Curine, an Alkaloid Isolated from *Chondrodendron platyphyllum* Inhibits Prostaglandin E_2 in Experimental Models of Inflammation and Pain

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Key words
- pain
- inflammation
- PGE_2
- curine
- *Chondrodendron platyphyllum*
- Menispermaceae

Abstract

Curine is a bisbenzylisoquinoline alkaloid that is isolated from *Chondrodendron platyphyllum*, a plant that is used to treat malaria, inflammation, and pain. Recent reports have demonstrated the antiallergic effects of curine at nontoxic doses. However, its anti-inflammatory and analgesic properties remain to be elucidated. This study investigated the anti-inflammatory and analgesic effects of curine in mice. We analyzed the effects of an oral treatment with curine in the formation of paw edema, vascular permeability, abdominal contortion, licking behavior, and hyperalgesia using different inflammatory stimuli. Curine significantly inhibited the formation of paw edema by decreasing vascular permeability, inhibited the acetic acid-induced writhing response, inhibited the licking behavior during inflammation but not during the neurogenic phase of the formalin test, and inhibited carrageenan-induced hyperalgesia. Finally, curine inhibited prostaglandin E_2 production *in vitro* without affecting cyclooxygenase-2 expression. The effects of curine treatment were similar to the effects of indomethacin, but were different from the effects of morphine treatment, suggesting that the analgesic effects of curine do not result from the direct inhibition of neuronal activation but instead depend on anti-inflammatory mechanisms that, at least in part, result from the inhibition of prostaglandin E_2 production. In conclusion, curine presents anti-inflammatory and analgesic effects at nontoxic doses and has the potential for use in anti-inflammatory drug development.

Supporting information available online at http://www.thieme-connect.de/products

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Introduction

Chondrodendron platyphyllum A.St. Hil (Miers) (Menispermaceae) is a medicinal plant found in northeast Brazil. This plant is popularly known as "abútua", "abútua grande", and "uva do mato" and has been used in folk medicine to treat a great variety of conditions, including malaria, fever, pain, edema, urethritis, cystitis, ulcers, and menstrual disorders [1,2]. According to Silva [3], *C. platyphyllum* has been used in conjunction with *Zanthoxylum articulatum* A.St. Hil. to compose the herbal medicine Uva do mato®, which is used to treat abdominal, urinary tract, and muscle cramps, and muscle pain. The phytochemical analysis of the *C. platyphyllum* root revealed that *C. platyphyllum* is rich in bisbenzylisoquinoline alkaloids, a group of natural compounds with interesting pharmacological properties, such as anti-inflammatory, antiallergic, and analgesic properties [4]. At least three alkaloids, including curine, isocurine, and 12-*O*-metilcurine, have been identified from this plant, and curine is the major constituent [5].

Previous studies have shown that the alkaloids extracted from *C. platyphyllum* are pharmacologically active [6]. Dias et al. [5] have demonstrated that curine (**Fig. 1**) and isocurine have a vasodilator effect and have suggested that the effects of curine were associated with the inhibition of calcium channels. Medeiros et al. [7] demonstrated that curine decreased intracellular Ca^{2+} transients in A7r5 cells, possibly through a direct blockade of L-type Ca^{2+} channels. Recently, we demonstrated the antiallergic effects of an oral treatment with curine using a mouse model of allergic asthma. The oral administration of curine significantly inhibited eosinophilic inflammation, eosinophil lipid body formation, cytokine production, and airway hyper-responsiveness (AHR) *in vivo*. Verapamil, a calcium channel antagonist, had similar antiallergic properties, and curine pretreatment

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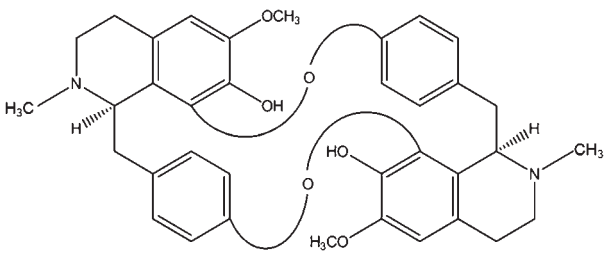

Fig. 1 Chemical structure of curine.

inhibited the calcium-induced tracheal contractile response *ex vivo*, suggesting that the mechanism by which curine exerts its effects is through the inhibition of a calcium-dependent response. Importantly, oral treatment with curine for seven consecutive days in doses up to 10-fold higher than the median effective dose (ED_{50}) did not induce changes in the hematologic or biochemical parameters. Additionally, the treatment did not induce the formation of gastric ulcers, and no physical or behavioral changes were observed, indicating that curine is not toxic under these conditions [8].

Despite the popular use of *C. platyphyllum* as an anti-inflammatory and analgesic plant, and the prominent antiallergic effect and low toxicity of curine, the scientific evidence of the analgesic effect of this compound remains to be provided. Such findings justify the characterization of the pharmacological properties of curine using models of inflammation and nociception. Considering the popular use and the pharmacological activity of curine in the absence of toxicity, the present work aimed to investigate its anti-inflammatory and analgesic effects in mice.

Results

We used two different inflammatory agents to evaluate the role of curine on edema formation. As shown in **Fig. 2A**, carrageenan injection significantly induced the formation of paw edema, which was significantly inhibited by the pretreatment with curine at 2.5 mg/kg or 10 mg/kg or indomethacin. However, the lower concentrations of curine did not reduce paw edema. Since curine at 2.5 mg/kg caused significant inhibition of the edema formation in this model, and based on the results obtained from the dose-response curve performed by Ribeiro-Filho et al. [8] in a mouse model of allergic asthma, we opted for this dose for our experiments. Similarly, zymosan stimulation induced the expressive formation of paw edema, which was significantly inhibited by curine treatment (**Fig. 2B**), demonstrating the inhibitory effect of curine on edema formation that is triggered by inflammatory agonists. To further examine the inhibitory effect of curine on edema formation, we analyzed the effects of curine in vascular permeability induced by acetic acid [9]. The intraperitoneal injection of acetic acid significantly induced more plasma extravasations than the vehicle in the control group, as attested by the optical density of Evans blue dye (**Fig. 3**). Curine and indomethacin significantly and similarly decreased vascular permeability compared to the non-treated animals, suggesting that the role that curine plays in edema formation is associated with the inhibition of vascular permeability.

Because we demonstrated the inhibitory effect that curine plays on edema formation, we attempted to characterize its effects on

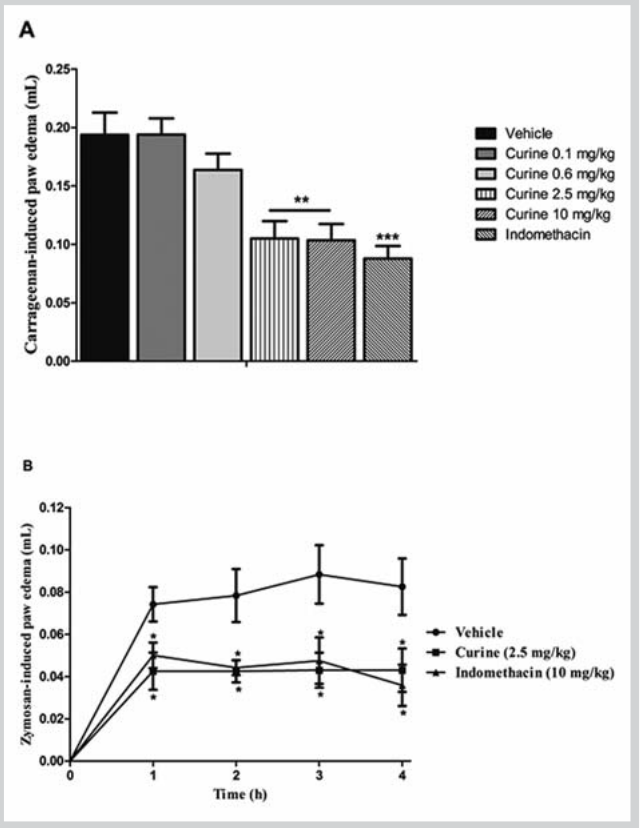

Fig. 2 Effect of curine on mouse paw edema. Swiss mice were orally pretreated with curine (0.1, 0.6, 2.5, or 10 mg/kg) or indomethacin (10 mg/kg). After 1 h, the animals were injected (s. c.) with 20 µL of carrageenan (500 µg/paw) or zymosan (200 µg/paw) solution into the left hind paw, and 20 µL of PBS was injected into the right hind paw. The results are expressed as the difference of volume between the left and right paw. **A** Carrageenan- or **B** zymosan-induced mouse paw edema. The results are expressed as the mean ± SEM from at least six animals. * Significantly different ($p < 0.05$) from the non-treated group.

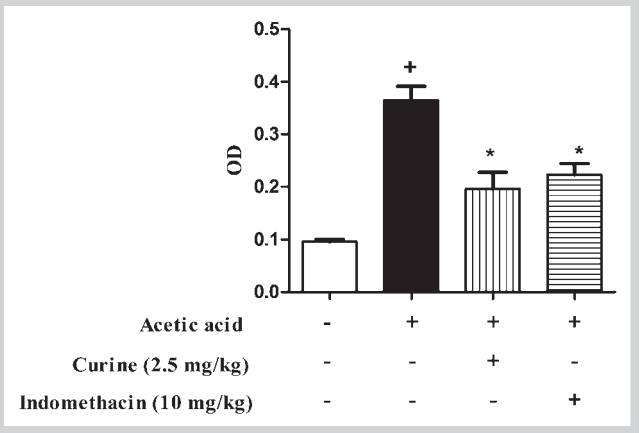

Fig. 3 Effect of curine on acetic acid-induced vascular permeability. Swiss mice pretreated (30 min before) with curine (2.5 mg/kg) or indomethacin (10 mg/kg) received an injection (i. p.) of 1% Evans blue solution (0.1 mL/10 g) followed (30 min later) by 0.6% acetic (i. p.). After 50 min, the animals were sacrificed and the peritoneal fluid was collected. Vascular permeability was expressed as Evans blue dye OD at 610 nm. The results are expressed as the mean ± SEM from at least six animals. + Significantly different ($p < 0.05$) from the non-stimulated group. * Significantly different from non-treated group.

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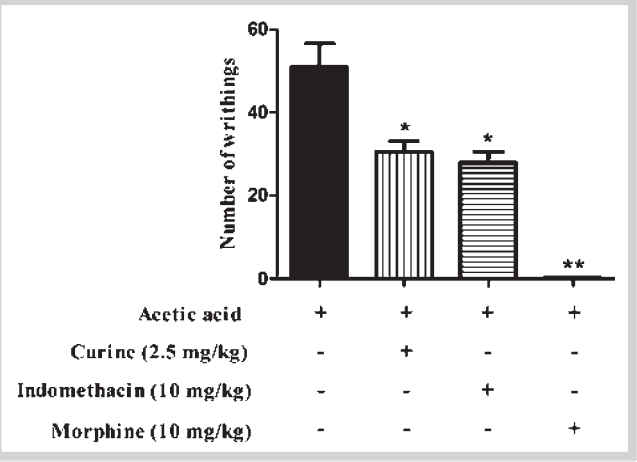

Fig. 4 Effect of curine on the acetic acid-induced writhing response. Swiss mice were treated (p.o.) with curine (2.5 mg/kg), indomethacin (10 mg/kg), or morphine (10 mg/kg). One hour later, the animals received an injection (i.p.) of 1.0% acetic acid (0.1 mL/10 g) and were placed in individual boxes for 20 min for analyses of the writhing response. The results are expressed as the mean ± SEM of the number of writhings from at least six animals. * Significantly different ($p < 0.05$) from the non-treated group. ** Significantly different from the non-treated or curine-treated group.

inflammatory pain. We analyzed the effect of curine in the writhing response induced by acetic acid. We used indomethacin, a nonsteroidal anti-inflammatory drug (NSAID), and morphine, a central acting analgesic drug, as standard pharmacological controls. As shown in **Fig. 4**, the writhing response was significantly inhibited by all of the treatments compared to the non-treated group. Interestingly, curine and indomethacin presented similar but only partially inhibitory effects, whereas the writhing response was completely inhibited by morphine, suggesting that curine presents an analgesic effect that is associated with anti-inflammatory mechanisms.

We used the formalin test to characterize the analgesic effect of curine in nociception mediated by neurogenic or inflammatory mechanisms. Formalin stimulation induces a biphasic response. The first phase is triggered in the first 5 min after the stimulus, causing direct neural activation and pain. The second phase occurs between 15–30 min after the stimulus and is triggered by the action of mediators released in the inflammatory reaction. Inhibitory mechanisms suppress pain between these phases [10]. Mice treated with curine or indomethacin presented no difference in the licking time in the neurogenic phase compared to non-treated mice (**Fig. 5A**), whereas the licking behavior was significant inhibited by morphine. However, the second phase was significantly inhibited by curine, indomethacin, and morphine (**Fig. 5B**), indicating that the analgesic effect of curine is dependent on anti-inflammatory mechanisms and is not a result of a direct inhibition of neuronal activation.

Hyperalgesia is characterized by an enhanced response to noxious stimuli and is a hallmark of inflammatory pain [11]. Because we demonstrated that curine inhibits the inflammatory phase of the formalin test, we used the hot plate test to confirm the inhibitory effect of curine on inflammatory pain triggered by carrageenan. **Fig. 6** shows that the challenge with carrageenan significantly decreased the heat withdrawal latency compared to the challenge with PBS in non-treated animals. However, there were no significant differences between the carrageenan challenge and the PBS challenge in animals treated with curine or indomethacin, supporting the potent analgesic role of curine in inflammatory conditions.

Based on the significant inhibitory effect of curine in hyperalgesia and the similarity between the curine and indomethacin effects in our experiments, we hypothesized that curine mechanisms might involve the inhibition of the production of prostaglandin E_2 (PGE_2), a key mediator of hyperalgesia in inflammatory conditions [12]. Therefore, we investigated the effect of curine on PGE_2 production *in vitro* using peritoneal macrophages stimulated with lipopolysaccharide (LPS). **Fig. 7A** shows that the LPS-stimulated cells presented a consistent increase in cyclooxygenase-2 (COX-2) expression that was not affected by curine pretreatments. However, the supernatants from curine-treated cells presented lower concentrations of PGE_2 compared to the supernatant of the non-treated cells (**Fig. 7B**), indicating that in this model, curine inhibits PGE_2 production without affecting the expression of COX-2. These findings suggest that the anti-inflammatory and analgesic effects of curine are, at least in part, dependent on PGE_2 production inhibition.

Discussion

In this study, we have demonstrated for the first time the anti-inflammatory and analgesic properties of curine, an active bisbenzylisoquinoline alkaloid isolated from *C. platyphylum*. We demonstrated that oral pretreatment with curine significantly inhib-

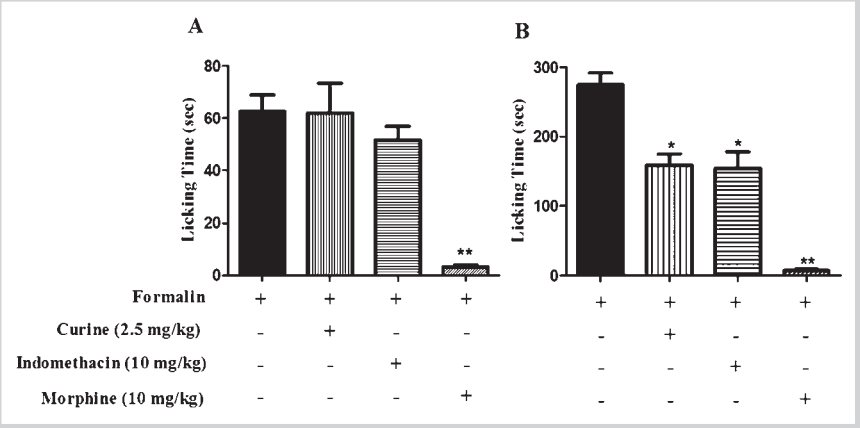

Fig. 5 Effect of curine on formalin-induced licking behavior. Swiss mice were orally treated with curine (2.5 mg/kg), indomethacin (10 mg/kg) or morphine (10 mg/kg). After 1 h, 20 µL of 2.5% formaldehyde solution (formalin) was injected into the right hind paw. The results are expressed as the licking time in the first 5 min (neurogenic phase, **A**) or between 15–30 min (inflammatory phase, **B**). The results are expressed as the mean ± SEM of the number of writhings from at least six animals. * Significantly different ($p < 0.05$) from the non-treated group. ** Significantly different from the non-treated or curine-treated group.

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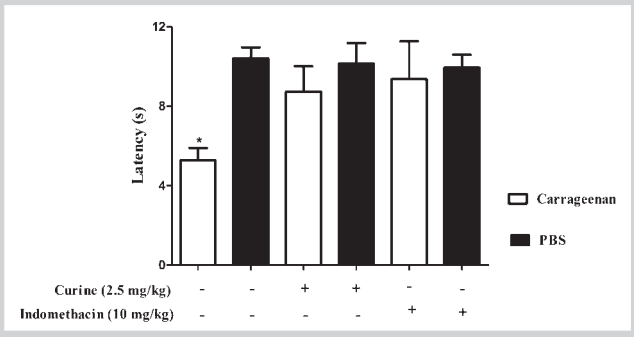

Fig. 6 Effect of curine on carrageenan-induced hyperalgesia. Swiss mice pretreated with curine (2.5 mg/kg) or indomethacin (10 mg/kg) were injected with carrageenan (25 µg/paw) into one hind paw. PBS was injected into the other paw as a negative control. Hyperalgesia was analyzed 4 h later using a hot plate test and is expressed as a decrease in the latency time of the carrageenan-challenged paw compared to the PBS-challenged paw. The results are expressed as the mean ± SEM of at least six animals. * Significantly different ($p < 0.05$) from the PBS-challenged group.

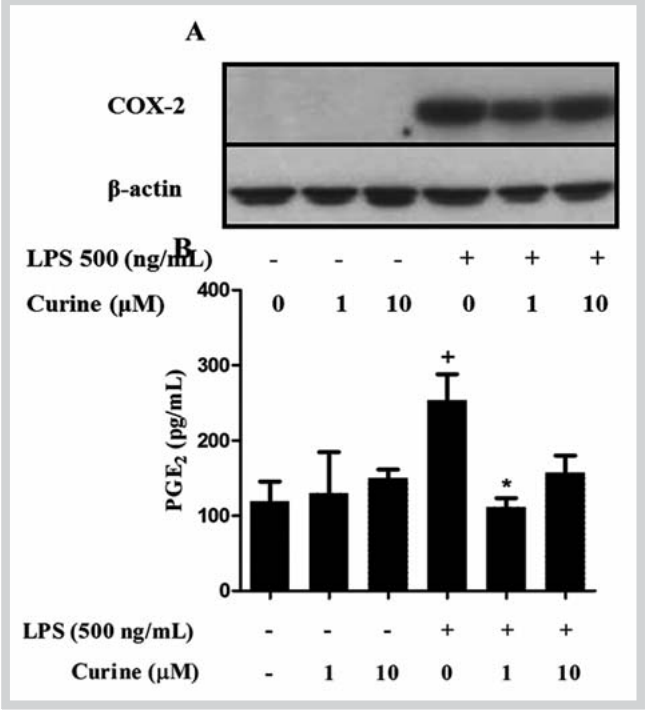

Fig. 7 Effect of curine on cyclooxygenase-2 expression and prostaglandin E_2 production. Peritoneal macrophages (5×10^5 cells/well) were incubated for 1 h with curine (1 or 10 µM) and stimulated with LPS (500 ng/mL) for 12 h. PGE_2 concentrations were determined using Duo-Set kits, and COX-2 expression was analyzed by Western blotting. **A** COX-2 expression. The results are representative of two different experiments. **B** PGE_2 concentrations. The results are expressed as the mean ± SEM of two different experiments.

its mouse paw edema induced by carrageenan and zymosan, and acetic acid-induced vascular permeability, indicating that curine plays an inhibitory role in edema formation that is associated with a decrease in vascular permeability. The administration of carrageenan, zymosan, and acetic acid is known to trigger a local inflammatory reaction with the release of inflammatory mediators that induce vascular changes that cause plasma extravasations and edema formation [13]. Here, we suggest that curine inhibits edema formation by modulating the inflammatory reaction. Previous studies have reported that curine is able to reduce leukocyte activation by decreasing the production of inflammatory cytokines in other models of inflammation [8]. Accordingly, the anti-inflammatory properties of other BBA have been previously described [14–18], and our group has demonstrated the antiallergic and anti-inflammatory properties of warifteine, a BBA isolated from *Cissampelos sympodialis* (Menispermaceae) [19,20].

Importantly, pretreatment with curine inhibited both the writhing response induced by acetic acid and the licking behavior in the inflammatory phase of the formalin test, demonstrating the analgesic properties of curine. However, curine did not inhibit the neurogenic phase of the formalin test, indicating that the analgesic effect of curine is associated with anti-inflammatory mechanisms and is not a result of the direct inhibition of neuronal activation. Accordingly, our experiments have shown that curine presents phenotypic outcomes similar to those observed for indomethacin, a NSAID, but different from morphine, a central acting analgesic drug. In addition, curine significantly inhibited the hyperalgesic response triggered by carrageenan, which is highly dependent on anti-inflammatory mechanisms [21]. Furthermore, several reports have indicated that hyperalgesia is essentially dependent on the action of PGE_2 [10]. Thus, considering that PGE_2 is importantly involved in the development of the inflammatory and nociceptive responses that were evaluated in the present study and that curine and indomethacin (whose analgesic effect is highly dependent on the inhibition of PGE_2 production) presented similar analgesic effects, we demonstrated that curine inhibited PGE_2 production without affecting COX-2 expression, indicating that the analgesic and anti-inflammatory effects of curine result, at least in part, from the inhibition of PGE_2 production. In fact, classical NSAIDs promote the inhibition of COX activity without alteration in the expression of this enzyme [22]. However, this effect could also be explained by the inhibition of other enzymes in the pathway of the arachidonic acid metabolism, particularly PLA_2, since it is a key enzyme in the cascade formation of both cyclooxygenase and lipoxygenase pathways [23]. Finally, some drugs are simultaneous inhibitors of 5-LO and COX [24], and so this mechanism could also explain the simultaneous inhibition of both prostaglandins and leukotrienes. We have recently reported the antiallergic effects of curine [8]. We demonstrated that the *in vivo* oral treatment with curine significantly inhibits eosinophilic inflammation, eosinophil lipid body formation, and AHR and cytokine production (IL-13 and eotaxin). Verapamil, a calcium channel antagonist, has similar antiallergic properties, and curine pretreatment inhibits the calcium-induced tracheal contractile response *ex vivo*, indicating that the antiallergic effect of curine is associated with the inhibition of the calcium influx. These results suggest that curine affects many inflammatory signaling pathways, including those involved in the production of PGE_2 [25]. Importantly, oral treatment with curine for seven consecutive days did not change hematologic (such as leukocytes, red blood cells platelets, hematocrit, and hemoglobin) or biochemical (such as alkaline phosphatase, alanine transaminase, aspartate transaminase, bilirubin, creatinine, creatinine kinase, cholesterol, glucose, total proteins, and uric acid) parameters. Additionally, curine treatment did not induce the formation of gastric ulcers, and no physical or behavioral changes were observed, indicating that curine is not toxic when used in doses

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up to 10-fold higher than the ED_{50} under these conditions [8]. These findings may have a therapeutic potential because curine has anti-inflammatory effects at nontoxic doses; therefore, curine could be an alternative for the development of novel safe and effective anti-inflammatory and analgesic drugs.

In conclusion, oral treatment with curine exhibits anti-inflammatory and analgesic effects through mechanisms that, at least in part, depend on the inhibition of PGE_2 production.

Materials and Methods

Curine purification

C. platyphyllum was collected in Santa Rita, Paraíba, Brazil. A voucher specimen was deposited in the herbarium of Prof. Lauro Pires Xavier (UFPB – João Pessoa, Brazil), number 3631-P, and was identified by Prof. Dr. Maria de Fatima Agra. *C. platyphyllum* bark (3.0 kg) was dried and pulverized in a Harley-type grinder and was extracted under exhaustive percolation with ethanol 95° GL for 3–4 days. The extract (150.0 g) was concentrated under vacuum at a temperature ranging from 50 °C to 60 °C to obtain the crude ethanol extract. This extract was then dissolved in 3% HCl, filtered through Celite (545 Fischer Scientific), and submitted to $CHCl_3$ extraction alternating with NH_4OH (pH 8) basification. After washing with water and $MgSO_4$, the solvent was evaporated, and this $CHCl_3$ extract became the total tertiary alkaloid fraction (TTA). The TTA was submitted to column chromatography (60 × 600 mm) on aluminum oxide using a step gradient of hexane, hexane-$CHCl_2$, and $CHCl_2$: MEOH. Fractions of 50 mL were collected and monitored by TLC using a step gradient of $CHCl_3$-MeOH 100 : 0 (3 L) and 97 : 3 (2 L). Fractions of 50 mL were collected for each system [hexane 100%, hexane : $CHCl_2$ (1 : 1), $CHCl_2$ (100%), $CHCl_2$: MEOH (99 : 1), $CHCl_2$: MEOH (8.5 : 1.5), and $CHCl_2$: MEOH (2.5 : 7.5)] yielding 60 fractions. Fractions 52–60 were monitored by TLC (system 6, $CHCl_2$: MEOH by hydroxylammonium atmosphere), and curine was obtained (0.031% of dry plant; purity > 98%) with a fusion point at 215 °C $[\alpha]_D$: −225° (MeOH, c = 0.04). No other compound was detected. The structure was established by spectroscopic data analysis of NMR ^{13}C and NMR ^{1}H ($CDCl_3$, 400 MHz) (**Figs. 1S** and **2S**, Supporting Information, respectively), and when compared to the literature data [26], it was demonstrated that the product was curine. The curine solution was prepared using 1 mg of the crystalin, 50 µL of 1 N HCl, and 500 µL of distilled water. The pH of 7–8 was adjusted with 1 N NaOH. The volume was completed to 1 mL with PBS.

Animals

Male or female Swiss mice and male C57Bl/6, mice weighing 25–30 g, were obtained from the Federal University of Paraiba and the Oswaldo Cruz Foundation breeding units, respectively. The animals were maintained with food and water *ad libitum* in a room with the temperature ranging from 22 °C to 24 °C and a 12-h light/dark cycle. This study was carried out in accordance with the recommendations of the Brazilian National Council of Control of Animal Experimentation (CONCEA). The protocols were approved by the Animal Welfare Committee of the Oswaldo Cruz Foundation (CEUA/FIOCRUZ protocol # L-033/09) and the Ethical Committee for Experimental Animals (CEPA N°0504/08, UFPB). Groups of five to ten animals were used in each experiment.

Treatments

For the *in vivo* experiments, the animals were orally (p.o.) pretreated with curine (2.5 mg/kg, purity 98%). Indomethacin (10 mg/kg, p.o., purity 99%, Sigma-Aldrich), an NSAID, and morphine (10 mg/kg, p.o., purity 99%, Sigma-Aldrich), a central acting analgesic drug, were used as standard pharmacological controls. PBS (p.o.) was used as a negative control. All treatments were performed 1 h before each challenge. For the *in vitro* experiments, cells were treated with curine (1 or 10 µM) 1 h before the stimulus. Non-treated cells received supplemented RPMI medium as detailed below.

Mouse paw edema induction

Acute inflammation of the mouse hind paw was induced as described [27,28]. Briefly, 1 h after the pretreatments, the Swiss mice were subcutaneously (s.c.) injected with 20 µL of carrageenan (500 µg/paw) or zymosan (200 µg/paw) into the plantar region of the left hind paw. As a control, PBS (20 µL) was injected into the right hind paw. The paw thickness of each animal was measured using a plethysmometer (Ugo Basile) at 0 and 2 h after the administration of the λ-carrageenan or at 0, 1, 2, 3, and 4 h after the administration of zymosan. The results are expressed as the differences of volume between the left and the right paw.

Acetic acid-induced peritoneal vascular permeability in mice

The peritoneal vascular permeability in mice was adapted from the procedure described by Whittle [9]. Briefly, 30 min after the pretreatments, the Swiss mice received an intraperitoneal (i.p.) injection of 1% Evans blue solution (0.1 ml/10 g) followed by 0.6% acetic (i.p.) 30 min later. The animals were sacrificed 50 min later. The peritoneal cavity was washed with PBS (10 mL), and the peritoneal fluid was collected. The vascular permeability was expressed as the optical density (OD) of Evans blue dye in an UV/VIS spectrophotometer at 610 nm.

Acetic acid-induced writhing response

Abdominal contortions were induced as previously described [29]. I.p. injections of acetic acid are known to induce abdominal writhing in mice followed by hind limb twitching, which are indicative actions of nociception [30]. One hour after the treatments, the animals received an injection (i.p.) of 1.0% acetic acid solution (0.1 mL/10 g). After being challenged, the animals were placed in individual boxes, and the abdominal constrictions were counted cumulatively over a period of 20 min. Nociception was expressed as the number of writhings.

Formalin test

The formalin test was used to analyze the licking behavior, which is characteristic in this nociceptive model. This procedure has been described by Hunskaar and Hole [10]. Briefly, 1 h after pretreatment, the Swiss mice were injected with 20 µL of 2.5% formaldehyde (formalin solution) into the right hind paw. The animals were placed in individual boxes and observed from 0–5 min (neurogenic phase) and 15–30 min (inflammatory phase), and the total time spent licking the injected paw (licking time) was recorded with a chronometer and considered a parameter of nociception.

Carrageenan-induced hyperalgesia

Hyperalgesia was analyzed using the hot plate test as previously described [11]. Briefly, mice were injected with 20 µL of carrageenan (25 µg/paw) into one hind paw 1 h after the pretreat-

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ments. The other hind paw was injected with an equal volume of PBS, which was used as a negative control. The animals were placed on a plate (IITC Life Science, Inc.) at 52 °C. The latency time of each paw was manually recorded with a chronometer for 30 s, and the following behaviors were considered: 1 – jump (i.e., all paws raised from the surface of plate), 2 – licking of a hind paw, 3 – shaking of a hind paw, 4 – lifting of a hind paw and spreading of the phalanxes, or 5 – rapid repeated lifting of the hind paws. Hyperalgesia was defined as a decrease in the withdrawal latency of the carrageenan-challenged paw compared with the PBS-challenged paw.

Peritoneal macrophage culture

Peritoneal macrophages from the C57Bl/6 mice were obtained four days after an injection of 4% thioglycollate by washing the peritoneal cavity with RPMI 1640 medium supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were adjusted to a concentration of 2×10^6/mL and were plated in 24-well culture plates (500 µL) at 37 °C in a 4% CO_2 atmosphere overnight. Following the incubation, the cells were pretreated with curine (1 or 10 µM) and were stimulated with LPS (500 ng/mL) 1 h later. Notably, 1 µM and 10 µM curine did not affect the cell viability.

Cyclooxygenase-2 expression and prostaglandin E_2 production analyses

Twelve hours after LPS stimulus, the supernatants from the cell cultures described above were collected and the PGE_2 production was analyzed using Duo Set kits, according to manufacturer's instructions (R&D Systems).Then, COX-2 expression was analyzed by Western blotting as follows: Briefly, the cells were washed with PBS buffer and homogenized with 10 mM Tris-HCl buffer (pH 7.4), 150 mMNaCl, 0.5% Triton X-100, 10% glycerol (v/v), 0.1 mM EDTA, 1 mM DTT, and a cocktail of protease inhibitors (Roche). The proteins from the cell homogenates were separated by polyacrylamide gels in the presence of 10% SDS at a constant current of 16 mA. Full-range rainbow (RPN800E, GE Healthcare Life Sciences) was used as a relative molecular mass standard. After gel separation, the samples were transferred at 200 mA for 120 min onto a nitrocellulose membrane using 25 mM Tris-HCl (pH 8.3) and 192 mM glycine at 4 °C. The membranes were blocked with TBS-0.t Tween 20 and 5% milk for 1 h, incubated with a polyclonal antibody (1 : 1000) raised against COX-2 (sc-1745, Santa Cruz Biotechnology) for 18 h, incubated in a secondary antibody (anti-goat IgG-HRP, Santa Cruz Biotechnology) for 1 h, and developed using the Super Signal West Pico chemiluminescent substrate (Thermo Fisher Scientific).

Statistical analyses

A one-way ANOVA and a Dunnett test, as a post-test, were used to determine the statistical significance in comparison to the control. Data are expressed as the mean ± standard error of the mean (SEM) of at least four independent experiments performed in duplicate or triplicate, as described above. P values of 0.05 or less were considered to be statistically significant. Statistical analyses were made using GraphPad Prism 5 Software (San Diego).

Supporting information

^{1}H and ^{13}CNMR spectra of curine are available as Supporting Information.

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Conflict of Interest

The authors state that they have no conflict of interest.

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