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ORIGINAL ARTICLE

# The anti-hyperalgesic and anti-inflammatory profiles of *p*-cymene: Evidence for the involvement of opioid system and cytokines

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**Abstract**

*Context*: Pain corresponds to the most frequent reason for visits to physicians, and its control by conventional drugs is accompanied by several side effects, making treatment difficult. For this reason, new chemical entities derived from natural products still hold great promise for the future of drug discovery to pain treatment.

*Objective*: The objective of this study was to evaluate the antinociceptive and anti-inflammatory profiles of *p*-cymene (PC), a monocyclic monoterpene, and its possible mechanisms of action.

*Materials and methods*: Mice treated acutely with PC (25, 50, or 100 mg/kg, i.p.) were screened for carrageenan-induced hyperalgesia and the inflammatory components of its cascade (30–180 min), carrageenan-induced pleurisy (4 h), and tail-flick test (1–8 h). Also, we observed the PC effect on the generation of nitric oxide by macrophages and the activation of neurons in the periaqueductal gray (PAG) by immunofluorescence.

*Results*: PC reduced ($p < 0.001$) the hyperalgesia induced by carrageenan, TNF-α, dopamine, and $PGE_2$. PC decrease total leukocyte migration (100 mg/kg: $p < 0.01$), neutrophils (50 and 100 mg/kg: $p < 0.05$ and 0.001), and TNF-α (25, 50, and 100 mg/kg: $p < 0.01$, 0.05, and 0.001, respectively), besides reducing NO production ($p < 0.05$) *in vitro*. PC produced antinociceptive effect in tail-flick test ($p < 0.05$), which was antagonized by naloxone, naltrindole, nor-BNI, and CTOP, and increased ($p < 0.001$) the number of c-Fos-immunoreactive neurons in PAG.

*Discussion and conclusion*: These results provide information about the anti-hyperalgesic and anti-inflammatory properties of PC suggesting a possible involvement of the opioid system and modulating some pro-inflammatory cytokines.

**Keywords**

Monoterpene, opioid system, pain, TNF-α

**History**

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## Introduction

Pain, recognized in some circumstances as a disease, is one of the most frequent reasons for visits to physicians and is among the most common reasons for taking medications. In addition, it is also a major cause of work disability. A significant portion of the world population is affected by some kind of pain, causing loss of good quality of life (De Sousa, 2011). Thus, treatment of painful disorders of various types continues to be a challenge to modern medicine.

In this context, natural products, particularly medicinal plants, have greatly contributed to the treatment of pain since the dawn of mankind. On account of that, new chemical entities derived from natural products still represent a great promise for the discovery of new drugs bearing pharmacological profiles and which are free of side effects, mainly in the treatment of pain disorders (McCurdy & Scully, 2005).

Within the broad range of compounds obtained naturally, terpenes have been gaining importance among the new chemical entities with potential for the development of novel analgesics. Constituting the superfamily of terpene compounds are the monoterpenes, which are widely found in essential oils of aromatic species and which bear an appreciable analgesic effect (De Sousa, 2011). Recently, Guimarães et al. (2013) found 27 monoterpenes with potential analgesic profile, suggesting that these are candidates for the

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development of new drugs for the treatment of painful conditions. In addition, therapeutic products with compounds belonging to this class presented several patent applications in the main patent office (Guimarães et al., 2014).

The *p*-cymene (PC) [1-methyl-4-(1-methylethyl) benzene] is a monocyclic monoterpene, found in plants of the genus *Protium*, *Thymus* and *Artemisia* (Pinto et al., 2010; Rather et al., 2012; Salas et al., 2012). Medicinal plants such as oregano, a favorite in cuisine worldwide, are a rich source of essential oil with a significant amount of PC (Kulisic et al., 2004). Recently, Quintans et al. (2013), Bonjardim et al. (2012), and Santana et al. (2011) suggested a variety of pharmacological effects of PC, including anti-inflammatory, antinociceptive, and anxiolytic profiles. However, there are no descriptions of their possible pharmacological mechanisms involved. Thus, we aimed to investigate the possible antinociceptive and anti-inflammatory effects of PC on different experimental protocols seeking to shed light on a possible pharmacological mechanism involved.

## Materials and methods

### Drugs and reagents

λ-Carrageenan, TNF-α, $PGE_2$, dopamine (DA), ethylenediamine tetra acetic acid (EDTA), lip polysaccharide (LPS), Griess reagent, Türk solution, and PC (99.7% purity) were purchased from Sigma (St. Louis, MO). Enzyme-linked immunosorbent assay (ELISA) for mouse quantitative determination of TNF-α was obtained from BD-Bioscience Pharmingen (San Diego, CA). Sodium nitrite ($NaNO_2$) was obtained from Merck, Darmstadt, Germany. Indomethacin and dipyrone were obtained from União Química (São Paulo, Brazil). Naloxone (non-selective antagonist of opioid receptors), naltrindole (δ-opioid receptor antagonist), and norbinaltorphimine (Nor-BNI; κ-opioid receptor antagonist) were purchased from Sigma Chemical Company (St. Louis, MO). D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide (CTOP; μ-opioid receptor antagonist) was purchased from Tocris Bioscience (Bristol, UK). Morphine was purchased from Cristália (Itapira, São Paulo, Brazil). Drugs were dissolved in physiological saline and administered through intraperitoneal (i.p.) or subcutaneous (s.c.) routes.

### Animals

Mice male Swiss (20–30) were obtained from the Central Animal Facilities of the Federal University of Sergipe and Gonçalo Moniz Research Center. In experiments, animal care and handling procedures were in accordance with the International Association for the Study of Pain guidelines for the use of animals in pain research (Zimmermann, 1983). These were housed in temperature-controlled rooms (22–25 °C), under a 12:12 h light:dark cycle, with access to water and food *ad libitum* until use. The Institutional Animal Care and Use Committee FIOCRUZ CPqGM 009/2011 and the Ethics Committee for Animal Research of the Federal University of Sergipe (UFS) (Protocol no. 19/11) approved the study. Every effort was made to minimize the number of animals used and any discomfort.

### Hyperalgesia induced by carrageenan, TNF-α, $PGE_2$, and dopamine

Mechanical hyperalgesia was tested in mice as previously described by Cunha et al. (2004, 2005) and Villarreal et al. (2009). The mice were treated with vehicle (saline + Tween-80 0.2% v/v, i.p.), PC (25, 50, or 100 mg/kg, i.p.), indomethacin (IND; 10 mg/kg, i.p.), or dipyrone (DIPY; 60 mg/kg, i.p.) ($n = 6$, per group). Thirty minutes after the treatment, 20 μL of carrageenan (CG, 300 μg/paw) was injected subcutaneously into the subplantar region of the hind paw, or important mediators involved in the inflammatory cascade of this phlogiston agent, such as TNF-α (100 pg/paw), $PGE_2$ (100 ng/paw), and dopamine (DA, 30 μg/paw). The degree of hyperalgesia was evaluated at 0.5, 1, 2, and 3 h after the injection of hyperalgesic agents.

Electronic anesthesiometer (Insight®, Ribeirão Preto, Brazil), with a pressure transducer coupled to a digital force detector that records the applied force in grams, was used to evaluate the hyperalgesia in mice. In this test, a gradual increase in pressure is applied to the hind paw until the withdrawal of the paw, followed by clear flinching movements. The force recorded in grams was automatically displayed. The intensity of the stimulus was obtained by averaging five measurements performed with minimal intervals of 3 min.

### Carrageenan-induced pleurisy

Pleurisy was induced by intrathoracic injection (i.t.) of carrageenan (CG, 300 μg; 0.1 mL diluted in sterile saline). The animals treated with PC (25, 50, or 100 mg/kg; i.p. $n = 6$) or vehicle (saline + Tween-80 0.2% v/v, i.p. $n = 6$) received injection of the inflammatory agent 30 min before. Four hours after, the mice were euthanized by stimulation in a $CO_2$ chamber; the pleural cavities were opened and washed with 1 mL of phosphate-buffered saline (PBS) (1×) containing EDTA (10 mM).

With samples collected in the pleural lavage and diluted (40×) in Türk solution and on a Neubauer chamber under an optical microscope, we performed the total leukocyte counts. The differential leukocyte analysis was performed under a light microscope with immersion oil objective in cytocentrifuged smears colored with May–Grunwald–Giemsa, where 100 cells per slide were counted. Four hours after injection of carrageenan, we assessed the amount of TNF-α produced in the pleural cavity. The recovered pleural lavage was centrifuged at 770 × *g* for 10 min. TNF-α was quantified on supernatant free of cells by means of enzyme immunoassay (ELISA) following the manufacturer's protocol (BD-Bioscience Pharmingen, San Diego, CA).

### Generation of nitric oxide by macrophages

A suspension of peritoneal macrophages ($5 \times 10^6$ cells/100 μL, in triplicates) was incubated in a 96-well microplate with 100 μL of PC (25, 50, or 100 μg/mL) or culture medium RPMI-1640 for 24 h at 37 °C with 5% $CO_2$. As a positive control, 100 μL of lipopolysaccharide solution (LPS, 1 μg/mL) was used. After incubation, aliquots of 100 μL of supernatant were mixed with 100 μL of Griess

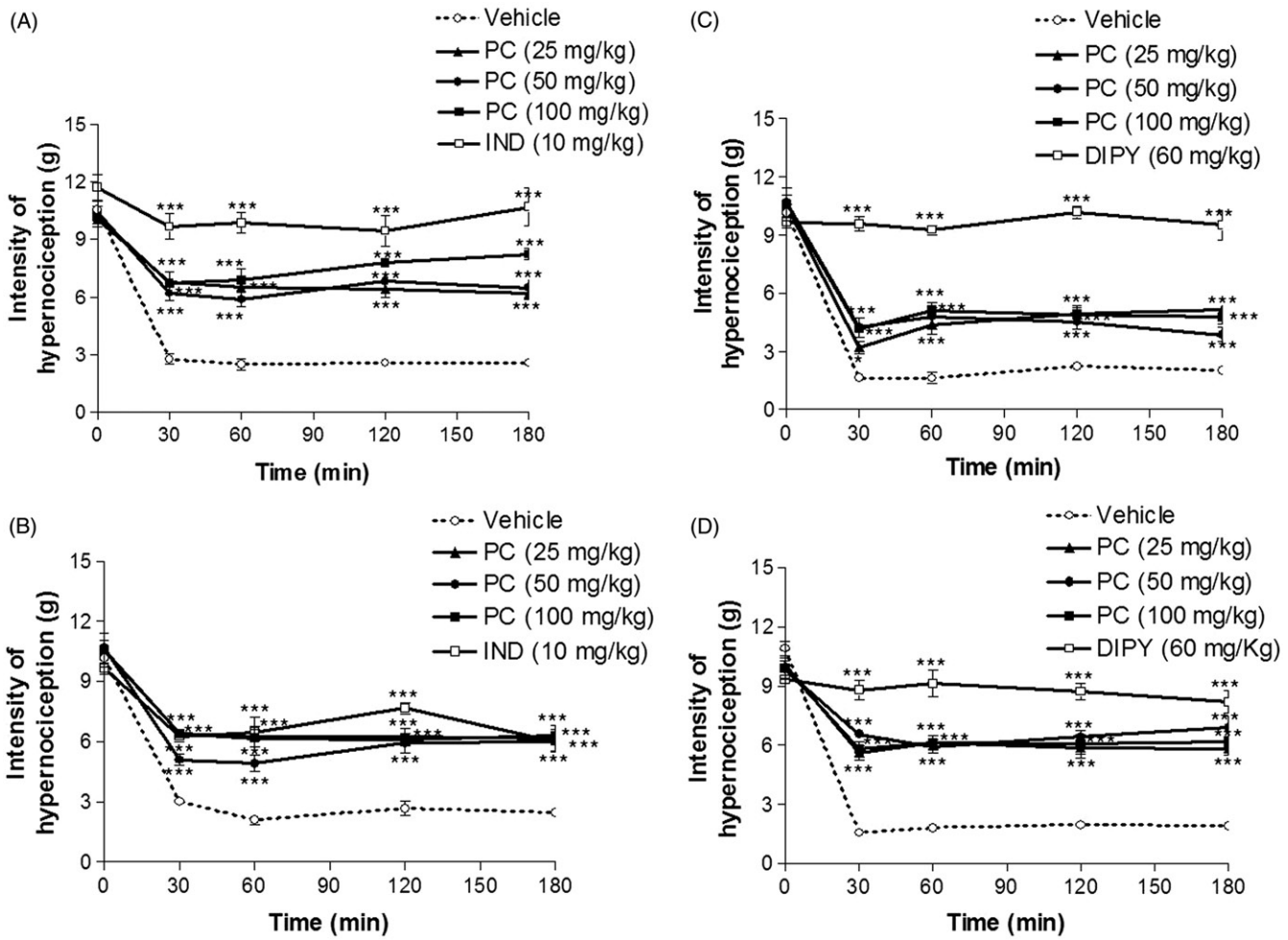


Figure 1. Effect of acute administration of vehicle, *p*-cymene (PC; 25, 50, or 100 mg/kg, i.p.), indomethacin (IND, 10 mg/kg, i.p.), or dipyrone (DIPY, 60 mg/kg, i.p.) on mechanical hyperalgesia induced by carrageenan (A), TNF-α (B), $PGE_2$ (C), or dopamine (D). Each point represents the mean ± S.E.M. of the paw withdrawal threshold (in grams) to tactile stimulation of the ipsilateral hind paw. ***$p < 0.001$ versus the control group (ANOVA followed by the Tukey test).

reagent (0.1% sulfanilamide, *N*-naphthyl-ethylenediamine 0.1%, and phosphoric acid 3%). After 10 min at room temperature, the absorbance was read at 540 nm in an ELISA reader. Data were expressed as concentration (in mM) of nitrite through the standard curve obtained previously with known molar concentrations of $NaNO_2$ in RPMI-1640 (Green et al., 1982).

### Tail-flick test

The tail-flick test in mice was conducted as described before (D'Amour & Smith, 1941), with minor modifications. Before the day of the experiment, each animal was habituated to the restraint cylinder for 5 consecutive days (20 min per day). On the experimental day, mice were placed in the restraint cylinder and the tail tip (2 cm) was immersed in a water bath at 48 °C ± 0.5 °C. The latency for the tail withdrawal reflex was measured. Each trial was terminated after 18 s to minimize the probability of skin damage. To determine the involvement of the opioid system in the PC-induced antinociception, mice were pre-treated with the non-selective opioid receptor antagonist naloxone (5 mg/kg, 15 min before PC administration), or with selective opioid receptor antagonists: naltrindole (δ-opioid receptor antagonist, 3 mg/kg, 5 min before PC administration), nor-BNI (k-opioid receptor antagonist, 0.5 mg/kg, 15 min before PC administration), or CTOP (μ-opioid receptor antagonist, 1 mg/kg, 5 min before PC administration). Tail-flick latency was measured before (baseline) and after treatments.

### Immunofluorescence

In the immunofluorescence protocol, mice ($n = 6$, per group) were perfused and the brains were collected and cryoprotected for immunofluorescence processing to Fos protein, 90 min after pretreatment with PC (25, 50, or 100 mg/kg, i.p.) or vehicle (saline + Tween-80 0.2% v/v, i.p.).

The whole brains were collected on slides of gelatinized glass frozen serial transverse sections (20 μm) and stored them at 80 °C until use. The sections were washed with phosphate buffer (0.01 M) saline isotonic (PBS) five times for 5 min and incubated with 0.1 M glycine in PBS for 10 min. Non-specific protein binding was blocked by the incubation of the sections for 30 min in a solution containing BSA (2%). After that, the sections were incubated overnight with rabbit anti-Fos sc-52 as primary antibodies (1:2000). Afterwards, the sections were incubated for 1 h with donkey anti-rabbit Alexa Fluor 594 as secondary antibodies (1:2000).

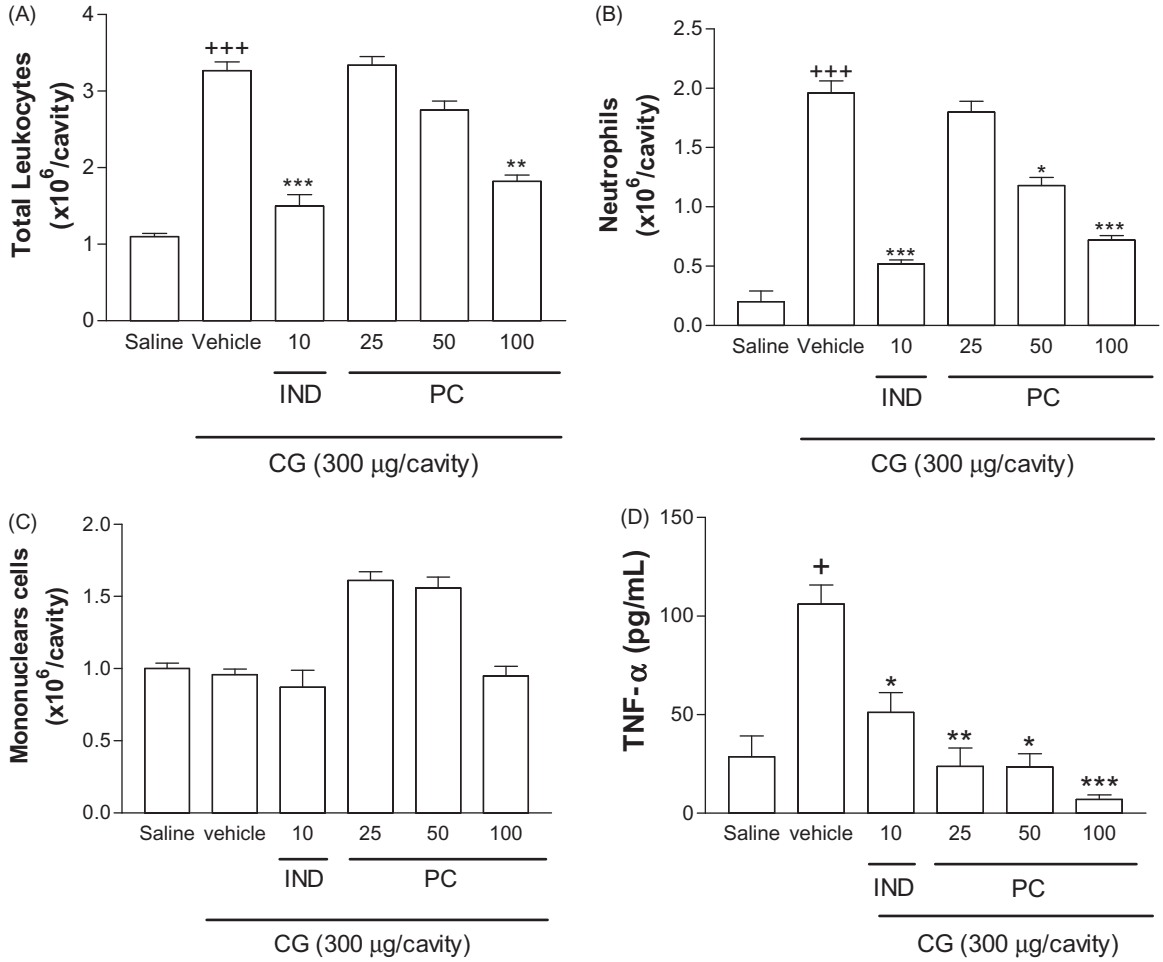


Figure 2. Effect of *p*-cymene (PC 25, 50, and 100 mg/kg; i.p.) or indomethacin (IND, 10 mg/kg; i.p.) on the inflammation induced by carrageenan (CG) in mouse pleurisy. The analyses were performed 4 h after carrageenan injection (300 µg/cavity) to evaluate the recruitment of total leukocytes (A), neutrophils (B), mononuclear cells (C), and to assess tumor necrosis factor-alpha (TNF-α) levels (D). Data were expressed as mean ± S.E.M., for a minimum of five animals. $+p<0.01$ and $+++p<0.001$ compared with the saline-injected mice; $*p<0.05$, $**p<0.01$, and $***p<0.001$ compared with the control group (vehicle) (ANOVA followed by the Tukey test).

The cover slip was mounted with Fluoromount G. As an immunofluorescence control for non-specific labeling, sections were incubated without primary antibody. After each stage, slides were washed with PBS five times for 5 min.

### Acquisition and analysis of images

The brain sections containing labeled neurons positive for Fos were acquired and classified into regions according to the Atlas (Paxinos & Watson, 2006). A photograph was taken bilaterally using a fluorescence microscope with digital camera (Axioskop 2 plus, CarlZeiss, Germany). Neurons were counted by means of the Image J® free software (National Institute of Health, Bethesda, MD) using a plug-in (written by the authors) that uses the same level of label intensity to select and count the Fos-positive cells.

### Statistical analysis

Data analyses were performed using the Graph Pad Prism 5.0 software (GraphPad Software, San Diego, CA). The data obtained were evaluated through one-way analysis of variance (ANOVA) followed by Tukey's test. Comparisons across three or more treatments were made through multivariate analysis of variance (ANOVA) with repeated measures to compare the groups over all times. The factors analyzed were treatments, time and treatment × time interaction. In the case of treatment × time interaction, one-way analysis of variance followed by a Bonferroni correction was performed for each time. In all cases, differences were considered significant if $p<0.05$.

## Results

Systemic pretreatment with PC, in all doses, reduced the hyperalgesia induced by CG (Figure 1A) and TNF-α (Figure 1B) (0.5, 1, 2, and 3 h; $p<0.001$) when compared with the animals of the control group. PC also reduced the hyperalgesia induced by $PGE_2$ (Figure 1C) (0.5 h: 25 mg/kg, $p<0.05$; 50 mg/kg and 100 mg/kg $p<0.001$; 1, 2, and 3 h: $p<0.001$, all doses) or dopamine (Figure 1D) (0.5, 1, 2, and 3 h; $p<0.001$, all doses).

PC also significantly suppressed the recruitment of total leukocytes to the pleural cavity at the higher dose (100 mg/kg, $p<0.01$) and neutrophils influx at doses of 50 mg/kg ($p<0.05$) and 100 mg/kg ($p<0.001$), as seen in Figure 2(A) and (B). However, PC did not reduce mononuclear cell counts

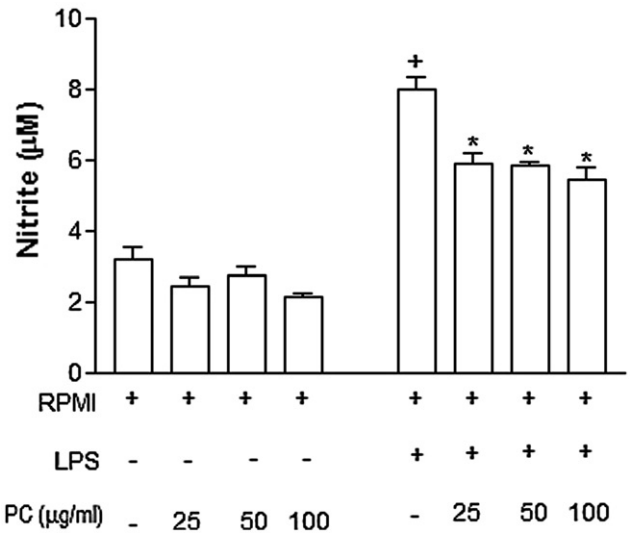


Figure 3. Effect of *p*-cymene (PC) on LPS-induced nitrite production by isolated murine macrophages. Cells were maintained in culture medium (RPMI) or pre-incubated with PC for 24 h and then treated with LPS (1 μg/mL) for 24 h. Nitrite levels in the supernatants were evaluated, and the results were expressed as concentration (in micromolar) of nitrite. Data were presented as the means ± S.E.M. of values obtained from triplicates and are representative of three experiments with similar results. $+p < 0.001$ compared with RPMI and $*p < 0.05$ compared with LPS (ANOVA followed by the Tukey test).

when compared to the control group (Figure 2C). PC (25 mg/kg, $p < 0.01$; 50 mg/kg, $p < 0.05$; and 100 mg/kg, $p < 0.001$) also significantly reduced the TNF-α levels in the pleural exudates collected at 4 h after carrageenan injection (Figure 2D).

The incubation of murine macrophages with LPS resulted in significant ($p < 0.05$) increase in the NO production in these cells, which was significantly reduced ($p < 0.05$) by all concentrations of PC tested (25, 50, and 100 μg/mL) (Figure 3).

Administration of PC (25–100 mg/kg) enhanced, in a dose-dependent manner, the reaction time in the tail-flick test (Figure 4; $p < 0.05$), an effect that lasted for 5 h. The administration of morphine (5 mg/kg, s.c.) caused a significant increase in the latency response through 3 h after administration (Figure 4; $p < 0.05$). The antinociception of the PC (100 mg/kg)-treated group was significantly higher ($p < 0.05$) relative to the morphine-treated group. This effect was completely antagonized in mice pre-treated with naloxone (5 mg/kg i.p.; 15 min before) (Figure 4B). Reinforcing this idea, the administration of the δ-opioid receptor antagonist naltrindole (3.0 mg/kg, s.c., 5 min before PC), μ-opioid receptor antagonist CTOP (1 mg/kg, i.p., 5 min before PC), or k-opioid receptor antagonist nor-BNI (0.5 mg/kg, s.c., 15 min before PC), antagonized the PC-induced antinociception (Figure 4C).

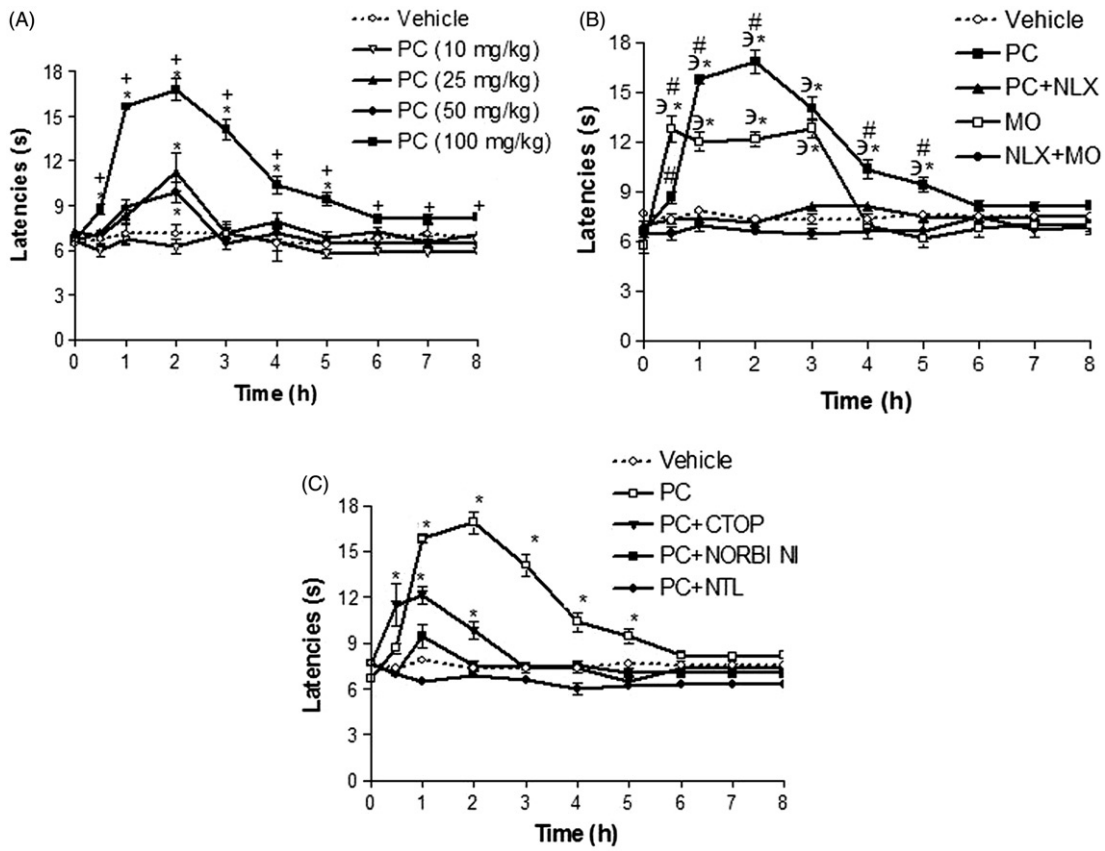


Figure 4. Antinociceptive effect of *p*-cymene (PC) in the tail flick test. The figure shows data of tail flick latencies represented in seconds, up to 8 h following treatment. (A) For the dose–response analysis, the effects of increasing doses of *p*-cymene (10–100 mg/kg) were tested. (B) Effects of naloxone (NLX; 5 mg/kg i.p.), a non-selective opioid receptor antagonist, on the antinociceptive effect of PC (100 mg/kg) or morphine (MO: 5 mg/kg s.c.). (C) Effects of μ-opioid receptor antagonist (CTOP; 1 mg/kg i.p.), k-opioid receptor antagonist (nor-BNI; 0.5 mg/kg s.c), and δ-opioid receptor antagonist (naltrindole; 3.0 mg/kg s.c.) on the antinociceptive effect of PC (100 mg/kg) or MO (5 mg/kg s.c.). Data are expressed as means ± S.E.M.; $n = 6$ mice per group. *Significantly different from the vehicle-treated group ($p < 0.05$); +significantly different from PC 50 mg/kg group ($p < 0.05$), #significantly different from antagonist-treated groups ($p < 0.05$), ɔsignificantly different from the morphine-treated group ($p < 0.05$).

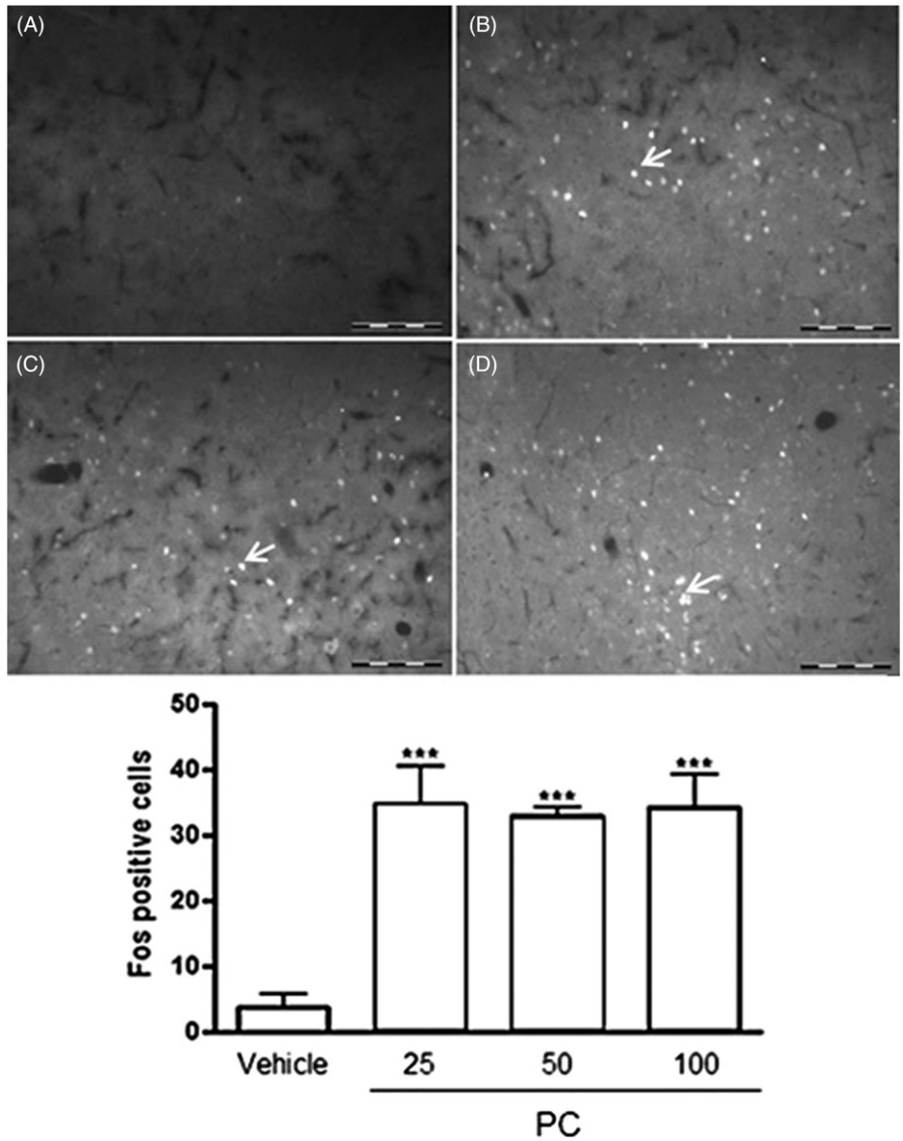


Figure 5. Immunofluorescence for Fos protein. The white arrows point neurons Fos positive in the periaqueductal gray. Vehicle (A) or PC (B: 25, C: 50, D: 100 mg/kg) were administered intraperitoneally 1.5 h before of the S.E.M. ($n = 6$, ±perfusion. Enlargement: 20×; scale 20 μm. Values represent in mean per group). ***$p < 0.001$ versus control (one-way ANOVA followed by Tukey's test).

Finally, we observed that mice treated with PC presented a significant increase ($p < 0.001$) in positively marked neurons in the periaqueductal gray (25 mg/kg: $34.7 \pm 5.6$; 50 mg/kg: $33.0 \pm 1.0$; 100 mg/kg: $34.4 \pm 4.9$) when compared with control (vehicle: $3.7 \pm 1.7$) (Figure 5).

## Discussion

The present study assessed the pharmacological effect of the PC, a monocyclic monoterpene, on central and inflammatory pain models. As reported previously (Guimarães et al., 2013, 2014), monoterpenes are a class of organic compounds naturally occurring and exhibiting appreciable pharmacological activities, especially analgesic and anti-inflammatory ones.

In the present series of experiments, it was demonstrated that PC reduced hyperalgesia induced by carrageenan, and important mediators are involved in the inflammatory cascade generated by this phlogistic agent. Injection of carrageenan into the plantar surface of mice produced inflammation and hyperalgesia through cytokine cascade, as TNF-α, released by resident or migrating cells, which leads to release of prostanoids and sympathomimetic amines (as dopamine), that stimulate peripheral A δ and C fiber sensory nerve terminals (Cunha et al., 1992, 2005; Dray, 1995).

Here, we demonstrated that PC may act inhibiting the development of inflammatory response through its effect on the hyperalgesia induced by carrageenan and TNF-α besides decreasing sensitization of the nociceptive fibers by $PGE_2$ and dopamine. These outcomes corroborated with a previous study, which suggested that PC is able to reduce response nociceptive through central and peripheral mechanisms (Bonjardim et al., 2012).

Subsequently, we investigated the effect of PC on cell migration due to the important role of this event on inflammation and hyperalgesia triggered by carrageenan (Cunha et al., 2008). PC showed a modulating effect on the migration of total leukocytes (100 mg/kg), neutrophils (50 and 100 mg/kg), and TNF-alpha release (all doses). Similarly, Xie et al. (2012) demonstrated that PC reduces production of TNF-α, IL-1β, and IL-6 induced by LPS, probably through NF-κB and MAPK inactivation (Xie et al., 2012).

NF-κB is a key part in the production of pro-nociceptives/inflammatory molecules and in the induction of the synthesis of nitric oxide synthase (iNOS), which promotes increased production of NO (Ferreira et al., 1997). In this sense, it was verified that PC can modulate the release of NO, which may be associated with the PC action on NF-κB (Xie et al., 2012).

Thus, on one hand, it can be seen that PC reduces carrageenan-induced hyperalgesia and TNF-alpha due to its ability to modulate the inflammatory response. On the other hand, considering that PC also reduced the hyperalgesia induced by the final mediators of the carrageenan cascade ($PGE_2$ and dopamine), which by themselves do not cause pain but sensitize nerve endings, the central effect of PC involved in the nociception control was assessed.

For this, the thermal model of the tail-flick test, considered to be a spinal reflex, but that could also involve higher neural structures (Jensen & Yaksh, 1986; Le Bars et al., 2001), was used. Treatment of animals with PC promoted an increase in the reaction time in the tail-flick test, which was reversed by the opioid antagonists used. Henceforth, the results presented herein suggested that this action could be related to the blockade of the neural transmission of pain, through opioid system modulation, corroborating with Santana et al. (2011). Additionally, all three opioid receptors (μ, δ, and κ) are present in the CNS and PNS on peripheral sensory nerve terminals (Lesniak & Lipkowski, 2011) and are able to produce potent clinically relevant central and peripheral analgesia (Machelska, 2000).

Considering that the antinociceptive effect of PC is mediated by the activation of opioid receptors, it is possible to propose the involvement of descending pain inhibitory pathways on this analgesic profile. Therefore, we demonstrated the effect of PC on the increase in the number of positively marked neurons in periaqueductal gray (PAG), an important region involved with pain modulation.

PAG is a midbrain region activated by opioid agonists, cannabinoids, and COX inhibitors (Ossipov et al., 2010). Furthermore, there is substantial evidence that the PAG and descendant projections comprise a neural circuit essentially mediated by endogenous and exogenous opioid analgesics (Loyd et al., 2007; Wang & Wessendorf, 2002), which significantly attenuated activity after the microinjection of opioid antagonists in this region (Bernal et al., 2007).

Therefore, our results suggest that PC has a significant ability by reducing the production of pro-inflammatory cytokine TNF-α, the migration of leukocytes, and the release of NO. Their effects also may involve the blocking of sensitization or direct activation of nociceptors by reducing mechanical hyperalgesia. Moreover, the demonstration of PC-induced antinociception through the opioid system was enhanced by the activation of the PAG, suggesting the involvement of descending pain-inhibitory mechanisms. These outcomes contribute to reinforce the relevance of this compound in the management of painful disturbances.

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## Declaration of interest

The authors report that they have no conflicts of interest. The authors alone are responsible for the content and writing of this paper. We would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil) and Fundação de Apoio à Pesquisa e Inovação Tecnológica do Estado de Sergipe (FAPITEC/SE/Brazil) for the financial support.

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