



Evaluation of antinociceptive and antiinflammatory effects of *Croton pullei* var. *glabrior* Lanj. (Euphorbiaceae)

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RESUMO: “Avaliação dos efeitos antinociceptivo e antiinflamatório de *Croton pullei* var. *glabrior* Lanj. (Euphorbiaceae)”. *Croton pullei* var. *glabrior* Lanj. (Euphorbiaceae) é uma liana, amplamente distribuída na Floresta Amazônica. Na medicina popular, diversas plantas do gênero *Croton* têm sido utilizadas com fins terapêuticos em patologias que envolvem dor e inflamação, o que justifica este trabalho. O objetivo deste estudo foi investigar as atividades antinociceptiva e antiinflamatória do extrato metanólico das folhas de *C. pullei* (MECP). O MECP reduziu, de forma dose-dependente, o número de contorções abdominais (1,2 %) em camundongos, sugerindo uma atividade antinociceptiva da planta. Por outro lado, o MECP não alterou significativamente a reatividade ao estímulo térmico no teste da placa quente e a reatividade à estimulação química na primeira fase do teste da formalina, indicando um mecanismo não-opioidérgico. O MECP reduziu a nocicepção na segunda fase do teste da formalina, inibiu o edema de orelha induzido pelo óleo de croton e reduziu a migração leucocitária no teste da peritonite induzida por carragenina, indicando uma atividade antiinflamatória. Apesar dos mecanismos responsáveis pelos efeitos da planta ainda não estarem completamente esclarecidos, estes resultados parecem justificar o uso medicinal potencial de *Croton pullei* var. *glabrior* Lanj. em patologias que envolvam dor e inflamação.

Unitermos: Antiinflamatório, antinociceptivo, *Croton pullei* var. *glabrior*, fitomedicina.

ABSTRACT: *Croton pullei* var. *glabrior* Lanj. (Euphorbiaceae) is a liana, vastly distributed in the Amazonian Forest. In the folk medicine, several plants of the *Croton* genus have been used with therapeutic purposes in pathologies that involve painful and inflammatory diseases which justify this work. The aim of this study was to investigate the antinociceptive and antiinflammatory activities of the *C. pullei* leaves methanol extract (MECP). MECP reduced in a dose-dependent manner the number of acetic acid-induced abdominal writhing (1.2%) in mice, suggesting an antinociceptive activity of the plant. On the other hand, MECP did not significantly modify the reactivity to the thermal stimulation in the hot-plate test and the reactivity to the chemical stimulation in the formalin test first phase, indicating a non-opioid mechanism. MECP reduced the formalin-induced nociception in the second phase, inhibited the croton oil-induced ear edema and reduced the leukocytes migration in the test of the carrageenan-induced peritonitis, indicating an antiinflammatory activity. Although the mechanisms that underlie these plant effects are not completely elucidated, these results appear to support the potential medicinal use of *Croton pullei* var. *glabrior* Lanj. against painful and inflammatory diseases.

Keywords: Antiinflammatory, antinociceptive, *Croton pullei* var. *glabrior*, phytomedicine.

INTRODUCTION

Pain is considered one of the most common complaints worldwide for which patients seek treatment. Along the history different treatments have been used to lessen pain. Nowadays, several antiinflammatory agents

are used to treat different types of pain associated or not to the inflammatory process. These agents are efficient in most cases, but the collateral effects are common, especially when they are used in chronic treatments. The most common collateral effect is gastrointestinal disturb (Peura & Goldkind, 2005). An approach to solve this

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problem was the use of selective COX-2 inhibitors with a low risk of gastrointestinal bleeding (Bombardier et al., 2000; Silverstein et al., 2000). Although these drugs are well tolerated, their cardiovascular safety has been questioned a few years ago (Mukherjee et al., 2001), and recently, rofecoxib was withdrawn from the market because of an increased risk of cardiovascular disease, mainly myocardial infarction and stroke (Singh, 2004).

Considering this situation, new drugs with different spectre of action or less adverse side effects have been searched. The research of medicinal plants can propitiate the discovery of new molecules with innovative mechanisms or less adverse side effects (McCurdy & Scully, 2005). Euphorbiaceae family is well known between the medicinal plants with more than 8,000 species vastly distributed in tropical and temperate regions of the world (Wilson et al., 1979; Agra et al., 2007). In this context, a genus with several species used in the folk medicine to treat different disorders is the genus *Croton*. Some of *Croton* species have been exhaustively studied with their medicinal and toxicological properties being scientifically proven (Souza et al., 2006; Costa et al., 2007; Perazzo et al., 2007; Salatino et al., 2007; Torrico et al., 2007). Other species of this genus were poorly studied, and their pharmacological properties and collateral effects are still unknown.

Croton pullei (Euphorbiaceae) is a liana that grows above other trees, distributed in tropical areas with vast distribution in the Amazon forest (Gallenmüller et al., 2001). Until this moment we have not found any report about the use of this species in the folk medicine. On the other hand, this plant belongs to a genus that is used commonly with medicinal purposes which justify this study. The aim of this work was to evaluate the effects of the *glabrior* Lanj. variety of *Croton pullei* in models of nociception and inflammation.

MATERIAL AND METHODS

Crude methanol extract

Croton pullei var. *glabrior* Lanj. leaves were collected in the city of Peixe-boi, State of Pará, (Amazon region - Brazil), in December 1997. Samples were authenticated by Dr. Ricardo de Souza Secco, a botanical from Emílio Goeldi Paranse Museum (MPEG), and voucher specimens were deposited at the herbarium of MPEG under MG-0151738 number. The methanol extract was obtained by percolation and then concentrated dry under reduced pressure, below 40 °C, to yield the methanol crude extract (MECP) (6.1%).

Drugs and reagents

Formalin, acetic acid, acetone (Merck AG,

Darmstadt, Germany), croton oil, indomethacin, carrageenan (Sigma Chemical Co., St. Louis, MO, USA), fentanyl (Janssen Pharmaceutical, Belgium), dexamethasone (Prodome, Brazil). MECP and indomethacin were diluted in tap water, whereas fentanyl and dexamethasone were diluted in saline.

Animals

Male adult Swiss mice weighting 25-30 g were used in all experiments. Animals were maintained on a 12-h light -dark cycle (lights on at 7:00 a.m.) at constant room temperature (23 ± 2 °C) with free access to food and water, except during the experiments. Control animals received tap water as vehicle (10 mL/kg, p.o.) and each animal was used just once. All experiments were carried out in accordance with current guidelines for the care of laboratory animals and ethical guidelines on the use of animals in pain research (Zimmermann, 1986). The experimental protocols were approved by the local Animal Care and Use Committee (005/2006/CEPEB/UFRRJ).

Acetic acid-induced writhing test

Groups from 6 to 9 mice were treated orally (p.o.) with vehicle, MECP (0.1 to 1.0 g/kg) or indomethacin (10 mg/kg) 60 min before intraperitoneal (i.p.) acetic acid injection (1.2%, 0.1 mL/10 g) and the number of writhes was counted for the following 30 min (Koster et al., 1959).

Hot-plate test

The latency (s) of heat stimulus (55 ± 0.5 °C) was measured every 30 min, starting 30 min before and up to 2 h after pre-treatment of 8 groups of mice with MECP (1.0 g/kg, p.o.), water or subcutaneous (s.c.) application of fentanyl (200 µg/kg) (D'Amour & Smith, 1941).

Formalin-induced nociception

Groups from 7 to 9 mice were orally treated with vehicle, MECP (1 g/kg) or indomethacin (10 mg/kg) 60 min prior to injection of the formalin solution (1.2 %; 20 µL/paw) into the plantar surface of the hind paw (i.p. injection). Another group was treated with fentanyl (200 µg/kg, s.c.) 15 min before these formalin nociceptive stimuli. The time that animals spent licking the injected paw was measured with a chronometer and was considered as an index of nociception. After the formalin injection, the initial nociceptive response peaked at about 5 min, denominated early phase, which

was followed by a second peak (late phase) that occurred at 15-30 min post injection (Hunnskaar et al., 1985).

Edema induced by croton oil in mouse ear

One hour after oral administration ($n = 7$) of water, MECP (1.0 g/kg) or indomethacin (10 mg/kg), each animal was treated with 20 μ L of freshly prepared croton oil (2.5% in acetone) on the inner surface of the right ear. The left ear was treated with the same volume of acetone (control). Four hours after treatments, mice were killed by cervical dislocation and a plug (6 mm in diameter) was taken from both the treated and untreated ears with a punch. We monitored the inflammatory response (edema) by weighing (mg) both plugs and testing the differences (Zanini Junior et al., 1992).

Carrageenan-induced peritonitis

Groups of 7 animals were treated with vehicle, dexamethasone (1 mg/kg, s.c.) or MECP (0.1-1.0 g/kg, p.o.), followed by 1% carrageenan (0.25 mL, i.p.) 1 h later. Four hours after the intraperitoneal injection, mice were killed and 2 mL of modified PBS (heparin, 10 IU/mL) were injected into the peritoneal cavity. Total cell counts were performed using a Neubauer chamber as described by Ferrández and Alcaraz (1991). The results were expressed as means \pm SEM of the numbers of total leukocytes ($\times 10^7$) per mL of peritoneal wash or percentage of inhibition of leukocyte migration compared to control group.

Statistical analysis

Data were statistically analysed by one-way ANOVA followed by the *Dunnett* multicomparison test. In the hot-plate test, the two-way ANOVA followed by the Bonferroni's test analysis was used. The values are reported as mean \pm standard error of the mean (SEM). P values less than 0.05 ($p < 0.05$) were considered to be significant.

RESULTS

Acetic acid-induced writhing test

Figure 1 shows that MECP (0.3 and 1.0 g/kg, p.o.) produced a significant ($p < 0.05$) dose-related inhibition of acetic acid-induced abdominal writhing by 33.6 and 65.5%, respectively, when compared to the control group. The major dose tested produced inhibition of abdominal constriction similar to positive control indomethacin (10 mg/kg), which produced an inhibition by 65.1%.

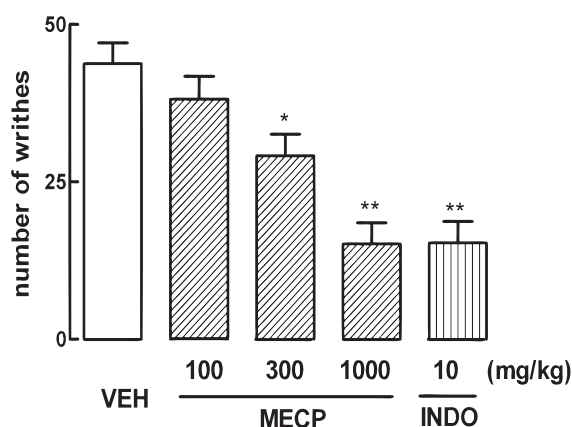


Figure 1. Effect of methanol extract of *C. pullei* (MECP) or indomethacin (INDO) on the acetic acid-induced writhing in mice. Values are expressed as Mean \pm SEM of the number of contractions registered for 30 min after the acetic acid injection. * $p < 0.05$; ** $p < 0.01$ when compared to vehicle control group (ANOVA, Dunnett's test as the *post hoc* test), $n = 6-9$.

Hot-plate test

The MECP was inactive in the hot-plate test at the highest dose effective in acid acetic abdominal writhing test (1.0 g/kg, p.o.). Fentanyl (200 μ g/kg s.c.), used as a reference drug, produced a significant antinociceptive effect at 30 min after administration of this drug when compared to control values (Figure 2).

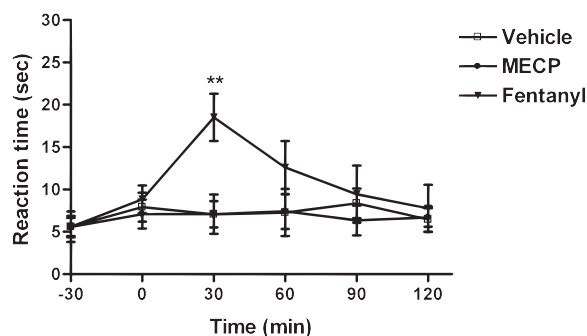


Figure 2. Effect of methanol extract of *C. pullei* (MECP; 1.0 g/kg p.o.) or fentanyl (200 μ g/kg s.c.) on the nociceptive response of mice in the hot-plate test. Values are expressed as mean \pm SEM of the latency for the nociceptive behavior. ** $p < 0.01$ when compared to vehicle control group (Two-way ANOVA, Bonferroni's test as the *post hoc* test), $n = 8$.

Formalin-induced nociception

In the first phase of formalin-induced nociception, the orally pre-treatment with MECP (1.0 g/kg) did not modify significantly the licking time of the animals when compared to control group. However, in

the second phase a significant reduction (71.5%) in the licking time could be observed after MECP treatment, with reduction comparable to that of indomethacin (10 mg/kg), which produced a reduction by 68.5% as showed in Table 1.

Croton oil-induced ear edema test

Table 2 shows that MECP (1.0 g/kg, p.o.) caused an inhibition of 72.4% in croton oil-induced ear edema ($p < 0.05$ vs control). Indomethacin (10 mg/kg), used as a reference drug, inhibited the ear edema by 58.1% when compared to control result.

Carrageenan-induced peritonitis

The results listed in Table 3 show that orally administration of MECP (0.1, 0.3 and 1.0 g/kg) produced a dose-related reduction of the number of leukocytes that migrated to the peritoneal cavity, with inhibition by 33.1, 60.1 and 76.7%, respectively. The highest dose of MECP inhibited leukocytes migration comparable to that induced by dexamethasone (1 mg/kg, s.c.) which produced 81.2 % of inhibition.

DISCUSSION

The antinociceptive effect of the MECP was tested in three models of nociception: acetic acid-induced abdominal writhing, hot-plate test and formalin-induced nociception. The treatment with MECP promoted dose-related antinociceptive effect in the acetic acid-induced abdominal writhing model. Although this model could be commonly used as a screening method to identify drugs with antinociceptive activity potential, several groups of drugs with different mechanisms of action can inhibit the abdominal writhing (Koster et al., 1959; Hendershot & Forsaith, 1959). Moreover, the involvement of different mediators such as prostaglandins (Deraedt et al., 1980), neurokinin A (Julia & Buéno, 1997) and CGRP (Friesse et al., 1997), for example, was described in this experiment which means that is not possible to suggest any mechanism to the antinociceptive effect of MECP, based only on this model. In the hot-plate test, MECP did not induce any antinociceptive effect. Since supraspinal and spinal opioid receptors play an important role in this assay (Schmauss & Yaksh, 1984) it is possible that MECP does not act on central opioid receptors or produce release of endogenous opioid peptides. This conclusion is reinforced by the fact that MECP did not reduce the licking time in the

Table 1. Effect of *Croton pullei* methanol extract on formalin-induced nociception in mice^a.

Group	Dose	n	Licking time (s)	
			Early phase	Late phase
Vehicle	10 mL/kg (p.o.)	9	97.2 ± 8.1	112.1 ± 18.6
MECP	1.0 g/kg (p.o.)	8	98.2 ± 10.4	31.9 ± 8.2**
Indomethacin	10 mg/kg (p.o.)	9	123.7 ± 13.3	35.3 ± 13.7**
Fentanyl	200 µg/kg (s.c.)	7	4.7 ± 2.2**	6.7 ± 3.2**

^a Values are Mean ± SEM of the time that animals spent licking the injected paw with formalin.

* $p < 0.01$ when compared to vehicle control group (ANOVA, Dunnet's test as the *post hoc* test).

Table 2. Effect of *Croton pullei* methanol extract on croton oil-induced ear edema in mice^a.

Group	Dose	Edema (mg)	Inhibition (%)
Vehicle	10 mL/kg (p.o.)	7,4 ± 1,3	-
MECP	1.0 g/kg (p.o.)	2,0 ± 0,6**	72.4
Indomethacin	10 mg/kg (p.o.)	3,1 ± 0,8**	58.1

^a Values are Mean ± SEM of differences in weight between right and left plugs ear of seven animals.

** $p < 0.01$ when compared to vehicle control group (ANOVA, Dunnet's test as the *post hoc* test).

Table 3. Effect of *Croton pullei* methanol extract on carrageenan-induced peritonitis in mice^a.

Group	Dose	Number of total leukocytes ($\times 10^7$)	Inhibition (%)
Vehicle	10 mL/kg (p.o.)	1.33 ± 0.18	-
MECP	0.1 g/kg (p.o.)	0.89 ± 0.09*	33.1
	0.3 g/kg (p.o.)	0.53 ± 0.07**	60.1
	1.0 g/kg (p.o.)	0.31 ± 0.03**	76.7
Dexamethasone	1 mg/kg (s.c.)	0.25 ± 0.05**	81.2

^a Values are Mean ± SEM of the number of the total leukocytes per mL of peritoneal wash after carrageenan injection in seven animals.

* $p < 0.05$

** $p < 0.01$ when compared to vehicle control group (ANOVA, Dunnet's test as the *post hoc* test).

first phase (neurogenic pain) of formalin test, which is highly sensitive to opioid agents (Hunnskaar & Hole, 1987). MECP reduced the licking time in formalin test second phase (inflammatory pain). This phase of the test is sensitive to non-steroid antiinflammatory drugs like indomethacin, used as reference drug (Hunnskaar & Hole, 1987). This result suggests that the antinociceptive effect of MECP could be related to antiinflammatory mechanisms.

The antiinflammatory hypothesis was tested in two models that evaluate different aspects of inflammatory process, the edema and the leukocyte migration. MECP reduced significantly the croton oil-induced ear edema by 72%, and also reduced in a dose-related manner the total leukocyte migration in the peritoneum after carrageenan stimulus. Production of exudates in these models is related to local release of vasoactive substances (histamine and kinins) and synthesis of prostaglandins (Morrow & Roberts II, 2001). Although migration of leukocytes could not be directly related to cyclooxygenase products, the process could be inhibited by some non-steroidal antiinflammatory compounds, indicating that many mechanisms may be implicated in its control (Mikami & Miyasaka, 1983). Steroidal antiinflammatory effects on the leukocyte migration may also be multifactorial since it could be related to the inhibition of phospholipase A2 (Morrow & Roberts II, 2001) as well as other mechanisms, for example, inhibition of TNF α and IL-1 β production which are potent triggers of many of the actions involved in leukocyte migration (Pereira et al., 2006). Since several mechanisms may be involved in the leukocytes migration more studies must be done to elucidate the mechanism of the antiinflammatory effect of MECP.

Previous work with the trunk wood and bark of *Croton pullei* var. *glabrior* Lanj. identified steroids such as sitosterol ester and stigmaterol, the triterpene lupeol and the diterpene kaurenoic acid (Abreu et al., 2001). The antiinflammatory and antinociceptive effects of stigmaterol (Santos et al., 1995; Garcia et al. 1999), beta-sitosterol and beta-sitosteryl-beta-D-glucoside (Villaseñor et al., 2002), kaurenoic acid (Block et al., 1998; Paiva et al., 2003) and lupeol, (Fernández et al., 2001a,b; Nikiéma et al., 2001) isolated from different vegetal species have been demonstrated in pharmacological models and may contribute to the effect of MECP.

Although beta-sitosterol, stigmaterol, kaurenoic acid and lupeol are potential compounds to justify the antinociceptive and anti-inflammatory actions of the MECP, these compounds were isolated from the trunk wood and bark of *Croton pullei* var. *glabrior* Lanj. (Abreu et al., 2001) and not from the leaves, material used in the present study. A bioassay-guided fractioning of this extract is now in progress to identify the bioactive substance(s) in the methanol extract of *C. pullei* as well as the mechanisms of action involved in the effects

described before.

ACKNOWLEDGMENTS

The authors thank University Centre of Barra Mansa and Brazilian National Research Council (CNPq) for financial support.

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