ORIGINAL ARTICLE

Anti-inflammatory Activity of Methanolic Extract and an Alkaloid from *Palicourea crocea* (Sw.) Roem and Schult

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Abstract— *Palicourea crocea* (Sw.) Roem. and Schult., "douradinha," are used by treat inflammation (edema). Croceaine A (PC-1) was isolated from *P. crocea* (MEPC) leaves and studied for its antioxidant and anti-inflammatory activity, as well as concentrations of constituents and acute toxicity. The phenols and polyphenolics compounds and HPLC/DAD were determined. The antioxidant activity were evaluated for DPPH, ABTS, and MDA. MEPC (300, 100, and 300 mg/kg) and PC-1 (10 and 30 mg/kg) were tested for anti-inflammatory effects in paw edema, pleurisy, cold sensitivity, and mechanical hyperalgesia. Acute toxicity is also described. MEPC contained high concentrations of phenolic and flavonoid compounds (≤ 800.35 mg/g), as well as caffeic acid, ferulic acid, rutin, and quercetin, revealed by HPLC-DAD analysis. MEPC displayed antioxidant activity against ABTS radicals ($IC_{50} = 68.5$ μg/mL) and MDA (74%). MEPC and alkaloid PC-1 demonstrated an anti-edematogenic effect in Cg-induced paw edema in 2 and 4 h, and also significantly reduced mechanical hyperalgesia, cold response to acetone in mice, at 3 and 4 h after injection, as well as leukocyte migrationin the pleurisy model. No toxicity was detected by MEPC. For the first time, *P. crocea* was evaluated for its antioxidant, systemic anti-inflammatory, and anti-hyperalgesic activities.

KEY WORDS: antioxidant; anti-hyperalgesic; croceaine A; douradina; edema; pleurisy.

INTRODUCTION

Palicourea crocea (Sw.) Roem. and Schult., known as "douradina", "douradão," and "douradão-do-campo," is used by Ribeirinhos in the North Araguaia microregion, Mato Grosso, Brazil, to make infusions or decoctions for general infections and anti-inflammatory (edema) [1], and is not considered a toxic plant [2–6]; however, some species in this genus are highly lethal, *i.e.*, *P. marcgravii* St. Hil. in Brazil contains a toxic organofluorine compound and is a very poisonous plant due to its acute toxicity and palatability [7–12]. Chemical studies of this species reported the isolation of alkaloids such as croceaines A and B, psychollatine, 3,4-dihydro-1-(1-β-glucopyranosyloxy-1, 4a, 5, 7a-tetrahydro-4-methoxycarbonylcyclopenta [c] pyran-7-yl)-β-carboline-N-2-oxide, brachycerine, and palicroceaine [13–15]. Studies with leaves have

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demonstrated allelopathic potential [16] and inhibition of carpogenic germination [17].

In the present work, we describe the isolation and identification of the monoterpene indole alkaloid, croceaine A (PC-1) (Fig. 1), which was previously isolated from *P. crocea* [13], but without scientific evidence of this potential therapeutic application of these compound and species, that collaborates with the popularity of *P. crocea*, for treatment of inflammatory diseases. Therefore, the main objective was bioguided study to assess the anti-inflammatory activity of *P. crocea*, as well as determine the concentrations of phenolic and polyphenolics compounds, HPLC/DAD, antioxidant, and toxicological analysis of the methanolic extract.

MATHERIALS AND METHODS

Animals

The animals obtained from Bioterio Central, UFGD (Dourados, Brazil), were kept under standard laboratory conditions in a controlled environment at 20 ± 2 °C (12 h light and 12 h dark cycle) with water and food *ad libitum*. The Institutional Ethics Committee at UFGD approved the procedures and protocols adopted in the study with authorization number 17/2017. Male *Swiss* mice (50 days, 25–35 g) were treated with vehicle (saline solution 0.9%, p.o.), MEPC (30, 100, and 300 mg/kg, p.o.), and PC-1 (10 and 30 mg/kg, p.o.) or injected with DEX (1 mg/kg; s.c.) for paw edema, mechanical hyperalgesia, and cold sensitivity assay. Three male *Wistar* rats (50 days old, 250–300 g)

were used for malondialdehyde assay. Female *Swiss* mice were treated with MEPC (30, 100, and 300 mg/kg, p.o.), PC-1 (10 and 30 mg/kg, p.o.), vehicle (saline solution), and DEX (1 mg/kg, subcutaneously, positive control), or the naive (negative control) group with sterile saline solution (0.9%) by intrapleural injection. Female *Wistar* rats (60 days old, 300 g) were used in acute toxicity.

Plant Material, Preparation of Extract, and Isolation of the Alkaloid

Aerial parts (branch and leaves) from *P. crocea* were collected in May 2016, in Dourados, MS, Brazil, and identified by Dr. Zefa Valdevina Pereira of the Federal University of Grande Dourados, UFGD. The voucher specimen 4487 was deposited at the Herbarium in the University. Dried and ground material (520 g) was extracted by maceration with methanol at room temperature for 15 days. After filtration, concentration under reduced pressure, and lyophilization, the methanolic extract of *P. crocea* (MEPC, 67 g, yield 12.9%) was obtained. Part of the MEPC (32 g) was dissolved in methanol:water (1:1) and partitioned with chloroform and ethyl acetate. The chloroform fraction was positive for alkaloids as determined by Dragendorff's reagent. Purification of the chloroform fraction by repeated column chromatography on silica gel and preparative TLC afforded the alkaloid PC-1 (67.8 mg). The isolated compound was identified by comparing spectroscopic data (^{1}H and ^{13}C NMR) with data from the literature [13].
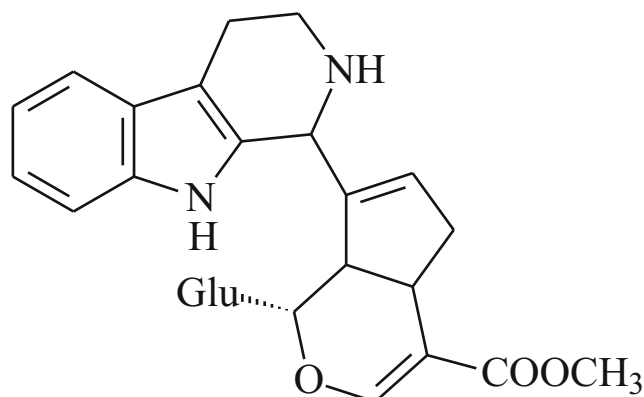
PC-1

Fig. 1. Chemical structure of PC-1.

Concentrations of Constituents

Total Phenolic Compounds

A total of 100 μL of MEPC (1 g/L in methanol) was mixed with 1.0 mL of distilled water and 0.5 mL of Folin–Ciocalteu's reagent (1:10 *v/v*). After mixing, 1.5 mL of 2% aqueous sodium bicarbonate was added and after 30 min; the absorbance was measured at 765 nm [16]. The total phenol concentration was expressed as gallic acid equivalents (GAE) in mg/g of extract. All assays were carried out in triplicate.

Total Flavonoids and Flavonols

A total of 500 μL of MEPC was mixed with 1.50 mL of ethanol (95%), 0.10 mL of aluminum chloride ($AlCl_3.6H_2O$, 10%), 0.10 mL of sodium acetate ($NaC_2H_3O_2.3H_2O$, 1 M), and 2.80 mL of distilled water. After incubation for 40 min, absorbance was measured at 415 nm. The flavonol concentration was determined by mixture of the 2 mL of MEPC, 2 mL $AlCl_3$ (2%, ethanol), and 3 mL of sodium acetate (50 g/L), followed by incubation for 2.5 h at 20 °C, and read at 440 nm. To calculate, we prepared a calibration curve to obtain the linear equation, using quercetin as standard, and the results were expressed as quercetin equivalents (QE) in mg/g of extract [16]. All assays were carried out in triplicate.

Condensed Tannins

MEPC was mixed with 5 mL vanillin-HCl (8% conc. aq. HCl and 4% vanillin in methanol), and after 20 min, the absorbance was read at 500 nm. We prepared a linear regression, using catechin as a standard, and the results were expressed as catechin equivalents (CE) in mg/g of extract [16]. All assays were carried out in triplicate.

HPLC/DAD

The extract (MEPC) was analyzed in an analytical LC (LC-6 AD, Shimadzu, Kyoto, Japan) system with a diode array detector (DAD) monitored at $\lambda = 200$–600 nm. The LC column was a C-18 (25 cm × 4.6 mm; particle size, 5 μm; Luna, Phenomenex, Torrance, CA, USA). In each analysis, the flow rate and the injection volume were set as 1.0 mL/min and 10 μL, respectively. All chromatographic analyses were performed at 23 °C. Elution was carried out using a binary mobile phase of water with 6% acetic acid and 2 mM sodium acetate (eluent A) and acetonitrile (eluent B). The following gradient was applied: 5% B (0 min), 15% B (30 min), 50% B (35 min), and 100% B

(45 min). The standards vanillic acid, *p*-methylbenzoic acid, caffeic acid, ferulic acid, p-coumaric acid, benzoic acid, cinnamic acid, rutin, sinapic acid, quercetin, luteolin, apigenin, and vanillin (Sigma, 97%) were prepared at the initial concentration of 100 μg/mL. The concentrations of compounds were determined by external calibration after dilutions were made in the range of 0.01–10 μg/mL.

Antioxidant Assays

Free Radical Scavenging Activity—DPPH and ABTS

Methanolic extract (MEPC) was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and azinobis-ethylbenzothiazoline-6-sulfonic acid (ABTS) reagents [16]. For the DPPH test, several concentrations of the samples in MeOH were added to 2 mL of freshly prepared DPPH solution (4.7 mg in 75 mL of MeOH). The mixture was shaken and left to stand at room temperature in the dark, and after 30 min, absorbance was measured at 517 nm. A DPPH solution without addition of the samples was used as control.

In ABTS assay, 7.0 mM ABTS and 140 mM potassium persulfate were mixed and kept in the dark for 16 h at ambient temperature. Before usage, the ABTS+ solution was diluted until the solution had an absorbance of 0.700 ± 0.05 at 734 nm with ethanol. Three milliliters of ABTS+ solution was added to 30 μL of solutions of different concentrations of the sample, and the absorbance was measured at 734 nm after 30 min. The BHT was used as positive control.

Malondialdehyde Assay—MDA

Malondialdehyde (MDA) was used for evaluation of lipid peroxidation [18] in rat brain homogenates after treatment with MEPC. Several aliquots of the MEPC were added to 3 mL of homogenate. After 1 h of incubation at 37 °C, 1.2 mL of trichloroacetic acid was added, and the homogenate sample was centrifuged to collect precipitated proteins. The supernatant was heated with 1 mL of an aqueous solution of 0.67% thiobarbituric acid for 15 min at 100 °C, and absorbance was measured at 535 nm. BHT was used as positive control.

Carrageenan-Induced Mice

Paw Edema

Seven groups of male mice ($n = 6$), totaling 42 animals, were orally (p.o.) treated with same doses of MEPC and PC-1 described before, vehicle (saline solution 0,9%)

or subcutaneously (s.c.) with the anti-inflammatory drug dexamethasone (1 mg/kg). After 1 h, the animals received a 50 µL subcutaneously injection of carrageenan (Cg, 300 µg) dissolved in sterile 0.9% saline into the right hind paw. The contralateral paw received only saline and was (3 and 4 h) used as the control. Edema was measured after 0.5, 1, 2, and 4 h with a plethysmometer (PANLAB Harvard) [19]. In this model, two other parameters of inflammation such as mechanical hyperalgesia [20] and cold sensitivity [21] were analyzed at 3 and 4 h after Cg administration. The cold sensitivity reaction, as indicated by paw licking, shaking, or rubbing the paw, was observed and recorded. The duration of the testing was 30 s.

Pleural Cell Migration and Protein Exudation

The doses of MEPC and PC-1 (dissolved in 0.9% saline) described before were also used in five groups ($n = 6$) of female mice by oral route (p.o.), while the vehicle group received 0.9% saline (p.o.), the naïve group received 0.9% saline (p.o.) but not receive carrageenan, and last group with the anti-inflammatory drug dexamethasone (1 mg/kg). Pleurisy was induced with 1% carrageenan (intrathoracic, 100 µL) as previously described by Vinegar et al. (1973) [22]. After 4 h, the animals were killed (isoflurane, 1.5%), thoracic cavity was washed with phosphate buffered saline (PBS, 1 mL), and the pleural exudate was collected, and an aliquot (20 µL) was diluted in Turk's solution (1:20) and used to determine the total number of leukocytes present in a Neubauer chamber. A portion of the exudates were centrifuged and the protein concentrations were determined by the Bradford method [23], to verify protein extravasation.

Acute Toxicity

The MEPC was administered by gavage, at a dose of 2000 mg/kg. Sequentially, at intervals of 48 h, the same dose was administered to four female rats, with a total of five treated animals, after 12 h of fasting. Animals were treated with vehicle (saline) in order to establish a comparative negative control group. Signs of the toxicity during the first 0.5, 1, 2, 4, 8, and 12 h and at every 24 h for 14 days were observed. Behavioral observations (reflexes, tremors, convulsions, lacrimation, cyanosis, salivation, piloerection, muscle tone, and motor coordination) and mortality were analyzed. Animals were weighed and subsequently euthanized, and the organs (heart, lung, spleen, liver, kidney) were removed, weighed, and examined macroscopically.

Statistical Analysis

Data are presented as the mean ± standard error of the mean. Difference among groups was evaluated by analyses of variance (one-way ANOVA) followed by the Newman–Keuls posttest. Statistical differences were considered significant at $p < 0.05$.

RESULTS

Chemical Constituents

The PC-1 was isolated as an amorphous solid from the chloroform fraction. ^{1}H and ^{13}C NMR data confirmed the presence of the monoterpenoid indole alkaloid croceaine A (PC-1), which was previously isolated from *P. crocea* [13, 14]. The extract (MEPC) indicated the presence of phenols (800.35 ± 9.45 mg GAE/g extract), flavonoids (719.40 ± 5.66 mg QE/g extract), flavonols (240.80 ± 12.39 mg QE/g extract), and condensed tannins (94.10 ± 15.20 mg CE/g extract).

The MEPC was subjected to HPLC-DAD to quantify the phenolic acids and flavonoids. The compounds caffeic acid ($tr = 8.64$ min; 54.6 mg/g), ferulic acid ($tr = 17.28$ min; 211.4 mg/g), rutin ($tr = 25.10$ min; 63.1 mg/g), and quercetin ($tr = 35.33$ min; 65.9 mg/g) were identified by comparing to standards (Fig. 2).

Antioxidant Activity

The MEPC displayed potent scavenging activity for ABTS radicals in a concentration-dependent manner, ranging from 5 to 250 µg/mL, with an IC_{50} value of 68.5 µg/mL, but did not show activity by the DPPH assay (> 250 µg/mL). The MDA assay, a product derived from lipid peroxidation, demonstrated decreased MDA production (74%), comparable to butylated hydroxytoluene (BHT), 84%.

Acute Toxicity

The assessment of acute toxicity was conducted for 14 days and no clinical signs of toxicity were observed at any dose. No deaths were reported. LD_{50} was greater than 2000 mg/kg indicating that MEPC has
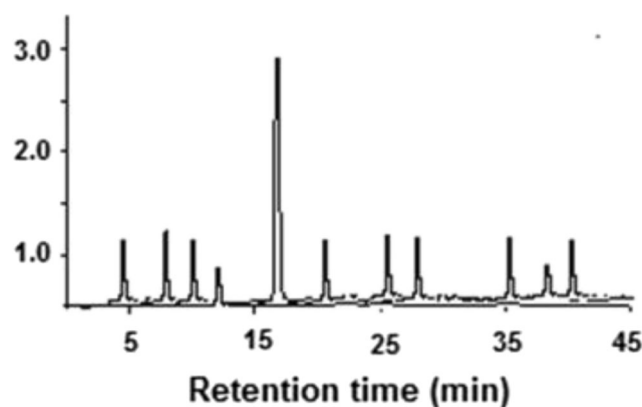
Fig. 2. Chromatogram of the MEPC by LC-DAD.

low toxicity as reported in a previous study [2–6]. Thus, *in vivo* studies were conducted to investigate

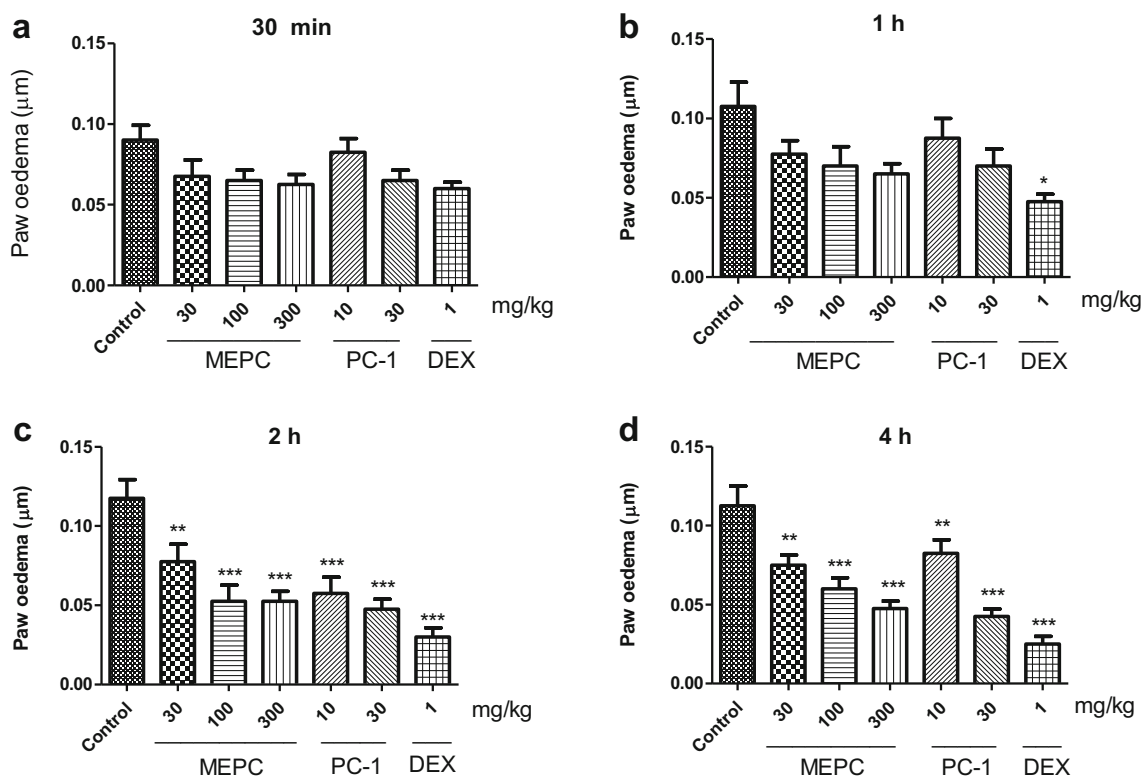their pharmacological properties and collaborate in part the popular use of the plant.

Fig. 3. a–d Effect of oral administration of MEPC and PC-1 on carrageenan-induced paw edema in mice. The animals received MEPC (30, 100, or 300 mg/kg, p.o.), PC-1 (10 or 30 mg/kg, p.o.), vehicle (control), or dexamethasone (DEX, 1 mg/kg, s.c.), and 1 h later, an intraplantar injection of carrageenan (50 μg/paw) was administered. Graphs represent the evaluation of the paw edema at 0.5, 1, 2, and 4 h, respectively, after carrageenan injection. Each bar represents the mean ± SEM of 6 animals. ANOVA/Newman–Keuls test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with the control group.
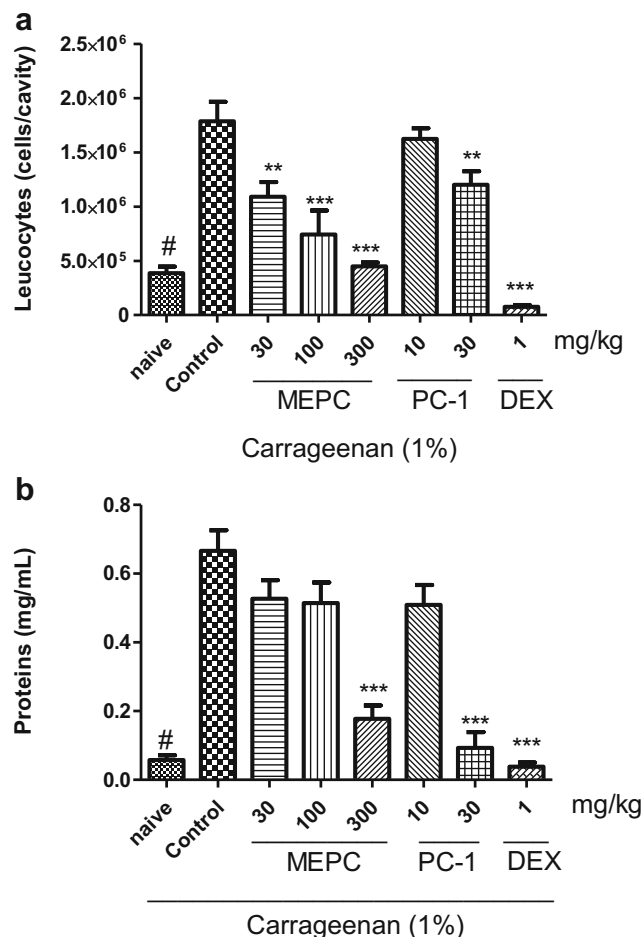
Fig. 4. Effect of oral administration of MEPC and PC-1 on leukocyte migration (**a**) and protein leakage (**b**) in the pleurisy test in mice. The animals received MEPC (30, 100 or 300 mg/kg, p.o.), PC-1 (10 or 30 mg/kg, p.o.), vehicle (control), or dexamethasone (DEX, 1 mg/kg, s.c.), and 1 h later, an intrathoracic injection of Cg (100 μl of a 1% solution/cavity). Naive group received an intrapleural injection of sterile saline instead of carrageenan. Each bar represents the mean ± SEM of 6 animals. ANOVA/Newman–Keuls test. $**p < 0.01$, $***p < 0.001$ compared with the control group.

Paw Edema

It was observed that the level of inhibition was of 55.32 ± 7% (100 and 300 mg/kg, MEPC), 34.04 ± 4% (30 mg/kg, MEPC), 59.57 ± 7% (30 mg/kg, PC-1), and 51.06 ± 6% (10 mg/kg, PC-1), at 2 h (Fig. 3c). At 4 h, the MEPC (30, 100, and 300 mg/kg) and PC-1 (10 and 30 mg/kg) showed inhibitions of 46.68 ± 5%, 46.66 ± 7%, 57.77 ± 8%, 42.22 ± 4%, and 62.20 ± 3%, respectively (Fig. 3d). The positive control (DEX) showed a significant reduction at all time points, with inhibition of 42 ± 5% at 0.5 h, 56 ± 9% at 1 h, 74 ± 5% at 2 h, and 77 ± 8% at 4 h (Fig. 3).

Pleural Cell Migration

The animals treated with MEPC at a dose of 30, 100, and 300 mg/kg showed a significant reduction in leukocyte migration induced by carrageenan in pleural exudate 39.23%, 58.72%, and 74.95%, respectively (Fig. 4a). The PC-1 showed reduction in dose of 30 mg/kg (33.11%) (Fig. 4a). Protein exudation was significantly decreased at doses of 300 mg/kg (73.42%) of EMPC and 30 mg/kg (86.04%) (Fig. 4b), demonstrating a dose-dependent effect.

Mechanical Hyperalgesia

The oral administration of MEPC (300 mg/kg) reduced the mechanical hyperalgesia in carrageenan treated animals by 100% at 3 and 4 h after injection, similar to results observed following treatment with DEX (positive control) (Fig. 5). The dose of 100 mg/kg also showed significant efficacy in reduction of mechanical sensitivity in mice, by 68% (3 h) (Fig. 5a) and 62% (4 h) (Fig. 5b).
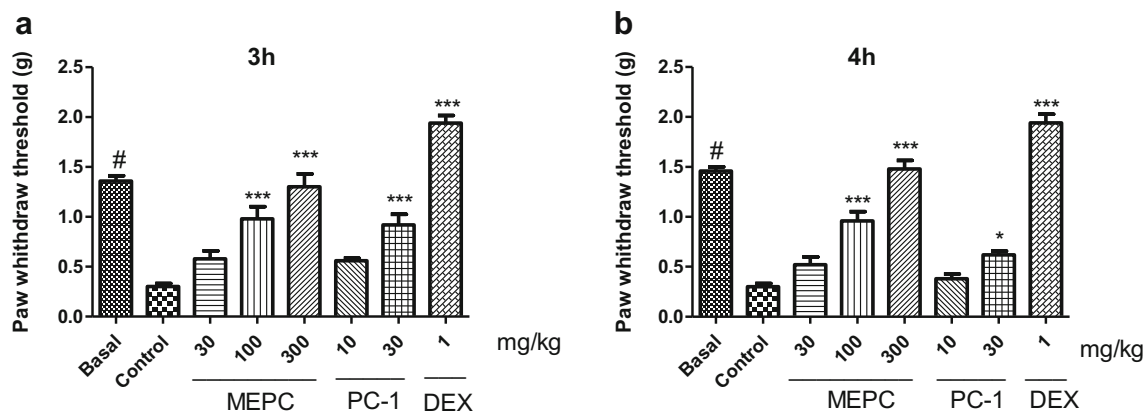
Fig. 5. Effect of oral administration of MEPC (30, 100, or 300 mg/kg, p.o.), PC-1 (10 or 30 mg/kg, p.o.), on mechanical hyperalgesia in mice. The animals received vehicle (control) or dexamethasone (DEX, 1 mg/kg, s.c.). In **a**, the mechanical hyperalgesia was measured with a digital analgesy meter for 3 and in **b**, 4 h after carrageenan administration. Each bar represents the mean ± SEM of 6 animals. ANOVA/Newman–Keuls test. $*p < 0.05$, $***p < 0.001$, $\#p < 0.001$ when compared with the control group.

The compound PC-1 at a dose of 30 mg/kg resulted in reduced mechanical sensitivity by 62% and 32% at 3 and 4 h after injection, respectively (Fig. 5a, b).

Cold Sensitivity

Was observed for 30 s, by paw licking, shaking, or rubbing. The maximum inhibition of cold sensitivity was measured to be $45 \pm 3\%$ and $42 \pm 4\%$ for MEPC (300 mg/kg) at 3 and 4 h after Cg injection, respectively, and $45 \pm 3\%$ after PC-1 (30 mg/kg) treatment, 3 h after carrageenan injection (Fig. 6a, b). The MEPC at a dose of 100 mg/kg (3 and 4 h) and PC-1 at a dose of 30 mg/kg (4 h) also reduced significantly the cold response to acetone in mice, showing inhibition ≥ 32% (Fig. 6a, b).

DISCUSSION

Alkaloids (Croceaine A) derived from the strictosidine (tryptamine–iridoid) were reported in *P. adusta* Standl. [24], *P. coriacea* (Cham.) K. Schum. [25, 26], *Psychotria umbellate* [27], and *P. acuminata* (Benth.) Borhidi [28]. These alkaloids are obtained *via* the metabolic route originating from tryptophan and a single precursor to strictosidine produced by the condensation of a molecule of tryptamine with secologanin, elaborated *via* geranyl, from mevalonic acid, and proposed by loganin (iridoid) [29] (Fig. 7).

The antioxidant activity can be directly related to the presence of phenolic and/or flavonoid compounds by number and position of free hydroxyl groups in the aromatic
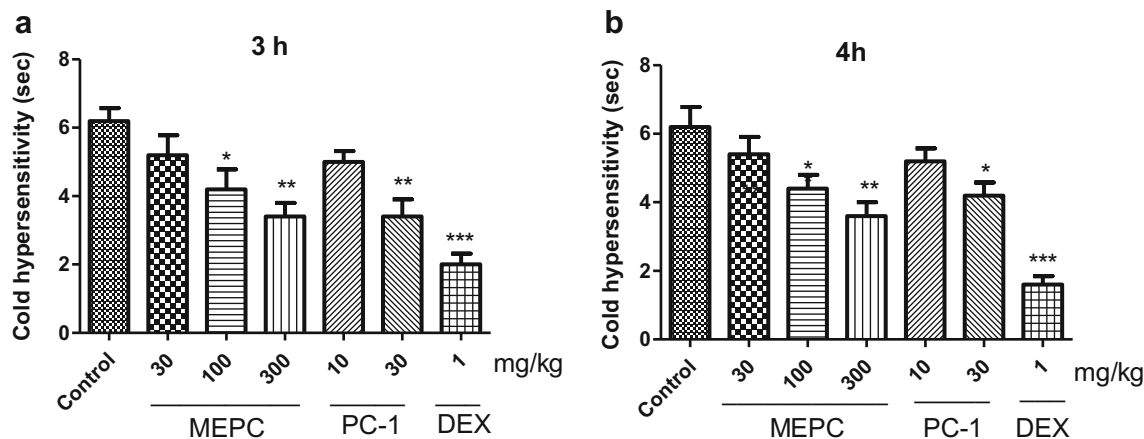

Fig. 6. Effect of oral administration of MEPC on the cold sensitivity induced by acetone in mice. The animals received MEPC (30, 100, or 300 mg/kg), PC-1 (30 or 10 mg/kg), control, or dexamethasone (DEX, 1 mg/kg, s.c.). In **a**, the cold sensitivity was measured 3 h and in **b**, 4 h after carrageenan administration. Each bar represents the mean ± SEM of 6 animals. ANOVA/Newman–Keuls test. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ compared with the control group.

structure, which could be a result of their hydrogen donating ability [30]. Three assays (DPPH, ABTS, and MDA) were employed to assess antioxidant activity of the MEPC, and the results have been demonstrated by the IC_{50} value, which corresponds to the concentration resulting in 50% inhibition. These results were superior to those previously reported by our research group, after assessing the content of phenolic and flavonoids compounds of the leaves of this species collected in 2014 [16] which may have been influenced by climatic conditions. The caffeic acid, ferulic acid, rutin, and quercetin compounds, presented for the first time in the MEPC, could be partly responsible for the measured antioxidant activity. The potent antioxidant activity of quercetin and rutin have been previously reported in the literature in different types of tests [31–33]. Caffeic and ferulic acid are examples of important natural phenolic antioxidants obtained from metabolism of phenylalanine and tyrosine in the Shikimate pathway in plants. They also have other therapeutic properties, such as UV-protection, anticarcinogenic activity, anti-inflammatory activity, cardiovascular protection, and protect against

neurodegenerative diseases, at least in part, due to their strong antioxidant activity which effectively neutralizes superoxide anion radicals and inhibits the lipid peroxidation [34–36]. These compounds showed these effects by virtue of the phenolic hydroxyl group in their structures.

The antioxidant potential of MEPC can be correlated to other important activities, such as anti-inflammatory activity. Studies have shown that reactive substances (EROs and ERNs) may be closely involved in the pathogenesis of inflammatory processes and that may exacerbate tissue damage [37]. Different models can be used to study the various events occurring in the development of the inflammation process, each one related to a type of tissue response, including signs and symptoms such as heat, flushing, tumor, edema, and pain. The inflammatory response is categorized into three distinct phases, each of which is apparently mediated different mechanisms, initially stage, of variable duration, where local vasodilation occurs and increased capillary permeability, followed by a subacute phase characterized by leukocyte and cell
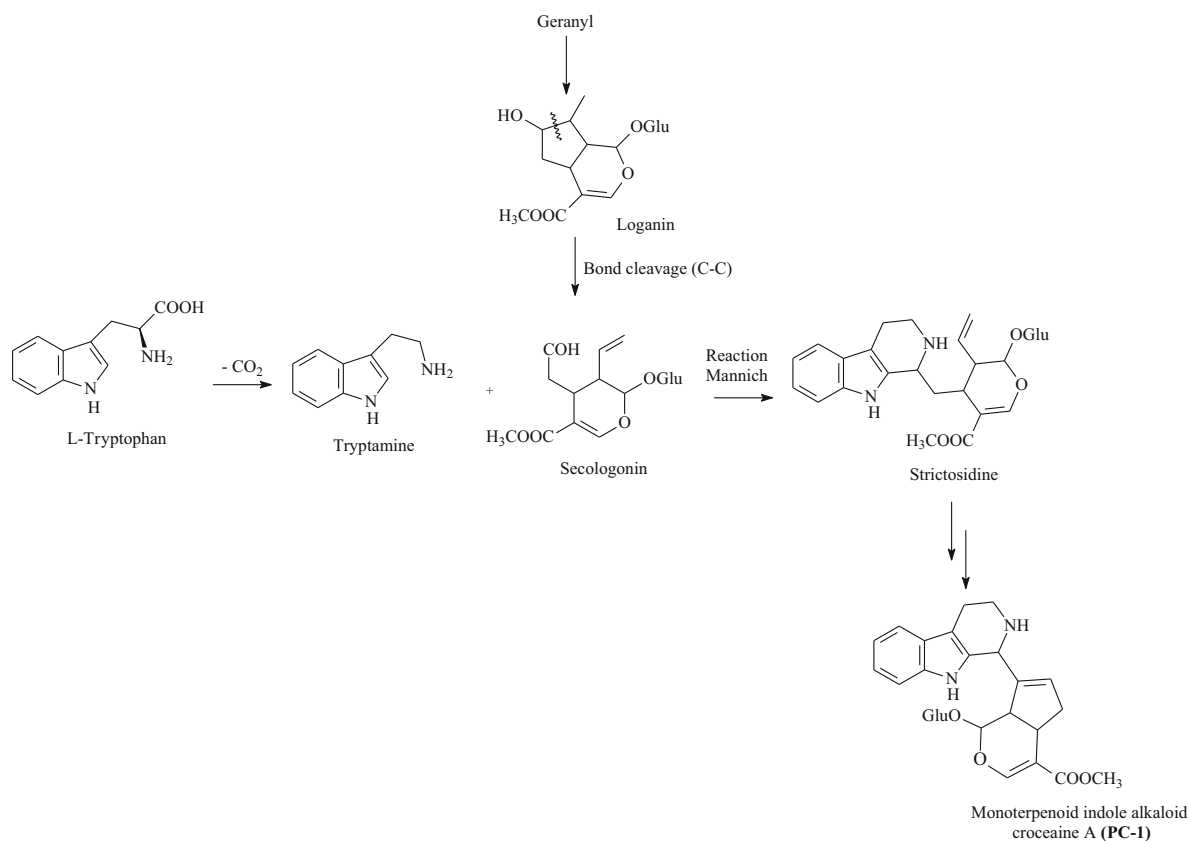

Fig. 7. Proposed biosynthetic route for the monoterpene indole alkaloid (PC-1) as precursor estrictosidine.

infiltration phagocytic and subsequently, tissue regeneration occurs or fibrosis.

The systemic anti-inflammatory effects of the MEPC and alkaloid isolated (PC-1) in acute inflammation was evaluated by induction of a carrageenan-induced paw edema model during 0.5, 1, 2, and 4 h after carrageenan administration. This is a classic test to evaluate the first phase of the inflammatory reaction with multiple mediators acting in sequence to produce an inflammatory response. The early phase (0–1 h) includes the release of histamine, serotonin, and bradykinin occurs, and later stage (1–6 h) includes an increased production of prostaglandins, COX-2 activation, and NO release [38–40]. This study demonstrates that administration of MEPC and PC-1 significantly decreases swelling 2 h after administration when compared with the control group, acting mainly on the later phase of the carrageenan-induced inflammatory response, likely because of a reduction in local vascular permeability. Thus, we concluded that *P. crocea* promotes a reduction in cell leakage, leading to the consequent reduction of proinflammatory mediators, which provides support to the traditional use of this plant for the treatment of edema processes. The pleurisy model was used to evaluate the second phase of the response, and is characterized by the migration of leukocytes and other phagocytic cells to the lesion site. These results, together with the paw edema test, reinforce the anti-inflammatory potential of this species.

Palicourea crocea showed anti-hyperalgesic properties, inhibiting mechanical hyperalgesia and cold sensitivity. Pharmacological studies performed in animal models by one of the previously reported compounds from *P. crocea*, psychollatine, demonstrated analgesic effects against algogenic stimuli [41], anxiolytic, antidepressant, and amnesic. In this study, psychollatine was able to modulate systems of different neurotransmitters, including NMDA, opioid, and 5-HT$_{2A/C}$ receptors [42, 43]. *In vitro* studies conducted by Passos et al. (2013) [44] reported that this compound was able to inhibit the activity of butyrylcholinesterase in range of 72.5% to 10^{-4} M.

The main results of this study show that the extract did not present signs of toxicity in acute test. This is the first evaluation of the biological activity of the plant *P. crocea*, demonstrating its potential anti-inflammatory and anti-hyperalgesic, in the acute phase, confirms the traditional use in the treatment of inflammatory conditions. This effect can be attributed in part to the presence of phenolic compounds and the monoterpenoid indole alkaloid croceaine A. Additional studies are needed to elucidate the mechanism of action responsible for this activity.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

Ethical Approval. This work was found to comply with the ethical concepts required by the National Council for Animal Experiments Control (CONCEA), with current legislation and other provisions of the ethics investigations that directly involve the use of animals and all protocols and procedures were approved according to the Ethics Committee on Animal Use the Federal University of Grande Dourados, Mato Grosso do Sul, Brazil (CEUA/UFGD) with authorization number 17/2017.

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