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Antinociceptive and Antiinflammatory Properties of the Ethanolic Extract of *Pouteria ramiflora* Roots

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SUMMARY. The present study investigated the antinociceptive and antiinflammatory activities of the ethanolic extract of *Pouteria ramiflora (EEPr)* roots, commonly known in Brazil as "curiola". The *EEPr* (50 or 100 mg/kg) produced inhibition of abdominal constrictions in mice and increased the reaction time in the hotplate test. The *EEPr* inhibited the inflammatory process induced by carrageenan in the rat air pouch model. When the rats were treated with 50 mg/kg of *EEPr*, the number of inflammatory cells harvested from the air pouch, nitric oxide metabolites levels and adenosine deaminase activity decreased by 65%, 77% and 68%, respectively. When they were treated with 100 mg/kg of *EEPr*, the inhibition was higher (81%, 89% and 95%) for the same analyzed parameters. The results of the experiments performed in the present study exhibit pronounced antinociception and also indicate that *EEPr* exerts antiinflammatory activity by interfering with the lymphocyte proliferation and NO production.

INTRODUCTION

The use of natural medicine for the treatment of many diseases is well disseminated worldwide. Some studies have focused on the history of plant use from an ethnopharmacology perspective. The rich flora of the Cerrado biome in Brazil has been poorly studied in order to evaluate the efficacy and therapeutic effects of crude extracts or isolated compounds obtained from a wide range of plant families. It has been demonstrated that some of the extracts or active principles obtained from plants have a broad spectrum of biological activities, including analgesic and anti-inflammatory properties. *Pouteria ramiflora* (Mart.) Radlk, commonly known as "guapeva currioloa" or "curiola", is an example of natural medicine as the treatment for a variety of illnesses, from which people eat the yellow fruits and use the roots to treat worms, dysentery, pain and inflammation. It is a perennial tree, widespread in the Brazilian savannah (Cerrado) [1]; however there is no scientific information that proves any analgesic and/or anti-inflammatory effects, using classical pharmacological assays [2].

The inflammatory response is complex and is mediated by a variety of signal molecules produced locally by mast cells, nerve endings, platelets and leucocytes. Some chemical mediators, such as adenosine deaminase enzyme (ADA) and nitric oxide (NO), can be used as inflammatory markers. ADA is an enzyme involved in the catabolism of purine bases, capable of catalyzing the deamination of adenosine, producing inosine in the process [3] being widely distributed in human tissues and found at higher concentrations in the lymphoid system; its main biological activity is detected in T lymphocytes [4,5]. Its main physiologic activity is related to lymphocytic proliferation and maturation [6-8]. NO has been suggested to modulate both acute and chronic inflammatory reactions and has a well-established role in the endothelial-dependent control of the vascular tone [9]. It is released from

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the endothelium and it is activated by the soluble guanylate cyclase [10-12] and responsible for the cytotoxicity of macrophages and neutrophils [13-14]. In addition to that, studies with carrageenan air pouch inflammation have been characterized by the presence of an increasing vascular permeability and the formation of an exudate with the accumulaton of polymorphonuclear leucocytes [15-19]. All these compounds could be related to the process of analgesia and/or inflammation and to the fact that *Pouteria ramiflora* could present pharmacological effects against those processes.

Despite the ethno-medicinal properties attributed to *Pouteria ramiflora,* very little pharmacological information about this plant has been reported to the best of our knowledge. Thus, we decided to evaluate the analgesic and anti-inflammatory effect of the ethanolic extract obtained from the roots of *Pouteria ramiflora,* by using classical pharmacological assays.

MATERIAL AND METHODS

Animals

Swiss male mice (30-35 g) and male Wistar rats (250 g) were obtained from the Evandro Chagas Institute's Animal Resources Center, Belém-Pará-Brazil. The experiments were carried out at the Molecular and Cellular Neurochemistry Laboratory (Federal University of Pará). They were randomly assigned to groups of 10 animals and maintained in plastic boxes, with food and water *ad libitum,* under a 12 h light/12 h dark cycle. The room temperature was maintained at 22 ± 1 °C. The animals were acclimatized to the laboratory for at least 1 h before the experiments which were carried out between 8:00 and 13:00 h, in order to avoid circadian influence. All experiments reported in this study were carried out in accordance with current guidelines for the care of laboratory animals and ethical guidelines for the investigation of experimental pain in conscious animals, established by Zimmerman [20]. All efforts were made to minimize the number of animals used and their suffering.

Preparation of the ethanolic extract of Pouteria ramiflora (EEPr) roots

The roots of *Pouteria ramiflora* were collected near Brasília, DF, Brazil, in 2001, and identified by Dr. José Elias de Paula (Instituto de Biologia-UnB). A voucher specimen was deposited in the Herbarium of the University of Brasília (voucher number J Elias de Paula 3671). The plant material was dried at room temperature and powdered in a knife mill. The powdered material was extracted (maceration) using hexane followed by ethanol, furnishing 5.87 g and 10.56 g of hexane and ethanol crude extracts, respectively.

Drugs

The drugs used include acetic acid (Merck, São Paulo, Brazil), carrageenan and indomethacin (Sigma Chemical Co., St. Louis, MO, USA), morphine hydrochloride (Cristalia-Brazil, São Paulo, Brazil). All substances used were dissolved in distilled water, with the exception of indomethacin that was dissolved in a 5 % $NaHCO_3$ solution.

Analgesia test

Abdominal constriction by intraperitoneal injection of acetic acid

Abdominal contraction, induced by intraperitoneal (i.p.) injection of acetic acid 1%, consisted of a contraction of the abdominal muscle together with a stretching of the hind limbs [21]. The animals were pretreated orally with indomethacin (10 mg/kg, used as positive control) or with the *EEPr* (50 or 100 mg/kg) 1 h before acetic acid injection. The control groups received distilled water at the same volume. After challenge, pairs of mice were placed in separate boxes and the number of abdominal constrictions was counted every 5 minutes over a 1 h period. Antinociceptive activity was expressed as the reduction in the number of abdominal constrictions, i.e. the difference between control animals and those pretreated with *EEPr* or indomethacin.

Hotplate test

The hotplate test was used to measure the response latencies according to the method described previously [22]. In the experiments the hotplate was maintained at 55 ± 1 °C. Before beginning the experiments, the basal reaction time response of all animals was taken. As soon as the procedure was finished, the animals were pretreated orally with distilled water (10 ml/kg) or *EEPr* (50 or 100 mg/kg) or intraperitoneally with morphine (10 mg/kg), and they were subsequently tested again every 30 min for a period of 3 h. For this test, they were put onto the heated surface of the plate at 55 ± 1 °C. The time taken for the initial response to the painful stimulus (elevation of the paws, licking or jumping) was used to define the response. In order

to minimize damage to the animals' paws, the cutoff time was 30 sec.

Antiinflamatory test

Male Wistar rats were randomly divided into 4 groups, each with 5 rats weighing 200-220 g. The induction of the air pouches was carried out as described by Tao *et al.* [23] The air used was removed carefully from laminar flow (VECO) to avoid contamination. The pouches were injected with 20 ml of air into the intrascapular area. The pouches were reinflated with 10 ml of air three and six days after the beginning of the procedure, and nine days after the first injection. On this last day, one control group was treated orally with vehicle alone and another control group was treated with indomethacin injected into the air pouches. The *EEPr*-treated group was administered with 50 or 100 mg/kg of extract diluted in distilled water, by oral route. These treatments were fulfilled one hour before the administration of 10 mg of carrageenan, diluted in 1 ml of saline. The animals were sacrificed by excess of anesthesia 16 h after carrageenan administration. A small incision was made in the pouch wall, and the contents of the air pouch were carefully removed using a sterile Pasteur pipette after injection of 3 ml of PBS containing EDTA. The volume of the exudates was obtained by subtracting 3 ml from the total volume of the fluid mixture. The total number of cells was counted in a Neubauer camera.

Cell culture

Three samples of each group, containing 150 µl of exudate were incubated in DMEM (D5280, Sigma) supplemented with 10 % fetal bovine serum (10270-106, GIBCO), with penicillin-streptomycin 10000/Uml (15140-122, GIBCO) on a 12-well culture plate previously treated with poly-L-lysine (PLL 100 µg/ml; P5899, Sigma). The culture plates were maintained in an atmosphere consisting of 5% CO_2 and 95 % O_2 at 37 °C. After 24 hours, five fields of the exudate culture were selected and counted randomly. The field was photographed using an Olympus camera.

Assay of nitrite

Griess reagent was prepared by mixing an equal volume of 0.1% naphtylethylen and 1% sulfanilamide (in 5% phosphoric acid). The samples of 500 µl of culture means (obtained from exudate culture) were mixed with the same volume of Griess reagent. The measures were made at a λ = 540 nm. The concentration of nitrite was determined by comparison with the results obtained from the curves of serial dilutions of sodium nitrite.

Assay of adenosine deaminase

The adenosine deaminase (ADA) activity in exudate was determined according to the colorimetric method of Rodrigues *et al.* [24] For these analyses, 250 µL of exudate was incubated for one hour at 37 °C in 0.5 ml of adenosine solution (1.55 x 10-4 M), buffered with 50 mM phosphate (9.5 mM, KH_2PO_4; 40.5 mM, K_2HPO_4. $3H_2O$, in distilled water), pH 7.4. For the colorimetric determination of ammonia, 1.5 ml of phenol catalyst solution (0.15 M, phenic acid; 0.3 mM, sodium nitroprussiate) and 1.5 ml of alkaline hypocloride solution was added. The absorbance was measured at 630 nm.

Statistical analysis

The statistical significance of differences between groups was expressed as means ± SEM. The statistical significance of differences between groups was obtained by means of analysis of variance followed by Newmann Keuls multiple comparison test. *P* values less than 0.05 ($p < 0.05$) were considered as indicative of significance.

RESULTS

Analgesia test

Abdominal constriction by intraperitoneal injection of acetic acid.

The results in Figure 1 show that indomethacin caused a dose-related inhibition of acetic acid-induced visceral nociceptive response. The treatment of animals with *EEPr* (100 mg/kg, i.p.), 1 h beforehand, also produced significant and complete inhibition (100%) of acetic acid-induced visceral nociceptive response in all analyzed periods.

Hotplate test

The results in Figure 2 show that the treatment of animals with morphine (10 mg/kg, i.p.) caused a marked increase in the latency of the animals in all analyzed periods according to assessment in the hotplate test. Under the same conditions, the *EEPr* (50 or 100 mg/kg, i.p.) also produced a significant increase in the latency of the animals in the hotplate assay, when compared to the control group injected with saline. The *EEPr* (50 mg/kg), showed an increase in the latency at the following times: 30 (15.31 ±

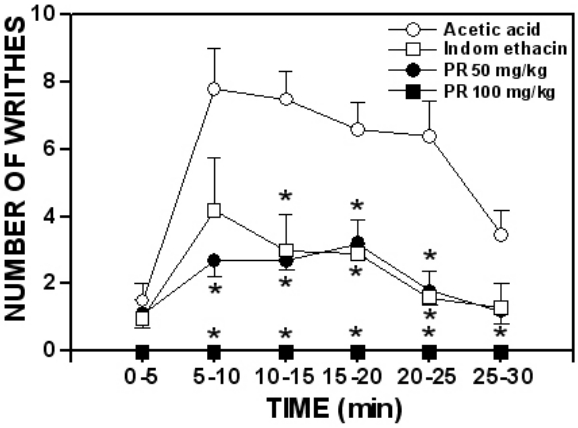

Figure 1. Effect of *EEPr* roots (50 or 100 mg/kg) or indomethacin (10 mg/kg), given by oral route, against acetic acid-induced visceral pain in mice. Each symbol represents the mean ± SEM of 10 animals. *p < 0.05 denotes the significant levels in comparison with control groups, injected with acetic acid (ANOVA, Newman Keuls test).

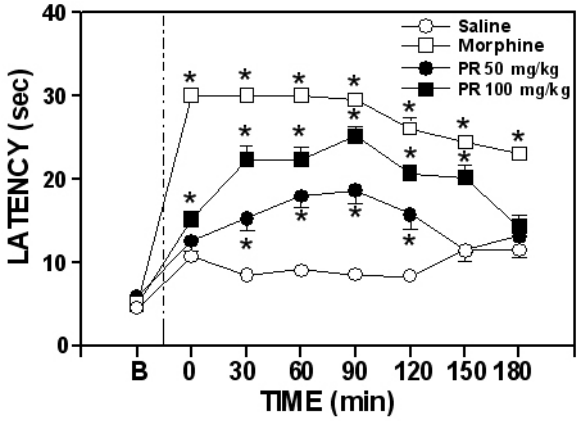

Figure 2. Effect of *EEPr* roots (50 or 100 mg/kg), given by oral route, or morphine (10 mg/kg), given intraperitoneally, on the hotplate test in mice. Each symbol represents the mean ± SEM of 10 animals. *p < 0.05 denotes the significant levels in comparison with control groups, injected with saline solution (ANOVA, Newman Keuls test). B=Basal reaction time (taken before *EEPr* or morphine administration); 0= starting time of the experiment (30 min after *EEPr* administration).

1.53), 60 (18.00 ± 1.27), 90 (18.68 ± 1.58) and 120 min (15.89 ± 1.90). The *EEPr* (100 mg/kg) showed an increase in the latency at the following time: 30 min after *EEPr* administration, represented by zero - 0 (15.20 ± 0.97), 30 (22.36 ± 1.74), 60 (22.43 ± 1.52), 90 (25.22 ± 1.21), 120 (20.71 ± 1.07) and 150 min (20.17± 1.62).

Antiinflammatory test

Cellular proliferation in the groups treated with EEPr

Numbers of inflammatory cells harvested from the air pouch after carrageenan administration were significantly suppressed by *EEPr* in comparison with vehicle + carrageenan- (Fig. 3.A) or indomethacin- (Fig. 3.B) treated groups. The total cell numbers were reduced to 65% and 81% when the animals were treated with the doses of 50 (Fig. 3.C) and 100 mg/kg (Fig. 3.D), respectively. Cellular migration takes part in host defense mechanisms against inflammatory reactions, when cells migrate from blood to air pouch. This process can be examined in cell cultures obtained from the air pouch exudates. *EEPr*-treated animals with doses of 50 or 100 mg/kg resulted in a decrease of the cellular migration, when compared to vehicle. The dose of 100 mg/kg was more efficient than 50 mg/kg in blocking the cellular migration (Fig. 4).

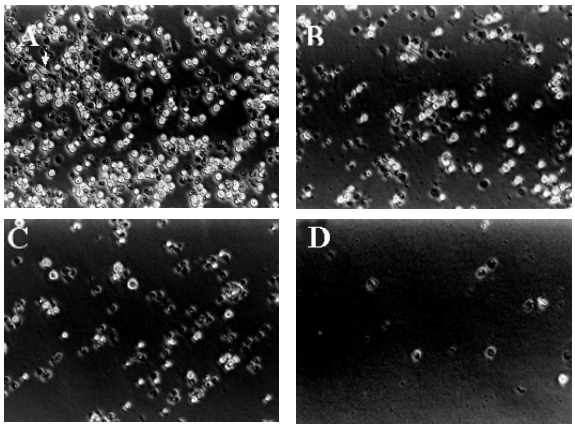

Figure 3. Inhibition of cellular migration in exudate cultures harvested from the air pouch after phlogistic agent administration, cultivated in DMEM: (**A**) carrageenan; (**B**) indomethacin; (**C**) *EEPr* 50 mg/kg and (**D**) *EEPr* 100 mg/kg. Scale bars = 25 µm.

Effect of EEPr on carrageenan-induced NO production

To corroborate the inhibitory effect *in vivo* of *EEPr* in the phlogistic process, it was deemed relevant to assess the role of NO, a chemical messenger that plays an important role in the vascular permeability (exudate volume) and cellular proliferation. NO was detected by measuring levels of nitrite. The rats were treated by oral injection with saline (1h before carrageenan administration) or with *EEPr* (50 or 100 mg/kg). Exudates were collected 16 h after carrageenan administration. As shown in Figure 5, the nitrite

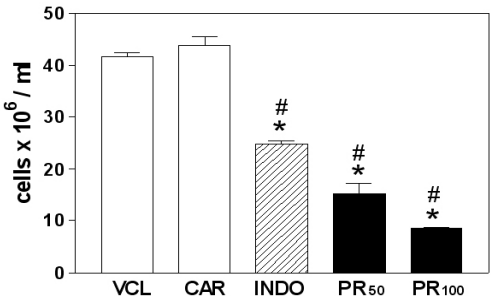

Figure 4. Total cell numbers in the vehicle (VCL), carrageenan (CAR), indomethacin (INDO) and *EEPr*-treated groups (PR_{50} or PR_{100} mg/kg). Each bar represents the mean ± SEM of the 5 rats in each group. *p < 0.05 compared with the vehicle-treated group; #p < 0.05 compared with the carrageenan -treated group (ANOVA, Newman Keuls test).

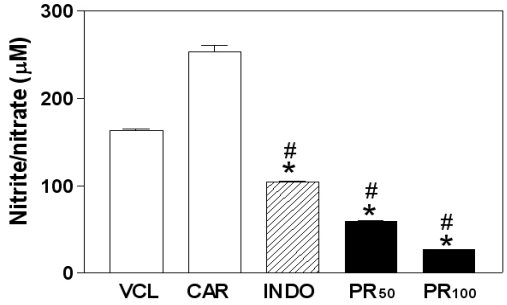

Figure 5. Nitrite/nitrate concentration in vehicle (VCL), carrageenan (CAR), indomethacin (INDO) or *EEPr*-treated groups (PR_{50} or PR_{100} mg/kg). Each bar represents the mean ± SEM for 5 rats in each group. *p < 0.05 compared with the vehicle-treated group; #p < 0.05 compared with the carrageenan-treated group (ANOVA, Newman Keuls test).

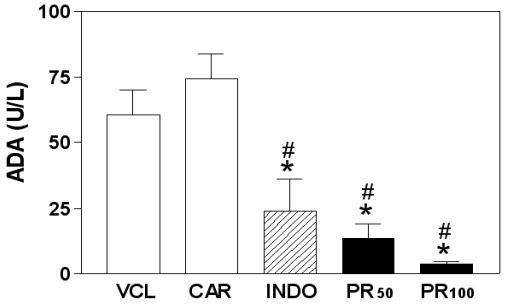

Figure 6. Adenosine deaminase enzyme (ADA) activity levels in vehicle (VCL), carrageenan (CAR), indomethacin (INDO) or *EEPr*-treated groups (PR_{50} or PR_{100} mg/kg). Each bar represents the mean ± SEM for 5 rats in each group. *p < 0.05 compared with the vehicle-treated group; #p < 0.05 compared with the carrageenan-treated group (ANOVA, Newman Keuls test).

level was 253 μM in samples induced by carrageenan, while the production of nitrite was significantly decreased to 59 μM and 27 μM in *EEPr*-treated animals with 50 and 100 mg/kg doses, respectively. Levels of nitrite had a marked decrease to 104 μM in the indomethacin-treated group.

In vivo inhibition of ADA activity by EEPr

The capacity of the *EEPr* to promote an inhibitory effect in T cells was examined by ADA activity. The rats were treated by intraperitoneal injection with saline (1 h before carrageenan administration) or with *EEPr* (50 or 100 mg/kg). Exudates were collected 16 h after injection of phlogistic agent. As shown in Figure 6, an increase in the ADA activity of the carrageenan-treated animals (74 U/L). In contrast to the stimulation found for *EEPr*-treated animals with 50 or 100 mg/kg, the enzyme activities were reduced to 68% and 95%, respectively. ADA activity had a decrease to 68% in the indomethacin-treated group when compared to the vehicle-treated group.

DISCUSSION

The present investigation clearly demonstrates that *Pouteria ramiflora* has antinociceptive and antiinflamatory effects against the pain induced by chemical (acetic acid), thermal (hotplate test) and inflammatory stimuli. The *EEPr* administered by oral route inhibited acetic acid-induced writhing in mice, showing a stronger analgesic activity. It has been suggested that acetic acid acts via the release of cytokines, such as TNF-α, interleukin-1β and interleukin-8, by resident peritoneal macrophages and mast cells [25-27] and by releasing endogenous mediators that stimulate the nociceptive neurons [28]. The results led to the hypothesis that *EEPr* plays a role in the inhibition of these different mediators. Furthermore, the *EEPr* can also have an additional and interesting mechanism of action which goes beyond its ability to inhibit proinflammatory cytokines and/or other mediators involved in the acetic acid model of pain. However, this possibility remains to be tested in future studies.

EEPr was also effective in the hotplate test, one the most traditional tests commonly used to assess narcotic analgesics. Other centrally acting drugs, including sedatives and muscle relaxants or psychotomimetics, have shown activity in this test [29]. In many circumstances, the affective aspect of pain goes beyond the immediate unpleasantness detected by this test. It is a simple

and sensitive method for studying central analgesic effects of drugs, which is based on a phasic stimulus of high intensity. *EEPr* (100 mg/kg) significantly attenuated the hotplate thermal stimulation, indicating significant protective effects on thermic pain stimuli.

We are reporting here for the first time that *EEPr* produces dose-related antinociceptive action in chemical (acetic acid-induced visceral pain nociception) and thermal (hotplate test) models of nociception in mice. The results, here presented, show that *EEPr* at the experimental doses presented no acute toxicity, since no changes were observed in behavioral responses analyzed 4, 24 or 48 h after *EEPr* administration, including involuntary movements, piloerection, stimulatory or sedative effects, respiratory depression or other signs. However, pharmacological and chemical studies are necessary to characterize the mechanisms of action responsible for the antinociceptive effect of *EEPr*. Furthermore, the antinociceptive action demonstrated in the present study supports, at least partly, the ethnomedical uses of this plant.

The antiinflamatory activities of *EEPr* were determined by the air-pouch experimental model in rats. These results provide support for the use of *EEPr* in relieving inflammation. Evidence presented here shows that *EEPr* is effective in producing dose-related and significant antiinflammatory action and potent immunosuppressive activities *in vivo*. The air pouch models of inflammation proved to be quite useful for determining the cell number, vascular permeability and levels of mediators at the inflammatory sites, since samples of tissue fluids can be collected easily from the inflammatory pouch [30,31]. Thus, it was possible to demonstrate a close correlation between mediator levels and exudates that was induced by carrageenan. The *EEPr* (100 mg/kg) showed a potent inhibitory action on the volume of exudate and in the total number of cells. The vascular permeability and macrophage's activity are related with the nitric oxide production mediated by endothelial NO synthase (eNOS) and inducible NO synthase (iNOS), respectively [14,32].

NO is an important mediator of inflammatory and other pathophysiological processes, and it is the most important of the endothelium-derived relaxing factors. After its formation, NO diffuses into vascular smooth muscle where it activates soluble guanylate cyclase [33-36], which in turn dilates the blood vessels and increases the volume of exudates. It is possible that the treatment with *EEPr* changes the air pouch's vascular permeability triggered by endothelial NOS activation, decreasing NO generated in response to carrageenan.

Furthermore, macrophage activation by inflammatory process upregulates the release of NO induced by iNOS and therefore contributes directly to inflammation [37,38]. We have demonstrated that *EEPr* inhibits production of the NO in macrophages, determined indirectly by nitrite levels. These results show that inhibition of the levels of NO by *EEPr* could be related with the blocking of the iNOS and consequently with the decrease of activation of the macrophage.

Evidence suggests that macrophages after their activation play a critical role in the activation of lymphocytes. ADA enzyme is widely distributed in human tissues and shows a higher activity in lymphocytes [4,5]. The role of ADA activity in the initial stages of T cell differentiation has been examined in patients with ADA deficiency [7]. The treatment of lymphoid malignancies with inhibitors of ADA has confirmed the ability of lymphocytes to interfere with the purine nucleoside metabolism [39]. Our data suggest that in the *EEPr*-treated group, ADA activity decreased in comparison with the values of the group-receiving vehicle. This result indicates that the *EEPr* exerts an inhibitory effect on the proliferation of T cells.

CONCLUSION

In summary, we have shown that *EEPr* produces dose-related analgesic and antiinflammatory actions. The mechanism by which *EEPr* produces those effects remains unclear. However, the effects shown in the present work support, at least partly, the ethno-medicinal uses of *Pouteria ramiflora*.

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