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α-Terpineol Reduces Mechanical Hypernociception and Inflammatory Response

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Abstract: α-Terpineol (TPN), a volatile monoterpene alcohol, is relatively non-toxic and one of the major components of the essential oils of various plant species. In this study, we tested for the antihypernociceptive activity of TPN (25, 50 or 100 mg/kg, i.p.) in mice using mechanical models of hypernociception induced by carrageenan (CG, 300 μg/paw) and the involvement of important mediators of its cascade signalling, such as tumour necrosis factor-α (TNF-α, 100 pg/paw), prostaglandin E_2 (PGE_2, 100 ng/paw) or dopamine (DA, 30 μg/paw). We also investigated the anti-inflammatory effect of TPN on the model of carrageenan-induced pleurisy and the LPS-induced nitrite production in murine macrophages. Pre-systemic treatment with TPN (25, 50 or 100 mg/kg, i.p.) inhibited the development of mechanical hypernociception induced by CG or TNF-α. A similar effect was also observed upon PGE_2 and DA administration. In addition, TPN significantly inhibited the neutrophil influx in the pleurisy model. TPN (1, 10 and 100 μg/mL) also significantly reduced ($p < 0.01$) nitrite production *in vitro*. Our results provide information about the antinociceptive and anti-inflammatory properties of TPN on mechanical hypernociception and suggest that this compound might be potentially interesting in the development of new clinically relevant drugs for the management of painful and/or inflammatory disease.

The primary function of pain is to protect the organism from potential tissue-damaging stimuli through the activation of spinal reflex withdrawal mechanisms. However, chronic pain states typically represented as inflammatory or neuropathic are quite different from acute pain conditions where pain persists even after the original injury has healed. Common clinical features that occur in inflammatory and/or chronic pain patients are abnormal painful sensations such as increased pain sensitivity (i.e. hyperalgesia) and pain in response to a non-nociceptive stimulus (i.e. allodynia) [1,2]. Moreover, currently available drugs that provide relief from these painful conditions are effective only in a fraction of the patients. In general, these drugs present low efficacy and numerous side effects [3]. In this context, natural products that present fewer side effects emerge as interesting therapeutic resources for the development of new drugs in the management of certain chronic pain states [2,4].

Monoterpenes are the major chemical constituents of the essential oils of medicinal plants having therapeutic properties [5,6]. Additionally, these compounds have various pharmacological properties such as anxiolytic, antinociceptive [6–8], sedative [9,10], antidepressant and anticonvulsant [8,10].

α-Terpineol (TPN) is a volatile monoterpene alcohol, a major component of the essential oils of various plant species, such as *Ravensara aromatica* ('Ravensara'), *Melaleuca qinquenervia* ('Niaouli'), *Myrtus communis* ('myrtle'), *Laurus nobilis* ('laurel'), *Croton sonderianus* ('Marmeleiro black', in the north-east of Brazil) and *Eucalyptus globulus* ('eucalyptus'), which are widely used in folk medicine and aromatherapy [4,11,12]. TPN is also used in perfumery, in cosmetic industries and in soap and household products (disinfectant sprays) [11]. It has insecticidal, antimicrobial, antispasmodic, anticonvulsant, antinociceptive and immunostimulant properties, and it increases the skin's permeability to soluble compounds [8,12–15].

Some recent studies have shown that monoterpenes demonstrate promising pharmacological activities, such as anti-inflammatory and analgesic properties [2,6,16–18]. But no studies have been found investigating the specific role of TPN in this regard. The present work, for the first time, explores the effects of TPN on the inflammatory hypernociception induced by carrageenan (CG), TNF-α, PGE_2 and dopamine. In addition, we evaluated the effect of TPN on the recruitment of leucocytes and production of TNF-α in carrageenan-induced pleurisy and its action on nitric oxide production *in vitro* by murine macrophages.

Materials and Methods

Drugs and reagents. λ-Carrageenan, tumour necrosis factor-alpha (TNF-α), prostaglandin E_2 (PGE_2), dopamine (DA), ethylenediamine tetraacetic acid (EDTA), lipopolysaccharide (LPS), Griess reagent, Türk solution, Tween-80 and α-terpineol (TPN, 98% purity) were purchased from Sigma (St Louis, Missouri, USA). Enzyme-linked immunosorbent assay (ELISA) for quantitative determination of TNF-α in mouse was purchased from BD-Bioscience Pharmingen (San Diego, CA, USA).

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Sodium nitrite ($NaNO_2$) was obtained from Merck (Rio de Janeiro, Brazil). Indomethacin and dipyrone were obtained from União Química (São Paulo, Brazil). The vehicle used to dissolve the TPN was saline + 0.2% Tween-80. The other substances were solubilized with distilled water or saline.

Animals. Male Swiss mice (28–32 g), 2–3 months of age, were obtained from the animal facility at Federal University of Sergipe (UFS). The animals were randomly housed in appropriate cages at 21 ± 2°C on a 12-hr light/dark cycle (lights on 6:00 a.m. to 6:00 p.m.) with free access to food (Purina®, São Paulo, Brazil) and water. Experiments were carried out between 9:00 a.m. and 2:00 p.m. in a quiet room. Nociceptive and inflammatory tests were carried out by the same visual observer, and all efforts were made to minimize the number of animals used as well as any discomfort. Experimental protocols were approved by the Animal Care and Use Committee (CEPA/UFS, 08/11) at the UFS, and handling procedures were in accordance with the International Association for the Study of Pain guidelines for the use of animals in pain research [19].

Hypernociception induced by CG, TNF-α, PGE_2 and dopamine. Mouse paw hypernociception was performed as previously described by Cunha *et al.* (2004, 2005) [20,21] and Villarreal *et al.*, (2009) [22]. The mice were divided into five groups (n = 6, per group), which were treated with vehicle (saline + Tween-80 0.2% v/v, i.p.), TPN (25, 50 or 100 mg/kg, i.p.), indomethacin (10 mg/kg, i.p.) or dipyrone (60 mg/kg, i.p.). Thirty minutes after treatment, 20 μL of CG (300 μg/paw), PGE_2 (100 ng/paw), DA (30 μg/paw) or TNF-α (100 pg/paw) were injected subcutaneously into the subplantar region of the hind paw. The degree of hypernociception was evaluated at 0.5, 1, 2 and 3 hr after the injection of hypernociceptive agents.

Measurement of mechanical hypernociception. Mechanical hypernociception was tested in mice as reported by Cunha [20]. In a quiet room, the mice were placed in acrylic cages (12 × 10 × 17 cm) with wire grid floors for 15–30 min. before starting the test. This method consisted of evoking a hind paw flexion reflex with a hand-held force transducer (electronic aesthesiometer; Insight®, Ribeirão Preto, São Paulo, Brazil) adapted with a polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The end-point was characterized by the withdrawal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was automatically recorded. The intensity of stimulus was obtained by averaging four measurements taken with minimal intervals of 3 min. The animals were tested before and after treatments.

Carrageenan-induced pleurisy. Pleurisy was induced by intrathoracic injection of carrageenan (300 μg, 0.1 mL) diluted in sterile saline. Control animals received the same volume of vehicle. The animals were pre-treated with TPN (25, 50 and 100 mg/kg, i.p.) or saline 30 min. before the injection of the inflammatory agent. Four hours after stimulation, the animals were killed in a CO_2 chamber, and the pleural cavities were opened and washed with 1 mL of PBS (1×) containing EDTA (10 mM).

Leucocyte evaluation. Total leucocyte counts collected in the pleural lavage were performed on a Neubauer chamber under an optical microscope. The samples were diluted (40×) in Türk solution. The differential leucocyte analysis was performed under a light microscope with immersion oil objective in cytocentrifuged smears coloured with May-Grunwald–Giemsa, where 100 cells per slide were counted.

Generation of nitric oxide by macrophages. A suspension of peritoneal macrophages (5×10^6 cells/100 μL) was incubated in a 96-well microplate with 100 μL of TPN (1, 10 and 100 μg/mL) or culture medium RPMI-1640 for 24 hr at 37°C with 5% CO_2. As a positive control, we used 100 μL of a LPS solution 1 μg/mL. After incubation, aliquots of 100 μL of culture supernatants were mixed with 100 μL of Griess reagent (0.1% sulphanilamide, 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 (μM) of nitrite through the standard curve obtained previously with known molar concentrations of $NaNO_2$ in RPMI-1640 [23,24]. The results were obtained from triplicate cultures.

Statistical analysis. Data were evaluated using GraphPad Prism (San Diego, California, USA) version 3.0, through the analysis of variance (ANOVA) followed by Tukey's test. The results are presented as mean ± SEM. In all cases, the differences were considered significant if $p < 0.05$.

Results

Effect of TPN on CG- or TNF-α-induced mechanical hypernociception in mice.

Injection of CG in the subplantar region of the mouse paw induced a marked hypernociception, characterized by decreased stimulus intensity of about 6–7 g at 30 min. after the injection, which remained throughout the 180 min. of assessment (fig. 1). TPN demonstrated antihypernociception in this model, as the mice treated with TPN (25, 50 or 100 mg/kg, i.p.) 30 min. before CG administration showed a significant reduction in mechanical hypernociception at all times evaluated when compared with control animals that received only the vehicle, except for 60 min. It is possible that the variability in time at 60 min. occurred owing to the increase in basal level of control group. However, TPN showed antihypernociceptive behaviour in the other times evaluated (fig. 1A). The group of animals that received saline in the subplantar region, instead of CG, showed no change in the threshold sensitivity to mechanical stimuli (data not shown).

The inhibitory effect of TPN on the mechanical hypernociception induced by TNF-α is shown in fig. 1. TPN (25, 50, and 100 mg/kg, i.p.) was able to reduce the mechanical hypernociception induced by TNF-α when compared with the animals of the vehicle group (fig. 1B).

Effect of TPN on dopamine- or PGE_2-induced mouse paw mechanical hypernociception.

The TPN antihypernociceptive effect on PGE_2- and dopamine-induced hypernociception is shown in fig. 2. TPN (25, 50 and 100 mg/kg) can reduce the mechanical hypernociception induced by PGE_2 and dopamine when compared with vehicle group animals (fig. 2).

Effect of TPN on CG-induced mouse pleurisy.

At the doses tested, TPN was not able to significantly suppress the recruitment of leucocytes to the pleural cavity of mice, as shown in fig. 3A. However, pre-treatment with TPN induced a significant inhibition in the neutrophils counts but not in the mononuclear cells counts when compared to the control group (fig. 3 B, C).

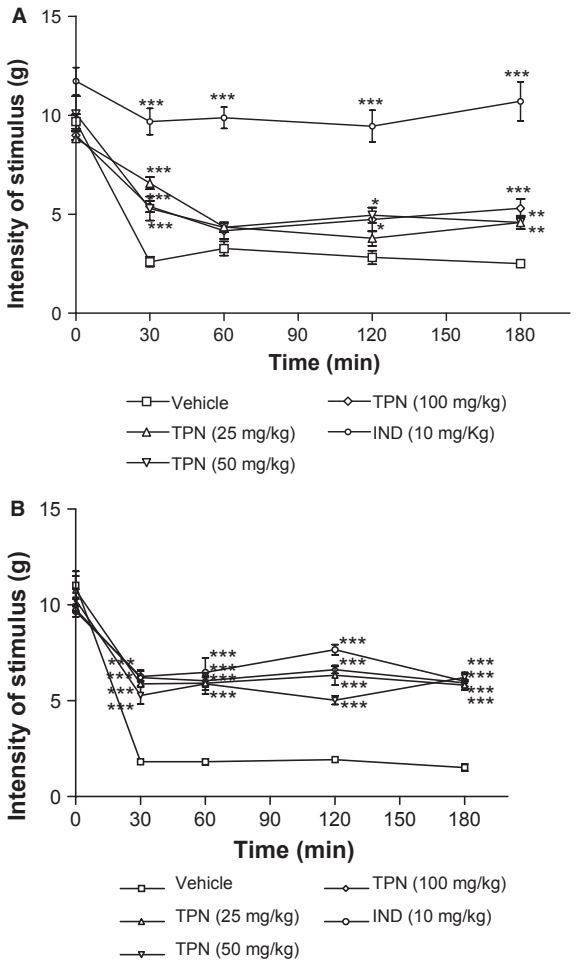

Fig. 1. Effect of acute administration of vehicle (saline + Tween-80 0.2% v/v, i.p.), α-terpineol (TPN; 25, 50 or 100 mg/kg, i.p.) or indomethacin (IND, 10 mg/kg, i.p.) on mechanical hypernociception induced by carrageenan (A) or tumour necrosis factor-α (B). Each point represents the mean ± SEM of the paw withdrawal threshold (in grams) to tactile stimulation of the ipsilateral hind paw. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ *versus* control group (ANOVA followed by Tukey's test).

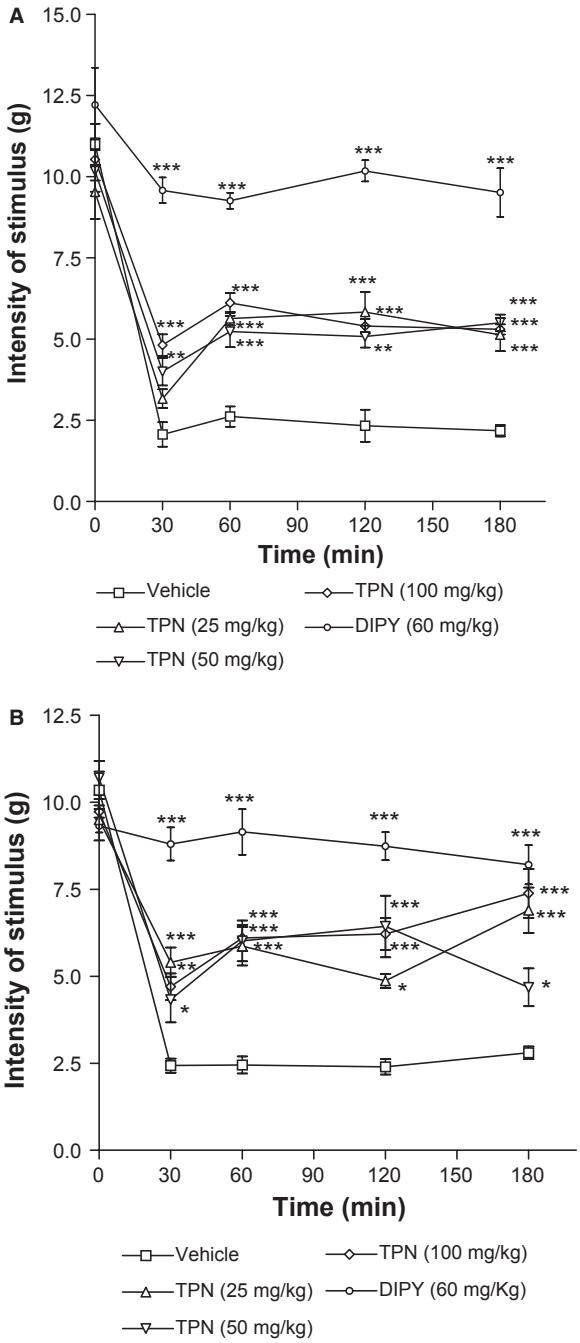

Fig. 2. Effect of acute administration of vehicle (saline + Tween-80 0.2% v/v, i.p.), α-terpineol (TPN: 25, 50 or 100 mg/kg, i.p.) or dipyrone (DIPY, 60 mg/kg, i.p.) on mechanical hypernociception induced by PGE_2 (A) or dopamine (B). Each point represents the mean ± SEM of the paw withdrawal threshold (in grams) to tactile stimulation of the ipsilateral hind paw. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *versus* control group (ANOVA followed by Tukey's test).

Effect of TPN on LPS-induced nitric oxide production by murine macrophages.

The effect of TPN on generation of nitric oxide by macrophages induced by LPS is shown in fig. 4. The incubation of murine macrophages with LPS resulted in a significant ($p < 0.001$) nitrite production by these cells. All concentrations of TPN tested (1, 10 and 100 μg/mL) significantly reduced ($p < 0.001$) the nitrite production.

Discussion

Pain is an important symptom of inflammatory disease. Sensitization of primary afferent nociceptors is a common denominator of all types of inflammatory pain, leading to states of hyperalgesia and/or allodynia, best described as hypernociception in animal models [25,26]. Carrageenan has been used as a tool for evaluating new drugs in rodents. The injection of carrageenan (CG) in mice induces mechanical hypernociception through a cascade of cytokines released by resident or migrating cells initiated by production of bradykinin [27]. This cascade leads to the release of inflammatory mediators such as prostaglandins and sympathetic amines, which cause the activation of fibre sensory nerve endings, types Aδ and C,

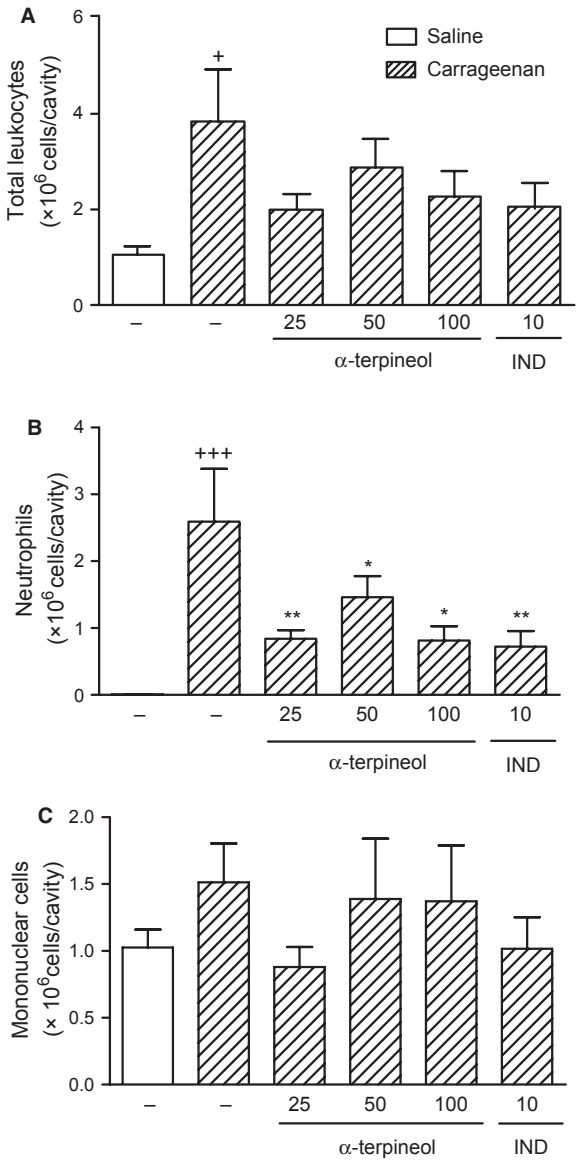

Fig. 3. Effect of α-terpineol (TPN: 25, 50 and 100 mg/kg, i.p.) and indomethacin (INDO, 10 mg/kg, i.p.) on the inflammation induced by carrageenan in mouse pleurisy. The analyses were performed 4 hr after carrageenan (300 μg/cavity) injection to evaluate the recruitment of total leucocytes (A), neutrophils (B) and mononuclear cells (C). Data were expressed as mean ± SEM of at least five animals. $^{+++}p < 0.001$ compared with the saline-injected mice; * $p < 0.05$ and ** $p < 0.01$ compared with the control group (vehicle) (ANOVA followed by Tukey's test).

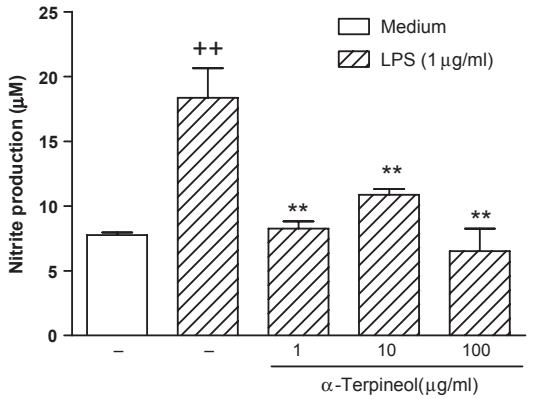

Fig. 4. Effect of α-terpineol on LPS-induced nitrite production by murine macrophages. Cells were maintained in culture medium (RPMI) or pre-incubated with α-terpineol for 24 hr and then treated with LPS (1 μg/mL) for 24 hr. Nitrite levels in the culture supernatants were evaluated, and the results were expressed as concentration (μM) of nitrite in the culture medium. Data were presented as the mean ± SEM of values obtained from triplicate cultures and are representative of three experiments with similar results. $^{++}p < 0.01$ compared with RPMI and ** $p < 0.01$ compared with LPS (ANOVA followed by Tukey's test).

increasing the local flow and vascular permeability by the release of neurokinin substance P and neurokinin A [21,28–31]. Therefore, CG-induced hypernociception causes a response characterized by decreased intensity of the stimulus to elicit a mouse paw withdrawal behaviour (expressed as the change in intensity), already well established in the literature [20,32].

According to Poole [33], inflammatory stimuli do not directly stimulate the release of primary hypernociceptive mediators, but their release is preceded by a cascade of cytokines. CG induces mechanical hypernociception through this cascade of cytokines [27], in which the first cytokine the TNF-α was released that subsequently triggers the release of other cytokines such as IL-1-beta [34,35]. This can lead to a neurogenic inflammation which contributes to the inflammatory process resulting in central and peripheral hyperalgesia.

To evaluate the effect of TPN in mechanical hypernociception induced by carrageenan and TNF-α, the model described by Cunha [21] was used. In this test, mice treated with TPN showed a reduced sensitivity threshold to mechanical stimuli, results also found for the mice that received indomethacin, a cyclooxygenase inhibitor. TPN was able to increase the mechanical threshold of hypernociceptive behaviour, probably by the inhibition of inflammatory mediators.

Our results are in agreement with previous work carried out by our group, which reported on the antinociceptive effect of TPN on models of central and peripheral pain, suggesting antinociceptive properties possibly by inhibiting the release of substance P and other inflammatory molecules such as serotonin, histamine, bradykinin and prostaglandins [13]. Additionally, TPN reduced nociception induced by glutamate injection to the mouse paw, with possible modulation in the transmission of nociceptive signals from the peripheral nervous system to the dorsal horn of the spinal cord. Glutamate evokes a pronounced nociceptive response mediated by neuropeptides released from C fibres, which can stimulate the production of a variety of intracellular second messengers and cytokines, such as NO, TNF-α and IL-1β, which act synergistically in the excitation of neurons [36]. The literature reports that TPN reversibly blocks the conduction of the action potential of the sciatic nerve in rats [37], which would influence the observed antinociceptive effect of TPN on the experimental models of hypernociception used in this study.

Cytokines, such as IL-1β, act in the release of prostanoids such as prostaglandins, while keratinocyte-derived chemokine (KC) acts in the release of sympathomimetic amines such as dopamine [21], triggering the activation of nociceptors and transmission impulse by primary nociceptive neurons. In addition, PGE_2 acts on the EP_2 receptors and dopamine acts on the metabotropic-type D_1 receptors, causing a reduction in the nociceptor threshold and increasing the neuronal membrane excitability [28,38,39]. Some drugs such as dipyrone have an analgesic action by inhibiting the final mediators of hypernociception, such as PGE_2 and dopamine, and consequently produce a direct down-regulation of the nociceptor activation [40].

On the basis of these findings, we investigated the possible effect of TPN on the hypernociception induced by PGE_2 and dopamine. It was observed that TPN was able to maintain the baseline nociceptive threshold. As the hypernociception induced by these mediators is independent of the final production of other inflammatory mediators or recruitment of cells, such as neutrophils [41], we cannot exclude the possibility that TPN interacts with these cells or even with other types of EP or dopamine receptors; consequently, the paths of its antinociceptive activity may be by action not only at the inflammatory level but also from a possible involvement of neuronal pathways.

To better investigate the potential anti-inflammatory effect of TPN, we performed a cell migration assay by carrageenan-induced pleurisy. Inflammation induced by carrageenan involves cell migration, exudation of plasma and production of mediators such as nitric oxide, prostaglandin E_2, interleukin (IL)-1β, IL-6 and TNF-α [42,43]. These mediators are capable of recruiting leucocytes, such as neutrophils, in various experimental models. The literature shows that mediators derived from arachidonic acid metabolism are involved in the mediation of cell migration induced by carrageenan [44]. In addition, other studies have shown that treatment with anti-inflammatory drugs such as dexamethasone, indomethacin and nimesulide are able to inhibit cell migration induced by carrageenan [45,46].

α-Terpineol showed an inhibitory profile on the cell migration of neutrophils by the statistically significant reduction in cell counts in the pleural cavity. One possible explanation for these results may be that TPN inhibits the synthesis of molecules involved in the inflammatory process, such as eicosanoids [13], probably by suppressing NF-κB signalling as demonstrated by Hassan [47].

Nitric oxide (NO) acts in the development and maintenance of hypernociception triggered by the injection of carrageenan, and NO mediates the inflammatory process, established in the literature [48,49]. Lipopolysaccharide (LPS) interacts with the liver macrophages by the LPS-binding protein (LBP). The LPS –LBP complex binds to receptors composed of type CD-14 and/or TLR4 (receptor 'Toll-like' receptor 4) [50–52], which activate the transcription factor NF-kB, inducing iNOS transcription and protein synthesis with a corresponding increase in NO production. Therefore, the expression of iNOS catalyses the formation of large amounts of NO, which plays a key role in the pathogenesis of a variety of inflammatory diseases. Therefore, the level of NO induced by iNOS may reflect the degree of inflammation and provides an indicator of inflammatory processes [53].

Our results show that TPN has an inhibitory activity on nitric oxide (NO) production in macrophages stimulated by LPS *in vitro*, probably by mechanisms dependent on the inhibition of the NF-kappaB, based on the findings of Hassan [47].

In summary, the data from this study support the hypothesis that α-terpineol has important anti-inflammatory and antihypernociceptive properties. These effects seem to be associated with the power of TPN to inhibit the cytokine cascade generated by carrageenan and/or decrease the production of inflammatory mediators, as well as inhibit NO release. Thus, α-terpineol can be an interesting candidate for the development of new drugs for treating painful conditions associated with inflammation.

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