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Inhalation of *Cedrus atlantica* essential oil alleviates pain behavior through activation of descending pain modulation pathways in a mouse model of postoperative pain

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ABSTRACT

Ethnopharmacological relevance: *Cedrus atlantica* essential oil (*Ca*EO) presents analgesic and anti-inflammatory sedative properties. However, it remains unknown whether *Ca*EO alleviates acute postoperative pain.

Materials and methods: Here, we investigated the effect of *Ca*EO on postoperative pain and its mechanisms related to the descending pain control in *Swiss* males mice induced by a plantar incision surgery (PIS) in the hindpaw.

Results: Inhalation of *Ca*EO (5′, 30′ or 60′) markedly reduced mechanical hypersensitivity. This effect was prevented by pre-treatment with naloxone or p-chlorophenylalanine methyl ester (PCPA, 100 mg/kg, i.p.)-induced depletion of serotonin. In addition, *p*-alpha-methyl-para-tyrosin (AMPT, 100 mg/kg, i.p.)-induced depletion of norepinephrine, intraperitoneal injection of the α_2-adrenergic receptor antagonist yohimbine (0.15 mg/kg, i.p.) or haloperidol (1 mg/kg, i.p.) an antagonist of dopaminergic (D1 and D2) receptors prevented the effect of *Ca*EO on hypersensitivity.

Conclusions: These findings suggest that *Ca*EO alleviates postoperative pain by activating the descending pain modulation pathways on the opioidergic, serotonergic, noradrenergic (α_2-adrenergic) and dopaminergic (dopamine D1 and D2 receptors) systems.

1. Introduction

Pain is an important parameter in patient recovery and satisfaction after surgery. For many patients, pain due to surgical incision can be severe despite treatment with conventional use of opioids, non-steroidal anti-inflammatory drugs (NSAIDs) and local anesthetics (Apfelbaum et al., 2003). Pain can be modulated by several areas distributed throughout the neuraxis exert a top-down modulation of pain sensation. This modulation is largely mediated by descending monoaminergic pathways that either inhibit or facilitate transmission of nociceptive information at the level of the dorsal horn (Gebhart, 2004; Millan, 2002; Benarroch, 2008; Mason, 2005). Monoamines, including serotonin, norepinephrine, and dopamine, act via different receptor subtypes to exert a complex modulation of neurotransmitter release from nociceptive afferents and excitability of dorsal horn neurons (Millan, 2002; Benarroch, 2008).

Opioids play an important role in pain transmission both by initiating the descending controls from the brainstem and by directly reducing the evoked activity of C and Aδ fibers in the dorsal horn via presynaptic opioid receptors. Thus, a significant antinociceptive effect mediated by opioid receptors in the nucleus raphe magnus (NRM) through its descending pain-modulating system has been reported recently in rats after repeated morphine treatment (Ma et al., 2006).

Activations of descending pain modulation (noradrenergic, serotonergic, dopaminergic and opioidergic pathways) by

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application of drugs or integrative therapies alleviate pain behavior in persistent pain models (Obata et al., 2007; Pertovaara, 2006) or in postoperative pain model (Onttonen and Pertovaara, 2000), and these are effective targets for therapeutic strategy.

The use of integrative and complementary therapies as adjuncts to traditional Western medical practice has significantly increased in the United States over the past two decades. Aromatherapy is the fastest growing integrative therapy (Buckle, 1997; Ehrlichman and Halpern, 1998; Buchbauer et al., 1993). Aromatherapy includes the inhaled, absorbed, or ingested use of essential oil extracts from plants and flowers for prophylactic medical care or active treatment (Buchbauer et al., 1993; Bouchra et al., 2003).

Essential oils are volatile, natural, complex compounds characterized by a strong odor and are formed by aromatic plants as secondary metabolites. Essential oils obtained from different species of Cedrus, such as *C. atlantica, C. deodora and C. libani*, have been reported by their use in aromatherapy to obtain many clinical benefits traditionally ascribed to genitourinary, musculoskeletal and cutaneous systems (Mojay, 2002, 2004; Sharma and Manhas, 2015; Lovell, 1998). Among important pharmacological properties that support their clinical use, we can name the anti-inflammatory and analgesic effects (Thabrew et al., 2003), as well as immunomodulatory (Shinde et al., 1999), antioxidant (Wu et al., 2015), antibacterial (Zeng et al., 2012) and insecticidal (Lamiri et al., 2001) activities. In terms of previous reports on the chemical composition of this plant, the oil from the wood of this species has been extensively studied, sesquiterpenoids of the himachalane type being the major components (Plattier and Teisseire, 1974; Teisseire and Plattier, 1974).

Regarding *Cedrus atlantica* ([Endl.] Manetti ex Carrière), also known as Cedarwood, it is not known whether inhalation of its essential oil (*Ca*EO) alleviates pain behavior in a mouse model of postoperative pain, and if so, how the descending inhibitory systems may be involved in this action. In the present study, we examined the effect of *Ca*EO inhalation on pain behavior and its mechanisms related to the descending opioid, serotonergic, noradrenergic or dopaminergic systems in a mouse model of postoperative pain, induced by a plantar incision surgery (PIS) in the plantar aspect of the foot of mice.

2. Materials and methods

2.1. Drugs

The following substances were used: *Cedrus atlantica* (Essential Oil,) (Penny Price Aromatherapy, Hinckley, England, batch no: CEDT1167); naloxone hydrochloride (a nonselective opioid receptor antagonist, experiment 3 (Tocris Cookson Inc., Ellisville, MO, USA); morphine hydrochloride (a nonselective opioid receptor agonist, experiment 3 (Merck, Darmstadt, Germany); p-chlorophenylalanine methyl ester (PCPA, inhibitor of the synthesis of serotonin, experiment 4), alpha-methyl-para-tyrosin (AMPT, an inhibitor of NE and dopamine synthesis), experiment 5) clonidine hydrochloride (a selective α-2-adrenergic receptors agonist, experiment 6), yohimbine hydrochloride (a selective α-2-adrenergic receptors antagonist, experiment 6), haloperidol (an antagonist of dopaminergic (D1 and D2), experiment 7, apomorphine (a dopamine D2 receptor agonist) experiment 7 (Sigma Chemical Co, St Louis, Missouri).

Other substances were dissolved in saline. Drugs were delivered by intraperitoneal route, a constant volume of 10 ml/kg body weight was injected. Appropriate control-treated groups also were assessed simultaneously. The doses of all substances used were chosen based on data in the literature (Martins et al., 2012, 2013) or were selected from preliminary experiments conducted in our laboratory.

2.2. Analysis of volatile components of the Cedrus atlantica essential oil

The oil was analyzed by gas chromatography (GC) to calculate the retention indexes (IR) and by chromatography–mass spectrometry (GC–MS) to obtain their mass spectra. The GC analysis was performed on SHIMADZU GC-14B equipped with and FID detector and a LM-5 (30 mx0.25 mm × 0.3 μm) capillary column. Oven temperature was programmed from 60 to 260 °C at 3 °C/min, and ending with 10 min at 260 °C; temperature of the injector 250 °C and detector 300 °C. Helium was used as carrier gas (1 ml/min) and split ratio 1:40. The injection volume was 0.1 μL of the oils and split ratio 1:40. The sample was dissolved in n-hexane. The GC/MS analyses were conducted on Agilent Technologies 5975 Series MSD (70 eV) instrument. A DB-5 column (30 m × 0.25 mm × 0.25 μm) was used with helium as the carrier gas at a flow of 1 mL/min. The conditions of the program column temperature were: 60–290 °C (3 °C/min) with isotherm at 290 °C to 5/min; injector temperature 260 °C, detector 280 °C. Sample volume injected was 1 μl. Split 1:10.

The components in the oil were identified by comparison of the calculated retention indices (IR), mass spectra obtained by CG–MS and literature data (Adams, 2007). The IR values of the components were obtained by co-injection of mixture of n-alkanes series (C5-C30) and using the equation reported by Van den Doll and Kratz (1963). The concentration was calculated using the individual peak areas for each substance.

2.3. Animals

All animal care and experimental procedures were carried out in accordance with the National Institutes of Health Animal Care Guidelines (NIH publications number 80-23). All experiments were approved by the Ethics Committee of the University of Southern Santa Catarina at Palhoça, Santa Catarina, Brazil (protocol number 12.014.4.06.IV) and were conducted using male Swiss mice (25–35 g). The animals were housed at 22 ± 2 °C under a 12 h light/12 h dark cycle (lights on at 6:00 a.m.) and with free access to food and water. Mice were acclimated to the laboratory for at least 1 h before testing that was carried out between 8:00 and 12:00 h. The number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of treatments (Zimmermann, 1983). Control animals received the same vehicle used to dilute the compounds.

2.4. Plantar incision surgery (PIS)

Here, we used a pre-clinical model of postoperative pain. Paw incision in rodents induces a variety of nocifensive behaviors that closely resemble the time course of postoperative pain in humans (Brennan et al., 1983). The PIS was performed as previously described (Martins et al., 2012, 2013; Pogatzki and Raja, 2003). Briefly, mice were anesthetized with 1–2% isoflurane delivered via a nose cone. After sterile preparation of the right hind paw, a 5-mm longitudinal incision was made through skin and fascia of the plantar surface using a number 11 scalpel blade. The incision started 2 mm from the proximal edge of the heel and extended toward the toes. After wound homeostasis, the skin was apposed with an 8.0 nylon mattress suture, and the wound was covered with 10% povidone-iodine solution. Control animals underwent a sham procedure. That is, they were subjected to anesthesia but not to PIS. Animals were allowed to recover in their cages, and sutures were removed on the first postoperative day.

2.5. Inhalation of Cedrus atlantica essential oil

Inhalation of *Cedrus atlantica* essential oil (*Ca*EO) was performed as previously described by Barocelli et al. (2004). The volume of 200 μl of CAEO, contained in a 10 ml glass baker, were positioned at the bottom of plastic cages (height 20 cm, width 30 cm, depth 20 cm) suddenly covered with plastic film during 30 min in order to saturate the ambient. At saturation, the concentration of the oils in the cage was 2.4 μl/l. Mice introduced into the cage were allowed to inhale oil vapors for controlled time periods (1, 5, 30 and 60 min) prior to performing the final experiments. Control animals were caged in the same conditions but in the absence of the tested oils (saline).

2.6. Evaluation of locomotor activity

Ambulatory behavior was assessed in an open-field test as previously described (Mazzardo-Martins et al., 2012). The apparatus consisted of a wooden box measuring 40×60x50 cm^3. The floor of the arena was divided into 12 identical squares. Mice inhaled *Ca*EO or saline for 30 min and the number of squares crossed with all paws (crossings) was counted in a 6-min session, 30 min beforehand inhalation.

2.7. Mechanical hypersensitivity

Mechanical hypersensitivity was measured as previously described by Martins et al., (2013). The animals were acclimated in individual clear boxes (9×7x11 cm^3) on an elevated wire mesh platform to allow access to the ventral surface of the hind paws. The right hind paw was stimulated with a constant pressure of 0.4 g von Frey filaments (VFF) (Stoelting, Chicago, USA). The response frequency to 10 applications was taken as the nociceptive behavior. The results are expressed as the percentage of withdrawal frequency. The day before surgery, the animals were subjected for testing to characterize the baseline response. Only animals that showed a response rate around 20% were selected.

2.8. Experiment 1: effect of inhalation of Cedrus atlantica essential oil on locomotor activity

In order to evaluate a possible non-specific muscle-relaxant or sedative effect of the extract, mice were subjected to the open-field test. Mice were subjected to PIS inhaled Saline or *Ca*EO for 0.5 h. Open-field test was performed 0.5 h after inhalations.

2.9. Experiment 2: inhalation of Cedrus atlantica essential oil on postoperative pain

To assess whether inhalation of *Ca*EO alleviates pain behavior in a mouse model of postoperative pain, mechanical hypersensitivity was assessed before (baseline), 24 h after PIS and at 0.5, 1, 2 and 3 h after *Ca*EO inhalation. In a separate series of experiments, we evaluated mechanical hypersensitivity before (baseline), 24 h after PIS and 0.5 h after CAEO inhalation at each day after PIS, for 6 days. Furthermore, in order to verify the effect *per se* of *Ca*EO inhalation, *naive* animals inhaled the oil for 6 consecutive days, being evaluated 0.5 h after the inhalation.

2.10. Participation of descending pain modulation pathways

2.10.1. Experiment 3: involvement of opioid system

To assess the participation of the opioid system in the antihypersensitivity produced by *Ca*EO inhalation, mice were subjected to PIS. Mechanical hypersensitivity was tested 24 h after PIS. Mice were pretreated with an intraperitoneal (i.p.) injection of vehicle (10 ml/kg) or naloxone (1 mg/kg) (Martins et al., 2012). After 20 min, animals inhaled Saline or *Ca*EO for 0.5 h. Mechanical hypersensitivity was evaluated using a von Frey monofilament (0.4 g) 0.5 h after inhalations.

Another group of mice was pretreated with vehicle (10 ml/kg, i.p.) or naloxone (1 mg/kg, i.p.) and, after 20 mn, received subcutaneous (s.c.) saline (10 ml/kg) or morphine (5 mg/kg) (Martins et al., 2012). These groups were assessed 30 min after vehicle or morphine treatment.

2.10.2. Experiment 4: involvement of serotoninergic system

To assess the possible contribution of descending serotonin endogenous system on the antihypersensitivity produced by *Ca*EO inhalation, we examined the effects of depletion of central norepinephrine using an inhibitor of serotonin synthesis (*p*-chlorophenylalanine methyl ester, PCPA). The animals were pretreated with an intraperitoneal injection of vehicle (10 ml/kg) or PCPA (100 mg/kg) once a day, for 4 consecutive days, 3 days before and 24 h after PIS (Martins et al., 2013). Twenty minutes after the last administration, animals inhaled vehicle or *Ca*EO for 0.5 h. Mechanical hypersensitivity was evaluated using a von Frey monofilament (0.4 g) 0.5 h after inhalations.

Another group of mice was pretreated with vehicle (10 ml/kg, i.p.) or PCPA (100 mg/kg, i.p.) and, after 20 min, received s.c. vehicle (10 ml/kg) or morphine (5 mg/kg) (Martins et al., 2013). These groups were assessed 0.5 h after vehicle or morphine treatments.

2.10.3. Experiment 5: involvement of noradrenergic system

To determine a possible involvement of the descending noradrenergic system in the on the antihypersensitivity produced by *Ca*EO inhalation, we examined the effects of depletion of central norepinephrine using an inhibitor of the synthesis of norepinephrine by inhibition of enzyme tyrosine hydroxylase, the alpha-methyl-para-tyrosin (AMPT). The animals were pretreated with an i.p. injection of vehicle (10 ml/kg) or AMPT (100 mg/kg) (Cunha et al., 2013). Four hours after administration, animals inhaled vehicle or *Ca*EO for 0.5 h. Mechanical hypersensitivity was evaluated using a von Frey monofilament (0.4 g) 0.5 h after inhalations.

Another group of mice was pretreated with vehicle (10 ml/kg, i.p.) or AMPT (100 mg/kg, i.p.) and, after 20 min, received s.c. vehicle (10 ml/kg) or morphine (5 mg/kg) (Cunha et al., 2013). These groups were assessed 0.5 h after vehicle or morphine treatments.

2.10.3.1. Experiment 6: involvement of α_2-adrenoceptors. In order to further clarify the role of the noradrenergic system, we conducted pharmacological experiments with adrenoceptor antagonists. Mice were subjected to PIS and mechanical hypersensitivity was tested 24 h after PIS. Mice were pretreated with an i.p. injection of vehicle (10 ml/kg) or yoimbine (0.15 mg/kg) (Martins et al., 2013). After 20 min, animals inhaled vehicle or *Ca*EO for 0.5 h. Mechanical hypersensitivity was evaluated using a von Frey monofilament (0.4 g) 0.5 h after inhalations.

Another group of mice was pretreated with vehicle (10 ml/kg, i.p.) or yoimbine (0.15 mg/kg, i.p.) and, after 20 min, received i.p. vehicle (10 ml/kg) or clonidine (0.1 mg/kg) (Martins et al., 2013). These groups were assessed 0.5 h after vehicle or clonidine treatments.

2.10.4. Experiment 7: involvement of dopaminergic system

In order to verify the role of the dopaminergic system, we conducted pharmacological experiments with haloperidol an antagonist at dopaminergic (D1 and D2) receptors (Naidu et al., 2003; Pandurangan et al., 2014). Mice were subjected to PIS and mechanical hypersensitivity was tested 24 h after PIS. Mice were pretreated with an i.p. injection of vehicle (10 ml/kg) or

haloperidol (1 mg/kg) (Naidu et al., 2003; Pandurangan et al., 2014). After 20 min, animals inhaled vehicle or *Ca*EO for 0.5 h. Mechanical hypersensitivity was evaluated using a von Frey monofilament (0.4 g) 0.5 h after inhalations.

Another group of mice was pretreated with vehicle (10 ml/kg, i.p.) or apomorphine (1 mg/kg, i.p.) and, after 20 min, received i.p. vehicle (10 ml/kg) or haloperidol (1 mg/kg) (Naidu et al., 2003; Pandurangan et al., 2014). These groups were assessed 0.5 h after vehicle or apomorphine treatments.

2.11. Statistical analysis

Results are presented as the mean ± standard errors of the mean for each group. Behavioral testing was analyzed using both one-way analysis of variance (ANOVA) following Student–Newman–Keuls test or two-way repeated measures ANOVA. A multi-comparison post hoc test was performed using the Bonferroni's test. Differences with a value of $P < 0.05$ were considered significant.

3. Results

3.1. Analysis of volatile components of the CaEO

Through the comparison of the mass spectra obtained by CG–MS, kovats indices (IR) ad literature data (Adams, 2007; Aberchane et al., 2004; Derwich et al., 2010; Boudarene et al., 2004) was possible identified some volatile constituents in the *Ca*EO. The results obtained with the analysis are demonstrated in Table 1 and Fig. 1. In total were identified 21 constituents (90.2%), in which 12 are sesquiterpenes hydrocarbons (83.6%) and nine are oxygenated sesquiterpenes (6.6%). The data obtained with the chemical analysis are in according with previous works reported in literature (Aberchane et al., 2004; Derwich et al., 2010; Boudarene et al., 2004).

Of the compounds analyzed, the sesquiterpenes hydrocarbons α-himachalene (16.6%), γ-himachalene (10.4%) and β-himachalene (46.4%) are the majority compounds, in which the component β-himachalene is the most abundant and represent practically the half of percentage of the oil composition.

3.2. Spontaneous locomotor activity

The C*a*EO did not affect the locomotor activity in the open-field test when compared with animals that inhaled vehicle (saline). The means ± SEM of crossed squares were 126.8 ± 20.0, 114.7 ± 23.5 and 107.6 ± 15.4 for the *naïve + vehicle*, PIS + vehicle and PIS + *Ca*EO (data not shown).

3.3. Inhalation of Cedrus atlantica essential oil alleviates post-operative pain

The results presented in Fig. 2 (panel A) show that acute inhalation of *Ca*EO for different times decreased mechanical hypersensitivity induced by PIS. Significant differences between groups (PIS + vehicle *vs* PIS + *Ca*EO 30′ or 60′) were observed at 0.5, ($P < 0.05$), 1 ($P < 0.05$), and 2 h ($P < 0.05$) after inhalations. Furthermore, *Ca*EO 5′ decreased mechanical hypersensitivity 0.5 h after inhalation. However, *Ca*EO 1′ had no effect on the response frequency and was not significantly different from the PIS + vehicle group (Fig. 2, panel A). In addition, the daily treatment of animals with *Ca*EO 30′ decreased the mechanical hypersensitivity induced by PIS when evaluated 0.5 h after treatment. This effect was evident until the fifth day of treatment (Fig. 2, panel B). In *naïve* mice the daily treatment of animals with vehicle 30′ or *Ca*EO 30′ had no effect on the response frequency and was not significantly different between groups were observed until the sixth day of treatments (Fig. 2, panel C).

Table 1
Chemical constitution of the *Cedrus atlantica* essential oil.

Components	RT[a] (min)	IR[b]	Concentration (%)
Iso-longifolene (**1**)	18.826	1387	0.6
β-cubebene (**2**)	18.944	1390	0.6
α-cedrene (**3**)	19.109	1409	0.5
α-himachalene (**4**)	20.098	1447	16.6
γ-Gurjunene (**5**)	20.593	1470	0.5
γ-himachalene (**6**)	20.798	1480	10.4
ar-curcumene (**7**)	20.860	1483	2.3
β-himachalene (**8**)	21.442	1510	46.4
α-dehydro-ar-himachalene (**9**)	21.615	1518	1.2
δ-cadinene (**10**)	21.819	1527	2.3
γ-dehydro-ar-himachalene (**11**)	21.976	1532	1.4
α-calacorene (**12**)	22.298	1548	0.8
Oxido himachalene (**13**)	23.076	1582	0.6
β-himachalene oxide (**14**)	23.602	1605	0.2
α-acorenol (**15**)	24.227	1631	0.8
himachalol (**16**)	24.757	1648	0.7
bulnesol (**17**)	25.055	1660	0.9
Z-γ-atlantone (**18**)	25.660	1682	0.6
deodarone (**19**)	25.801	1687	1.5
E-γ-atlantone (**20**)	25.927	1692	0.9
Z-α-atlantone (**21**)	26.155	1701	0.4
Total			**90.2**

[a] RT, retention time.
[b] RI, retention index.

3.4. Involvement of the opioid system

Since CAEO 30′ showed a significant effect on mechanical hypersensitivity for up to 2 h (Fig. 3, panel A), we focused on the 30′ time point, a period of inhalation (treatment) at which *Ca*EO had no significant effect locomotor activity. Administration of naloxone (opioid antagonists) by themselves did not alter paw mechanical hypersensitivity (Fig. 3, panel A and B). Systemic administration of naloxone, 20 min before mobilization of the *Ca*EO 30′, prevented the decreased in the mechanical hypersensitivity resulting from the inhalation (Fig. 3, panel A). Percentage of response frequency values for the group pre-treated with naloxone via i.p. route was significantly higher when compared to vehicle plus *Ca*EO group, 30 min after inhalations (Fig. 3 panel A).

The results depicted in Fig. 3 panel B, in experiment positive control, the pretreatment of mice with naloxone given 20 min before, prevented the antihypersensitivity caused by morphine compared with group control (vehicle + morphine).

3.5. Involvement of the serotoninergic system

Fig. 4 panel A shows that the pre-treatment of mice with PCPA was able to prevent the reduction of mechanical hypersensitivity elicited by inhalation of *Ca*EO 30′. PCPA without *Ca*EO 30′ had no effect on the increased response frequency being similar to i.p. injection of vehicle without *Ca*EO 30′. The results presented in Fig. 4 panel B show that the pretreatment of mice with PCPA, prevented the reduction of mechanical hypersensitivity caused by morphine (used as a positive control).

3.6. Involvement of the noradrenergic system

Fig. 5 panel A shows that the pre-treatment of mice with AMPT was able to prevent the reduction of mechanical hypersensitivity caused by inhalation of *Ca*EO 30′. AMPT without *Ca*EO 30′ had no effect on the increased response frequency being similar to i.p.

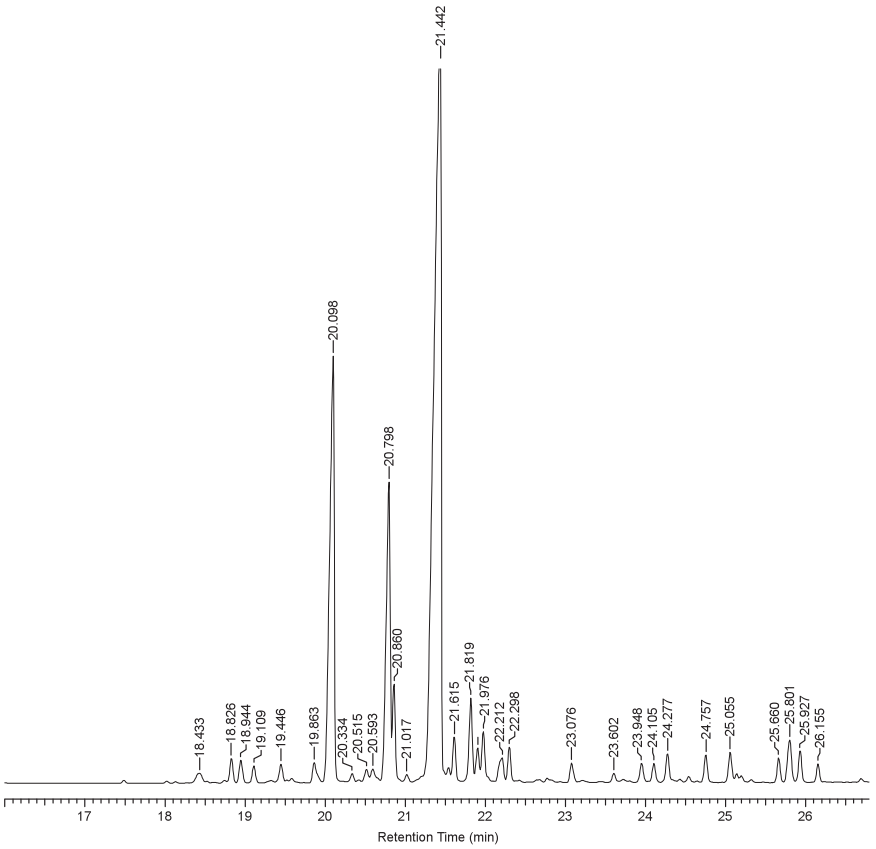

Fig. 1. Chemical profile of *Cedrus atlantica* essential oil.

injection of vehicle without *Ca*EO 30′. The results presented in Fig. 5 panel B show that the pretreatment of mice with AMPT, prevented the reduction of mechanical hypersensitivity caused by morphine (used as a positive control).

We also determined the role of α_2-adrenoceptors on the anti-hypersensitivity effect of *Ca*EO 30′ in the postoperative pain model. We administered a selective α_2 antagonist (yohimbine) systemically (Fig. 5, panel C). The pretreatment with yohimbine had no effect on response frequency, and the thresholds were similar to those of i.p. injections of vehicle without *Ca*EO 30′ (Fig. 5, panel C and D). In addition, the response frequency values for the groups pretreated with yohimbine were significantly higher compared with those of the vehicle+*Ca*EO 30′ group, 0.5 h after inhalation (Fig. 5, panel C and D). Furthermore, the results presented in Fig. 5 show panel D that the pretreatment of mice with yohimbine, prevented the reduction of mechanical hypersensitivity caused by clonidine (used as a positive control).

3.7. Involvement of the dopaminergic system

Fig. 6 panel A shows that the pre-treatment of mice with haloperidol prevented the reduction of mechanical hypersensitivity induced by inhalation of *Ca*EO 30′. The results presented in Fig. 6 panel B show that the pretreatment of mice with haloperidol, also

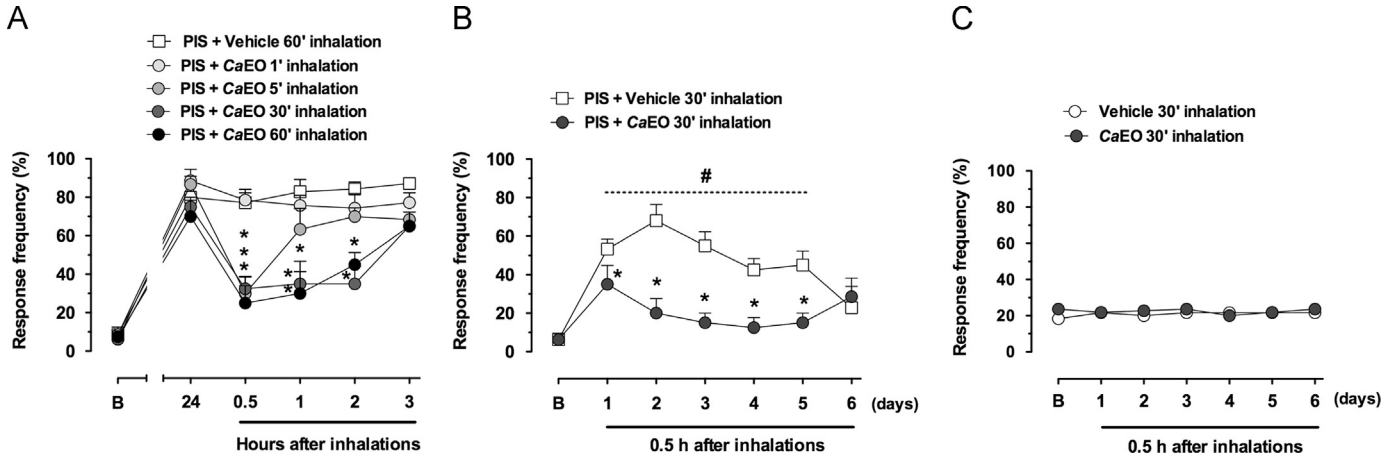

Fig. 2. Effect of *Ca*EO inhalation on mechanical hypersensitivity after plantar incision surgery in the paw of mice. Acute *Ca*EO inhalation in 1, 5, 30 or 60- min twenty-four hours after surgery (panel A). In operated animals, the daily treatment of animals with 30-min *Ca*EO inhalation (panel, B). In *naïve* mice the daily treatment of animals with vehicle or *Ca*EO 30-min (panel C). Each point represents the mean of 8 animals and vertical lines show the SEM. The symbols denote a significant difference of $^{\#}P < 0.05$, when compared with basal (B) condition or $^{*}P < 0.05$, when compared with control alone group. *Ca*EO: *Cedrus atlantica* essential oil; B: baseline analysis.

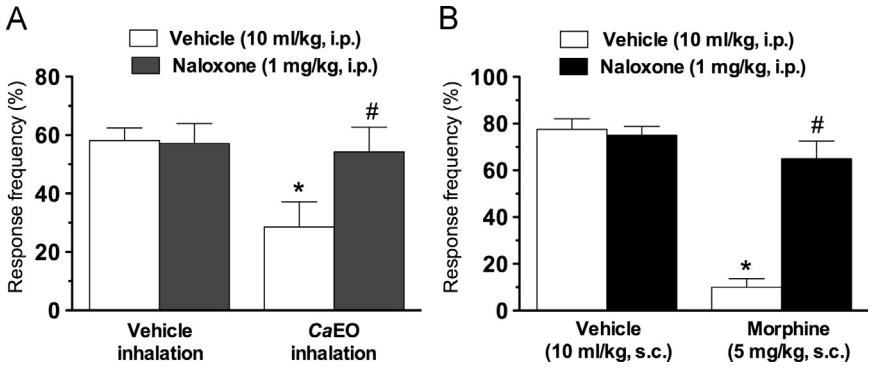

Fig. 3. Involvement of the opioid system in antihypersensitivity effect caused by *Ca*EO inhalation. Intraperitoneal (i.p.) pre-treatment with naloxone (1 mg/kg, i.p., panel A-B) on the antihypersensitivity effect of *Ca*EO inhalation (panel A) or morphine (5 mg/kg, s.c., panel B). Each point represents the mean of 8 animals and vertical lines show the SEM. The symbols denote a significant difference of $^{*}P < 0.05$ when compared with Vehicle + Vehicle inhalation (control) group; $^{\#}P < 0.05$ when compared with Vehicle + *Ca*EO inhalation, alone group. *Ca*EO: *Cedrus atlantica* essential oil.

prevented the reduction of mechanical hypersensitivity caused by apomorphine (used as a positive control). Haloperidol without *Ca*EO 30′ had no effect on the increased response frequency being similar to i.p. injection of vehicle without *Ca*EO 30′.

4. Discussion

Aromatherapy involves the therapeutic use of essential oils extracted from various parts of aromatic plants with the intent to calm, balance, and rejuvenate mind, body, and spirit (Robins, 1999). It is currently used in the management of depression, stress-related disorders, anxiety, some cognitive disorders, insomnia and pain (Perry and Perry, 2006). Beneficial effects on pain of inhaled essential oils in animals and in humans have been reported (Barocelli et al. 2004; Salamati et al., 2014; Nagata et al., 2014). The genus *Cedrus*, is native to the Atlas Mountains of Algeria and Morocco, is a tree up to 40 m high, with broad level branches. Essential oils have been widely used in traditional medicine. In relation to *Cedrus atlantica*, among others, anti-inflammatory and analgesic effects have been registered (Thabrew et al., 2003). However, analgesic effect on postoperative pain, as well as the possible mechanisms implicated on this, has not been investigated yet. Thus, the present study demonstrated the efficacy and safety of inhalation of *Ca*EO on postoperative pain.

This study showed for the first time that the inhalation of *Ca*EO displayed antihypersensitivity effect on postoperative pain evoked by plantar incision surgery (PIS) in mice. The inhalation of *Ca*EO did not alter spontaneous locomotor activity in the time that caused significant antihypersensitivity effect. Furthermore, here we demonstrated that inhalation of *Ca*EO reduces pain behavior through activation of descending pain modulation pathways. To our knowledge this is the first report of this kind in the literature.

Phytochemical analysis of the *Ca*EO reveled that sesquiterpenes hydrocarbons α-himachalene (16.6%), γ-himachalene (10.4%) and β-himachalene (46.4%) are the majority compounds, in which the component β-himachalene is the most abundant and represent practically the half of percentage of the oil composition. These data are in agree with reported by Aberchane and co-authors (2004) which described the chemical composition of *Ca*EO and identified α-himachalene (7.4–16.4%), γ-himachalene (5.1–8.6%), β-himachalene (23.4–40.4%) and (E)-α-atlantone (5.2–29.5%) as the majority compounds.

In the present study, we attempted to characterize some of the mechanisms through which the inhalation of *Ca*EO exerts its antihypersensitivity effect in a mouse model of postoperative pain. Previous studies have shown that the activation of the descending inhibition pathway results in analgesia after surgery. Descending control arises from a number of supraspinal sites, including the midline periaqueductal gray-rostral ventromedial medulla (PAG-RVM) system, and the more lateral and caudal dorsal reticular nucleus (DRt) and ventrolateral medulla (VLM) for inhibition of pain sensation includes projections from various brainstem nuclei to the spinal cord dorsal horn via the dorsolateral funiculus (DLF). More specifically, DLF fibers are comprised of serotonergic projections from the raphe nuclei, dopaminergic projections from the ventral tegmental area (VTA), and noradrenergic projections from the locus coeruleus (LC). These descending fibers suppress pain transmission at the nociceptive spinal cord neurons presumably by hyperpolarizing afferent sensory neurons using endogenous

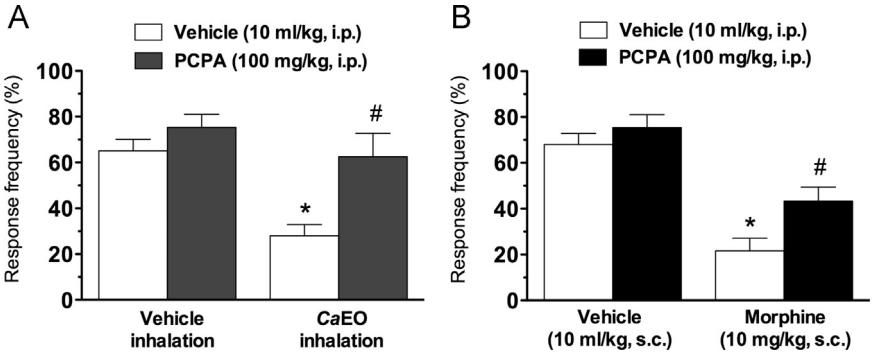

Fig. 4. Involvement of the serotoninergic system in antihypersensitivity effect caused by *Ca*EO inhalation. Intraperitoneal (i.p.) pre-treatment with PCPA (100 mg/kg, i.p., panel A and B) on the antihypersensitivity effect of *Ca*EO inhalation (panel A) or morphine (5 mg/kg, s.c., panel B). Each point represents the mean of 8 animals and vertical lines show the SEM. The symbols denote a significant difference of $^{*}P < 0.05$ when compared with Vehicle + Vehicle inhalation (control) group; $^{\#}P < 0.05$ when compared with Vehicle + *Ca*EO inhalation, alone group. *Ca*EO: *Cedrus atlantica* essential oil.

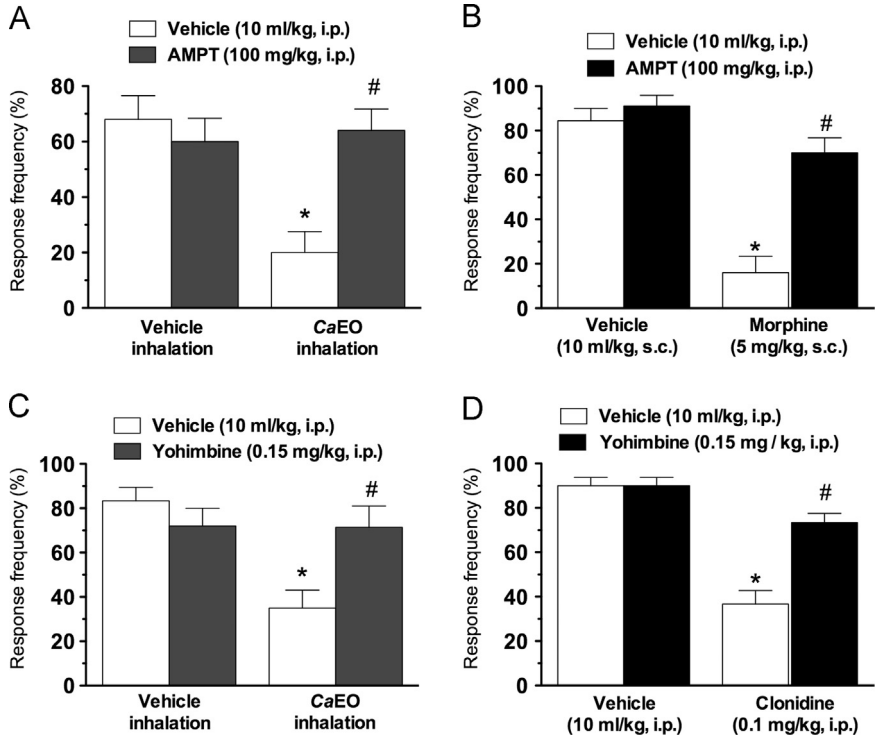

Fig. 5. Involvement of the noradrenergic system in antihypersensitivity effect caused by *Ca*EO inhalation. Intraperitoneal (i.p.) pre-treatment with AMPT (100 mg/kg, i.p., panel A and B) on the antihypersensitivity effect of *Ca*EO inhalation (panel A) or morphine (5 mg/kg, s.c., panel B). The pre-treatment with yohimbine (0.15 mg/kg, i.p., panel C and D) on the antihypersensitivity effect of *Ca*EO inhalation (panel C) or clonidine (0.1 mg/kg, i.p., panel D). Each point represents the mean of 8 animals and vertical lines show the SEM. The symbols denote a significant difference of $^{*}P < 0.05$ when compared with Vehicle + Vehicle inhalation (control) group; $^{\#}P < 0.05$ when compared with Vehicle + *Ca*EO inhalation, alone group. *Ca*EO: *Cedrus atlantica* essential oil.

opioids, or serotonin and norepinephrine as principal inhibitory mediators (Millan, 2002; Marks et al., 2009).

The results reported here indicate, to our knowledge for the first time, that pretreatment of animals with naloxone, a non-selective opioid receptor antagonist, at a dose that produced no significant effect on postoperative pain, completely prevented the antihypersensitivity effect of inhalation of *Ca*EO. The opioid system plays a very important role in the control of pain. Opioid receptors are widely expressed in the central and peripheral nervous systems and in numerous nonneuronal tissues (Iwaszkiewicz et al., 2013). There are several areas of the CNS that, directly or indirectly, are activated by nociceptive inputs, are targets of opioids, and participate in the central modulation of pain (Basbaum and Fields, 1984). Therefore, we suggest that inhalation of *Ca*EO could releases endogenous opioids like encephalin, endorphin or others that are responsible for antihypersensitivity effect.

The present study also showed that the monoaminergic system probably also is involved in the antihypersensitivity effect of *Ca*EO inhalation. This conclusion is derived from the fact that 1) depletion of endogenous serotonin with the tryptophan hydroxylase inhibitor PCPA, at a dose known to decrease the cortical content of serotonin and to significantly prevent the morphine antihypersensitivity, largely antagonized the antihypersensitivity action of *Ca*EO inhalation; 2) depletion of endogenous norepinephrine (NE) and dopamine with an inhibitor of NE and dopamine synthesis AMPT, at a dose known to decrease the cortical content of NE and dopamine and to significantly prevent the morphine antihypersensitivity, largely antagonized the antihypersensitivity action of *Ca*EO inhalation; 3) selective or non-selective antagonists of α_2 or D1 and D2 receptors, namely yohimbine and haloperidol, respectively, consistently prevented the antihypersensitivity caused by inhalation of *Ca*EO when analyzed

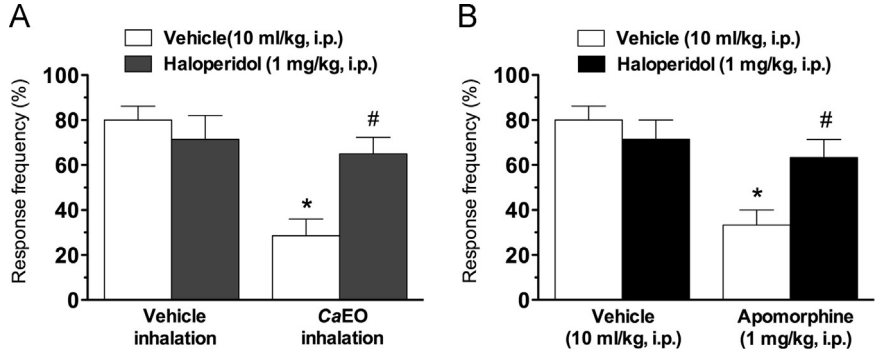

Fig. 6. Involvement of the dopaminergic system in antihypersensitivity effect caused by *Ca*EO inhalation. Intraperitoneal (i.p.) pre-treatment with haloperidol (1 mg/kg, i.p., panel A and B) on the antihypersensitivity effect of *Ca*EO inhalation (panel A) or apomorphine (1 mg/kg, i.p., panel B). Each point represents the mean of 8 animals and vertical lines show the SEM. The symbols denote a significant difference of $^{*}P < 0.05$ when compared with Vehicle + Vehicle inhalation (control) group; $^{\#}P < 0.05$ when compared with Vehicle + *Ca*EO inhalation, alone group. *Ca*EO: *Cedrus atlantica* essential oil.

in the behavior pain. Additionally, it will be relevant to assess whether intratecal administration of the antagonists should prevented the antihypersensitivity caused by inhalation of *Ca*EO, as a manner to better clarify the participation of these neurotransmitters released in the spinal cord to the the studied phenomena. This should be considered in future studies.

Essential oil components might be absorbed into the circulatory system, reach the CNS, and act on receptors of neurotransmitters to induce the effects (Kagawa et al., 2003). However, in inhalation aromatherapy, aroma component molecules are dissolved in the mucus on the olfactory mucosa and stimulate olfactory cells, and then the aroma information is converted to electric signals and transmitted to the olfactory nerve and olfactory bulb (OB) (Amir et al., 1999). Notably, it has been reported that exposure to essential oil increases Fos expression in the olfactory system (main olfactory bulb, anterior olfactory nucleus and piriform cortex) and the limbic system (amygdala, infralimbic cortex and entorhinal cortex) in rats. Neural circuit processing of odor information in the OB is regulated by afferent axons from olfactory receptor neurons (Amir et al., 1999).

In summary, the present results provide convincing evidence that *Ca*EO inhalation exerts a rapid onset, relatively long-lasting and pronounced systemic antihypersensitivity against pain behavior in a mouse model of postoperative pain at a inhalation time that does not interfere with the locomotor activity. In view of these results, we can speculate that the *Ca*EO inhalation may elicit an antihypersensitivity effect either by releasing endogenous opioid, serotonin, NE and/or dopamine that can bind to their specific their receptors, or by interacting with their receptors. However, other studies are necessary to further substantiate this assertion. Thus, our data support the hypothesis that inhalation of *Ca*EO reduces behavior pain through activation opioidergic, serotonergic, noradrenergic and dopaminergic systems.

Conflict of interests

The author(s) declare(s) that there is no conflict of interests regarding the publication of this article.

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Glossary

Aromatherapy: includes the inhaled, absorbed, or ingested use of essential oil extracts from plants and flowers for prophylactic medical care or active treatment;
Essential oils: are substance volatile, natural, complex compounds characterized by a strong odor and are formed by aromatic plants as secondary metabolites;
Plantar incision surgery: is a pre-clinical (animal) model of postoperative pain.