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Antinociceptive and Antiedematogenic Acctivities of Essential Oil of *Croton argyrophylloides*

Kildere Marques-Canuto[2,3], Ariclécio Cunha de Oliveira[1], Felipe Carmo de Moura[1,2], Juliana Magalhães da Cunha Rêgo[2,3], Erika Clemente Lima Machado[3], Andrelina Noronha Coelho de Souza[1]

[1]*(Superior Institute of Biomedical Science, Ceara State University, Brazil)*
[2]*(Institute of Biomedicine, Faculty of Medicine, Ceara Federal University, Brazil)*
[3]*(Ceara Estacio Center University, Brazil)*

ABSTRACT*:* *Croton argyrophylloides* Muell. Argl (*Euphorbiaceae*), is a bush that natively grows in drought regions of Northeast of Brazil. Various essential oils of crotons of Northeastern Brazil, have demonstrated pharmacological efficacy. Since Preliminary experiments done in our laboratory have suggested that Essential oil of *Croton argyrophylloides* (EOCa) has analgesic activity, we undertook the present study aiming to characterize the antinociceptive effect of EOCa. EOCa was extracted from the leaves by steam destilation and found to be constituted predominantly of alpha-pinene, spatulenol, beta-felandrene and trans-caryophyllene. At the writhing test, after EOCa, dosed at 30, 100, 300, 600 and 1000 mg/Kg, the number acetic acid-induced contortions was significantly ($p \leq 0.05$) reduced to 44.3 ± 5.97, 32.1 ± 2.93, 23.7 ± 4.80, 22.5 ± 2.45 and 18.0 ± 1.97, respectively, of control (69.5 ± 3.36). At the hot plate test, EOCa, at 300 mg/Kg, significantly increased the latency time for nociception above the control value. This latency alteration remained significantly increased during 180 min. In summary, this study has demonstrated that EOCa has analgesic activity as an important pharmacological effect. This activity was induced at doses, far below LD50, which lends potential therapeutic importance to EOCa.

Keywords*:* Analgesic; Antiedematogenic; *Croton argyrophylloides*; Essential oil; Hot plate test

I. INTRODUCTION

Croton argyrophylloides Muell. Argl (*Euphorbiaceae*), popularly called "marmeleiro prateado", is a bush that natively grows in drought regions of Northeast of Brazil ("caatingas" and "carrascos"). Its popular name "prateado" is originated from the silver-like color of the inferior side of its leaves. *C. argyrophylloides* produces an essential oil (yield 1% leaf dry weight) that is constituted predominantly of alpha-pinene, spatulenol, beta-felandrene and trans-caryophyllene [1].

Many plants of the genus Croton are widely employed in folk medicine of Brazilian Northeast to treat many illnesses [2]. The pharmacological studies with the essential oil extracted from other Crotons of the "caatingas" and "cerrados", have shown a wealth of pharmacological effects. Thus, *Croton zehntneri* and *Croton nepetaefolius*, which are popularly used for treatment of intestinal problems, yield essential oils that are documented to bear antispasmodic [3, 4] and analgesic activity [5, 6], besides other activities related to its relaxant action on smooth muscle [7, 8, 9].

Since other essential oils of crotons of Northeastern Brazil, such as those of *C. zehntneri* and *C. nepetaefolius*, have demonstrated pharmacological activity, we decided to investigate whether the essential oil *C. argyrophylloides* (EOCa) has analgesic efficacy. Preliminary experiments done in our laboratory have shown that EOCa has antinociceptive activity. Due to that we undertook the present study aiming to characterize the antinociceptive effect of EOCa.

II. MATERIAL AND METHODS

Croton argyrophylloides leaves were collected in the vicinity of Viçosa, Ceará, Brazil. EOCa was extracted from fresh chopped plant leaves by steam distillation and analysed chemically as previously described [10]. Briefly: freshly chopped plant leaves were placed in a glass flask, connected at one end to a glass vessel filled with water and at the other end to a water-cooled condenser. The water was heated to boiling, and the steam percolated through the chopped leaves and collected in the condenser. After condensation, the aqueous phase with its solutes separated from an oily phase, the essential oil.

The composition of EOCa from leaves of *Croton argyrophylloides* collected at Viçosa, Ceará, Brazil and used in this study was determined by gas chromatography and mass spectrometry. It contained (in % oil weight): alpha-pinene, 20.6, spatulenol, 13.3, trans-caryophyllene, 8.26, beta-felandrene, 10.65, beta-elemene,

2.67, 1,8-cineole, 2.3, sabinene, 3.9, beta-pinene, 1.26, para-cimene, 2.95, 4-terpineol, 2.20, aromadendrene, 2.02, alpha-humulene, 2.25, allo-aromadendrene, 2.02, gamma-muurolene, 2.61, beta-selinene, 2.33, alpha-selinene, 2.64, alpha-muurolene, 2.37, gamma-cadinene, 2.37, delta-cadinene, 3.08 and trans-nerolidol, 2.85; not identified, 6.29 %.

III. ANIMALS

All experiments were done in accordance with the recommendations of the IASP (International Association for the Study of Pain). The experimental protocols were approved by the Animal Care and Use Committee of our Institute in accordance with the guidelines formulated by State University of Ceará on the Care and Use of laboratory animals for experimentation (process number 04464068-4). All experiments related to nociception used Swiss mice (20-25g/Kg b.w.). The experiments to study the antiedematogenic activity were done in Swiss mice (those using carrageenan) or wistar rats (those using dextran).

IV. EXPERIMENTAL PROCEDURE

Test for antinocioception were performed as folows

Writhing test was performed according to Koster et al. [11]. Briefly, 0.1 mL/10 g body weight (b.w.) of an aqueous acetic acid solution (0.6 % v/v) was administered by intra-peritoneal injection. Abdominal contortions were counted during a 20 minutes period, starting 10 minutes after acetic acid injection. Sixty minutes before administration of acetic acid the animals were treated either with the vehicle alone (control) or with a given dose of EOCa (10-1000 mg/Kg in 0.1 mL/10g b.w., p.o.).

Hot plate test was done according to O'Callaghan and Holzman [12] with minor modifications. Briefly, a mouse was placed over a plate maintained at a 55.0 $\pm$ 0.5 °C and the latency of its reaction to this nociceptive stimulus (number of seconds before it licked its hind paw or jumped) was quantified. A 30 s cut-off time was used to minimize tissue damage. Only mice, which in a pre-test had showed a hot plate reaction time between 8 and 12 sec. were used in this test. Mice were then treated with only vehicle (0.1% tween 80 aqueous solution, 0.1 mL/10g b.w., p.o.), or with doses of essential oil (10-300 mg/Kg b.w., p.o.) or morphine (10 mg/Kg b.w., s.c.). The latency of the reaction to nociception was measured immediately after pharmacological agent administration (zero experimental time) and then at each 60 min up to 240 min.

Formalin test was done as previously described by Hunskaar et al. [13]. Briefly, 20 microL of a solution (2.5% v/v) of formalin in water was injected in the plantar region of the right hind paw. The time that the animal spent licking the paw during the first 5 min (early phase) and from 15 to 30 min (late phase) post-injection, served as measures of sensitivity. EOCa (10-600 mg/Kg b.w., p.o.) solubilized in vehicle (0.1% tween 80, aqueous solution) or only vehicle (control) was administered (p.o.) with orogastric canula. Test was done at an ambient temperature of 22-26°C and care was taken to exclude environmental disturbances (high temperature, noise, excessive movement) that might interfere with animal response [14].

In order to investigate the possible muscle relaxant or central neurodepressant effects of EOCa, mice underwent the rota-rod test following procedure previously described [15]. Briefly, the animals were evaluated for the time spent before falling from the bar of the rota-rod apparatus (INSIGHT, Model RR-2002, São Paulo-Brazil) which consisted of a cylindrical bar (25 mm diameter) subdivided into six compartments, rotating at 22 revolutions per minute. Each final quantitative evaluation of a given animal at a given experiment was the average time the animal stood on the cylinder during three subsequent trials. The animals were trained and selected 24 h previously by eliminating those mice that did not remain on the bar for at least 30s uninterruptedly at any trial. EOCa (300-1000 mg/Kg b.w., p.o.) or same volume of vehicle (0.1 mL/10g b.w., p.o.) was tested only in those mice that were able to reproduce this performance previously to drug administration, at morning of the test. Agents were administered orally 60 min before testing. Results are expressed as the time during which animals (n = 8 per dose) remained on the rota-rod bar. The cut-off time used was 60 s.

EOCa solutions were prepared daily by vigorous manual agitation (3-5 min) or by vortexing the EOCa in vehicle (a solution of 0.1 % v/v tween 80 in distilled water) and administered (p.o.) with orogastric canula. The final EOCa concentration was selected so as to inject a constant volume of 0.1 mL solution /10 g, b.w. Morphine was obtained from Cristália (Rio de Janeiro, RJ, Brazil), naloxone, carrageenan, dextran, indomethacin and tween 80 from Sigma Chemical Co. (St. Louis, MO, USA), formalin and acetic acid was from Merck Darmstadt – Germany.

Statistical analysis

Results are presented as mean ± S.E.M. (n), with n indicating the number of experiments. Values were analyzed using Student's t test, ANOVA, or a non parametric test as appropriate, and were considered significant at $p \leq 0.05$.

V. RESULTS

EOCa antinociceptive activity

The number of contortions induced by acetic acid intra-peritoneal injection was 69.5 ± 3.36 (18) in controls. After EOCa, dosed at 30, 100, 300, 600 and 1000 mg/Kg b.w., this number was significantly ($p \leq 0.05$, ANOVA) reduced to 44.3 ± 5.97 (8), 32.1 ± 2.93 (8), 23.7 ± 4.80 (8), 22.5 ± 2.45 (8) and 18.0 ± 1.97 (9), respectively. The effect of EOCa was smaller than the effect of indomethacin (10 mg/Kg b.w., i.p.), which reduced the number of contortions to 1.3 ± 1.33 (6) (Fig. 1).

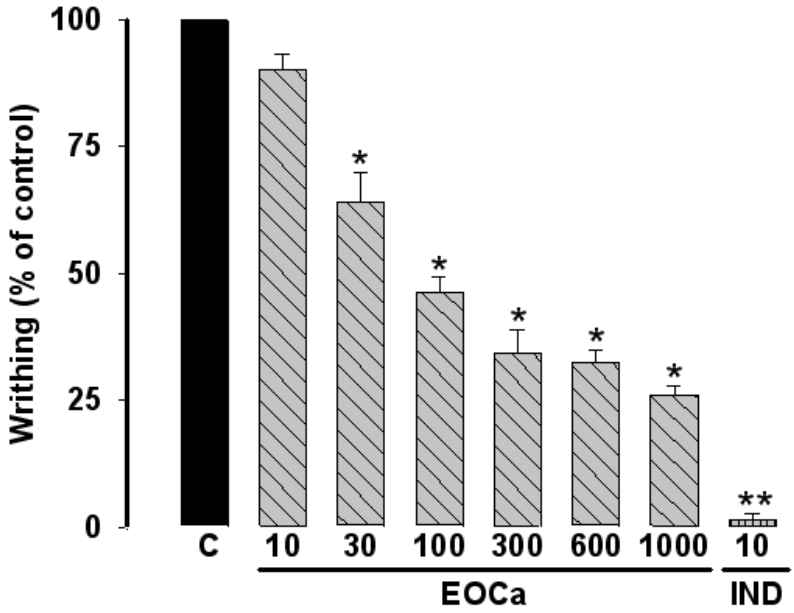

Fig. 1 Effects of the essential oil of *Croton argyrophylloides* (EOCa) on acetic acid (0.6%) induced writhing in mice. Ordinate values refer to writhing (% of control contortions number) upon the administration of acetic acid (i.p., 0,1 mL/10g b.w. of 0.6% v/v sol. in water) to animals previously dosed with EOCa (p.o.) or indomethacin (IND; i.p., 10 mg/Kg b.w.) or saline (C, control; p.o., 0,1 mL/10g b.w.). Data are reported as means ± SEM ($8 \leq n \leq 10$). *, $p < 0.05$ and **, $p < 0.01$ when compared with control group (ANOVA, Dunn's test).

As evaluated by the hot plate test, EOCa, at 300 mg/Kg b.w., significantly increased the latency time for nociception above the control value (9.1 ± 0,59 s (8)). This latency alteration remained significantly increased during a period of observation of 180 min (Fig. 2). Dosed at 10 and 30 mg/Kg b.w., EOCa had no effect. For comparison, the effect of morphine (10 mg/Kg b.w., s.c.) was also evaluated. The latency increase induced by 300 mg/Kg b.w. EOCa, although statistically significant, was smaller than that induced by 10 mg/Kg b.w. morphine.

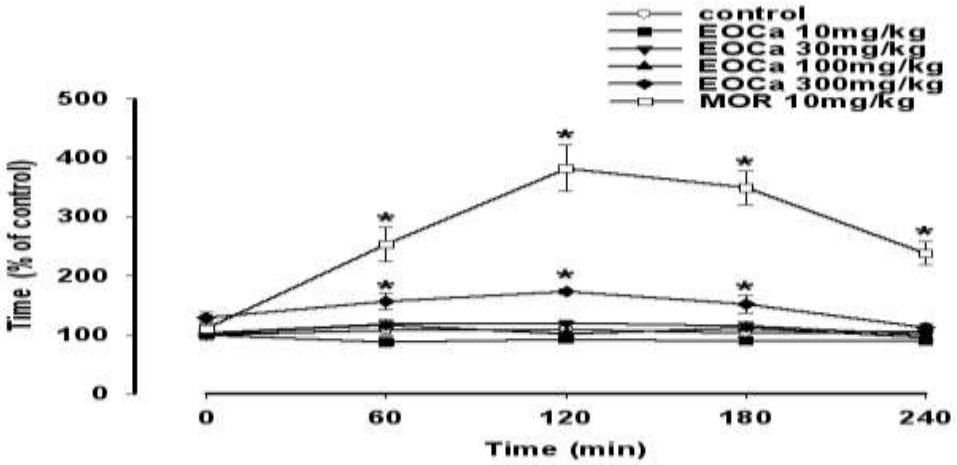

Fig. 2 Effects of the essential oil of *Croton argyrophylloides* (EOCa) on thermal nociception in mice submitted to the hot plate test. Abcissa shows time (min) after oral administration of EOCa (p.o.) or morphine (MOR; s.c.) or saline (C, control; p.o., 0,1 mL/10g b.w.). Ordinate, latency time (% of control) for the response to thermal stimulation (55 ± 0.5ºC). Data are reported as means ± SEM (n=8) for each agent dose. *, significantly different from control at a given time ($p < 0.05$ ANOVA, Dunn's test).

In the formalin test, at 30, 100 and 300 mg/Kg b.w. EOCa, significantly reduced ($p \leq 0.05$, ANOVA) to 60% (n = 8), 37% (n = 15) and 32% (n = 10), respectively, the number of seconds that mice spent licking their paws in the second phase (control: 149.5 ± 3.09 s (15)) of the response to formalin (Fig. 3a). At 10 mg/Kg b.w., the values of second phase and, at 10-300 mg/Kg b.w. EOCa, those values of the first phase of formalin test underwent no significant reduction. Morphine (10 mg/Kg b.w., s.c.) significantly reduced both phases of formalin test (Fig. 3). The analgesic effect of morphine on both phases of formalin test was reversed significantly by naloxone (5mg/Kg b.w., i.p.). Pretreatment with naloxone could not alter the antinociceptive activity of 100 mg/Kg EOCa (Fig. 3b).

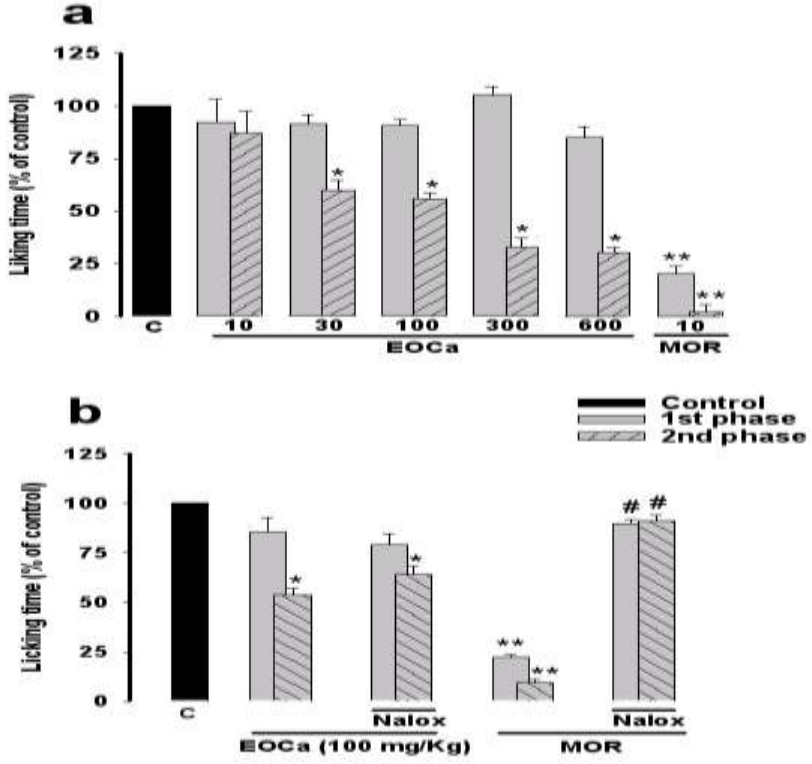

Fig. 3 Effects of the essential oil of *Croton argyrophylloides* (EOCa) on nociceptive response to intraplantar injection of formalin (1%). In a and b, ordinate values refer to the time (% of control) mice spent licking the paw at the two phases of formalin test. In a, quantification of licking time started 60 min after animals were dosed with EOCa (p. o.) or 30 min after morphine (MOR; s.c., 10 mg/Kg b.w.) or saline (C, control; p. o., 0,1

mL/10g b.w.). In b, naloxone (nalox, i.p., 5 mg/Kg b.w.) was administered 15 min before EOCa or MOR). Data are reported as mean ± SEM (n=8). * and **, $p \leq 0.05$ and $p \leq 0.01$, respectively, when compared with control and #, $p \leq 0.05$, when compared with morphine without naloxone group (ANOVA, Dunn's test).

Absence of motor central neurodepressant EOCa-induced activity

In the rota rod test, treatment of mice with 300, 600 and 1000 mg/Kg b.w. EOCa, did not significantly affect the motor response of animals. The mean performances in the rota rod test were 45.5 ± 2.31 s (control value 46.9 ± 2.22 s), 44.4 ± 2.43 (control 42.1 ± 3.53 s) and 43.9 ± 2.66 s (control 46.0 ± 2.65 s) in mice dosed with 300, 600 and 1000 mg/Kg b.w. EOCa, respectively (n = 8, each group).

EOCa antiedematogenic activity

In rats, EOCa, at a dose (100 mg/Kg b.w., p.o) which showed medium magnitude inhibitory effect on nociception, inhibited 46.4 ± 5.51 (8), 35.6 ± 4.28 (8) and 25.5 ± 6.92 % (8) of control (0.46 ± 0.051mL (8)) paw edema induced at the 30th, 60th and 120th min, respectively, after dextrana intradermal injection. Ciproheptadine (5mg/Kg b.w., p.o.) fully inhibited this edema during the 180 min of observation period.

In 8 mice, at 100 mg/Kg, p.o., EOCa reduced 46, 55, 71 and 83 % of control (26.11 ± 2.74 microL (8) paw edema induced at the 60t^{h}, 120th, 180th and 240th min, respectively, after carrageenan intradermal injection (Figure 4).

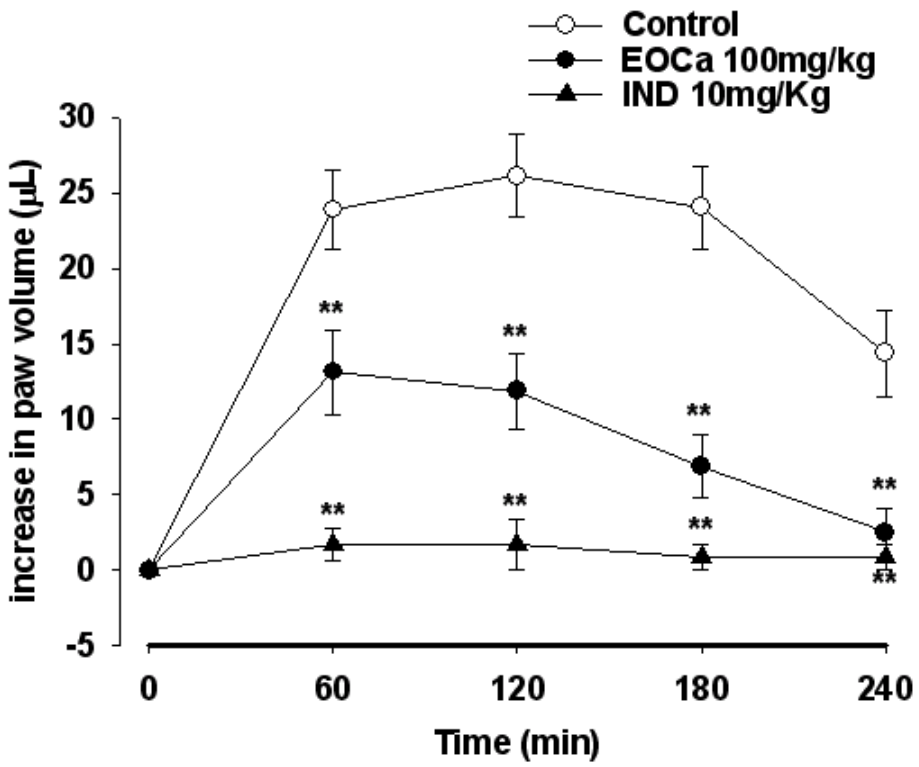

Fig. 4 Effects of the essential oil of *Croton argyrophylloides* (EOCa) on the carrageenan-induced rat paw edema. Abcissa, time (min) after intraplantar injection of carrageenan (1% w/v); ordinate, paw volume (microL). Data are reported as means ± SEM (n=6). **, $p \leq 0.01$, when compared with control group (ANOVA, Dunn's test).

VI. DISCUSSION

The most important discovery of the present study is that EOCa has antinociceptive and antiedematogenic activities which occurs in a range of doses much smaller than those for acute toxicity. This is a novel finding to best of our Knowledge. The positive results at various tests for antinociception, such as the writhing, the hot plate and formalin tests demonstrate that the essential oil of *Croton argyrophylloides* possesses antinociceptive activity. This effect is unlikely to be mediated through opioid receptors, since it is not altered by naloxone [16].

It is proposed that the writhing test is a general test that includes the demonstration of neural and anti-inflammatory components of nociception [17]. The effectiveness at the hot plate test of the larger concentration (300 mg/Kg) indicates that the analgesic agent acts primarily at the spinal medulla and/or higher central nervous system levels or by an indirect mechanisms [14, 18]. This result therefore suggests that EOCa, at least at higher concentrations (>300 mg/Kg b.w.) includes a component of central mechanism. At 30-300 mg/Kg, EOCa was not effective at the first phase of formalin test, attributed to a peripheral neural mechanism [19], which suggests no participation of this third mechanism in the antinociception induced by this essential oil at the range of doses here investigated. On the other hand, EOCa, at 30-300 mg/Kg, showed a positive result at the second phase of the formalin test, which is attributed to inflammatory activity and/or alteration of central processing [14, 20]. Thus, the effectiveness at this phase of formalin test is in accordance with a central mechanism or with an indirect effect, via anti-inflammatory action.

Since contortions induced by intra-peritoneal injections of acetic acid are said to originate, amongst other factors, from the pain of an inflammatory reaction [21, 22], taken together, the effectiveness of EOCa at the writing and the second phase of the formalin test suggest that anti-inflammatory activity as an important component of the mechanism of action of this essential oil [14, 18]. This suggestion is reinforced by the fact that, at the hot plate test, EOCa was effective only at the higher concentrations (> 300 mg/Kg) whilst at the writing and second phase of formalin tests it was effective from 30 to 300 mg/Kg. This suggestion was confirmed by the anti-edematogenic activity of EOCa here documented to hold for the two types of edema tested.

The two types of edemas here employed differ in many aspects. Dextran-induced swelling is promoted primarily by histamine and 5-hydroxytryptamine [23, 24]. and that induced by carragenan is caused predominantly by prostaglandins and BK [25, 26, 27]. No matter these differences, they have many steps in common, which hampers identifying with precision the inflammatory mediators whose activity is affected by the EOCa. The investigation of the mechanism of action of anti-edematogenic activity is currently under investigation in our laboratory.

The data showing the concentration-dependent antinociceptive activity on the various tests represents a profile of activity similar to other essential oils of crotons of northeastern Brazil. Similarly to EOCa, the essential oil of *Croton zehntneri* (EOCz) and *Croton nepetaefolius* (EOCn) showed similar pharmacological potency to block nociception [5, 6]. EOCa, however, more uniformly, as far as dose-response relationship is concerned, blocked the responses of writhing test and second phase of formalin test, since with EOCa this relationship is the same for both tests. Moreover, whilst EOCa showed no effect on the first phase of formalin test even at 300 mg/Kg, EOCn and EOCz did it, which suggest that EOCa might bear less potency for its central antinociceptive activity. What is most important in the comparison is that, although the antinociceptive activity of both three agents occur at the same range of doses, unpublished data from our laboratory have shown that the LD50 is much higher for EOCa (> 8 g/Kg) than for EOCz and EOCn (3.5 g/Kg). This suggests a much higher antinociceptive therapeautic index for EOCa than for EOCn and EOCz. Such a high therapeutic index places EOCa as a potentially useful candidate for human therapeutic use.

Croton argyrophylloides has received no use by the people of northeast of Brazil. Our laboratory has demonstrated with *in vivo* studies that EOCa has analgesic activity as an important pharmacological effect. It also has demonstrated that EOCa is an agent to be considered atoxic regarding acute toxicity. Since EOCa analgesic activity was induced at doses, far below LD50, this effect is potentially therapeutically useful, and this essential oil deserves further pharmacological investigation.

VII. Acknowledgments

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