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Original Article

Antinociceptive effect of aqueous extracts from the bark of *Croton guatemalensis* Lotsy in mice

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Abstract

Croton guatemalensis Lotsy (CGL), known as "copalchi" in Chiapas, Mexico, is used for the treatment of fever, abdominal pain and malaria and also as a remedy for chills and for treating rheumatism. The aim of this study was to evaluate whether aqueous extracts from the bark of this plant possesses indeed antinociceptive properties by using two different animal models of nociception, the acetic acid-induced writhing test and the hot plate model. The results showed that i.p. administration of this extract (0, 100, 200 and 400 mg/kg) 30 min prior testing had significant dose-dependent antinociceptive effects in the acetic acid-induced writhing test and that the reduction of writhings (85.5 % as compared to the control) at the highest dose tested is similar to that exhibited by dipyrone (250 mg/kg). This effect was not reversed by naloxone, a non-selective opioid receptor antagonist, suggesting that the endogenous opioid system does not underlie the antinociceptive effects of CGL in the acetic acid-induced writhing test. No effects were however observed in the hot-plate model. Our results indicate that aqueous extracts from *Croton guatemalensis* bark contain pharmacologically active constituents endowed with antinociceptive activity. It is suggested that cyclooxygenase inhibition might be at least partially involved in the antinociceptive effects of this extract.

Keywords: *Croton guatemalensis*; Antinociception; Acetic acid-induced writhing test

INTRODUCTION

Croton is a genus of Euphorbiaceae, a widespread group of plants found in tropical and subtropical regions of the world. Many species of this genus have been widely used in traditional medicine to treat several diseases (1-7). Accordingly, some *Croton* species such as *C. cajucara* Benth (5), *C. celtidifolius* Baill (6), *C. cuneatus* Klotz. (8), *C. malambo* (9), *C. sonderianus* (10), *C. zehntneri* (11), *C. nepetaefolius* (12) and *C. crassifolius* (13) have been reported to possess antinociceptive effects and anti-inflammatory properties.

Croton guatemalensis Lotsy (CGL), popularly known as "copalchi" in Chiapas, Mexico (14) is a widespread shrub largely employed in this region as a living fence and its bark is used for treatment of fever, abdominal pain and malaria (2,15). In Guatemala, such a bark is valued as a remedy for chills and as an additive to baths for treating rheumatism (16).

However, since in spite that CGL has been widely used in traditional medicine as an antinociceptive compound the basis for its empirical use in the management of pain has never been scientifically verified. In this work, we have investigated in mice whether or not aqueous extracts from CGL displays antinociceptive activity in two acute nociception models, the acetic acid-induced writhing paradigm and the hot plate test, a chemical and a physical based nociceptive paradigms respectively. Experiments designed to understand its putative antinociceptive action were also carried out.

MATERIALS AND METHODS

Collection of plant material

Fresh plants of *C. guatemalensis* were collected in April 2013, from the Pacú,

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Suchiapa Municipality of Chiapas, Mexico. The collected specimens were identified and authenticated by Biol. Teresa Guadalupe Cabrera Cachón, Curator from the herbarium "Dr. Faustino Miranda", Chiapas, Mexico, where a voucher specimen with the number 45377 was deposited.

Extract preparation

Chopped barks of CGL were dried in a shade room for a month before being powdered. For extraction, 25 g of powder were dissolved in 100 ml of purified water and heated to boiling under stirring. After cooling, the mixture was filtrated and freeze-dried yielding 0.425 g of dried powder (1.7%) from the original plant material. CGL extracts were reconstituted in distilled water before being injected to the animals.

Phytochemical screening

Phytochemical screening of the CGL extract was performed as described by Evans (17) with minor modifications. To detect the presence of flavonoids few drops of diluted NaOH (0.1 N) were added to 1 ml of a concentrated extract. An intense yellow color which disappears upon the addition of few drops of 1 N hydrochloric acid indicated the presence of flavonoids in the extract. Saponins were identified in a sample of 1 ml of CGL extract by shaking it after the addition of 20 ml distilled water. A foam layer at the top of the extract indicated the presence of saponins. In order to detect steroids a sample of the freeze-dried extract (1 mg powder) was deposited at the bottom of a test tube and dissolved with chloroform (10 ml). An equal volume of concentrated (97.3%) sulfuric acid was carefully added by the walls of the tube and vigorously shacked to form a two layer suspension. The upper layer of the suspension becomes red and the sulfuric acid layer at the bottom of the tube takes a yellow color which exhibits a weak green fluorescence. The presence of steroids in the CGL extract is indicated by changes occurring at the lower layer of the suspension. The presence of tannins was detected by adding a few drops of a 10 % ferric chloride solution to a diluted CGL extract sample. Tannins are identified by the development of either a blue-black or green to greenish black coloration. To detect terpenoids 2 ml of chloroform were mixed with 1 ml of CGL extract. Terpenoids were identified by the appearance of reddish to brown coloration at the interface of the two layers formed after the addition of a few drops of concentrated (97.3%) sulfuric acid. Alkaloids were identified in 1 ml of the extract by the formation of a turbid orange color following the addition of 2 ml of Dragendroff's reagent.

Animals

Experiments were conducted using adult male ICR mice (25–30 g). Mice were housed in a controlled environment (temperature 23 ^{o}C; lights on 07:00–19:00 h) with water and food (Purina, Mexico) available ad libitum. Animals were donated by the biotherium of Laboratorio Estatal de Salud, Chiapas, Mexico.

Chemicals and drugs

The chemicals and drugs used in this study were acetic acid (Merck, Darmsted, Germany), dipyrone (Hoechst, Mexico), naloxone hydrochloride (Sigma Chemical Co., St Louis, Mo, USA) and morphine (PISA, Mexico). Drugs and extracts were dissolved in distilled water. Stock solutions of each compound were prepared, and from there working solutions having proper concentrations were obtained to inject the animals. All compounds were administered intraperitoneally (i.p.) in a total volume of 10 ml/kg. Animals from the control group received an equal volume of water (18).

Behavioral evaluation

All experiments reported in this study were performed in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigation of experimental pain in conscious animals (19). Behavioral experiments were carried out in a sound attenuated room. The room was dimly illuminated and was equipped with video-recording facilities. The behavioral evaluation was carried out in a manual way. All evaluations were conducted between 10:00 and 18:00 h. The apparatuses used for all

behavioral tests were cleaned with detergent and dried before each trial. In all experiments animals were assigned to each group at random. Animals were used only once.

Antinociceptive activity

Acetic acid-induced writhing test

Five groups of mice were used: A control group was treated (i.p.) with distilled water and 3 additional groups with a different CGL extract dose (100, 200, 400 mg/kg; i.p.). A separate group of mice was treated with dipyrone (250 mg/kg; i.p.) and used as a positive control. Each group contained 5 mice. The test was performed essentially as described by Hajhashemi and coworkers (20) and Rejón-Orantes and colleagues (21). Acetic acid (0.6%; v/v) was administered (i.p.) at the beginning of the test in a volume of 10 ml/kg. Writhing responses involving contraction of the abdominal wall and pelvic rotation followed by hind limb extension were counted during the whole duration of the test (20 min). Both, CGL extract and the reference analgesic drug dipyrone were injected 30 min before the acetic acid administration. Percentage inhibition of the antinociceptive effect of the extract was calculated using the following expression:

$$\% \text{ Inhibition} = \frac{(\text{Mean number of writhes (control)} - \text{Mean number of writhes (test)})}{\text{Mean number of writhes (control)}} \times 100$$

Involvement of opioid receptors in the antinociceptive effects of Croton guatemalensis extracts

In order to verify the involvement of the opioidergic system on CGL-induced antinociception, in the acetic acid-induced writhing test either CGL extract (400 mg/ kg; i.p.) or morphine (10 mg/kg; i.p.) were injected to separate groups of animals that had been previously treated (15 min before) with either naloxone (5 mg/kg; i.p.) or distilled water. Since naloxone 5 mg/kg; i.p. was able to induce effective antinociceptive effects following treatment with *Melanostomamela bathricum* extracts (22) this dose was chosen for our studies. Distilled water (control group), CGL extract (400 mg/mg) (CGL extract group) and morphine (10 mg/kg; i.p.) (morphine group) were also administered to additional groups of mice that were previously treated with vehicle. Behavioral evaluation started 30 min after the last administration. Each group contained 6 mice.

Hot plate test

Male mice were distributed into 5 groups (5 animals in each group): a control group which was treated with distilled water, 3 groups treated with a different CGL dose (100, 200, 400 mg/kg, i.p.) and an extra group, which was used as a positive control, with morphine (10 mg/kg; i.p.) (23). This test was performed as described by Rejón-Orantes and coworkers (21). Animals were placed on a hot plate (UgoBasile, Hot/Cold plate 35100) set at 55 ± 0.5 °C and their pain responses (hind-paw licking or animal jumping) were assessed. The time elapsed between the placement of the animal in the platform of the apparatus and its pain response was recorded as the response latency. Such latency was determined both before testing and at 30, 60 and 90 min after the administration of CGL extract, the reference drug or the vehicle.

Motor activity

Both, exploration of an open field and the rotarod test were carried out to disclose any possible CGL extract-induced muscle relaxant or central depressant effects which may had influence the effects of the extract in the nociceptive models.

Open field test

This method has been used to evaluate the locomotor activity of rodents (24). In this experiment 4 groups of animals (6 animals in each group) were used. Distilled water was administered to the control group. Test groups received CGL extracts (100, 200 and 400 mg/kg, i.p.). The open-field test was performed in a glass box of 48 × 48 × 30 cm^3 with transparent walls and floor. The floor of the box was divided with lines painted in black forming squares of 12 × 12 cm^2. Thirty min after treatment mice were placed in the center of the box and their locomotor activity was registered for 5 min. An observer blind to the treatments registered both the number of lines crossed by the animals and their rearings (25).

Rotarod test

Motor coordination was evaluated using the rotarod test. Four groups (5 animals in each group) were previously trained to remain for 3 min on a rolling rod (3 cm, diameter) rotating at 8 rpm. For the test, the control group received purified water and the other groups were given CGL extracts (100, 200 and 400 mg/kg, i.p.). Thirty min after either both water or CGL extracts administration animals were placed on the rolling rod and the number of falls experienced by the mice during the procedure (3 min) was registered (26).

Acute toxicity

Different doses of CGL extract (50, 100, 200, 300, 400, 800 mg/kg; i.p.) were administered to different groups of mice and their mortality were recorded for 48 h (27).

Statistical analysis

Non parametric results are presented either as medians with their respective interquartile range. Whiskers represent maximum and minimum values within the sample. Parametric results (hot-plate experiment) are presented as means ± S.E.M. Kolmogorov-Smirnov test was used to ascertain whether or not the sample under analysis was normally distributed. A two-way ANOVA followed by Bonferroni test for multiple comparisons was used to analyze the results in the hot plate using extract dose as one factor and time elapsed for of the evaluation of the response following the administration of the extract as the other factor. Non parametric analysis was assessed using the Kruskal-Wallis test followed when required by either the Mann-Whitney U-test or the Dunn's Multiple Comparison Test. Global dose-response effects of CGL extracts in the acetic acid-induced writhing test were further assessed by the Jonckheere-Terpstra test for ordered alternatives. An alpha value of $P<0.05$ was considered statistically significant. Statistical parameters were computed using GraphPad Prism statistical software (GraphPad Software, Inc. Version 6). Jonckheere-Terpstra test was computed by using the IBM SPSS Statistics package (Version 22).

RESULTS

Phytochemical screening

Qualitative phytochemical analysis of CGL extracts revealed that they were enriched in flavonoids and saponins (Table 1).

Antinociceptive activity

Acetic acid-induced writhing test

Overall effects of CGL extract administration were statistically significant (Kruskal-Wallis statistic = 18.20, df = 4/20; $P<0.001$). As can be observed in the Fig. 1, i.p. CGL extract administration at doses of 200 and 400 mg/kg, reduced (57.2 and 83.5 % respectively) the number of acetic acid-induced writhings in comparison with the control group. The effects were dose-dependent (Jonckheere-Terpstra test for ordered alternatives, P = 0.00009) and at the highest dose used (400 mg/kg) CGL extract showed an anti-nociceptive activity comparable to that shown by dipyrone (90.7 % inhibition), an established antinociceptive drug.

Table 1. Phytochemical constituents of the aqueous extracts from *C. guatemalensis* bark.

Biochemicals	Existance
flavonoids	+
saponins	+
steroids	-
Tannins	-
alkaloids	-
Terpenoids	-

+; Presence, -; Absence

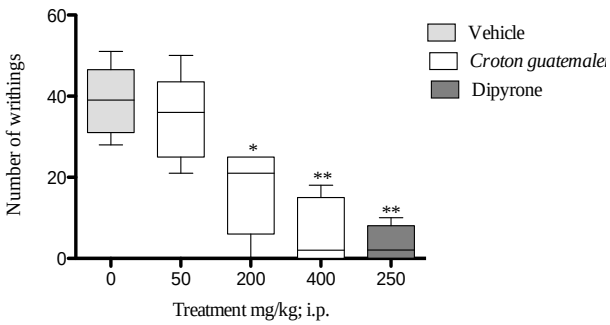

Fig. 1. Antinociceptive effects of aqueous extracts from *Croton guatemalensis* on the acetic acid-induced writhings in mice. Both *Croton guatemalensis* extracts and dipyrone used as a positive control produced an inhibition of abdominal writhings. Values are presented as medians with their respective interquartile range.Whiskers represent maximum and minimum values within the sample. Jonckheere-Terpstra test showed significant (P = 0.00009) global dose-dependent effects of the CGL treatment. Kruskal-Wallis test (P<0.05) was followed by the Mann-Whitney U-test as a post-hoc test. *P<0.05, **P<0.01 versus vehicle. N = 5.

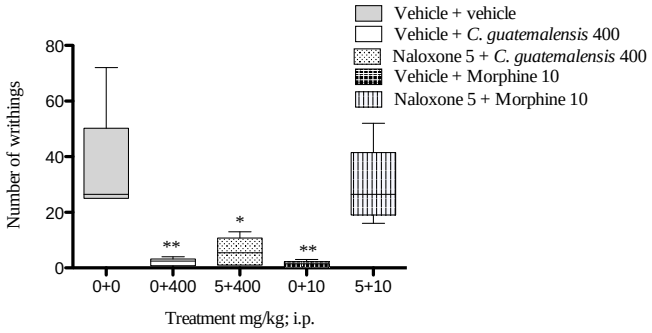

Fig. 2. Effects of naloxone on the *Croton guatemalensis*-induced reduction of abdominal writhings. Morfine, a μ-receptor agonist, was used as positive control. Both, *Croton guatemalensis* extracts and morphine administration reduced the number of abdominal writhings but only the effects of morphine were antagonized by naloxone. Values are presented as medians with their respective interquartile range. Whiskers represent maximum and minimum values within the sample. Kruskal-Wallis test (P<0.05) was followed by Dunn's multiple comparison test. *P<0.05, **P<0.01 versus vehicle. N = 6.

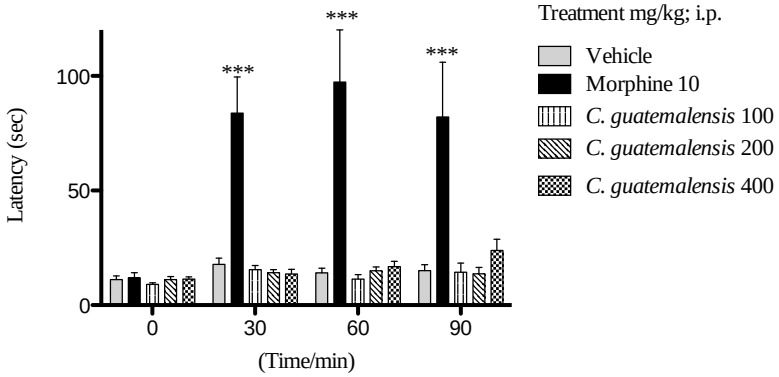

Fig. 3. Effects of aqueous extracts from *Croton guatemalensis* on the response latency of mice subjected to the hot-plate test. Only morphine used as a positive control increased the response latency as measured 30, 60 and 90 after *Croton guatemalensis* treatment. Results are means ± SEM. Two-way ANOVA was followed by the Bonferroni test for multiple comparisons as a post-hoc. ***P<0.001 versus the vehicle. N = 5.

Involvement of opioid receptors in the antinociceptive effects of Croton guatemalensis extracts

As shown in Fig. 2, naloxone (5 mg/kg; i.p.) an opioid receptor antagonist was able to block the antinociceptive effects of morphine in the acetic acid-induced writhing test but failed to antagonize the antinociceptive effects of the CGL extract (Kruskal-Wallis statistic = 22.14, df = 4/25; P<0.0002).

Hot-plate test

Two-way ANOVA showed overall significant effects both on treatment ($F_{4,80}$ = 48.07; P<0.0001) and on the time of testing ($F_{4,80}$ = 6.85; P<0.0006) as well as for the interaction between these two factors ($F_{4,80}$ = 16.36; P<0.0001). These effects were however exclusively accounted for the morphine treatment since no significant effects on any of the other variables were found (Bonferroni test) when CGL extract-treated groups (100, 200, 400 mg/kg; i.p.) were compared against their respective control group (Fig. 3).

Motor activity

Open-field test

No effects of CGL extract were observed both in the number of crossings (Kruskal-Wallis statistic= 6.233, df = 3/20, P = 0.1008)

and rearings (Kruskal-Wallis statistic = 6.756, *df* = 3/20, *P* = 0.0801) at any dose (100, 200, 400 mg/kg; i.p.) used in this test (Fig. 4).

Rotarod test

No effects in the number of falls (Kruskal-Wallis statistic = 1.481, *df* = 3/16, P = 0.6866) was observed in the rotarod test following CGL extract treatment (100, 200, 400 mg/kg; i.p.) (Fig. 5).

Acute toxicity

No mortality was observed up to 48 h following the systemic (i.p.) treatment of mice with a single dose (50, 100, 200, 300, 400, 800 mg/kg) of CGL extract (data not shown, N = 6).

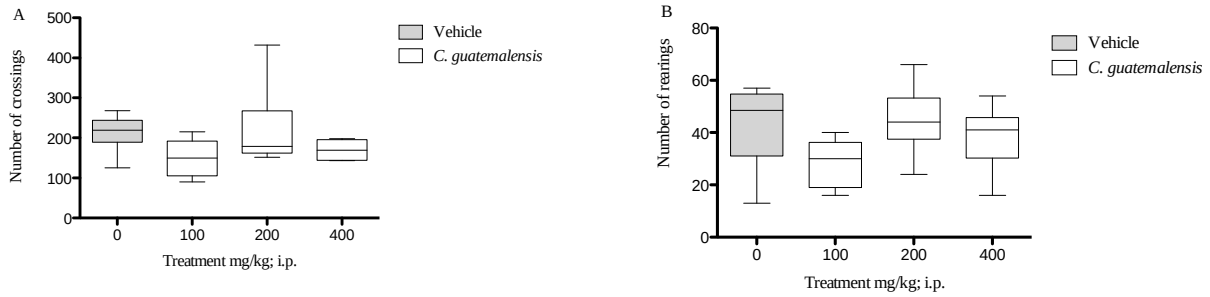

Fig. 4. Effects of aqueous extracts from *C. guatemalensis* in the open field test. Treatment with extracts from *Croton guatemalensis* modified neither A; the number of line crossings nor B; the number of rearings. Values are presented as medians with their respective interquartile range. Whiskers represent maximum and minimum values within the sample. Kruskal-Wallis test: *P*>0.05, N = 6.

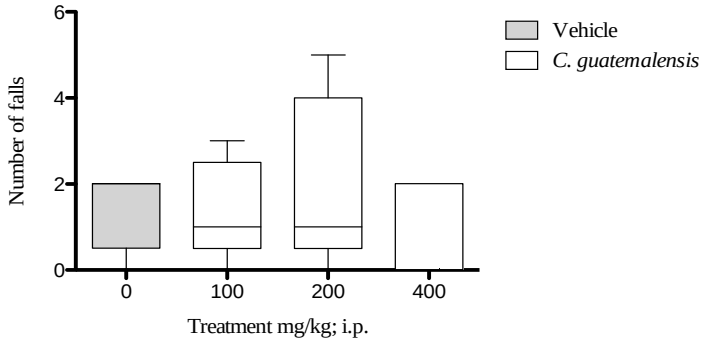

Fig. 5. Effect of aqueous extracts from *Croton guatemalensis* in the rotarod test. Treatment with extracts from *C. guatemalensis* failed to modify the number of the falls.Values are presented as medians with their respective interquartile range. Whiskers represent maximum and minimum values within the sample. Kruskal-Wallis test: *P*>0.05, N = 5.

DISCUSSION

The present study was conducted to assess whether the alleged antinociceptive effect of *C. guatemalensis* Lotsy, known as "Copalchi" in Mexican folk medicine (2,14,15) has any actual neurobiological basis and to get an insight of the mechanism of its putative effects.

Putative antinociceptive properties of CGL were studied using both the "acetic acid-induced writhing test" and the "hot plate test", two different animal models, which allow the assessment of noxious responses towards either a chemical or a physical stimulus respectively (28). Acetic acid-induced writhing test is used for detecting peripheral analgesia whereas the hot-plate test is more sensitive to centrally acting analgesics (29). In this work, it is reported that in mice aqueous extracts from the bark of *C. guatemalensis* Lotsy significantly reduced the number of writhings induced by the injection of acetic acid in a dose-dependent manner.

In contrast with the results obtained in the acetic acid-induced writhing test, no effects of

CGL extract were found in the hot-plate test. The reason why CGL extract have antinociceptive effects in the acetic acid-induced writhing test but not in the hot-plate test is not clear. Thus, in order to get an insight into the mechanism, which may underlie the antinociceptive properties of CGL extract, the involvement of opioid mechanisms was assessed in both paradigms. Our results show that although both CGL extract and morphine exhibited antinociceptive effects in the “acetic acid-induced writhing test”, naloxone, an opioid antagonist, was only effective in blocking the effects of morphine but not those of CGL extract suggesting that the opioid system is not involved in the antinociceptive effects of CGL extract. Moreover, in line with this suggestion only morphine displayed antinociceptive effects in the “hot-plate test”.

On the other hand, since both CGL extract and dipyrone, a non-steroidal anti-inflammatory agent, showed antinociceptive effects in the acetic acid-induced writhing test it may be feasible that inhibition of cyclooxygenase in peripheral tissues accompanied by a reduction of PGE2 synthesis (30) may be involved in the antinociceptive effects of CGL extract. Consistent with this suggestion, it is known that acetic acid induces pain by liberating pain mediators such as arachidonic acid, which is produced via the cyclooxygenase, and participates as a substrate in the prostaglandin biosynthesis (31). Since our results showed that CGL extract is enriched in flavonoids and it has been reported that these compounds inhibit prostaglandin synthesis and produce antinociceptive activity (32), it is tempting to speculate whether inhibition of prostaglandin synthesis mediated by flavonoid compounds present in our extracts may underlie their anti-nociceptive effects. It will of considerable interest for the future to isolate such flavonoid compounds from CGL extract and to evaluate their putative analgesic effects.

To our knowledge studies on the psychoactive properties of different genus of the plant studied here are rather scarce. However, *C. celtidifolius* a plant having the same genus of CGL has been shown to produce a decrease in spontaneous motor activity (33). In contrast no effects of CGL extract on both locomotion and motor coordination, as measured respectively in the open-field test and in the rotarod paradigm, were observed in this work suggesting that plants having the same genus may display a different pharmacological profile. Finally, it is important to remark that this lack of motor effects preclude the presence of confounding effects in our behavioral motor observations. In line with this relative lack of toxic effects no acute toxicity was observed in this work at those doses having antinociceptive effects.

CONCLUSION

The results of this work support the traditional use of CGL as analgesic in human diseases associated with pain and suggest that although its antinociceptive mechanism seems to be unrelated to classical opioid receptor stimulation it may at least partially involve a cycloxygenase inhibition. Further studies will be however needed to isolate from CGL extract the active principle(s) responsible of its antinociceptive effects.

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