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Evidence for the involvement of TNF-α and IL-1β in the antinociceptive and anti-inflammatory activity of *Stachys lavandulifolia* Vahl. (Lamiaceae) essential oil and (-)-α-bisabolol, its main compound, in mice

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Abstract

Ethnopharmacological relevance: *Stachys lavandulifolia* Vahl (Lamiaceae) is a medicinal plant widely used in Turkey and Iranian folk medicine due to its analgesic and anti-inflammatory properties, but little is known about its essential oil.

Aim of this study: We studied the antinociceptive and anti-inflammatory effects of *S. lavandulifolia* essential oil (EOSl) and (-)-α-bisabolol (BIS), its main compound, in algogen-induced orofacial nociceptive behavior in mice, and assessed the possible involvement of pro-inflammatory cytokines in these profiles.

Materials and Methods: The GC-FID and GC-MS analysis of EOSl demonstrated the presence of (-)-α-bisabolol (56.4%), bicyclogermacrene (5.3%), δ-cadinene (4.2%) and spathulenol (2.9%) as the main compounds. Male Swiss mice were pretreated with EOSl (25 or 50 mg/kg, p.o.), BIS (25 or 50 mg/kg, p.o.), morphine (3 mg/kg, i.p.) or vehicle (saline 0.9% with two drops of tween 80, 0.2%), before formalin- (20 µl, 2%), capsaicin- (20 µl, 2.5 µg) or glutamate- (20 µl, 25 Mm) injection into the right upper lip (perinasal area) in mice. The anti-inflammatory profile of EOSl or BIS (50 mg/kg) was assessed by the inflammatory response induced by carrageenan (2% in 0.2 mL) in mice (pleurisy model).

Results: Our results showed that p.o. treatment with EOSl and BIS displayed significant inhibitory ($p<0.05$ or $p<0.01$ or $p<0.001$) effects in different orofacial pain tests on mice, but BIS proved to be more effective, significantly reducing nociceptive behavior in all tests including both phases of the formalin test. The analgesic effect is not related to any abnormality since EOSl- or BIS-treated mice exhibited no performance alteration in grip strength. Moreover, EOS1 and BIS exhibited a significant anti-inflammatory effect ($p<0.001$) in the pleurisy model of inflammation, which seems to be related to a significant reduction ($p<0.05$) of the pro-inflammatory cytokine TNF-α in BIS treatment, and of the pro-inflammatory cytokine IL-1β ($p<0.01$) in EOS1 treatment.

Conclusion: Our results corroborate the use of *S. lavandulifolia* in traditional medicine as an analgesic and anti-inflammatory, which seems to be related to (-)-α-Bisabolol, the main compound of EOSl.

Keywords: *Stachys lavandulifolia;* (-)-α-bisabolol; facial pain; oils, volatile; sesquiterpenes, citokynes.

Introduction

Orofacial pain is a symptom related to a heterogeneous group of disorders. These are highly prevalent and debilitating conditions that cause ongoing pain in the head, face and neck region. These conditions represent a challenge for clinicians since the orofacial region is complex and, therefore, pain can arise in a multifactorial manner (Ganzberg, 2010; Romero-Reyes and Uyanik, 2014).

A wide variety of medicines classes are commonly used as analgesics for orofacial pain, including nonsteroidal anti-inflammatory agents (NSAIDs), muscle relaxants, opioids, and antidepressants, particularly the tricyclic antidepressants (TCAs) and the selective serotonin-norepinephrine reuptake inhibitors (SNRIs), and anti-epileptic drugs (AEDs) (Arnold et al., 2004; Collins et al., 2000; McQuay et al., 1995, 1996). However, side effects and toxicity are apparent with long-term administration (Becker, 2010; Dionne, 2001; Ganzberg, 2010).

In recent years, there has been a constant search for alternative drugs with greater efficacy and safety in reducing inflammatory and neuropathic pain, in order to stop the transition from acute to chronic pain (Dengler et al., 2014; Ji et al., 2014). In this regard, natural products, mainly essential oils and terpenes, have been shown to be an important source of new drugs with significant biological activity (Brito et al., 2013; Guimarães et al., 2012; Lima et al., 2013; Quintans-Júnior et al., 2010; Siqueira-Lima et al., 2014; Siqueira et al., 2010).

Stachys lavandulifolia Vahl (Lamiaceae), a type of *Stachys* also known as Mountain Tea (Chay-e-Kouhi), is a medicinal plant widely used in Turkish folk medicine because of its antimicrobial, analgesic and anti-inflammatory properties. This plant is also used as herbal tea and in Iranian folk medicine (Minae et al., 2015). Studies have demonstrated that *S. lavandulifolia* possess anxiolytic (Rabbani et al., 2005, 2003), antidepressant, antibacterial (Fooladvand and Fazeli-nasab, 2014), antimicrobial, antioxidant (Işcan et al., 2012), analgesic and anti-inflammatory effects (Hajhashemi et al., 2007). In general, the essential oils of various populations of *S. lavandulifolia* were rich in monoterpenoids and sesquiterpenoids (Pirbalouti and Mohammadi,

2013), such as (-)-α-bisabolol (BIS). *S. lavandulifolia* is used in folk medicine to treat pain and inflammation, including for toothache and other orofacial problems (Mosaddegh et al., 2012). However, little is known about its essential oil, and its components that can vary with ecotype (genetically distinct geographic variety), environmental conditions and geographic origin (Pirbalouti and Mohammadi, 2013).

BIS (Fig. 1), a monocyclic sesquiterpene alcohol and the main compound of our oil sample (see results section), is also present in chamomile (*Matricaria chamomilla*) and has been identified as the main component responsible for most pharmacological actions of its essential oil. (Guimarães et al., 2013; Kamatou and Viljoen, 2009). This compound has been widely used as an ingredient in dermatological and cosmetic formulations such as aftershave creams, hand- and body-lotions, deodorants, lipsticks, and baby care products (Kamatou and Viljoen, 2009). Additionally, BIS has been seen as a promising compound to treat inflammatory pain, as it appears to be effective in the inhibition of proinflammatory cytokines, such as TNF-α and IL-6, (Maurya et al., 2014; Rocha et al., 2011a)

Thus, we aimed to study the antinociceptive and anti-inflammatory effects of *S. lavandulifolia* essential oil (EOSl) and (-)-α-bisabolol (BIS), its main compound, in orofacial algogen-induced nociceptive behavior and in response to inflammatory stimuli (evoked by carrageenan) in mice. We also assessed the possible involvement of the inhibition of pro-inflammatory cytokines (TNF-α and IL-1β) in the pharmacological profile of EOSl and BIS. The obtained results could be valuable for the development of new functional drugs.

Material and Methods

Drugs and Reagents

The drugs and reagents used in this study were morphine sulphate from Dimorf-Cristalia (Itapira, SP, Brazil), glutamate, capsaicin, ethanol, dimethyl sulphoxide (DMSO), and indometacin from Sigma (St. Louis, MO, USA), naloxone from Res. Biochemicals Inc. (Natick, MA, USA),

diazepam from União Química (São Paulo, SP, Brazil) and formaldehyde from Merck (Kenilworth, NJ, USA). The vehicle was 0.2% Tween 80 from Sigma (St. Louis, MO, USA) dissolved in saline solution (NaCl 0.9%). Tumor necrosis factor-alpha (TNF-α) and Interleukin-1-beta (IL-1β) levels were measured in the mice using ELISA kits purchased from BD-Bioscience Pharmingen (San Diego, CA, USA).

Plant material

Stachys lavandulifolia var. *lavandulifolia* was collected from Campus-Yukselen village road, Konya, Turkey in the flowering season (June 2014). Taxonomic identification of the plant material was confirmed by the senior taxonomist Dr. Murad Aydin Sanda, Department of Field Crops, Igdir University. The voucher specimen has been deposited at the Herbarium of the Department of Biology, Selcuk University, Konya, Turkey.

Isolation and analysis of the essential oil

The air-dried and powdered aerial parts of the plant (100 g) were submitted to water-distillation for 5 h using a British-type Clevenger apparatus (ILDAM Ltd., Ankara-Turkey). The obtained essential oil was dried over anhydrous sodium sulphate and, after filtration, stored at +4°C until tested and analyzed.

The essential oil sample was analyzed by gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC/MS) techniques. The GC/MS analysis was carried out with an Agilent 5975 GC-MSD system coupled to an Agilent 7890A GC (Agilent Technologies Inc., Santa Clara, CA). HP-Innowax FSC column (60 m x 0.25mm, 0.25μm film thickness) was used with helium (purity 99.99%) as a carrier gas (1.2 mL/min). The GC oven temperature was kept at 60°C for 10 min and then programmed to increase to 220°C at a rate of 4°C/min, before being kept constant at 220°C for 10 min and then to increase to 240°C at a rate of 1°C/min. The split ratio was used at 40:1. The injector temperature was at 250°C, mass spectra were recorded at 70 eV. Mass range was from 35 to 450 m/z. GC-FID analysis was carried out by

simultaneous auto-injection using an Agilent 7693A series autosampler; 1μL of essential oil diluted with n-hexane (10%, v/v) was injected into GC/MS system. The GC analysis was carried out using an Agilent 7890A GC system. In order to obtain the same elution order with GC/MS, simultaneous triplicate injections were made using the same column and same operational conditions. The FID temperature was 300°C.

The identification of constituents was achieved on the basis of a retention index determined by co-injection with reference to a homologous series of n-alkanes (C_8-C_{30}), under the same experimental conditions. Further identification was carried out by comparison of their mass spectra with those from NIST 05 and Wiley 8th version, as well as by comparison of their retention indices with literature values.

Animals

Young-adult male Swiss mice (28-33 g) were used for all experiments. The mice were housed in controlled temperature (23 ± 1ºC) and lighting (lights on: 6 AM to 6 PM) conditions and had free access to food and water. All procedures described in the present work were approved by the Animal Research Ethics Committee of the Federal University of Sergipe (CEPA/UFS # 72/2015) and were in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 85–23, revised 1996; http://www.nap.edu/readingroom/books/labrats/index.html). All experimental protocols were carried out in a double-blind manner to avoid any influence on the results and we also avoided any unnecessary discomfort to the animals.

Formalin test

Orofacial nociception was induced in the mice by injection (s.c.) of 20 μl of 2 % formalin into the right upper lip (perinasal area) (Luccarini et al., 2006), with adaptations (Quintans-Júnior et al., 2010). This volume and the concentration percentage of formalin were selected from pilot studies that showed a nociceptive-related biphasic behavioral response (face-rubbing) of great

intensity at periods of 0–5 min (first phase) and 15–40 min (second phase). Nociceptive behavior was quantified at these periods by measuring the time (sec.) that the animal spent face-rubbing the injected area with its fore or hind paws. To assess the effects of test drugs, groups of different mice (n = 6, per group) were pre-treated with vehicle (saline 0.9% with Tween 80 0.2%, p.o., the solvent for EOSl and BIS); EOSl (25 and 50 mg/kg, p.o.) or BIS (25 and 50 mg/kg, p.o.) 1 h before the local injection of formalin. Morphine (MOR, 3 mg/kg, i.p.), administered 0.5 h before the algogen was included as a positive control.

Capsaicin-induced nociception test

Orofacial pain was induced by capsaicin or glutamate in mice as previously described (Quintans-Júnior et al., 2010). The mice (n = 6, per group) were injected with 20 µl of capsaicin (2.5 µg) or 40 µl of glutamate (25 mM) subcutaneously into the right upper lip (perinasal area), using a 27-gauge needle. Capsaicin was dissolved in ethanol, dimethyl sulphoxide and distilled water (1:1:8). In pilot studies, mice manifested pain-related face-rubbing behaviour after the injection of capsaicin or glutamate with a high intensity at the 10–20 min. period. Therefore, nociception quantification was performed at this period, measuring the time (sec.) that the animals spent face-rubbing the injected area with the fore or hind paws. EOSl or BIS (25 and 50 mg/kg, p.o.) or vehicle (saline 0.9% with Tween 80 0.2%, p.o.) were given to the animals as described in the formalin test 1 h before the local injection of the algogen (capsaicin or glutamate). Morphine (MOR, 3 mg/kg, i.p.), administered 0.5 h before the algogen was included as a positive control.

Glutamate-induced nociception test

In an attempt to provide information on any interaction of EOSl or BIS with the glutamate system, we investigated whether they were able to antagonize glutamate-induced orofacial nociception in mice. The procedure was similar to that previously described (Beirith et al., 2002a) but with some alterations. A volume of 40 µl of glutamate (25 µM) was injected into the right upper lip. Animals were observed individually for 15 min. after the glutamate injection. Quantification of nociception was performed by measuring the time (sec.) that the animal spent face-rubbing the

injected area with its fore or hind paws. Animals (n = 6, per group) were pretreated with vehicle (saline 0.9% with Tween 80 0.2%, p.o.); EOSl or BIS (25 and 50 mg/kg, p.o.) or MOR (5 mg/kg; i.p.), 1 or 0.5 h before the local injection of glutamate.

Grip strength meter

A grip strength test was performed using a grip strength meter (Model EFF-305, Insight, Ribeirão Preto, SP, Brazil), as previously described (Meyer et al., 1979), which measures forelimb grip strength only. Mice were placed on the plate and were horizontally pulled by the tail with increasing force until unable to grasp the trapeze and the grip was broken. The instrument digitally captured and displayed the peak pull-force achieved. Muscle strength was defined as the peak weight (g) indicated on the display. One hour after the administration of EOSl or BIS (50 mg/kg, p.o.) or vehicle (saline 0.9% with Tween 80 0.2%, p.o.) each animal was tested on the grip strength meter.

Carrageenan-induced pleurisy

Different groups of mice were subjected to increasing oral doses of *S. lavandulifolia* essential oil (EOSl, 50 mg/kg, p.o.) or (-)-α-Bisabolol (BIS, 50 mg/kg, p.o.) dissolved in saline 0.9% with Tween 80 0.2%. The vehicle given was saline 0.9% with Tween 80 0.2%, and the positive control group received 10 mg/kg of indomethacin the same via 1 h before carrageenan pleural injection. Pleurisy was induced in the mice by intrapleural administration of 100 µL of 1 % (w/v) carrageenan suspension in sterile saline solution (de Oliveira et al., 2012). A specially adapted 13 x 5 needle was introduced into the right side of the thoracic cavity for injection of the carrageenan solution. Four hours after the induction of pleurisy, the animals were euthanized, and the pleural inflammatory exudate was collected through pleural lavage with 1 mL of PBS containing ethylenediaminetetraacetic acid (EDTA; 10 mM). The exudate volume was measured, and an aliquot of 50 µL was diluted with Turk's solution (1:20). The total leukocytes were counted in a Neubauer chamber, examining four external quadrants, using a light microscope (Vinegar et al., 1973).

Determination of IL-1β and TNF-α levels in the pleural fluid

Exudate levels of cytokines IL-1β and TNF-α were determined 4 h after the induction of pleurisy by carrageenan injection as previously described (Brito et al., 2012; de Santana et al., 2015). The assays were carried out using commercial colorimetric ELISA kits (BD-Bioscience Pharmingen, San Diego, CA, USA) following the manufacturer's recommendations. The ELISA has a lower detection limit of 5 pg/mL.

Statistical analysis

Values are expressed as mean ± S.E.M. The data obtained were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. In all cases, differences were considered to be statistically significant when $p<0.05$. All statistical analyses were performed using the software Graph Pad Prism 5 (Graph Pad Prism Software Inc., San Diego, CA, USA).

Results and Discussion

The essential oil obtained from *S. lavandulifolia* (EOSl) was analyzed by GC-FID and GC-MS techniques. The essential oil revealed the presence of thirty-four components, representing 89.9% of the total oil. All the identified volatile components are listed in Table 1. As can be seen in Table, α-bisabolol (56.4%) was determined as the major component, followed by bicyclogermacrene (5.3%), δ-cadinene (4.2%) and spathulenol (2.9%). The total ion chromatogram and MS spectral data for α-bisabolol are shown in Figure 2. EOSl is characterized by a high concentration of sesquiterpenes. Several compositional studies performed on the essential oil of *S. lavandulifolia* (Aghaei et al., 2013; Khadivi-Khub et al., 2014; Meshkatalsadat et al., 2007) have identified its major component to be different compounds including: β-phellandrene, bicylogermacrene, germacrene D, δ-cadinene, and α-pinene etc. These differences observed in volatile constituents may be associated with climatic, geographical, genetic and other factors. Similar findings have been observed by several researchers for different essential oils (Dhouioui et al., 2016; You et al., 2015).

S. lavandulifolia is a medicinal plant widely used as an antimicrobial, analgesic and anti-inflammatory in folk medicine of countries in Western Asia, including in the treatment of toothache and other problems like periodontitis (Minae et al., 2015). Despite its important and widespread ethnopharmacological uses, little is known about the properties of its essential oil. Thus, we aimed to evaluate the antinociceptive and anti-inflammatory effects of *S. lavandulifolia* essential oil (EOSl) and (-)-α-Bisabolol (BIS), the main compound, in orofacial nociceptive behavior induced by formalin, glutamate or capsaicin in mice. Moreover, we also evaluated the possible involvement of IL-1β and TNF-α in their analgesic and anti-inflammatory profiles. Our results showed that p.o. treatment with EOSl and BIS displayed significant inhibitory ($p<0.05$; $p<0.01$; $p<0.001$) effects in different orofacial pain models in mice. BIS significantly reduced nociceptive behavior and inflammatory response, these effects seeming to be mediated by the inhibition of pro-inflammatory cytokines.

The administration of EOSl and BIS produced a significant reduction in the face-rubbing behavior induced by formalin (Fig. 3). Only the higher dose of BIS (50 mg/kg, p.o.) produced significant inhibitory (35.50 ± 4.405; $p<0.001$) effects on nociceptive face-rubbing behavioral response in the first phase of the formalin test when compared to the control group (86.00 ± 2.951) and to EOSl (25 mg/kg, p.o.; 69.50 ± 3.128) group (Fig. 3A). However, all doses of EOSl or BIS (25 and 50 mg/kg, p.o.) produced significant inhibitory effects (91.50 ± 13.07 and 87.50 ± 9.293; $p<0.01$ or 79.50 ± 6.967 and 45.50 ± 13.71; $p<0.001$, respectively) on the nociceptive face-rubbing behavioral response in the second phase of the formalin test when compared to the control group (141.5 ± 13.33) (Fig. 3B).

Acute pretreatment with EOSl or BIS, by oral route, caused an antinociceptive effect in the formalin test in mice, which was evidenced by a decrease in face-rubbing behavior particularly during the second phase. Surprisingly, BIS at a dose of 50 mg/kg produced a reduction in pain

behavior in the first phase, in contrast to the results of the study by Rocha et al. (Rocha et al., 2011b) with the same dose but using the paw formalin test. This may be explained by the idea that, according to Luccarini et al (2006), formalin administered into the orofacial region can strongly sensitize the sensory receptors, producing the conduction of painful impulses through the trigeminal nerve, making the model more sensitive than the formalin-induced paw test. Our results for BIS corroborate this hypothesis (Luccarini et al., 2006; Sessle, 2011).

Moreover, the biphasic component of formalin-induced nociception reflects different underlying mechanisms. The first phase is related to the direct chemical stimulation of nociceptive nerve endings, which reflects centrally mediated pain with the release of substance P (Le Bars et al., 2001; Raboisson and Dallel, 2004), while the second phase depends on a combination of inputs from nociceptive afferents, due to the release of excitatory amino acids, PGE2, nitric oxide (NO), tachykinin, kinins and other peptides (Capuano et al., 2009).

Some studies have proposed that inhibition by monoterpenes in both phases of the formalin test may be associated with the blockade of the voltage-dependent Na^+ channels, causing a stabilization of the excitable membrane (de Sousa et al., 2006; Gonçalves et al., 2008) or by the involvement of the descending modulatory pain systems (Brito et al., 2015, 2013; Guimarães et al., 2014). Moreover, Alves et al. (Alves et al., 2010) have demonstrated that the decreased nervous excitability elicited by BIS might be caused by an irreversible blockade of voltage dependent sodium channels, and it is therefore possible to suggest that the observed antinociceptive response of EOSl and BIS is related to this effect.

Following the rational hypothesis of modulation of both phases of the formalin test, BIS has been described as having an anti-inflammatory effects in edema reduction (Leite et al., 2011; Rocha et al., 2011a; Tomić et al., 2014); reducing neutrophil degranulation; decreasing leukocyte migration; increasing protein extravasations and the amount of TNF-α in the peritoneal cavity in response to carrageenan (Rocha et al., 2011a).

Moreover, Kim et al. (Kim et al., 2011) showed that BIS inhibited lipopolysaccharide (LPS)-induced production of nitric oxide (NO) and prostaglandin E2 (PGE2) reduced the expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) genes, and reduced LPS-induced activation of AP-1 (ERK and p38) and NF-κB promoters. Chen et al. (Chen et al., 2010) observed that the detection of an accumulation of nuclear wild-type p53 and up-regulated expression of NF-kappaB indicated that these two key regulators, which have a transcriptional decision-making function in various signaling pathways, might also play a role in BIS-induced apoptosis. Thus, these pharmacological properties of EOSl and BIS may involve the inhibition of inflammatory mediators and have also been implicated in the antinociceptive effect demonstrated in our study.

In order to better understand the pharmacological profile of EOSl and BIS, we assessed them in capsaicin- and glutamate-induced orofacial pain tests. In the capsaicin test, all doses of EOSI or BIS (78.50 ± 12.68 and 63.00 ± 9.495; $p<0.05$; $p<0.01$ or 59.50 ± 13.57 and 48.50 ± 11.48; $p<0.001$, respectively) inhibited nociceptive behavior in mice when compared to a control group (133.5 ± 12.60) (Fig. 4).

Capsaicin has a selective action on the sensory fibers that convey pain sensations, and elicits axon reflex vasodilatation resulting from the activation of the capsaicin vanilloid receptors (TRPV1). This induces an influx of cations which produces depolarization and excitation of neurons causing the release of neuropeptides such as tachykinins, substance P, calcitonin gene-related peptide, excitatory amino acids (aspartate and glutamate), nitric oxide, and pro-inflammatory mediators from the peripheral terminals (Sakurada et al., 1992). Capsaicin injection is also able to increase the excitability of the spinal and trigeminal nociceptive neurons (Pelissier et al., 2002). This model is interesting in relation to the possible involvement of vanilloid-receptors in a pharmacological mechanism involved in the new analgesic drugs (Holanda Pinto et al., 2008; Quintans-Júnior et al., 2010).

The effects observed in the formalin and capsaicin tests may be a result of an inhibition of substance P release, or by a direct blocking action on its receptor neurokinin-1 (NK-1) (Holanda Pinto et al., 2008), as studies have demonstrated that the activation of NK-1 receptors, through NK-1 antagonist administration, blocked the second-phase formalin test (Luccarini et al., 2003). On the other hand, Leite et al. (Leite et al., 2012) have shown that BIS does not appear to act as a TRPV1 agonist but may possibly induce a modulatory influence on other vanilloid-receptors.

Waning et al. (Waning et al., 2007) demonstrated that the capsaicin-sensitive transient receptor potential vaniloid 1 (TRPV1), which plays an important role in pain transduction, is one of the Ca^{+2} influx channels involved in cell migration. It was also shown that TRPV1 activation increases the expression of the TNF receptor 1 (TNFR1) by a ROS-dependent mechanism (Ma et al., 2009). Thus, the antinociceptive effect demonstrated by EOSl and BIS in the capsaicin test seems to be a result of its possible inhibition of neurotransmitters involved in the activation of sensory fibers responsible for the transmission of nociceptive stimuli.

Additionally, the results also showed that oral administration of EOSl (50 mg/kg) and BIS (25 and 50 mg/kg) produced a significant (53.00 ± 5.057; $p<0.05$, 40.00 ± 7.990; $p<0.001$, 34.50 ± 4.527; $p<0.001$, respectively) inhibition of nociceptive behavior induced by injection of glutamate into the right upper limb when compared to the control group (84.00 ± 9.580) (Fig. 5).

Glutamate is present in both the central and peripheral terminals of trigeminal and dorsal root ganglion neurons. Noxious stimulation of primary afferent fibers results in the release of glutamate from the peripheral as well as the central terminals of trigeminal and spinal afferent fibers (Keast and Stephensen, 2000; Lam et al., 2005). This nociceptive response caused by glutamate seems to involve peripheral, spinal, and supra-spinal sites. Moreover, it has been reported that glutamate injection evoked pronounced nociceptive responses, which are mediated by neuropeptides (Substance P) released from C fibers, and activate glutamate receptors (e.g. the NMDA receptor). This can stimulate the production of a variety of intracellular second messengers,

such as Nitric Oxide (NO) and pro-inflammatory cytokines, including TNF-α and IL-1β, which act synergistically in the excitation of the neurons (Beirith et al., 2002b).

Moreover, Leite et al. (Leite et al., 2012) observed that when mice were pretreated with L-NAME, a nitric oxide synthase inhibitor and the 5-HT_3 antagonist, ondansetron, apparently a potentiated/ additive response occurred, which needs further analysis. Studies have demonstrated that the excitatory amino acid receptors are involved in nociceptive primary afferent transmission, both in the development and maintenance of painful responses (Carlton et al., 2001; Coggeshall and Carlton, 1997). Thus, the inhibition of glutamate-induced nociception by EOSl and BIS treatment can be associated with its interaction with the glutamatergic system.

Previous studies have suggested that CNS depression and nonspecific muscle relaxation effects can reduce the response of motor coordination which might invalidate the results found in behavior testes (de Almeida et al., 2004; de Sousa et al., 2006). Thus, we sought to demonstrate that the doses used in our study were unable to produce any motor deficit. Our results demonstrated that the analgesic effect is not related to any motor abnormality since EOSl- or BIS-treated mice exhibited no performance alteration on the grip strength meter when compared to the control group (Fig. 6). Interestingly EOSl-treated animals demonstrated a significant increase in strength in the grip test, which may be related to a direct effect on skeletal muscle or because of direct stimulation of the CNS (Suokas et al., 2014).

As some of our results suggested an anti-inflammatory profile for EOSl or BIS, we sought to characterize it better using the carrageenan-induced pleurisy test. EOSl or BIS exhibited a significant anti-inflammatory effect ($p<0.001$) in the pleurisy test (Fig. 7). Moreover, this effect is related to inhibition of the pro-inflammatory cytokines TNF-α and IL-1β as EOSl (50 mg/kg) produced a significant reduction ($p<0.05$ and $p<0.01$) in both cytokines. However, although BIS-treated mice had a significantly reduced ($p<0.05$) TNF-α level in pleural inflammatory exudate, it was ineffective in regards to IL-1β (Fig. 8 e 9).

Cell recruitment during inflammation depends on the orchestrated release of local mediators that are responsible for local vascular and tissue changes as well as for the recruitment of host defense cells (Luster et al., 2005). The inflammation induced by carrageenan involves cell migration, plasma exsudation and production of mediators, such as nitric oxide, prostaglandin E2, IL-1β, IL-6 and TNF-α (Loram et al., 2007a, 2007b). These mediators have been shown to be able to recruit leukocytes, such as neutrophils, in several experimental models. Since EOSl and BIS inhibited the leukocyte migration induced by the injection of carrageenan, this suggests that these activities may inhibit the synthesis of many pro-inflammatory mediators whose involvement in cell migration is well established. So, this seems to corroborate the use of *Stachys lavandulifolia* in folk medicine to treat inflammatory processes, which are related to the formation of plasma exsudation and production of mediators (Rahzani et al., 2013).

Cytokines are small regulatory proteins produced by white blood cells and a variety of other cell types. Currently, it is widely accepted that cytokines constitute a link between cellular injuries and signs of inflammation (Dinarello, 1998; Faccioli et al., 1990; Verri et al., 2006). IL-1β is a pro-inflammatory cytokine that induces the production of other inflammatory mediators involved with cellular recruitment, fever, acute phase protein release, increase of vascular permeability, and hyperalgesia (Dinarello, 1998). Considering the important role of IL-1β during the inflammatory response, it may be suggested that the anti-inflammatory effect of EOSl, but not that of BIS, is related to an ability to inhibit this cytokine. As previously described by Maurya et al (Maurya et al., 2014) BIS is able to inhibit 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced production of pro-inflammatory cytokines (TNF-α and IL-6) in macrophage cells. In addition, It is known that IL-1β release precedes the production of the final inflammatory mediators, such as prostaglandins (Cunha et al., 2005; Verri et al., 2006). Both IL-1β and TNF-α up-regulated COX-2 protein expression and the subsequent production of prostaglandins (Chang et al., 2003). Based on the relevant anti-inflammatory action of non-steroidal anti-inflammatory drugs and their

cyclooxygenase inhibitory effect, it is accepted that prostaglandins are important contributors to inflammatory response. Since the inhibition of IL-1β and TNF-α release might lead to the inhibition of prostanoid production, it is possible that EOSl and BIS act by preventing the production of the final mediators of inflammation.

Corroborating this hypothesis, the effect of BIS on PGE_2 levels and COX-2 expression were demonstrated by Kim et al. (Kim et al., 2011). They demonstrated that BIS can reduce the expression of cyclooxygenase-2 (COX-2) and the PGE_2 levels in the paw after complete Freund's adjuvant (CFA) stimuli. Since the COX-2/PGE_2 pathway is a key event during different inflammatory conditions, the reduction of PGE2 levels and COX-2 expression can be responsible for the anti-inflammatory effects of BIS, which certainly contributes to the anti-inflammatory effect of EOSl.

Taken together, the results presented herein strongly suggest that *S. lavandulifolia* essential oil and BIS modulate neuropathic and inflammatory pain in the test models of orofacial pain induced by glutamate, capsaicin and formalin. The precise mechanisms through which EOSl and BIS exerts their action are currently under investigation, but could be related to modulation of pro-inflammatory molecule production. Thus, our results corroborate the traditional medicine use of *S. lavandulifolia* as an analgesic and anti-inflammatory, which seems to be related to the (-)-α-Bisabolol, the main compound of EOSl. All these findings also suggest that EOSl and BIS might represent important tools for the management of orofacial pain and inflammatory disorders.

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TABLES

Table 1. Volatile components in essential oil of *S. lavandulifolia*

No	RI[a]	Compound	%	Identification Method
1	1215	β-Phellandrene	0.1	RI,MS
2	1500	Pentadecane	0.2	RI,MS
3	1501	α-Copaene	1.3	RI,MS
4	1531	β-Bourbonene	0.5	RI,MS
5	1600	Hexadecane	0.3	RI,MS
6	1601	β-Elemene	0.1	RI,MS
7	1614	β-Caryophyllene	1.0	RI,MS
8	1670	(Z)- β-Farnesene	0.8	RI,MS
9	1700	Heptadecane	0.3	RI,MS
10	1704	γ-Muurolene	0.2	RI,MS
11	1706	p-Menth-1-en-8-ol	0.1	RI,MS
12	1715	Borneol	0.1	RI,MS
13	1729	Germacrene D	2.3	RI,MS
14	1738	β-Bisabolone	1.9	RI,MS
15	1757	Bicyclogermacrene	5.3	RI,MS
16	1776	δ-Cadinene	4.2	RI,MS
17	1786	(E)-α-Bisabolone	0.2	RI,MS
18	1800	Octadecane	0.2	RI,MS
19	1802	Selina-3,7(11)-diene	1.8	RI,MS
20	1900	Nonadecane	0.4	RI,MS
21	1943	α-Calacorene	0.1	RI,MS
22	2000	Eicosane	0.5	RI,MS
23	2100	Heneicosane	1.0	RI,MS
24	2147	Spathulenol	2.9	RI,MS
25	2153	Valeranone	1.0	RI,MS
26	2166	α-Bisabolol oxide B	0.7	RI,MS
27	2200	Docosane	0.8	RI,MS
28	2237	α-Bisabolol	**56.4**	RI,MS
29	2248	α-Eudesmol	0.8	RI,MS
30	2254	α-Cadinol	1.4	RI,MS
31	2258	β–Eudesmol	0.8	RI,MS
32	2300	Tricosane	0.9	RI,MS
33	2400	Tetracosane	0.1	RI,MS
34	2500	Pentacosane	0.6	RI,MS
		Total identified	**89.9**	

[a]Retention index relative to n-alkanes on HP-innowax capillary column

FIGURES

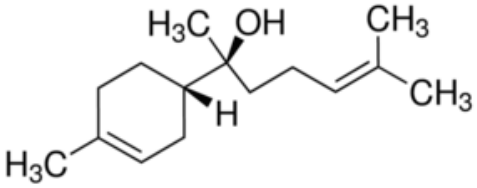

Figure 1. Molecular structure of (-)-α-bisabolol (*IUPAC name*: 6-methyl-2-(4-methylcyclohex-3-en-1-yl)hept-5-en-2-ol).

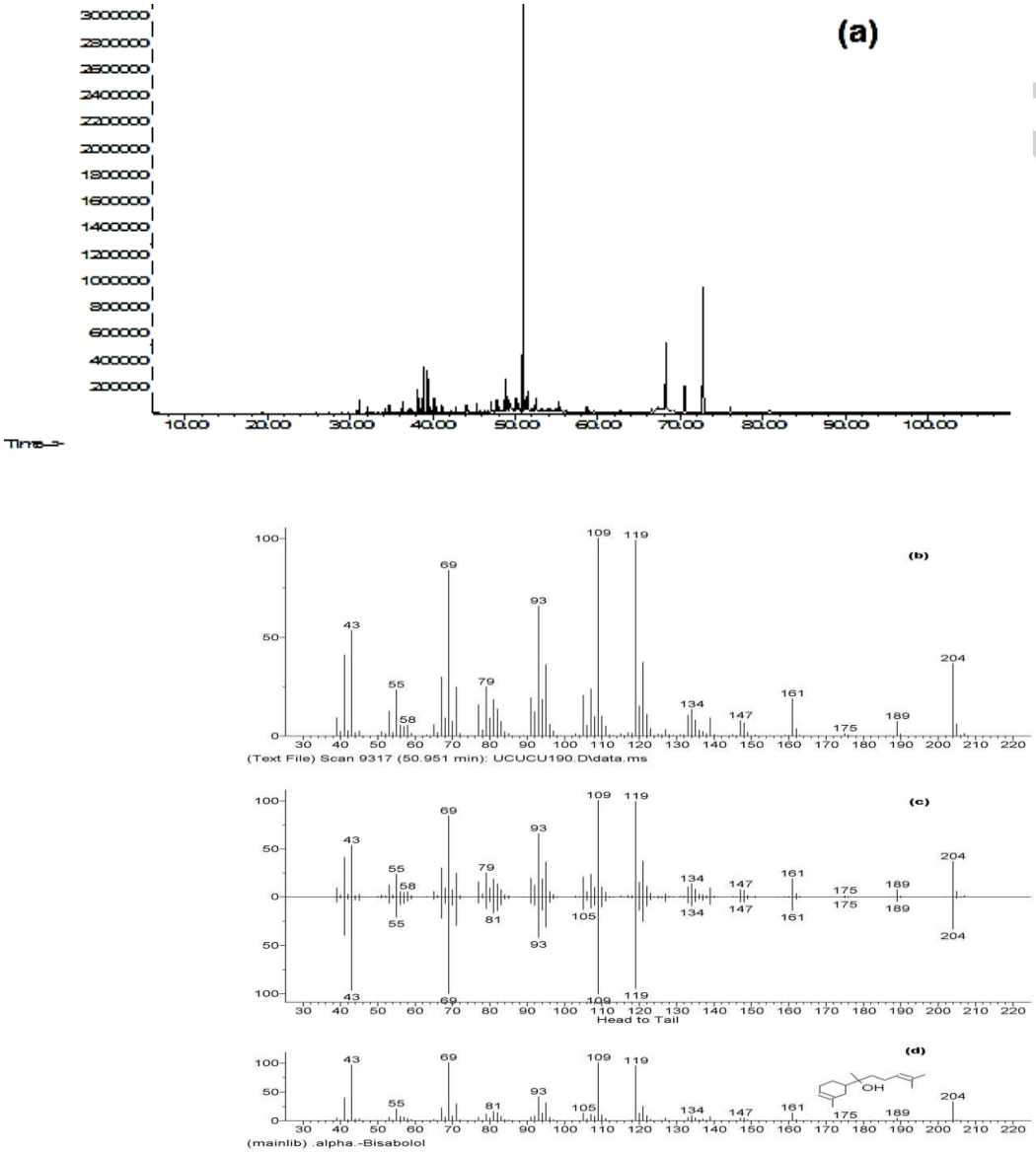

Figure 2. Total ion chromatogram of EOSl (a) and the mass spectral data for α-bisabolol (in the EOSl (b), the library match (c) and in the NIST library (d))

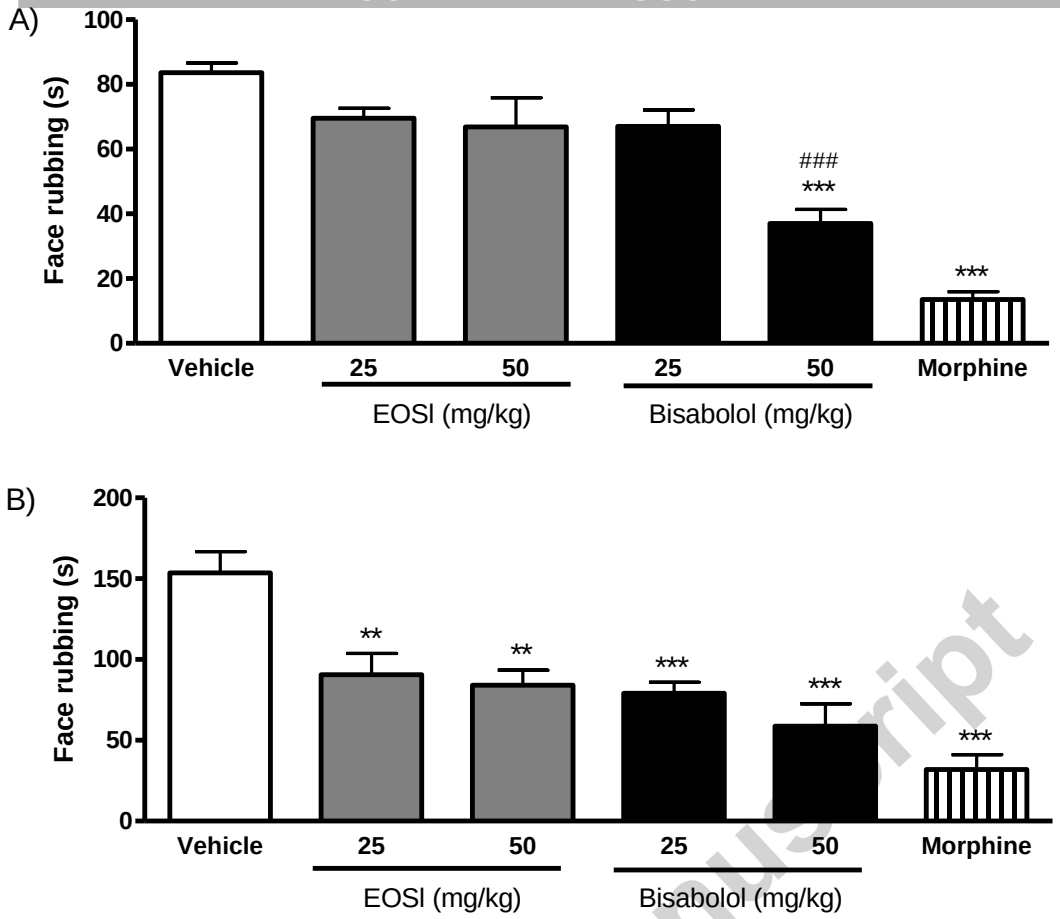

Figure 3. Effects of *S. lavandulifolia* essential oil (EOSl) and (-)-α-Bisabolol (BIS) on formalin-induced orofacial nociceptive behavior. Vehicle (control), EOSl (25 and 50 mg/kg), BIS (25 and 50 mg/kg) or MOR (3 mg/kg) were administered orally or intraperitoneally 1 or 0.5 hr before formalin injection. (A) First phase (0–5 min.) and (B) second phase (15–40 min.) of the formalin test. Values represent mean ± S.E.M. (n=6, per group). **p<0.01; ***p<0.001 versus control $^{###}$p<0.001 versus EOSl (25 mg/kg) (one-way ANOVA followed by Tukey's test).

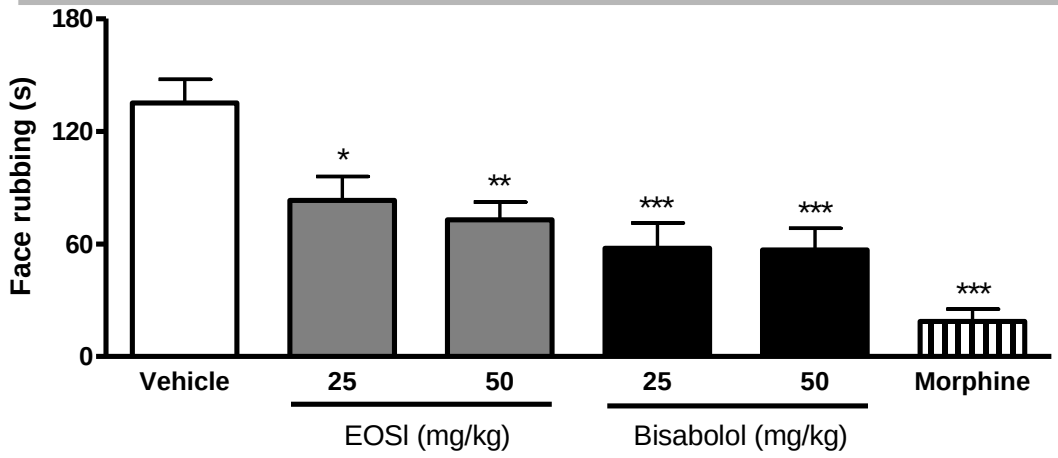

Figure 4. Effects of *S. lavandulifolia* essential oil (EOSl) and (-)-α-Bisabolol (BIS) on capsaicin-induced orofacial nociceptive behaviour. Vehicle (control), EOSl (25 and 50 mg/kg), BIS (25 and 50 mg/kg) or MOR (3 mg/kg) were administered orally or intraperitoneally 1 or 0.5 hr before capsaicin injection. Values represent mean ± S.E.M. (n=6, per group). *p<0.05; **p<0.01; ***p<0.001 versus control (one-way ANOVA followed by Tukey's test).

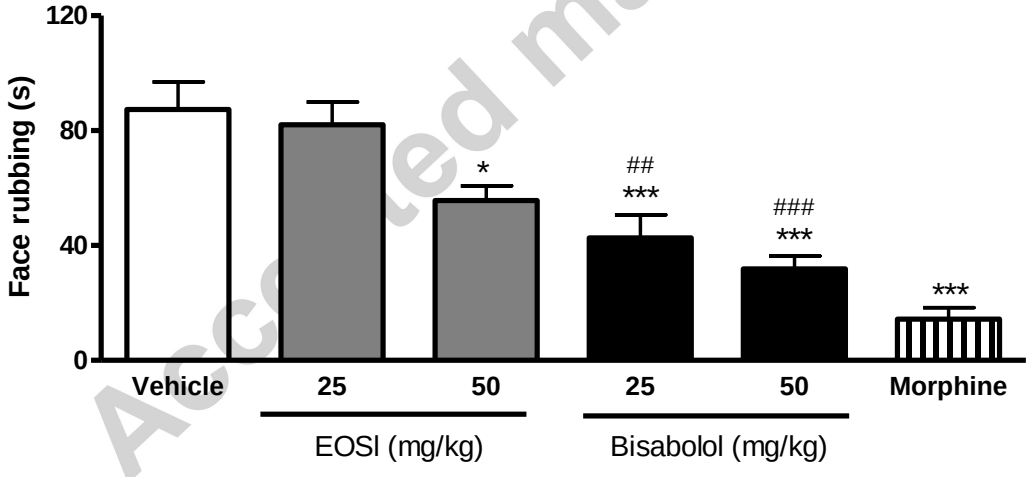

Figure 5. Effects of *S. lavandulifolia* essential oil (EOSl) and (-)-α-Bisabolol (BIS) on glutamate-induced orofacial nociceptive behaviour. Vehicle (control), EOSl (25 and 50 mg/kg), BIS (25 and 50 mg/kg) or MOR (3 mg/kg) were administered orally or intraperitoneally 1 or 0.5 hr before formalin injection. Values represent mean ± S.E.M. (n=6, per group). *p<0.05; ***p<0.001 versus control $^{\#\#}$p<0.01; $^{\#\#\#}$p<0.001 versus EOSl (25 mg/kg) (one-way ANOVA followed by Tukey's test).

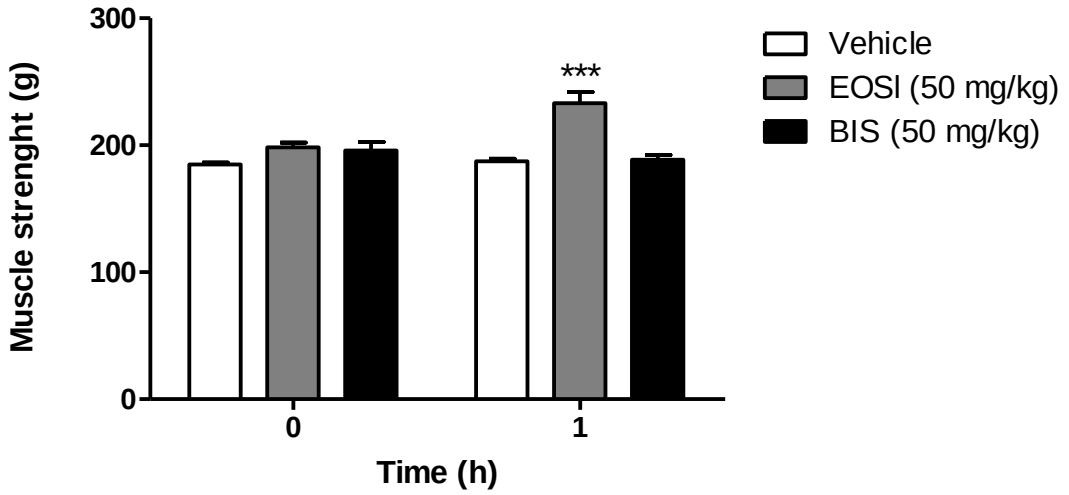

Figure 6. Effects of *S. lavandulifolia* essential oil (EOSl) and (-)-α-Bisabolol (BIS) on grip strength meter. Vehicle (control), EOSl (50 mg/kg) or BIS (50 mg/kg) were administered orally 1 h before. Values represent mean ± S.E.M. (n=6, per group). ***p<0.001 versus control (one-way ANOVA followed by Tukey's test).

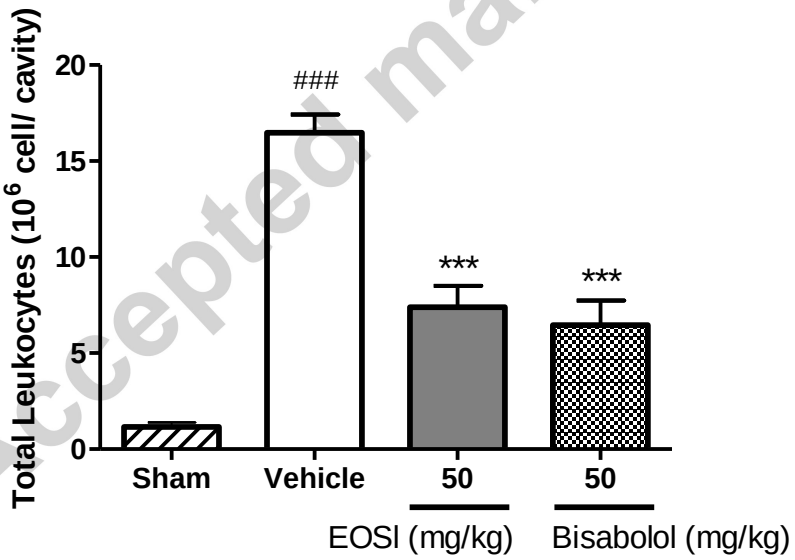

Figure 7. Effects of *S. lavandulifolia* essential oil (EOSl) and (-)-α-Bisabolol (BIS) on carrageenan-induced pleurisy. Vehicle (control), EOSl (50 mg/kg) or BIS (50 mg/kg) were administered orally 1 h before carrageenan injection. Values represent mean ± S.E.M. (n=6, per group). ***p<0.001 versus control, ###p<0.001 versus sham (one-way ANOVA followed by Tukey's test).

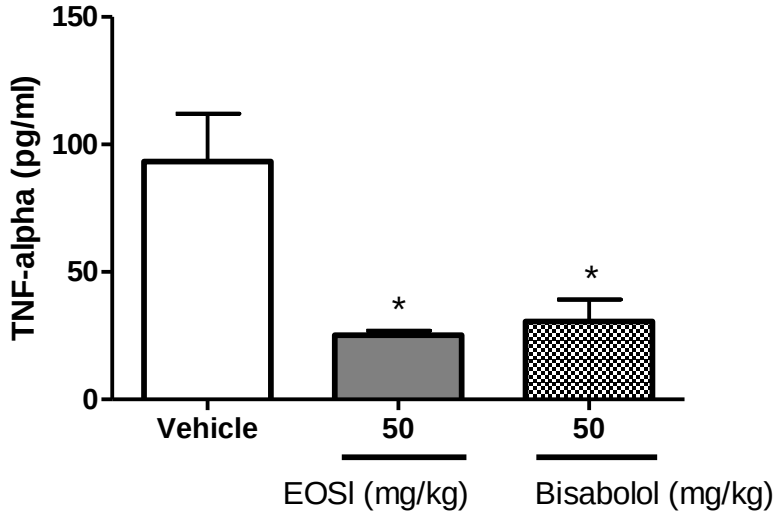

Figure 8. Effects of *S. lavandulifolia* essential oil (EOSl) and (-)-α-Bisabolol (BIS) on TNF-alpha levels. Vehicle (control), EOSl (50 mg/kg) or BIS (50 mg/kg) were administered orally 1 h before carrageenan injection. Values represent mean ± S.E.M. (n=6, per group). *p<0.05 versus control (one-way ANOVA followed by Tukey's test).

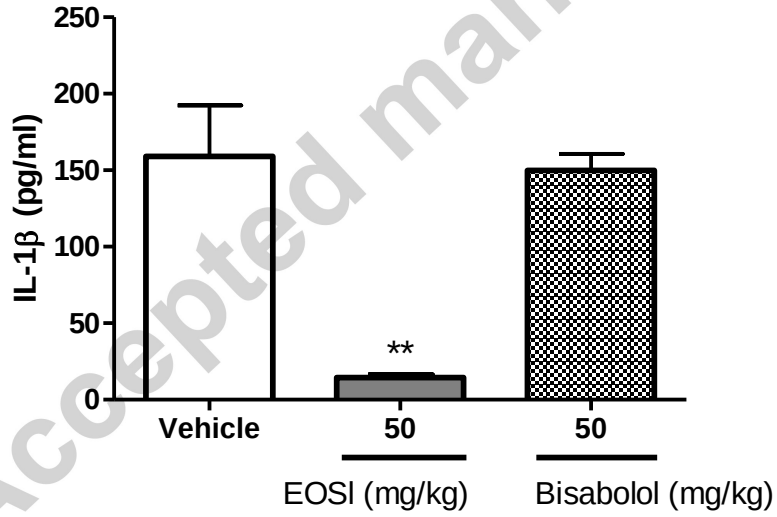

Figure 9. Effects of *S. lavandulifolia* essential oil (EOSl) and (-)-α-Bisabolol (BIS) on IL-1beta levels. Vehicle (control), EOSl (50 mg/kg) or BIS (50 mg/kg) were administered orally 1 h before carrageenan injection. Values represent mean ± S.E.M. (n=6, per group). **p<0.01 versus control (one-way ANOVA followed by Tukey's test).

Graphical abstract