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Research Paper

Antinociceptive activity of the monoterpene α-phellandrene in rodents: possible mechanisms of action

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Abstract

Objectives The aim of this work was to investigate the antinociceptive property of α-phellandrene (α-PHE) in experimental nociception models and possible mechanisms involved.

Methods Mass spectrometry was used to evaluate the purity and molecular mass of α-PHE. Macrophages from mice peritoneal cavity were used in an MTT test. Rodents were used in tests of chemical and mechanical nociception. In the study of the mechanisms, the animals were treated with pharmacological tools and then submitted to the glutamate test.

Key findings α-PHE purity was 98.2% and molecular mass 136.1 Da. α-PHE did not show cytotoxicity. In the writhing and capsaicin tests, α-PHE promoted the antinociceptive effect in all evaluated doses (minimum dose 3.125 mg/kg). In the formalin test, α-PHE (50 mg/kg) was effective in inhibiting both phases. In the glutamate test, the monoterpene (12.5 mg/kg) decreased the nociceptive response. In carrageenan-induced hyperalgesia, α-PHE (50 mg/kg) decreased the hypernociception index. In the study of the mechanisms involved, pretreatment with naloxone reversed the α-PHE antinociceptive effect, the same occurred with glibenclamide, L-arginine, atropine and yohimbine. α-PHE did not show muscle relaxant activity or central depressant effects in open field and rota rod tests.

Conclusions α-PHE has an antinociceptive effect and it possibly involves the glutamatergic, opioid, nitrergic, cholinergic and adrenergic systems.

Introduction

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.[1] Pain is subjective and its perception depends on the individual's psychological state.[2] Hence, anxiety[3] and depression[4] can elevate its perception. It has adverse effects on well-being and quality of life, resulting in reduced physical and emotional functions.[5]

The management of pain requires an effective analgesic drug associated with few side effects, and appropriate therapy should offer a combination of efficacy and safety for each individual patient. There are several therapeutic approaches to treating pain and the currently available and most widely prescribed are non-steroidal anti-inflammatory drugs (NSAIDs), opioids and synthetic drugs with narcotic properties that target different components of the peripheral and central nervous system.[6] These drugs have important side effects (NSAIDs, gastric disorders; opioids, tolerance and dependence) that limit their use, especially in chronic pain.[7]

The research and development of analgesic drugs are very important and natural products seem to be a good source of biomolecules, especially the secondary metabolites from medicinal plants. Among these metabolites essential oils are an important class that presents several pharmacological properties, among these, analgesic, anti-inflammatory, bactericidal, virucidal, fungicidal, antiparasitical, anti-malarial, anti-cancer, antioxidant, anticonvulsant and antifungal activity.[8–10]

These compounds are extracted by steam distillation of plants or cold pressing of citrus fruit pericarp and characterized as a complex liquid chemical mixture, that has common

physical-chemical properties (low molecular weight, volatile at room temperature and lipophilic). Because of the extraction mode, they contain a variety of molecules such as terpenes, terpenoids, phenol-derived aromatic components and aliphatic components. Essential oils are used in the pharmaceutical, sanitary, cosmetic (personal care), agricultural and food industries.[11]

The terpenes (monoterpenes and sesquiterpenes) are a large and varied class of hydrocarbons derived biosynthetically from units of isoprene. They represent one of the most diverse classes of secondary metabolites from plants. Over 30 000 compounds have been identified, from fragrances and antibiotics to insect repellents.[12]

Among many other essential oil constituents, we found the monoterpene alpha-phellandrene (α-PHE) (5-isopropyl-2-methyl-1,3-cyclohexadiene), present in varied concentrations, among these specimens: *Lippia affinis sidoides* Cham.,[13] *Curcuma zedoaria* Christm. (14.93%),[14] *Foeniculum vulgare* Mill.,[15] *Xylopia aromatica* L. (2.2–6.4%),[16] *Rosmarinus officinalis* L. (0.1–0.4%),[17] *Eucalyptus dives* Schauer (17.4%), *Eucalyptus staigeriana* F.v. Muell. (8.8%),[18] *Pinus taeda* L., *Pinus virginiana* Mill.[19] and *Citrus limon* L.[20]

α-PHE is also part of the essential oil chemical constituents of several plant species that present analgesic and anti-inflammatory actions, including *Matricaria chamomilla* L.[21] and *Zingiber officinale* Roscoe.[22] So, it is very important to study this compound and its contribution to its pharmacological profile, since specimens rich in this monoterpene have analgesic and anti-inflammatory effects. The aims of this work were to investigate the cytotoxicity and, for the first time, the potential as an analgesic drug of α-PHE in experimental nociception models in rodents, as well as some possible mechanisms involved.

Materials and Methods

The monoterpene alpha-phellandrene

α-Phellandrene (Figure 1) can occur in two enantiomeric forms, (−)-α-phellandrene and (+)-α-phellandrene. The α-PHE used in this study was obtained commercially from Sigma-Aldrich (St. Louis, MO, USA), being a mixture enriched by 80% of (−)-α-phellandrene (manufacturer data).

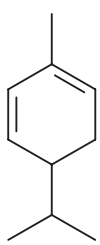

Figure 1 Structure of the monoterpene α-phellandrene.

Mass spectrometry was used to evaluate the purity and exact molecular mass of α-phellandrene Amazon SL equipment model from BrukerDaltonics (Bremen, Germany). The sample was diluted using water–acetonitrile–formic acid (4 : 100 : 1). Mass spectra were acquired in a mass range of m/z 70–160 Da. MS/MS was carried out in manual mode with fragmentation of the precursor ion by CID (collision-induced dissociation) using He as the collision gas. Precursor ions were selected within an isolation width of 2 u and scans were accumulated with variable RF signal amplitudes. The m/z scale of the mass spectrum was calibrated using the external calibration standard G2421A electrospray '*tuning mix*' from Agilent Technologies (Santa Rosa, USA).

Animals

The acute pain tests were carried out on male Swiss mice ($n = 6$–9), 20–30 g, and male Wistar rats ($n = 6$–9), 180–200 g, reared at the Medicinal Plants Research Center of the Federal University of Piauí. The animals were housed at 24 ± 2°C under a 12-h light–dark cycle and with free access to food and water. Animals were acclimatized to the laboratory for at least 1 h before testing and were used only once throughout the experiments. The protocols were performed after their approval by the Institutional Ethics Committee (Animal Ethics Committee/UFPI, n°. 090/2010, 12/17/2010) and were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigation of experimental pain in conscious animals.[23]

Assessment of cytotoxicity

To evaluate the possible mammalian cell cytotoxicity the MTT (3-[4,5-dimethylthiazol-2-yl]−2,5-diphenyltetrazolium bromide) test was performed. Macrophages obtained from peritoneal cavity of Swiss mice were used. Macrophages were removed by administering 8.0 ml of sterile phosphate-buffered saline (PBS) pH 7.4, at 4°C to the abdominal cavity. Macrophages were then added to sterile cell culture plates, at a concentration of 1×10^5 cells per well in RPMI 1640 medium (Sigma, St Louis, USA). α-PHE at concentrations of 400, 200, 100, 50, 25, 12.5, 6.25 and 3.12 μl/ml were used in this test.[24]

Antinociceptive tests

Acetic acid-induced abdominal writhing

The procedure was similar to a previously described method.[25] Swiss mice ($n = 6$–9) were pretreated with saline vehicle (0.1 ml/10 g) and α-PHE (3.125, 6.25 and 12.5 mg/kg, p.o.) 60 min before the intraperitoneal administration of 0.75% acetic acid, then, the total number of writhings was counted over a period of 20 min. The strength of the elicited

antinociceptive effect was compared with that of an effective dose of morphine (5 mg/kg, s.c.) administered 30 min before the acetic acid injection.

Formalin test

Mice were orally given α-PHE (25 and 50 mg/kg) or saline vehicle (10 ml/kg) 1 h before the test. Morphine (5 mg/kg) was administered subcutaneously 30 min before the test and used as a positive control. The right hind paw was injected with formalin (20 µl, 2%) in the intraplantar region. Nociception was evaluated by quantifying paw licking time during the first 5 min (first phase) and at 15–30 min (second phase).[26,27]

Capsaicin test

Mice were given α-PHE (3.125, 6.25 and 12.5 mg/ kg p.o.). Control animals received saline orally or morphine (5 mg/ kg). Morphine was administered subcutaneously 30 min before the test and used as positive control. One hour after these treatments, the right hind paw was injected with capsaicin (2 µg/paw) prepared in 5% Tween solution and 2% ethanol. Nociception was assessed immediately after injection and quantified by paw licking time during a 5-min period.[28,29]

Glutamate test

The procedure used was similar to the one previously described.[30] Mice received an intraplantar injection of glutamate (20 µmol/paw) 60 min after administration of saline vehicle (0.1 ml/10 g) or α-PHE (6.25, 12.5 and 25 mg/kg p.o.) and 30 min after the administration of MK 801 (0.03 mg/kg, i.p.) as a positive control. Animals were observed individually for 15 min following glutamate injection. The amount of time spent licking the injected paw was taken to indicate nociception. Trying to elucidate the mechanism by which α-PHE induces antinociception, mice were pre-treated intraperitoneally with naloxone (2 mg/kg), glibenclamide (3 mg/kg), L-arginine (600 mg/kg) or yohimbine (0.15 mg/kg), or subcutaneously with atropine (0.1 mg/kg). The doses of antagonists were selected based on previous results from our laboratory.[31]

Mechanical hypernociception

Mechanical hypernociception was evaluated in rats as previously reported.[32] In a quiet room, rats were placed in acrylic cages (12 × 20 × 17 cm) with a grid floor, 15–30 min before the beginning of the test. The test consisted of evoking a hind paw flexion reflex with a hand-held force transducer adapted with a 0.7 mm^2 polypropylene tip (AVS Projects). The investigator was trained to apply the tip to the hind foot pad with gradual increase in pressure. The stimulus was automatically discontinued and its intensity recorded when the paw was withdrawn. The rats were tested before and after treatments. The results are expressed by Δ withdrawal threshold (in grams, g), which was calculated by subtracting the average of the last three measurements after the treatments from the average of three measurements before treatments.

Carrageenan-induced inflammatory hypernociception

The inflammatory hypernociception was evaluated by Digital Von Frey test. Wistar male rats were orally given α-PHE (25 and 50 mg/kg), vehicle or indometacin (10 mg/kg), 1 h before carrageenan 1% (100 µg/0.1 ml) injection in the right hind paw. The response was evaluated at different times: basal and 1, 2, 3, 4, 5, 6 and 24 h after the stimulus. The *Δ* withdrawal threshold was calculated at each time.[33]

Measurement of motor performance and locomotor activity

The open field test involved an acrylic box with transparent walls and black floor (30 cm × 30 cm × 15 cm), and its base was divided into nine quadrants of equal area. One day before the experiment, mice were placed in the arena for adaptation. The mice were treated with α-PHE (50 mg/kg, p.o.), vehicle (10 ml/kg, p.o.) or diazepam (4 mg/kg, i.p.) 0.5 and 1 h before being taken individually to the open field and the number of squares crossed with all paws (crossings) was counted in a 5 min session. The Rota Rod (Model RR – 2002, Insight equipment) consisted of a 2.5 cm-diameter bar, subdivided into four compartments by 25 cm-diameter disks, rotating at 14 revolutions per minute. The mice were selected 24 h previously by eliminating those mice that did not remain on the bar for three consecutive periods of 60 s. Mice were treated with α-PHE (50 mg/kg, p.o.), vehicle (10 ml/kg, p.o.) or diazepam (4 mg/kg, i.p.) 0.5 and 1 h beforehand. Results are expressed as the time (s) that mice remained on the rota rod. Cut-off time used was 60 s.[34,35]

Statistical analysis

The results were expressed as the mean ± SEM and analysed by one-way analysis of variance followed by post-hoc Bonferroni test. Differences between groups were considered significant when $P < 0.05$ (GraphPad Prism software 4.0).

Results

Mass spectrometry

As shown in Figure 2, the exact molecular mass of the monoterpene was 136.1 Da. The sample purity was 98.2% calculated from MS analyses.

Cytotoxicity (MTT test)

The α-PHE in all concentrations tested did not show cytotoxicity in the MTT test when compared with control group. The

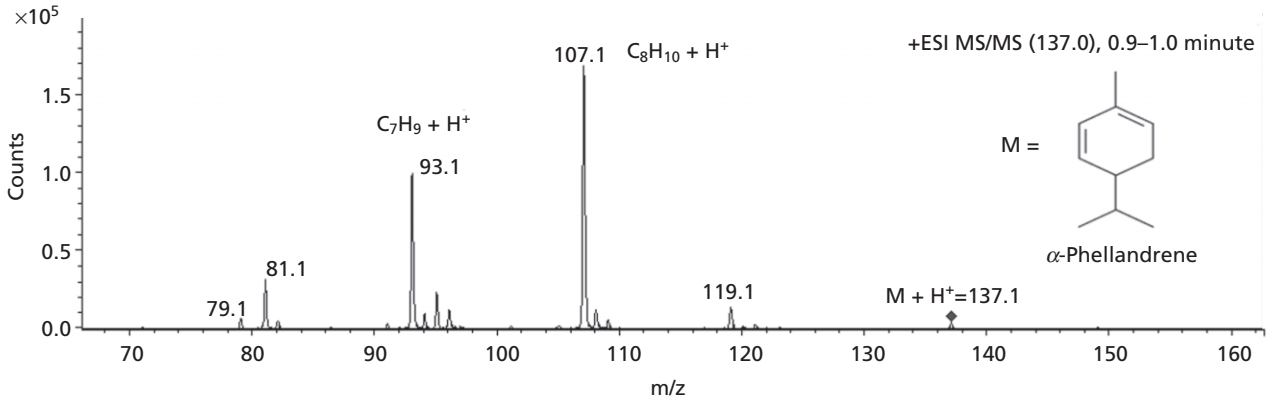

Figure 2 ESI+ MS/MS spectra of α-phellandrene (2-methyl-5-(1-methylethyl)-1,3-cyclohexadiene) showing the fragmentation from [M + H⁺] = 137.01. [M] represents the structure and MS/MS the fragment ion 93.1 and 107.1 Dalton are corresponding typical ion for the molecule.

Table 1 Antinociceptive effect of the monoterpene α-phellandrene on acetic acid-induced writhing and capsaicin-induced nociception in mice

		Acetic acid-induced writhing		Capsaicin test	
Treatment	Dose (mg/kg)	Number of writhings	Inhibition (%)	Licking time (s)	Inhibition (%)
Vehicle	–	71.88 ± 3.46	–	51.23 ± 4.02	–
Morphine	5	6.17 ± 0.91***	91.42	5.30 ± 0.40***	89.65
α-Phellandrene	3.125	42.83 ± 3.19***	40.41	22.86 ± 2.67***	55.38
	6.25	35.83 ± 4.34***	50.15	26.16 ± 2.89***	48.94
	12.5	40.00 ± 2.73***	44.35	24.51 ± 2.86***	52.16

Mice were treated with α-phellandrene (α-PHE) 60 min (p.o.) before writhing and capsaicin test. Data represent the mean ± SEM of 6–9 mice. ***$P < 0.001$ compared with vehicle.

cell viability calculated in percentage (%) in relation to the control group (100%), obtained for each concentration, was 105.8 ± 1.37% (3.12 µl/ml), 105.3 ± 0.77% (6.25 µl/ml), 115.5 ± 0.28% (12.5 µl/ml), 112.9 ± 3.14% (25 µl/ml), 109.0 ± 5.62% (50 µl/ml), 108.1 ± 2.54% (100 µl/ml), 104.2 ± 1.55% (200 µl/ml) and 101.0 ± 3.83% (400 µl/ml).

Acetic acid-induced abdominal writhing

Injection of acetic acid into mice develops writhing after 20 min. After oral administration of α-PHE at doses of 3.125, 6.25 and 12.5 mg/kg a significant inhibition of writhing was observed at all doses compared with vehicle, indicating an antinociceptive effect (Table 1).

Formalin test

Injection of formalin develops a biphasic licking response on the injected paw of mice. The first phase (neurogenic phase) occurs 5 min after injection and the second phase (inflammatory phase) occurs between 15 and 30 min after formalin injection. As shown in Table 2, α-PHE at 50 mg/kg (p.o.) significantly reduced the time the mouse licked its stimulated paw in both testing phases when compared with vehicle, while the dose of 25 mg/kg p.o. did not reduce the antinociceptive effect in the first or second phases.

Capsaicin test

The effect of α-PHE against capsaicin-induced nociception in mice is shown in Table 1. A significant reduction in time length spent on licking the paw was observed in mice administered with α-PHE in all doses tested (3.125, 6.25, 12.5 mg/kg), compared with vehicle, indicating that α-PHE had an antinociceptive effect in neurogenic pain.

Glutamate test

The result of this test indicates that α-PHE produced a dose-dependent reduction of the licking and biting behaviour in glutamate-induced nociception in mice as shown in Figure 3. The doses of 12.5 and 25 mg/kg (p.o.) showed an antinociceptive effect, relative to vehicle, which corresponds to inhibition of 33.60% and 57.94%, respectively.

Carrageenan-induced inflammatory hypernociception

α-PHE 50 mg/kg significantly reduced (Figure 4) the Δ withdrawal threshold (g) in the Digital Von Frey test at times of 1 h

Table 2 Antinociceptive effect of the monoterpene α-phellandrene in the formalin-induced nociceptive response in mice

		Licking time (s)			
Treatment	Dosage (mg/kg)	0–5 min	Inhibition (%)	15–30 min	Inhibition (%)
Vehicle	–	85.32 ± 6.02	–	109.21 ± 10.48	–
α-PHE	25	102.55 ± 9.37	–20.19	98.90 ± 8.04	9.44
	50	51.90 ± 10.25*	39.17	27.81 ± 7.29***	74.53
Morphine	5	13.85 ± 4.13***	83.77	8.81 ± 3.33***	91.93

Mice were treated with α-phellandrene (α-PHE) 60 min (p.o.) before formalin test. Data represent the mean ± SEM of 6–9 mice. *$P < 0.05$; ***$P < 0.001$ compared with vehicle.

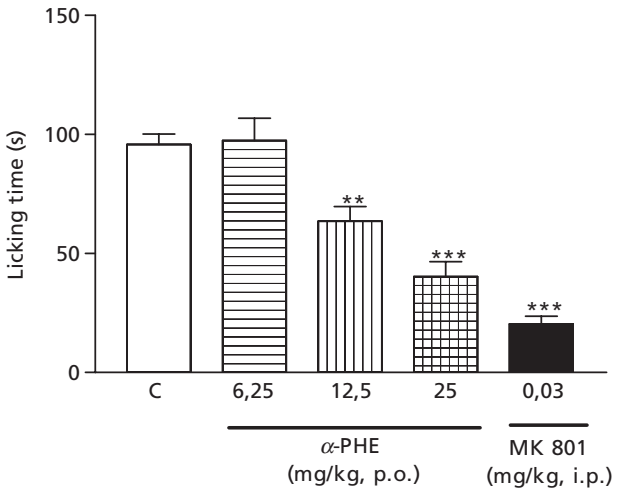

Figure 3 Effect of the monoterpene α-PHE (6.25–25 mg/kg, p.o.) against glutamate-induced nociception in mice. Each column represents the mean ± SEM of 6–8 mice. C, control mice treated with vehicle. **$P < 0.01$; ***$P < 0.001$ compared with control groups (one-way analysis of variance followed by Bonferroni's test).

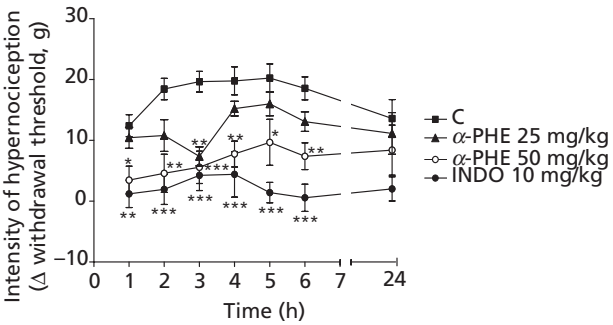

Figure 4 Effect of the monoterpene α-PHE (25–50 mg/kg) on hypernociception induced by carrageenan in rats. Each time represents the mean of the intensity of hypernociception (g) ± SEM of 6–8 rats. C, control rats treated with vehicle. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with control groups (one-way analysis of variance followed by Bonferroni's test).

($\Delta = 3.47 \pm 2.28$, $P < 0.05$), 2 h ($\Delta = 4.58 \pm 3.14$, $P < 0.01$), 3 h ($\Delta = 5.57 \pm 2.66$, $P < 0.001$), 4 h (7.75 ± 2.13, $P < 0.01$), 5 h (9.67 ± 3.78, $P < 0.05$) and 6 h (7.38 ± 2.18, $P < 0.01$), after carrageenan administration (inflammatory hypernociception inductor agent), when compared with vehicle 1 h ($\Delta = 12.40 \pm 1.80$), 2 h ($\Delta = 18.43 \pm 1.75$), 3 h ($\Delta = 19.63 \pm 1.69$), 4 h ($\Delta = 19.77 \pm 2.30$), 5 h ($\Delta = 20.23 \pm 2.32$) and 6 h ($\Delta = 18.54 \pm 1.88$). The α-PHE (25 mg/kg) significantly reduced only the 3 h value ($\Delta = 7.35 \pm 1.58$, $P < 0.01$), when compared with vehicle. All the groups tested did not show significant reduction when compared with vehicle 24 h after the stimulus ($P > 0.05$).

Evaluation of the mechanism of action

To assess the possible participation of the opioid, nitric oxide (NO), cholinergic and adrenergic systems in the antinociceptive effect of α-PHE, mice were pre-treated with naloxone (2 mg/kg i.p.), glibenclamide (3 mg/kg i.p.), L-arginine (600 mg/kg i.p.), atropine (0.1 mg/ kg s.c.) or yohimbine (0.15 mg/kg i.p.), 30 min before α-PHE (12.5 mg/kg). As shown in Figure 5a, naloxone (opioid antagonist) significantly reversed the antinociceptive effect of α-PHE, indicating the possible participation of opioid system. Glibenclamide (blocker of K^+_{ATP} channels) (Figure 5b), L-arginine (substrate of NO formation) (Figure 5c), atropine (muscarinic antagonist) (Figure 6a) and yohimbine (adrenergic antagonist) (Figure 6b) all reversed the antinociceptive effect of α-PHE, indicating the possible participation of K^+_{ATP} channels, NO, cholinergic and adrenergic systems, respectively.

Measurement of motor performance and locomotor activity

To evaluate any non-specific muscle-relaxant or sedative effects of α-PHE, mice were submitted to the open-field and rota-rod test. In the test of open field, the α-PHE at dose of 50 mg/kg (38.57 ± 6.13) did not alter the frequency of movement of animals when compared with vehicle (32.33 ± 3.73). Treatment with α-PHE (50 mg/kg, p.o.) did not alter the length of time the animals stayed in the bar in Rota Rod test for a period of 1 min (59.33 ± 0.37) compared with vehicle (59.88 ± 0.12).

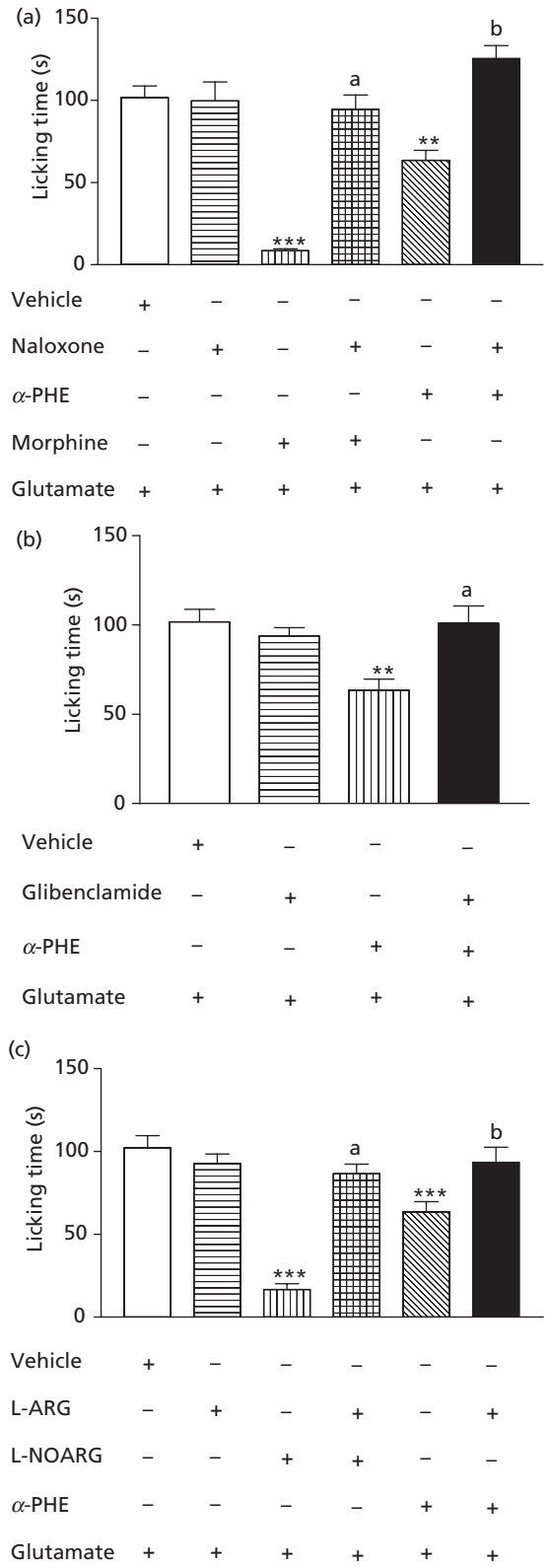

Figure 5 Effect of the monoterpene α-PHE (12.5 mg/kg, p.o.) against the action of naloxone (2 mg/kg, i.p.) (a), glibenclamide (3 mg/kg, i.p.) (b), L-arginine (600 mg/kg, i.p.) (c) and vehicle on glutamate-induced nociception (20 µl, 20 µmol/paw) in mice. Data represent mean ± SEM of 6–9 mice. The symbols indicate the level of significance: $^{**}P < 0.01$; $^{***}P < 0.001$ compared with vehicle, $^{a}P < 0.001$ compared with the morphine group, $^{b}P < 0.001$ or $P < 0.01$ compared with α-PHE group; (Figure 5b, $^{**}P < 0.01$ compared with vehicle, $^{c}p < 0.01$ compared with α-PHE group; Figure 5c, $^{***}P < 0.001$ compared with vehicle, $^{d}P < 0.001$ compared with L-nitroarginine (L-NOARG), $^{e}P < 0.01$ compared with α-PHE group; + treatment present; – missing treatment (one-way analysis of variance, Bonferroni's test).

Discussion

The results of this study demonstrate, for the first time, that α-phellandrene, a terpenoid present in many essential oils, produces an antinociceptive effect in inflammatory and acute nociception models. It is effective following oral administration at very low doses.

In pharmacological tests the drug purity is extremely important to its effect, so to confirm the purity and exact molecular mass, mass spectrometry was performed. It was the first time this monoterpene has been analysed directly by mass spectrometry ion trap ESI+ without passing through gas chromatography (GC) coupled with Free Induction Decay (FID)/ion trap mode. This analysis shows a considerable purity of the compound (98.2%).

The α-PHE did not show any cytotoxicity against mammalian cells (macrophages from mouse peritoneal cavity) in the MTT test; this finding corroborated literature reports in regards to the low oral toxicity in rats (Lethal Dose (LD50) = 5700 mg/kg).[36]

α-PHE was able to reduce the acetic acid-induced writhing reaction in all doses tested. This response has been largely used as screening tool for assessment of analgesic and anti-inflammatory properties of new agents as well as a typical model for visceral inflammatory pain.[37] Local irritation provoked by a test agent in the intraperitoneal cavity triggers a variety of mediators, such as bradykinin, substance P and prostaglandins (PG), especially PGI_2, PGE_2 and PGF_2, as well as some cytokines such as interleukin (IL)-1β, tumour necrosis factor (TNF)-α and IL-8.[38–40] The antinociceptive effect of α-PHE could be related to synthesis and/or release inhibition of mediators that promote nociception in the nervous terminations, similarly to NSAIDs, suggesting a peripheral analgesic action. This method shows good sensitivity to central and peripheral analgesic drugs, but poor specificity because the abdominal writhing response may be suppressed by muscle relaxants and other drugs, leaving scope for the misinterpretation of results.[41]

The α-PHE was effective in inhibiting the first and second phases of the formalin test. This test is believed to resemble

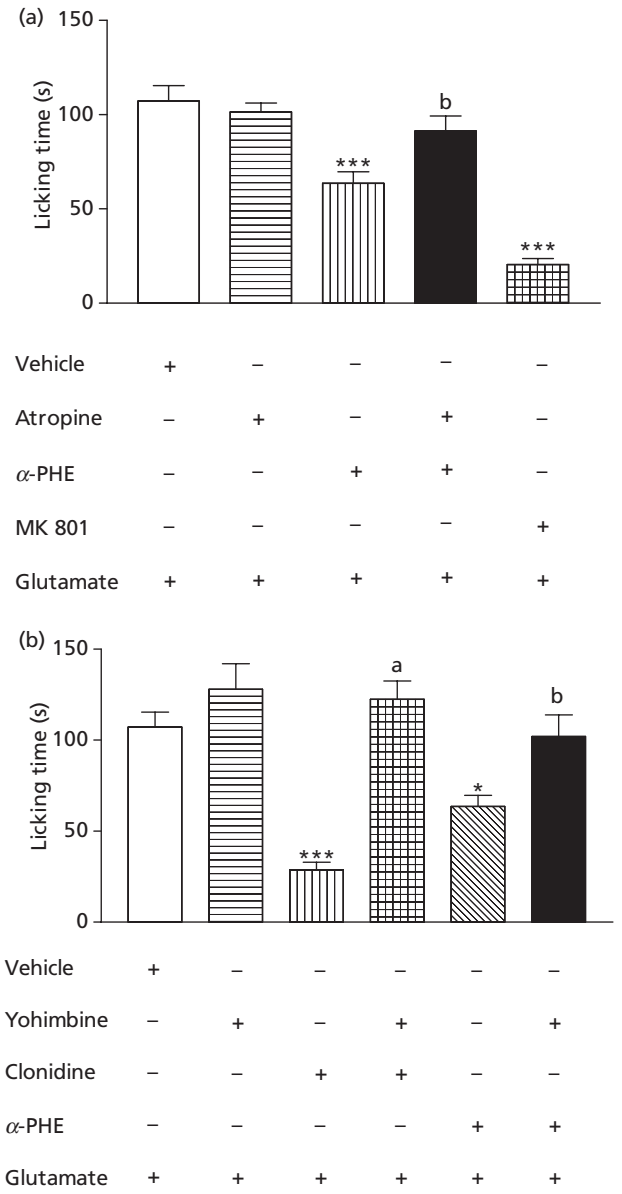

Figure 6 Effect of the monoterpene α-PHE (12.5 mg/kg, p.o.) against the action of atropine (0.1 mg/kg s.c) (a), yohimbine (0.15 mg/kg i.p.) (b) and vehicle on glutamate-induced nociception (20 µl, 20 µmol/paw) in mice. Data represent mean ± SEM of 6–9 mice. The symbols indicate the level of significance (*$P < 0.05$; ***$P < 0.001$ compared with vehicle (c), $^{a}P < 0.001$ compared with the clonidine group, $^{b}P < 0.05$ compared with group α-PHE; + treatment present; – missing treatment)(one-way analysis of variance, Bonferroni's test).

clinical pain more closely compared with others that employ mechanical or thermal stimuli.[42] The formalin test is a model of nociceptive response in two distinct phases involving different mechanisms. The first phase (neurogenic pain) results from the direct chemical stimuli of myelinated and unmyelinated nociceptive afferent fibres, mainly C fibres, which can be suppressed by opioid analgesic drugs like morphine.[43,44] The second phase results from the action of inflammatory mediators in peripheral tissues, such as prostaglandins, serotonin, histamine and bradykinin, and from functional changes in the neurons of the spinal dorsal horn that, in the long term, promote facilitation of synaptic transmission at the spinal level.[45,46] The dose levels required to produce the suppression had no interference in the patterns of performance or motor activity on open field and rota rod tests.

Putting together the ability of α-PHE to produce antinociceptive effect in the acetic acid-induced writhing and the formalin test, these findings point to a likely peripheral analgesic action of α-PHE. In line with these results, the effect of the α-PHE on the neurogenic phase of the formalin-evoked response was supported by the data obtained in the capsaicin test. Capsaicin evokes nociception by activating the vanilloid receptor, also known as TRPV1, and mediates the release of several neuropeptides, excitatory amino acids (glutamate and aspartate), NO and pro-inflammatory mediators from the peripheral and central terminals of primary sensory neurons that can critically contribute to nociceptive processing.[47]

Another interesting finding of this study is that α-PHE produced a dose-dependent inhibition of nociceptive response caused by intraplantar injection of glutamate into the mouse hind paw. The glutamate-induced response appears to involve peripheral, spinal and supra-spinal sites of action and is greatly mediated by both activation of *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (non-NMDA) receptors, as well as by NO release or by some NO-derived substances, because the elevation of intracellular calcium concentration activates neuronal NO synthase.[48,49] The release of NO eventually increased the synthesis/release of pro-inflammatory mediators such as cytokine, reactive oxygen species as well as prostanoids that result in enhancing the inflammatory reaction.[30] The present study strongly suggests that, at least in part, the antinociceptive activity induced by α-PHE in glutamate test could be due to its interaction with the glutamatergic system or its ability to inhibit NO production. The reversion of the α-PHE antinociceptive effect by the pretreatment with L-arginine (substrate for NO formation) corroborated this fact.

In the present study, we also demonstrate an anti-hypernociceptive effect of α-PHE in the carrageenan-induced hypernociception. Carrageenan activates a sequential cytokine cascade in rats, that begins with TNF-α which stimulates two distinct pathways; IL-1β which in turn activates cyclooxygenase to produce prostanoids and the release of sympathetic amines.[50] These substances are ultimately responsible for nociceptor sensitization.[51] This result confirms the findings in formalin test (Second phase/ inflammatory pain) and acetic acid-induced writhing (prostanoids production).

In an attempt to elucidate the possible antinociceptive mechanism of α-PHE, animal were pre-treated with several drugs that interfere with different systems, as naloxone, glibenclamide, L-arginine, atropine or yohimbine on glutamate-induced nociception in mice.

The mechanism of action for α-PHE seems to be, at least in part, from a direct action on the opioid receptors, since pre-treatment of animals with the nonselective opioid antagonist naloxone reverted the antinociceptive activity. The α-PHE antinociceptive effect was also antagonised by pre-treatment with glibenclamide (a blocker of K^+_{ATP} channels), suggesting that opioid system via K^+_{ATP} channels is likely to be involved in α-PHE antinociception.

As previously commented on the glutamate test our result demonstrates the participation of the NO pathway in the antinociceptive effect resulting from α-PHE treatment. This result is also in agreement with studies suggesting that NO plays an important role as nociceptive mediator.[52]

Our results also indicate the participation of cholinergic receptors in this process, since atropine (nonselective muscarinic antagonist) inhibited the antinociceptive effect of α-PHE. In fact, other evidence demonstrates that the cholinergic system has a therapeutic potential against some clinical pain states. Painful stimuli are known to increase acetylcholine in the spinal cord, which in turn results in the increased release of inhibitory transmitters and decreased release of excitatory transmitters, and this, in part, mediates its antinociceptive effect.[53]

Besides the aforementioned mechanisms, the antinociceptive action of α-PHE also involves the sympathetic system, since yohimbine (α-2 receptor antagonist) reversed the antinociceptive effect of the monoterpene. In fact, several reports describe that through the stimulation of their receptors on the dorsal horn, α2-receptor agonists produce an antinociceptive effect, through the pre-synaptic connection (Aδ and C nociceptors), decreasing the release of neurotransmitters, and act post-synaptically evoking hyperpolarization of neurons in the spinal cord.[54]

Although the present data do not provide a clear-cut mechanism, the observed antinociception can justify the wide usage of several essential oils that contain α-PHE for the clinical treatment of local pain conditions. On the other hand, this monoterpene seems to be a promising molecule as an analgesic drug, taking into consideration that an ideal analgesic should offer a combination of efficacy and safety.

Conclusions

The results of this study provided, for the first time, convincing evidence that oral administration of the monoterpene α-PHE exerted pronounced antinociception when assessed in chemical-induced nociception models and mechanical hypernociception in rodents. Some of the possible mechanisms of action involve the opioid system via K^+_{ATP} channels, the glutamatergic system via the L-arginine/NO pathway, and an interaction of the monoterpene with the cholinergic (muscarinic receptors) and adrenergic (α-2 receptors) systems.

The α-PHE did not show any cytotoxicity against mammalian cells or interference in the patterns of motor activity in open field and rota rod tests.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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