

Article

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p-Cymene reduces orofacial nociceptive response in mice

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Abstract: This study investigated the possible antinociceptive effect of *p*-cymene in different tests of orofacial nociception. The animals (mice) were pretreated (*i.p.*) with *p*-cymene (25, 50, 100 mg/kg), morphine (5 mg/kg), or vehicle (0.2% Tween 80+saline), and were then subsequently administered, subcutaneously into their upper lip: formalin, capsaicin, and glutamate. The nociceptive behavior response was characterized by the time in s that the mice remained rubbing the orofacial region, for a period of 40 min in the formalin test (first phase, 0-6 min; and second phase, 21-40 min), and for 42 and 15 min in the capsaicin and glutamate tests, respectively. To verify the possible opioid involvement in the antinociceptive effects, naloxone (*i.p.*) was administered into the mice 15 min prior to the pretreatment with *p*-cymene (100 mg/kg). Finally, whether or not the *p*-cymene evoked any change in motor performance in the Rota-rod test was evaluated. The results showed that the treatment with *p*-cymene, at all doses, reduced ($p < 0.001$) the nociceptive behavior in all nociception tests. The antinociceptive effect of *p*-cymene was antagonized by naloxone (1.5 mg/kg). Additionally, mice treated with *p*-cymene did not show any change in motor performance. In conclusion, *p*-cymene attenuated orofacial nociception, suggesting an involvement of the opioid system in this effect. Thus, *p*-cymene might represent an important biomolecule for management and/or treatment of orofacial pain.

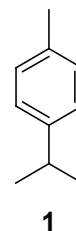
Introduction

Orofacial pain accounts for over 25% of all complaints in respect to pain (Sessle, 1987). Orofacial pain refers to conditions related to oral and face structures (Lipton et al., 1993), and stands out from other somatic pain due to its high intensity and prevalence, which often prevents an individual from performing his/her daily activities normally (Barros et al., 2009).

A variety of drugs are used for the pharmacological treatment of orofacial pain, such as the opioid and non-opioid analgesics, non-steroidal anti-inflammatory drugs (NSAID), corticosteroids, anxiolytics, antidepressants, and anticonvulsants (Dionne, 2001), but the side effects and high costs of these drugs have led to a constant search for alternative drugs from medicinal plants with analgesic and anti-inflammatory properties.

p-Cymene (*p*-isopropyltoluene, **1**) is a monoterpene biological precursor of carvacrol and one of the main constituents of the essential oil from species

of *Protium*, with more than 80% of these species found in the Amazon region (Siani et al., 1999). Recent studies have shown that species such as *Protium heptaphyllum* (Aubl.) Marchand, *P. kleinii* Cuatrec., Burseraceae, *Hyptis pectinata* (L.) Poit., Lamiaceae and *Zataria multiflora* Boiss., Lamiaceae, rich in *p*-cymene, show antinociceptive activity in rodents (Oliveira et al., 2005; Otuki et al., 2001; Bispo et al., 2001; Ramezani et al., 2004).



In addition, other monoterpenes such as menthol, linalool, citronellal, and carvone have shown antinociceptive activity in animal models of nociception (Melo et al., 2010; Galeotti et al., 2002; Peana et al., 2006; Gonçalves et al., 2008; Quintans-Júnior et al.,

2010).

Since *p*-cymene-containing natural products and others monoterpenes have been used by to alleviate pain and inflammation-related processes it is believed that *p*-cymene can be an effective agent in the management of orofacial pain. With this in mind, this study investigated the antinociceptive potential of *p*-cymene in models of orofacial nociception induced by formalin, capsaicin, and glutamate.

Material and Methods

Drugs

For the experiments the following drugs were used: *p*-cymene (99.7% purity; Sigma, USA), morphine hydrochloride (Merck, Brazil), naloxone (Res. Biochemicals Inc., USA), formaldehyde PA (Reagen, Brazil), capsaicin (Sigma, USA), diazepam (União Química, Brazil). Vehicle was 0.2% Tween 80 (Sigma, USA) dissolved in saline.

Animals

The experiments were performed with male Swiss mice (25-30 g), from the Central Animal Facility of the Federal University of Sergipe. The animals were kept four to five per cage (41 x 34 x 18 cm), at a temperature of 22-24 °C, in a light controlled room (12 h/12 h light/dark cycle, lights on at 6:00 a.m.), with water and food *ad libitum*. All procedures were in compliance with the guidelines of the International Association for the Study of Pain (IASP) (Zimmermann, 1983) and were approved by the Ethics Committee on Animal Care at UFS (Protocol N° 03/2009).

Experimental nociceptive tests

Formalin test

The induction of nociception in the orofacial formalin test was done by subcutaneous injection of formalin (20 µL, 2%) into the upper lip of mice (n=8/group) according to previous studies (Luccarini et al., 2006). The administration of formalin in the orofacial region induces a biphasic behavioral response (Cadet et al., 1993). The first phase (Phase 1) occurs within 0-5 min after administration of the nociceptive substance; this phase is followed by a latency period of about 10 min. Then, a second phase (Phase 2) occurs with duration of about 25 min (15 to 40 min). The nociceptive behavior assessed during the two phases was the time that the animals remained rubbing the orofacial region.

The animals were pretreated systemically (intraperitoneal route) with vehicle (0.2% Tween

80+saline), *p*-cymene (25, 50, 100 mg/kg), and morphine (5 mg/kg) 30 min before injection of formalin. The possible involvement of the opioid system in the antinociception produced by *p*-cymene was tested by administration of naloxone (1.5 mg/kg), an opioid antagonist, 30 min before administration of the above mentioned drugs.

Capsaicin test

This orofacial nociception test was induced by subcutaneous administration of capsaicin as described previously by Pellisier (Pellisier et al., 2002). Mice (n=8) received a subcutaneous injection of capsaicin (20 µL, 2.5 µg diluted in DMSO 25% in distilled water) into the upper lip. In pilot studies performed in our laboratory, the animals showed the behavior of rubbing the orofacial region with greater intensity for a period of 42 min after the injection of capsaicin. Therefore, the quantification of nociception was performed during this period, measuring the time (s) that the animals exhibited the nociceptive behavior of rubbing the orofacial region with the hind or front paw. The *p*-cymene, morphine, and vehicle were administered in a similar manner and at the same doses as the previous test.

Glutamate test

This orofacial nociception test was induced by subcutaneous administration of glutamate into the upper lip of mice as described previously by Quintans-Júnior et al. (2010). Mice were divided into five groups (n=8) and treated intraperitoneally with vehicle, *p*-cymene (25, 50, 100 mg/kg), or morphine 30 min before the injection of 40 µL of a glutamate (25 mM) solution in the upper lip. Then, the animals were observed for a period of 15 min, starting immediately after the injection of the glutamate. The quantification of nociceptive response was performed during this period, measuring the time (s) that the animals exhibited the behavior of rubbing the orofacial region with the hind or front paw.

Motor performance test

Rota-rod test

To assess the possible interference of *p*-cymene on animal motor performance, the rota-rod test was performed. Initially, the mice able to stay on the rota-rod for more than 180 s (7 rpm) were selected 24 h before the experiment. Then, the animals were divided into five groups (n=8), with which we performed the following treatments: *p*-cymene (25, 50, 100 mg/kg, *i.p.*), vehicle (control), and diazepam (1.5 mg/kg, *i.p.*);

after 30, 60, and 120 min each animal was submitted to the rota-rod and the results are expressed as the time (s) the animals remained on the rotating rod after the treatments mentioned above. The maximum time of permanence of the animal on the rota-rod was 180 s.

Statistical analysis

Data were analyzed using GraphPad Prism 3.0 Software. Analysis of variance (ANOVA) was performed, followed by Dunnett's and Tukey's post-test to determine, respectively, which doses differed statistically from the control group and among them. For all tests the level of significance was set at 5%.

Results

Formalin test

The results showed that pretreatment with *p*-cymene (at all doses tested) and morphine produced antinociception, evidenced by a statistically significant difference ($p < 0.001$) for the time that the animal remained rubbing the orofacial region, in both phases of testing (Figure 1). It was also demonstrated that naloxone (1.5 mg/kg, *i.p.*) reversed the antinociception induced by morphine and *p*-cymene (100 mg/kg) in both phases of testing.

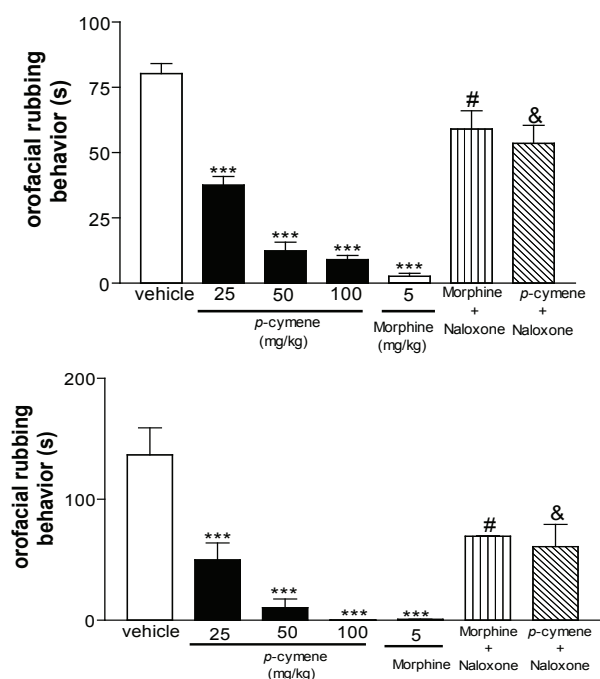


Figure 1. Effect of pretreatment of animals with vehicle, *p*-cymene (25, 50 and 100 mg/kg) or morphine (5 mg/kg) in the nociception induced by formalin. A indicates the first phase (0 to 5 min) and B the second phase (15 to 40 min) of formalin test *** $p < 0.001$ represents significant differences

when compared to the control group (ANOVA followed by Dunnett). # $p < 0.001$ indicates significant difference compared with morphine and & $p < 0.001$ compared with the *p*-cymene (100 mg/kg), (ANOVA followed by Tukey).

Capsaicin test

According to Figure 2, the pretreatment with *p*-cymene caused a statistically significant decrease ($p < 0.001$) of nociceptive behavior induced by administration of capsaicin at all doses (25, 50, and 100 mg/kg). The effect of the highest dose was similar to morphine.

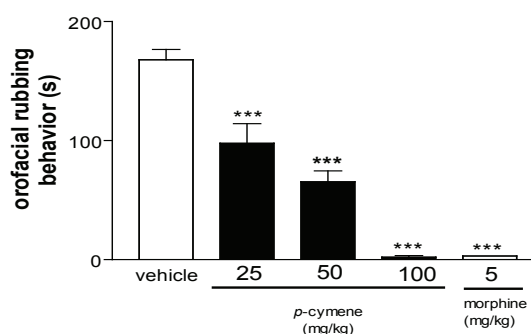


Figure 2. Effect of pretreatment of animals with vehicle, *p*-cymene (25, 50 and 100 μ g/kg) or morphine (5 mg/kg) in the nociception induced by injection of capsaicin (20 μ L) in the orofacial region (n=8). *** $p < 0.001$ represents significant differences when compared to the control group (ANOVA followed by Dunnett).

Glutamate test

Systemic treatment of animals with *p*-cymene, at doses of 25, 50, and 100 mg/kg significantly reduced the nociception induced by administration of glutamate in the upper lip (Figure 3).

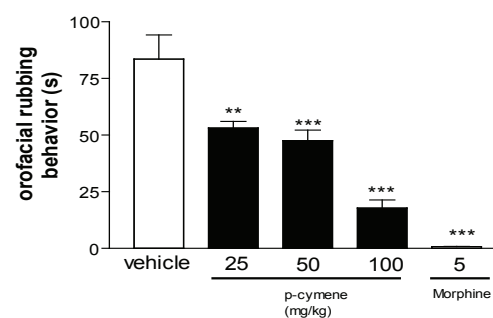


Figure 3. Effect of pretreatment of animals with vehicle, *p*-cymene (25, 50 and 100 mg/kg) in the nociception induced by injection of glutamate (40 μ L/25 mM) in the orofacial region (n=8). ** $p < 0.01$ *** $p < 0.001$ represents significant differences when compared to the control group (ANOVA followed by Dunnett).

Rota-rod test

Pretreatment of mice with *p*-cymene (25, 50, and 100 mg/kg) or vehicle did not induce changes in motor performance, since the animals remained on the rotating rod for 180 s at 30, 60, and 120 min (data not shown in figure). As expected, diazepam (1.5 mg/kg), a CNS depressant drug, reduced the latency time of falling at 30, 60, and 120 min.

Discussion

This study demonstrated the antinociceptive effect of *p*-cymene by behavioral tests of nociception (formalin, capsaicin, and glutamate tests) in mice. The use of formalin in the orofacial region induces a biphasic nociceptive response (Luccarini et al., 2006). The first phase (0-5 min) corresponds to the direct stimulation of nociceptors, predominantly C fibers, with the participation of substance P, bradykinin, and glutamate (Shibata et al., 1989). This phase is only sensitive to analgesics acting on the CNS (Hunskar et al., 1985). The first phase is followed by an interphase, which lasts about fifteen minutes and results from active inhibition of the excitability of nociceptors (Tjolsen et al., 1992). The second phase, which starts twenty-one min after formalin injection, is derived from two components: central sensitization of nociceptors and second order neurons, and the action of inflammatory mediators such as histamine, serotonin (5-HT), bradykinin, and prostaglandins released as a result of tissue injury (Henry et al., 1999). This second phase of nociception could be inhibited by both NSAID and opioid drugs (Hunskar et al., 1985). The results of the present study showed that *p*-cymene significantly reduced the nociceptive behavior in the 1st and 2nd phases of the formalin test, suggesting therefore an action in both neurogenic and inflammatory pain. It was also verified that the non-selective opioid antagonist naloxone reversed the antinociceptive effect of *p*-cymene (100 mg/kg), suggesting an effect mediated by the opioid system.

Just as formalin, capsaicin is also an important irritant used to induce nociception. Capsaicin is used as a pharmacological tool for the study of nociception through direct activation of nociceptive fibers, especially type C (Sakurada et al., 1992). Capsaicin applied to skin, muscle, and other tissues has been shown to produce inflammation, to activate and to sensitize trigeminal and spinal small-diameter nociceptive afferents, as well as dorsal horn neurons. It also evokes nociceptive behavior in animals and intense pain, hyperalgesia, and referred pain in humans (Hu et al., 2005; Lam et al., 2009a; Lam et al., 2009b).

The activation of nociceptors by capsaicin occurs through its binding to vanilloid receptors (TRPV1), which induces the influx of cations producing depolarization and excitation of neurons that cause the release of neuropeptides such as tachykinins, substance P, calcitonin gene-related peptide, excitatory amino acids (aspartate and glutamate), nitric oxide, and proinflammatory mediators from the peripheral terminals (Sakurada et al., 1992). In this context, the antinociceptive effect observed for *p*-cymene in the capsaicin test may be a result of its possible inhibition of neurotransmitters involved in activation of sensory fibers responsible for transmission of nociceptive stimuli.

A number of monoterpenes have also been described as agonists or antagonists of different members of the TRPV channel family (Xu et al., 2005; 2006; Vogt-Eisele et al., 2007). The ability of camphor, a naturally occurring monoterpene produced by Camphor Laurel (*Cinnamomum camphora*), to modulate sensations of warmth in humans has been attributed to its ability to activate TRPV3 (Moqrich et al., 2005). TRPV3 is expressed in keratinocytes, the dorsal root ganglia, the brain, and spinal cord (Vogt-Eisele et al., 2007). It has been implicated in hyperalgesia, inflamed tissues, and possibly skin sensitization (Xu et al., 2005). Vogt-Eisele et al. (2007) demonstrated that some of the effects described for the terpenoids may be related to the activation of vanilloid receptors. The mechanism of action of camphor, menthol, thymol, and carvacrol, for example, is related to activation of TRPV3 receptors. *p*-Cymene did not significantly activate these receptors.

Glutamate is present in both central and peripheral terminals of trigeminal and dorsal root ganglion neurons. Noxious stimulation of primary afferent fibers results in the release of glutamate from the peripheral as well as central terminals of trigeminal and spinal afferent fibers (Keast & Stephensen, 2000; Lam et al., 2005). In addition, glutamate injection into the rat masseter muscle or temporomandibular joint (TMJ) reflexly evokes a dose-dependent increase in jaw muscle electromyographic (EMG) activity (Cairns et al., 1998; Cairns et al., 2001a; Cairns et al., 2001b, Cairns et al., 2002) and central sensitization of trigeminal brainstem nociceptive neurons (Lam et al., 2009b). Similarly, glutamate injection into the human masseter muscle causes pain and mechanical hyperalgesia that may be attenuated by co-injection of a NMDA receptor antagonist (Cairns et al., 1998).

This nociceptive response caused by glutamate seems to involve peripheral, spinal, and supra-spinal sites, and its action is mediated by NMDA (*N*-methyl-D-aspartate) and non-NMDA receptors (Beirith et al., 2002). Several studies have demonstrated that

the excitatory amino acid receptors are involved in nociceptive primary afferent transmission, both in the development and maintenance of painful response (Coggeshall & Carlton, 1997). Thus, the suppression of glutamate-induced nociception by *p*-cymene treatment can be associated with its interaction with the glutamatergic system (Ferreira et al., 1999). Batista et al. (2008) demonstrated that linalool, a monoterpene compound prevalent in the essential oils of various aromatic plant species, possesses antinociceptive properties in mice. This effect involves peripheral and spinal sites of action and seems to be mediated by interaction with ionotropic glutamatergic-dependent mechanisms, via NMDA receptors.

Previous studies have suggested that the CNS depression and the nonspecific muscle relaxation effect can reduce the response of motor coordination and might invalidate the behavior tests' results (Almeida et al., 2004; De Sousa et al., 2006). Our results revealed that all mice treated with *p*-cymene, at the doses evaluated, did not have any performance alteration in the Rotarod test. In addition, visually as well, there has been no interference with motor coordination of animals, thus eliminating a nonspecific muscle-relaxing effect of *p*-cymene.

Conclusions

Based on our results, the monoterpene *p*-cymene proved to have antinociceptive activity that does not interfere with motor coordination of animals, and this pharmacological action may modulate both neurogenic and inflammatory pain. Additionally, it seems, at least in part, that the antinociceptive action of *p*-cymene involves the opioid system. These results suggest and support the use of *p*-cymene as a promising substance for the management of orofacial pain; however, further studies are necessary to prove its mechanism of action.

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