Brazilian Journal of Medical and Biological Research (2002) 35: 1215-1219
ISSN 0100-879X Short Communication

Antinociceptive effects of the essential oil of *Croton nepetaefolius* on mice

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Abstract

Croton nepetaefolius Baill. is an aromatic plant native to the northeast of Brazil where it is extensively used in folk medicine as a sedative, orexigen and antispasmodic agent. In the present study the antinociceptive effects of the essential oil of *C. nepetaefolius* (EOCn), administered orally, were evaluated in male Swiss mice (20-25 g). In the acetic acid-induced writhing test, EOCn (100 and 300 mg/kg; N = 14 and N = 12, respectively) was effective at the highest dose. In the hot-plate test, EOCn at 30 and 300 mg/kg, but not at 3 mg/kg, significantly increased the latency at all observation times up to the 180th min (N = 12 for each dose). In the formalin test, EOCn significantly reduced paw licking in the second phase of the test at 100 mg/kg (N = 12), but decreased it in both phases at 300 mg/kg (N = 12). At 30 mg/kg, the effect of EOCn did not differ from control values in either phase of the formalin test (N = 6). Pretreatment with naloxone (5 mg/kg, *ip*) significantly reversed the analgesic effect of morphine (5 mg/kg, *sc*) on both phases, but not that of EOCn at 300 mg/kg (N = 6) on both phases of the formalin test. The data show that orally administered EOCn promotes a dose-dependent antinociceptive effect whose mechanisms remain to be elucidated.

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Presented at the XV Annual Meeting of the Federação de Sociedades de Biologia Experimental, Caxambu, MG, Brazil, August 23-26, 2000.

Research supported by CNPq, CAPES, and Colégio Christus.

Received April 12, 2000
Accepted July 4, 2002

Key words
- *Croton nepetaefolius*
- Essential oil
- Analgesic
- Antinociception
- Formalin test
- Hot-plate test

The aromatic plant *Croton nepetaefolius* Baill., popularly called "marmeleiro vermelho", is abundant in northeastern Brazil where it grows natively. *C. nepetaefolius* is widely used in folk medicine as a stomachic, a carminative and for the treatment of intestinal colic (1).

Despite its popularity as an herbal remedy, *C. nepetaefolius* has received little scientific attention. Available studies have dealt only with the chemical composition of the essential oil of *C. nepetaefolius* (EOCn) and its pharmacological effects on intestinal, respiratory and vascular smooth muscle and on arterial pressure (2-6). While EOCn is present in relatively large quantities in the leaves of the plant (1% yield) (7), pharmacological investigations have shown that it has potent antispasmodic activity, consistent with its use in folk medicine. It is not known, however, whether EOCn has other biological activities.

It has been reported that essential oils are biologically active agents (8). Thus, EOCn might induce other effects of possible therapeutic use. In the present study, we determined whether EOCn has antinociceptive activity.

C. nepetaefolius leaves were collected in September, 1998, in the vicinity of Viçosa,

CE, Brazil. Its botanical identity was confirmed by Dr. Francisco J. Abreu Matos (Laboratório de Produtos Naturais, Universidade Federal do Ceará (UFC)) and by Dr. Raymond Harley (Royal Botanic Garden, Kew, UK), and a voucher specimen (number 11096) is deposited in the Herbarium Prisco Bezerra (UFC).

EOCn was prepared and kindly provided by the Department of Physics and Chemistry of the Universidade Estadual do Ceará (UECE) and analyzed in the Laboratório de Produtos Naturais of UFC. EOCn was extracted from freshly chopped plant leaves by steam distillation and analyzed chemically as previously described (1). 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 was separated from an oily phase, the essential oil. The composition of EOCn from leaves of *C. nepetaefolius* used in the present study was determined by gas chromatography and mass spectrometry. It contained 31.5% 1,8-cineole, 17.2% caryophyllene E, 10.3% methyleugenol, 6.6% sabinene, 4.1% α-terpineol, 2.8% elemicin, 2.6% 1,3,5-trimethoxybenzene, 2.1% α-humulene, 1.8% α-pinene, 1.5% ß-caryophyllene oxide, 4.5% other identified minor (<1.0%) constituents, and 15.0% not identified constituents.

All experiments were carried out in male Swiss mice (20-25 g body weight) deprived of food, but with free access to drinking water for 12 h prior to the experiments. Animals were provided by the vivarium of UFC.

Tests for antinociception were performed as follows: the writhing test was performed according to the method of Koster et al. (9). Briefly, 0.1 ml/10 g body weight of a 0.8% (v/v) acetic acid solution in saline was administered by intraperitoneal injection. Abdominal contortions were counted during a 20-min period, starting 10 min after acetic acid injection (10). Antinociceptive activity was expressed as the reduction of the number of abdominal constrictions.

The hot-plate test was performed by the method of Jacob et al. (11). Briefly, a mouse was placed on a plate maintained at 50.0 ± 1ºC and the latency of its reaction to this nociceptive stimulus (number of seconds before it licked its hind paw or jumped) was quantified, with an interruption time ≤45 s. Only mice which in a pretest showed a hot-plate reaction time ≤20 s were used in this test. The latency of the reaction to nociception was measured at time 0 (60 min after pharmacological agent administration) and then at 30-min intervals up to the 180th min.

The formalin test was carried out as described by Hunskaar et al. (12). Briefly, 20 µl of a 1% (v/v) solution of formalin in saline was injected into the subplantar region of the right hind paw. The time the animal spent licking the paw during the first 5 min (early phase) and from 20 to 25 min (late phase) post-injection served as measures of sensitivity (10). The test was done at ambient temperature of 22-26ºC and care was taken to exclude environmental disturbances (high temperature, noise and excessive movement) that might interfere with the animal's response (13).

In the writhing, hot-plate and formalin tests, EOCn solubilized in vehicle (a solution of 0.1% Tween 80 in sterile saline) or only vehicle (control) was administered orally with an orogastric cannula 60 min before initiation of noxious stimulation. Morphine was administered subcutaneously, while indomethacin and naloxone were administered by the intraperitoneal route.

Morphine was obtained from Cristália (Rio de Janeiro, RJ, Brazil), Tween 80, naloxone and indomethacin from Sigma (St. Louis, MO, USA), and acetic acid and formalin were from Reagen (Rio de Janeiro, RJ, Bra-

zil). EOCn solutions were prepared daily by vigorous manual shaking (3-5 min) or by vortexing the EOCn in vehicle. The final EOCn concentration was selected so as to inject a constant volume of 0.1 ml solution/10 g body weight.

Results are reported as means ± SEM (N), with N indicating the number of animals. Data were analyzed by the Student *t*-test, ANOVA, or nonparametric tests as appropriate, and were considered significant at P≤0.05.

The number of contortions induced by intraperitoneal injection of acetic acid was 56.8 ± 4.99 in controls (N = 11). After EOCn, dosed at 100 and 300 mg/kg body weight, this number was 47.4 ± 1.78 (N = 14) and 41.1 ± 3.46 (N = 12), respectively, and after indomethacin (25 mg/kg, administered 30 min prior to noxious stimulation) the number was reduced to 21.8 ± 4.90 (N = 6). Only the alterations induced by EOCn dosed at 300 mg/kg and by indomethacin were significant (P≤0.05, ANOVA, Dunnett test).

As evaluated by the hot-plate test, EOCn, at 30 and 300 mg/kg, significantly increased the latency time for nociception above the control value throughout the period of observation (Figure 1). At 3 mg/kg body weight, EOCn had no effect. The latency increase induced by 30 and 300 mg/kg EOCn was similar to that induced by 10 mg/kg morphine, although the effect of the oil was longer lasting at both doses (P≤0.05, ANOVA, Dunnett test).

In the formalin test, at 300 mg/kg, EOCn significantly reduced (P≤0.05, ANOVA, Bonferroni *t*-test) by 37 and 58% (N = 12) the number of seconds the mice spent licking their paws in the first and second phase (control: 54.5 ± 4.03 and 39.7 ± 5.04 s, N = 12), respectively, of the response to formalin (Figure 2A). At the dose of 100 mg/kg, EOCn significantly (P≤0.05, ANOVA, Bonferroni *t*-test, N = 12) reduced (48%) only the second phase. At 30 mg/kg no significant reduction occurred. The analgesic effect of morphine (5 mg/kg) on both phases of the formalin test was significantly reversed by naloxone (5 mg/kg) (Figure 2B). Naloxone, however, did not alter the effect of EOCn (300 mg/kg) on the formalin test (Figure 2B) (P≤0.05, ANOVA, Bonferroni *t*-test).

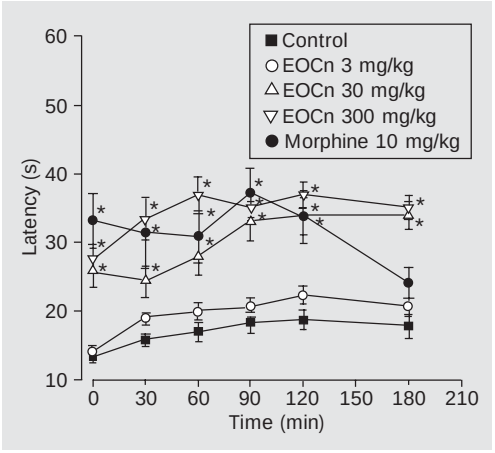

Figure 1. Antinociceptive effect of the essential oil of *Croton nepetaefolius* (EOCn) in the hot-plate test. Each point represents the mean ± SEM, indicated by vertical bars. Abscissa, time in min (0 time: 60 min after *per os* administration of EOCn or 30 min after subcutaneous administration of morphine); ordinate, latency time (s) for the response to thermal stimulation (50.0 ± 1ºC). N = 12 for each EOCn dose and N = 6 for morphine. *P≤0.05 compared to control (ANOVA, Dunnett test) at a given time.

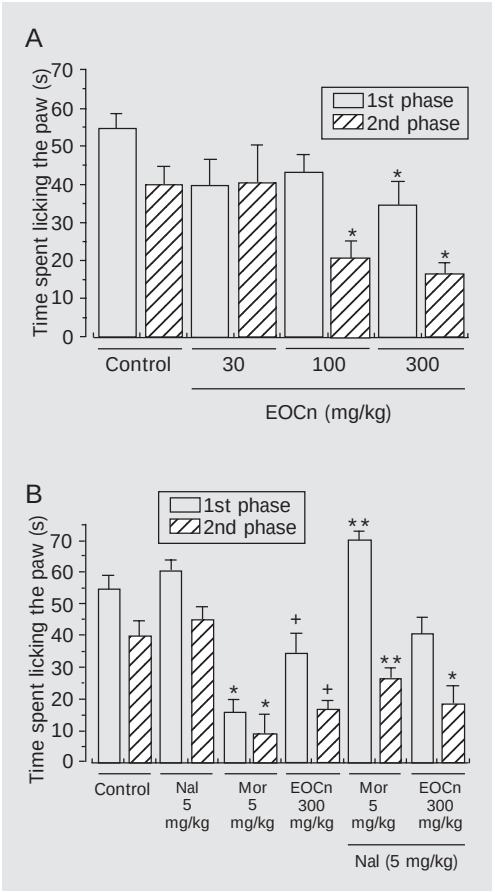

Figure 2. Effect of the essential oil of *Croton nepetaefolius* (EOCn) on the nociceptor stimulation by intraplantar injection of formalin (1%). Data are reported as means ± SEM, with the SEM indicated by vertical bars. Morphine (Mor) was administered subcutaneously and EOCn (*per os*) 30 and 60 min prior to noxious stimulation, respectively. Naloxone (Nal) was administered intraperitoneally 10 min prior to morphine or EOCn administration. *P≤0.05 compared to control, **P≤0.05 compared to morphine alone, and $^{+}$P≤0.05 compared to control but not in the presence of naloxone (ANOVA, Bonferroni *t*-test).

The results of the hot-plate and formalin test demonstrate that EOCn possesses antinociceptive activity. We propose that the effectiveness of EOCn in the hot-plate test indicates that the analgesic agent acts primarily at the spinal medulla and/or higher central nervous system levels or by an indirect mechanism (12-14). This result therefore might suggest that EOCn includes a component of action with a central mechanism. EOCn, at 100 mg/kg, showed antinociception in the second phase of the formalin test, and noxious stimulation of this phase is attributed to inflammatory activity and/or alteration of central processing (13-15). Thus, the effectiveness during this phase of the formalin test is consistent with a central mechanism or with an indirect effect via an anti-inflammatory action. At 300 mg/kg EOCn was also effective during the first phase of the formalin test, an event attributed to a peripheral neural mechanism (16), which suggests the participation of this third mechanism in the antinociception induced by this higher dose of the essential oil. If there is central mechanism participation, it is not likely to involve opiate receptors, since naloxone (11), which blocked the effect of morphine, did not alter EOCn-induced antinociception.

Experiments from our laboratory which were not included in the results of the present study showed that 300 mg/kg EOCn did not alter the motor performance of mice in the Rotarod test (17). This absence of effect on the Rotarod test and the writhing test at doses smaller than 300 mg/kg EOCn suggests that this essential oil has no general depressant action. The effect of EOCn on the writhing test, although significant for 300 mg/kg, was of less importance in the range of doses, since it reduced the control value only by 27.5%, while in the second phase of the formalin test it produced a more marked reduction. This decreased EOCn-induced effect on the writhing test came as a surprise, since as a broad-spectrum investigation tool to identify analgesic agents this test was expected to be more sensitive to EOCn activity. So far, we do not have an explanation for this effect. The data presented here, although suggestive of involvement of primarily neural mechanisms, do not preclude a component of the antinociceptive effect of EOCn due to an anti-inflammatory activity secondary to an inhibitory action on the phlogogenic activity of autacoids such as histamine and/or serotonin. The elucidation of the mechanism of EOCn-induced antinociceptive action, which was not the aim of the present study, needs further investigation.

Regarding the contribution of EOCn constituents to its antinociceptive activity, 1,8-cineole is likely to participate in the induction of this effect, since 1,8-cineole, in the 100-400 mg/kg oral dose range, has been reported to bear antinociceptive activity (18), and 35% of the EOCn weight corresponds to 1,8-cineole. Furthermore, in agreement with the mechanism of action of EOCn, 1,8-cineole was found to act by a non-opioid mechanism (18). Methyleugenol (19) and α-terpineol (20) have been reported to be central and peripheral neural depressants, respectively, which raises the suspicion that they might act as antinociceptive agents. For the other constituents we found no report in the literature on either antinociceptive or neural activity.

C. nepetaefolius is used by the people of northeastern Brazil as a sedative, orexigen and antispasmodic agent. Our laboratory has confirmed in *in vitro* studies that EOCn has antispasmodic activity (2,6), which is consistent with the use of the plant in folk medicine. In the present study we detected analgesic activity as an additional pharmacological effect of EOCn. Unpublished experiments from this laboratory have shown that the LD_{50} of EOCn (administered *per os*) is >3 g/kg body weight. Since the analgesic activity of EOCn was induced at doses far below the LD_{50}, this effect is of potential therapeutical use, and this essential oil deserves further pharmacological investigation.

Acknowledgments

We are grateful to Dr. Afrânio A. Craveiro and Iracema Lacerda Machado for performing the chemical analysis of EOCn. We thank Dr. Steven D. Aird for editing the manuscript.

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