WAY0.5: WAY100635, 0.5 mg/kg, s.c.) receptors, alone and combined with the *T. lucida* crude extract (100 mg/kg, i.p.). One-way ANOVA followed by Dunnett's test, *$P < 0.05$, **$P < 0.01$, $n = 6$ repetitions.

[30] and central mechanisms like 5-HT_{1A} receptors [6] or opioid receptors, both implicated in the descending inhibitory pathway of pain [14,31]. In agreement to these studies, antinociceptive effects of the *T. lucida* crude extract were prevented in the presence of antagonists of the endogenous opioids and serotonin 5-HT_{1A} receptors in both phases of the formalin test, mainly at central level suggesting activation of the descending pathway to control pain perception.

WAY-100635 is an antagonist useful to characterize 5-HT_{1A} receptor-mediated functional responses since it possesses high level of specificity on this receptor [32]. In our study, pretreatment with WAY-100635 avoided the analgesic-like response in rats receiving only the *T. lucida* extract suggesting the implication of these serotonin receptors that have also been related to human pain control as observed by using positron emission tomography (PET) imaging [33]. Since more than one pool showed significant antinociceptive effect, it is possible than multiple mechanisms of action are involved in the final antinociceptive response of the extract. It will be interesting to explore in the future other bioactive compounds and associated mechanisms of action to reinforce the potential of this species in the therapeutic.

Affection of the gastric mucous by NSAIDs is a well-known collateral adverse effect as observed in our reference drug ketorolac. This unwanted effect is because of the greatest selectivity for cyclooxygenase 1 (COX 1), an enzyme involved in the prostaglandins production and the protection of the gastric mucous [34]. In our study, ethanol crude extract of *T. lucida* did not produced gastric damage at antinociceptive doses, on the contrary it protected and prevented ulcer damage in the presence of an NSAID like ketorolac. Our data agree with a preventive gastric damage instead of ulceration preliminary reported for the *T. patula* essential oil [35]. These results reinforce the medicinal use of Tagetes species for gastrointestinal diseases.

In conclusion, all data of this study together with literature give evidence of the analgesic activity of this plant species without producing the most common adverse effect of analgesic anti-inflammatory drugs in the pain therapy.

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Declaration of Competing Interest

Authors declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.05.079.

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