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Artículo Original / Original Article

Antinociceptive and anti-inflammatory effect of *Lippia pedunculosa* Hayek essential oil and its β-cyclodextrin inclusion complex

[Efecto antinociceptivo y antiinflamatorio del aceite esencial de *Lippia pedunculosa* Hayek y su complejo de inclusión de β-ciclodextrina]

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Abstract: *Lippia pedunculosa* Hayek (EOLp) presents tripanocid and amebicid effects. However essential oil needs to be further studied in experimental models of analgesia and inflammation once the prevalence of pain in the population generates great suffering and disability and the drugs most often used have undesirable side effects. We also evaluated whether the inclusion complex formulation EOLp/β-cyclodextrin (β-CD) was able to improve the antinociceptive activity of the EOLp alone. Data were evaluated by analysis of variance (ANOVA), followed by Tukey's test. Differences were considered significant if $p<0.05$. EOLp presented better antinociceptive effect when compared to the EOLp/β-CD inclusion complex. Thus, cyclodextrins appear not to be efficient for essential oils with peroxide substances. However, in peritonitis, EOLp reduced total leucocyte migration and IL-1β levels in the peritoneal fluid, which confirmed its anti-inflammatory effect. The observed effects suggest that EOLp is the best promising option for the treatment of inflammation and pain-related disorders.

Keywords: Essential oil; *Lippia pedunculosa*; Cyclodextrin; Pain; Inflammation.

Resumen: *Lippia pedunculosa* Hayek (EOLp) presenta efectos tripanocidas y amebicidas. En este trabajo se estudia su aceite esencial en modelos experimentales de analgesia e inflamación una vez que la prevalencia del dolor en la población genera un gran sufrimiento y discapacidad, y los medicamentos que se usan con mayor frecuencia tienen efectos secundarios indeseables. También se evalúa si la formulación del complejo de inclusión EOLp/β-ciclodextrina (β-CD) fue capaz de mejorar la actividad antinociceptiva de la EOLp sola. Los datos se evaluaron mediante análisis de varianza (ANOVA), seguido de la prueba de Tukey. Las diferencias se consideraron significativas si $p<0,05$. EOLp presentó un mejor efecto antinociceptivo en comparación con el complejo de inclusión EOLp/β-CD. De esta manera, las ciclodextrinas parecen no ser eficientes para aceites esenciales con sustancias de peróxido. Sin embargo, en peritonitis, EOLp redujo la migración total de leucocitos y los niveles de IL-1β en el líquido peritoneal, lo que confirma su efecto antiinflamatorio. Los efectos observados sugieren que EOLp es una buena y prometedora opción para el tratamiento de la inflamación y los trastornos relacionados con el dolor.

Palabras clave: Aceite esencial; *Lippia pedunculosa*; Ciclodextrina; Dolor; Inflamación

INTRODUCTION

In most diseases such as autoimmune, gastrointestinal, neurodegenerative, and respiratory diseases, cancers, and infections; inflammation and pain are both cause and consequence. To relieve pain and to turn the treatment of disease into a less traumatic process is critical, and in the pharmaceutical search for adequate solutions, has a long history. The drugs most often used to treat pain and inflammation, despite their well-known adverse effects, are the non-steroidal anti-inflammatory (NSAIDs), and opioids (Pang *et al*., 2012; Lenardão *et al*., 2016). This situation has inspired researches with essential oils and their components which could become new analgesic medicines to treatment for painful disorders (De Sousa, 2011; Guimarães *et al*., 2013), including orofacial pain (Damasceno *et al*., 2016; Tomaz-Morais *et al*., 2017; Melo *et al*., 2017; Siqueira-Lima *et al*., 2017).

Essential oils (EOs) and monoterpenes present a series of limitations like low solubility in water, slow dissolution rate, instability, and short half-life (Siqueira-Lima *et al*., 2014). Yet, formation of cyclodextrin (CD) complexes has been successfully used to improve the therapeutic properties of non-polar natural products (Pinho *et al*., 2014; de Oliveira *et al*., 2015; Rodrigues *et al*., 2017). CDs are cyclic oligosaccharides composed of either: 6, 7, 8, or 9 glucopyranose units, respectively (α-, β-, γ- or δ-CD). They present a relatively hydrophilic surface and a lipophilic central cavity (Li & Loh, 2008; Hwang *et al*., 2012; Andrade *et al*., 2017). CDs are an important new class of pharmaceutical excipients, which promotes the formation of inclusion complexes with these molecules, which has improved stability, solubility and bioavailability of nonpolar substances, as well as transforming essential oils (liquids) into powders of easy dispersion in water, facilitating the control of its volatility (Siqueira-Lima *et al*., 2014).

β-cyclodextrins (β-CD(s)) have been extensively used to form inclusion complexes with EOs from: *O. basilicum (*Nascimento *et al*., 2014), *Lavandula viridis, Lavandula pedunculata, Thymus lotocephalus* (Costa *et al*., 2015), *Hyptis pectinata* (Menezes *et al*., 2015), *Cymbopogon winterianus* (Santos *et al*., 2015), and *Lippia grata* (Siqueira-Lima *et al*., 2014), and thus improve their analgesic profile.

The genus *Lippia* (Verbenaceae) is distributed across the South and Central Americas and tropical Africa (Terblanché & Kornelius, 1996). There are almost 200 species, some of which are already used to promote analgesic and anti-inflammatory effects (Pascual *et al.*, 2001; Siqueira-Lima *et al*., 2014). *Lippia pedunculosa* Hayek, popularly known as "*Pai-pedro*", is a shrub found in the northeast (Alagoas and Sergipe), and southeastern (São Paulo) regions of Brazil (Santos, *et al*., 2009; Silva *et al.*, 2013). Certain biological properties of the essential oil from the leaves of *Lippia pedunculosa* (EOLp) have already been described. *In vitro* assays have demonstrated promising trypanocidal (Menezes *et al*., 2014) and amoebacidal activity (de Amorim Santos *et al*., 2016), this due to the presence of the monoterpenes rotundifolone (71.7%) and (R)–limonene (21.8%); the principal compounds in EOLp (Santos, *et al*. 2014). Several studies have attempted to improve the pharmacological profile of *Lippia* species essential oils using a β-CD inclusion complexes (Fernandes *et al*., 2004; Marreto *et al*., 2008; Arana-Sánchez *et al*., 2010; Siqueira-Lima *et al*., 2014). Our collaborators started the evaluation of the analgesic profile of EOLp with a preliminary formalin test which indicated a possible antinociceptive effect (Menezes *et al*., 2018).

So, the aim of this study was to confirm the antinociceptive and anti-inflammatory effect of EOLp and a β-CD inclusion complex containing *L. pedunculosa* leaf essential oil (EOLp/β-CD) in experimental protocols of pain and inflammation in mice and investigate whether EOLp/β-CD complexing improves the pharmacological activity of isolated EOLp as well as evaluating potential pain modulation interactions involving important neurotransmitter pathways.

MATERIALS AND METHODS

Plant material and essential oil

Fresh *L. pedunculosa* leaves were collected in October 2013 in *Poço Redondo*, Sergipe, in northeastern Brazil (S 09º 40' 46" W 37º 39' 41"). *L. pedunculosa* was identified by Dr. Ana Paula do Nascimento Prata (Federal University of Sergipe - Herbarium, HFUS); a voucher specimen has been deposited (HFUS 23159).

Pharmacological Procedures

Animals

Male Swiss mice (25–35 g), 2–3 months of age were used. They were randomly kept in appropriate cages at 21 ± 2°C in a 12-hr light/dark cycle (light from 06:00 am to 06:00 pm) with free access to food

(Purina®, São Paulo, SP, Brazil), and water. All experiments were carried out between 01:00 pm and 05:00 pm, nociception tests were performed by the same visual observer in a double-blind study.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Experimental protocols were approved by the Animal Care and Use Committee of the Federal University of Paraíba (0159/2015)**.**

Evaluation of motor activity

The motor activity was evaluated in a rota-rod apparatus (Dunham & Miya, 1957). Initially, mice were selected 24 h previously by eliminating animals unable to remain on the rota-rod apparatus (Insight ©, Ribeirão Preto, SP, Brazil) for a period of 60s (7 rpm). Then, the selected animals were then divided into four groups (n = 6, per group) and treated p.o. with vehicle, EOLp, or EOLp/β-CD (400 mg/kg, p.o.). At 1, 1.5, and 2 h each animal was placed on a rota-rod apparatus, and the time (in seconds) completed on the bar was measured for up to 180 sec.

Acetic acid-induced writhing

This study was performed according to Koster & Anderson (1959), Mice (n=6, per group) were pre-treated with EOLp (200, 300, 400 mg/kg, p.o.), EOLp/β-CD (200, 300, 400 mg/kg, p.o.), morphine (6 mg/kg, i.p.), or the EOLp dilution vehicle (Tween-80 5%, p.o.). After 1 h, the mice received a 1% acetic acid injection (i.p.), which caused writhing characterized by extension of the hind limbs and contraction of the abdominal muscles. The number of abdominal writhes was observed for a period of 15 min; after a latency period of 5 min.

Formalin-induced nociception

The procedure described by Hunskaar & Hole (1987) was used. Nociception was induced by injecting (s.c.) 20 μL of 2% formalin in the subplantar region of the right hind paw. The mice (n=6, per group) had previously received EOLp (200, 300, 400 mg/kg, p.o.), EOLp/β-CD (200, 300, 400 mg/kg, p.o.), morphine (6 mg/kg, i.p.), or vehicle 1h prior to the injection of formalin. They were individually placed in mirrored chambers (25 X 25 X 25 cm), and the time (in seconds) spent licking their injected paws was immediately recorded. Nociception was quantified for 5 min after formalin injection (first phase, neurogenic) and again at 15-30 min (second phase, inflammatory).

Formalin, glutamate, and capsaicin-induced orofacial nociception

Orofacial nociception protocols were performed on the mice by s.c. injection of 20 μL 2% formalin, 40 μl of glutamate (25 mM), or capsaicin (20 μL, 2.5 μg) into the right upper lip (perinasal area) using a 27-gauge needle. Mice (n = 6, per group) were treated with either vehicle, EOLp, EOLp/β-CD (300 mg/kg, p.o.), or morphine (6 mg/kg, i.p.) at 1h before specific algogen administrations. Formalin test: The neurogenic phase occurs within 0–5 min after the administration of formalin and is followed by a latency period of about 10 min. An inflammatory phase occurs within 15-40 min after formalin administration. Glutamate test: Mice were observed individually for 15 min following glutamate injection. Capsaicin test: Animals were assessed during the 10-20 min after injection of capsaicin. The animals were observed individually in mirrored chambers (25 X 25 X 25 cm) to allow an unobstructed view of the orofacial region. For the three tests, the nociceptive behavior was assessed during the period of time in which the animals continued rubbing the orofacial region.

Hot Plate Test

The animals were pre-selected, and those considered suitable presented a pain response time of less than 10s when placed on the hot plate apparatus (Insight ©, Ribeirão Preto, SP, Brazil) at 55 ± 1°C (Eddy & Leimbach, 1953). The selected mice were pre-treated with vehicle, EOLp, EOLp/β-CD (400 mg/kg, p.o.), or morphine (6 mg/kg, i.p.); and they were individually placed on the hot plate at 0.5, 1, and 2h after initial treatment. The registered parameter was the latency time to jump or lick the hind paws. In order to minimize animal paw tissue destruction, time on the plate did not exceed 30s.

Carrageenan-induced peritonitis test

Leukocyte migration was induced by intraperitoneal administration of carrageenan (1%, 300 μL) in mice (n=6) treated 1h earlier with either vehicle, EOLp (200, 300, and 400 mg/kg, p.o.), or dexamethasone (2 mg/kg, i.p.). Four hours after administration of the stimulus, the mice were euthanized, and the

peritoneal cavity was washed with 2 mL of saline. From the peritoneal fluid an aliquot was removed for a total leukocyte count. The results were expressed as the number of leukocytes/mL.

Dosage of TNF-α and IL-1β in the peritoneal fluid

Four hours after carrageenan administration to groups pre-treated with vehicle, EOLp (200, 300 and 400 mg/kg, p.o.), or dexamethasone (2 mg/kg, i.p.), peritoneal liquid was removed and centrifuged at 1500 rpm/5 min/4°C. The supernatant was removed for measurement of TNF-α and IL-1β using sandwich cytokine ELISA performed according to the manufacturer's instructions (Boster Biological Technology CO., Ltd, Pleasanton, CA, USA). The cytokine levels were calculated from standard curves and expressed as total quantity per milliliter (pg/mL).

Statistical analysis

The data obtained were evaluated using the GraphPad Prism (v 4.00) software (San Diego, CA, USA); by one and two-way analysis of variance (ANOVA); followed by Tukey's test. Differences were considered significant if $p<0.05$.

RESULTS

EOLp effect on motor coordination

Treatment with EOLp (200, 300, and 400 mg/kg, p.o.) did not alter the rotating bar permanence times: observed at 60 (178.8 ± 0.8; 146.3 ± 19.3; 157.5 ± 14.7s), 120 (174.5 ± 3.6; 152.0 ± 11.4; 173.0 ± 3.5s), and 180 (176.3 ± 2.4; 176.0 ± 1.4; 150.5 ± 11.1s) minutes from initial treatment; as compared to the controls (180.0 ± 0.0; 176.0 ± 1.6; 171.0 ± 3.4s)(Figure Nº 1).

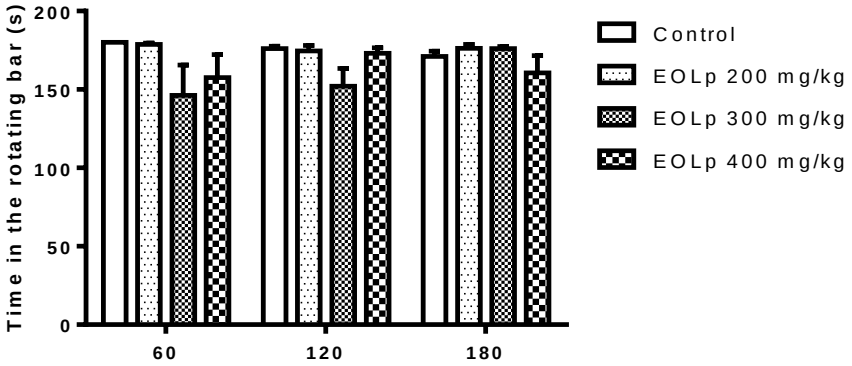


Figure Nº 1

Effect of EOLp administration on motor coordination. Mice were pretreated with vehicle, or EOLp (200, 300 and 400 mg/kg, p.o.); and at 60, 120 and 180 min later were placed on a rotating rod. Each column represents the mean ± S.E.M. (n = 6). Statistical analysis: ANOVA "one-way", followed by Tukey's test

EOLP and EOLp/β-CD effects on acetic acid test induced contortions

EOLp in doses of 300 and 400 mg/kg presented a significant reduction (11.3 ± 1.7; 5.7 ± 2.2) in the number of writhings induced by acetic acid as compared to the control group (34.5 ± 2.0). EOLp/β-CD did not reduce contortions at any of the tested doses. Morphine, as expected decreased the number of writhes (2.0 ± 0.5) (Figure Nº 2).

EOLP and EOLp/β-CD effects on formalin-induced nociception

The EOLp in doses of 300 (47.1 ± 4.7) and 400 (21.1 ± 7.2) mg/kg presented a significant reduction in the paw licking time for the first phase when compared to the control group (74.5 ± 3.1). Morphine (6 mg/kg), as expected decreased the same parameter (21.7 ± 2.4) (Figure Nº 3.1). During the second phase of the test, EOLp in doses of 300 (20.1 ± 7.2) and 400 (0.2 ± 0.1) mg/kg also reduced paw licking time significantly when compared to the control group (123.9 ± 8.4). Morphine (6 mg/kg), as expected decreased the same parameter (26.0 ± 5.1) (Figure Nº 3.2). The animals treated with EOLp (200 mg/kg), and EOLp/β-CD (at all doses) did not present reduced paw licking times.

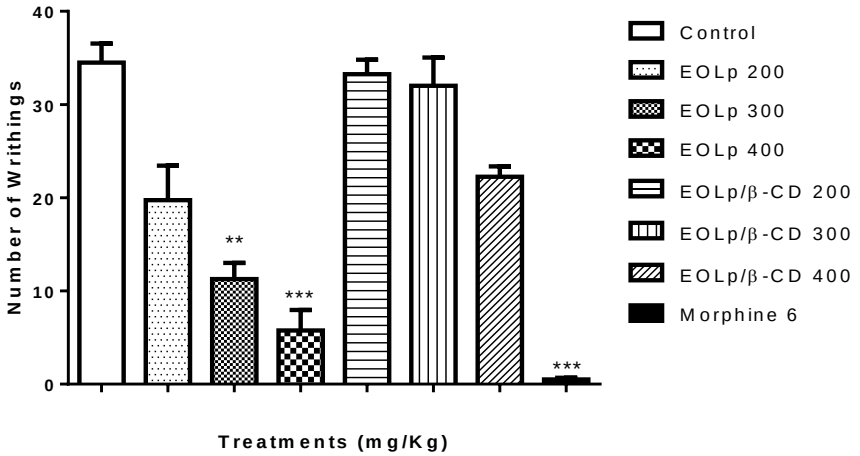


Figure Nº 2

Effect of EOLp and EOLp/β-CD treatment on abdominal contortions induced by acetic acid tests. Mice were pretreated with either vehicle, EOLp (200, 300 and 400 mg/kg, p.o.), EOLp/β-CD (200, 300 and 400 mg/kg, p.o.), or morphine (6 mg/kg, i.p.). Each column represents mean ± S.E.M. (n = 6) (ANOVA "one-way"– Kruskal-Wallis followed by Dunn's test) ** *p*<0.01, ***p<0.001 vs control

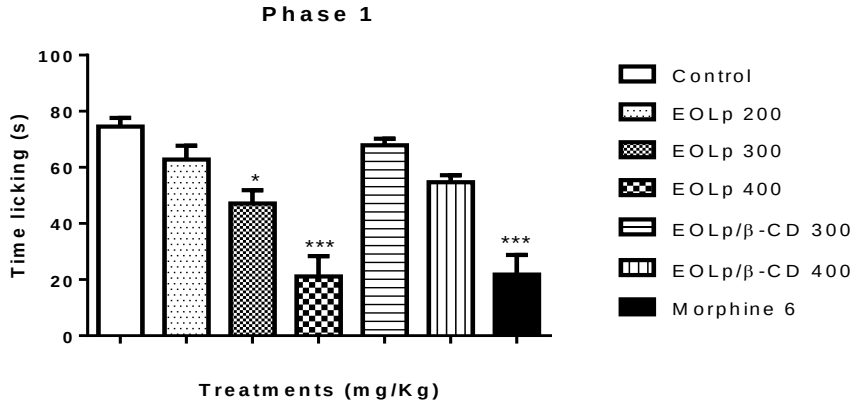


Figure Nº 3.1

Effect of EOLp and EOLp/β-CD treatments on the first phase of the formalin-induced nociception test. Mice were pretreated with vehicle, EOLp (200, 300 and 400 mg/kg, p.o.), EOLp/ β-CD (200, 300 and 400 mg/kg, p.o.), or morphine (6mg/kg, i.p.). Each column represents mean ± S.E.M. (n = 6) (ANOVA "one-way"– Kruskal-Wallis followed by Dunn's test) * *p*<0.05, ***p<0.001 vs control

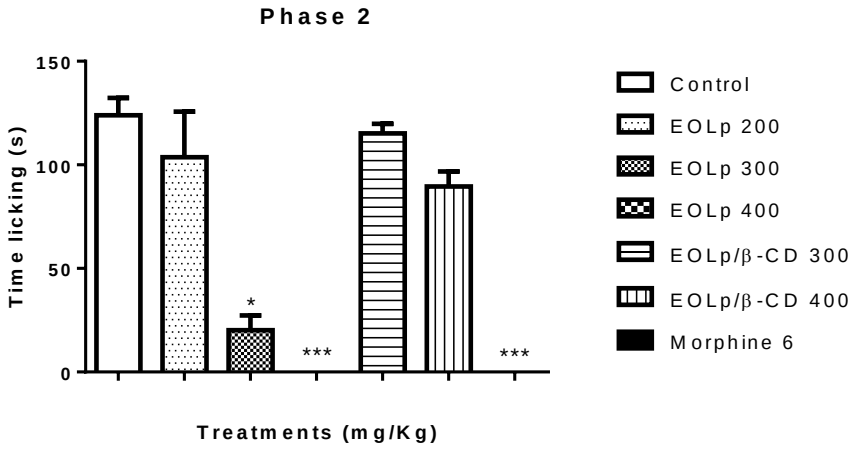


Figure Nº 3.2

Effect of EOLp and EOLp/β-CD treatment on the second phase of the formalin-induced nociception test. Mice were pretreated with vehicle, EOLp (200, 300 and 400 mg/kg, p.o.), EOLp/β-CD (200, 300 and 400 mg/kg, p.o.), or morphine (6 mg/kg, i.p.). Each column represents mean ± S.E.M. (n = 6) (ANOVA "one-way"– Kruskal-Wallis followed by Dunn's test) *p<0.05, ***p<0.001 vs control

Formalin, glutamate, and capsaicin-induced orofacial nociception

Administration of EOLp (300 mg/kg) produced a reduction in face-rubbing behavior induced by formalin in both phases (p<0.05; p<0.001), glutamate (p<0.01), and capsaicin (p<0.001), when compared with the control groups (Table Nº 1). Conversely, these outcomes were not observed for EOLp/β-CD; which was unable to inhibit formalin, glutamate, or capsaicin-induced orofacial nociception behavior. As expected, morphine was able to reduce nociceptive behavior in all analyses.

Table Nº 1
Effect of EOLp, EOLp/β-CD, and morphine on formalin, glutamate, and capsaicin-induced orofacial nociception.

Treatment	Dose (mg/kg)	Formalin test		Glutamate test[1]	Capsaicin test[1]
		0-5min[1]	15-40min[1]		
Control	-	59.1 ± 3.9	101.8 ± 9.4	70.9 ± 3.5	103.0 ± 3.6
EOLp	300	15.6 ± 5.2[a]	27.0 ± 5.0[c]	18.4 ± 4.2[b]	5.1 ± 1.4[c]
EOLp/β-CD	300	23.1 ± 6.4	85.1 ± 12.3	40.6 ± 7.3	57.2 ± 8.6
Morphine	6	5.5 ± 3.2[c]	2.1 ± 0.5[c]	7.6 ± 0.8[c]	3.4 ± 1.0[c]

[1]Values represent mean ± S.E.M.

[a]p< 0.05; [b]p<0.01 [c]p<0.001 Kruskal-Wallis followed by Dunn's test), significantly different from control group

EOLP and EOLp/β-CD effect - hot plate test

After treatment with EOLp the results at doses of 200, 300, and 400 mg/kg did not increased latency when compared to the control. As expected, morphine increased the reaction time for all times tested (Figure Nº 4).

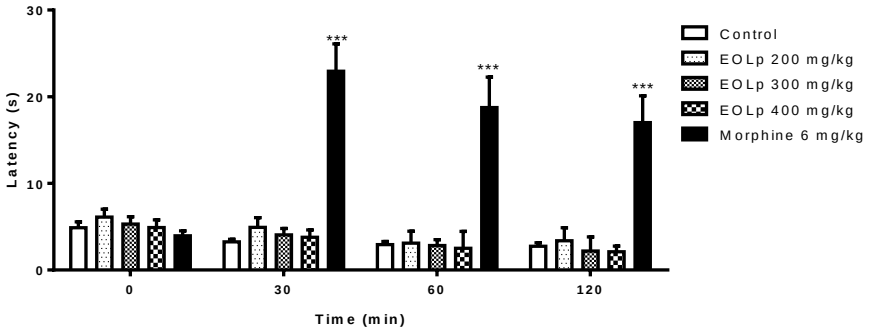


Figure Nº 4

Effect of EOLp administration during the hot plate test. Mice were pretreated with vehicle, EOLp (200, 300 and 400 mg/kg, p.o.), or morphine (6 mg/kg, i.p.). Measurements were performed at 30, 60, and at 120 min after treatment. Each column represents mean ± S.E.M. (n = 6). Statistical analysis: ANOVA "one-way" followed by Tukey's test. ******p*<0.001 vs control group

EOLp effect on carrageenan-induced peritonitis test

Treatment with EOLp 1h before carrageenan administration (1%, 300 µL) inhibited leukocyte migration at doses of 300 and 400 mg/kg (4.3 ± 0.8; 2.3 ± 0.2) when compared to the control (12.0 ± 1.1). As expected, dexamethasone reduced leukocyte migration (4.5 ± 1.0) (Figure Nº 5).

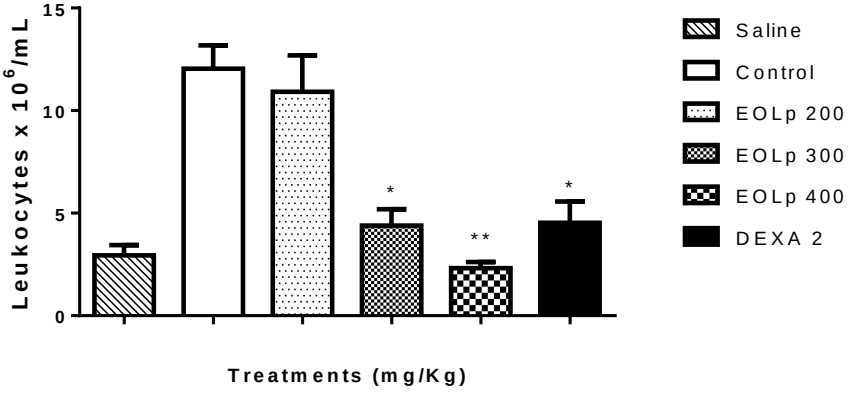
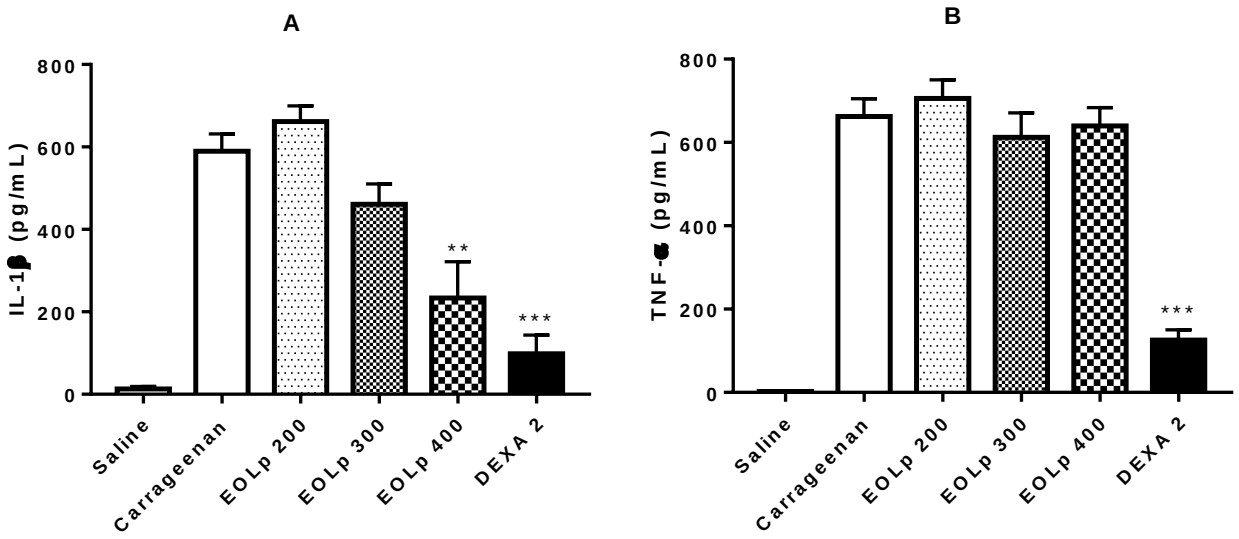


Figure Nº 5

Effect of EOLp on total leukocyte migration in carrageenan-induced peritonitis, by injection of carrageenan (1%, 300 µL) into the mice peritoneal cavities at 1 hour after administration of either vehicle, EOLp (200, 300 and 400 mg/kg, p.o.), or dexamethasone (DEXA, 2 mg/kg i.p.). Each column represents mean ± S.E.M. (n = 6). Statistical analysis: ANOVA "one-way" followed by Dunnett's test. ****p*<0.05, *****p*<0.01 versus control group

EOLp effect on TNF-α and IL-1β

Four hours after carrageenan administration, EOLp at a dose of 400 mg/kg (233.6 ± 87.9 pg/mL) reduced (p<0.01) IL-1β levels compared to the control group (589.4 ± 42.1 pg/mL). However, when evaluating TNF-α levels, we observed that EOLp (at doses of 200, 300, and 400 mg/kg) was unable to promote reductions (705.4 ± 45.0; 612.3 ± 58.4; 639 ± 44.0 pg/mL) when compared to the control group (662.5 ± 42.4 pg/mL). Dexamethasone reduced both IL-1β (98.0 ± 45.7 pg/mL) and TNF-α (125.7 ± 24.3pg/mL) levels (Figures No. 6a & 6b).



Figures No. 6a & 6b

EOLp effect on levels of IL-1β (A) and TNF-α (B) in the peritoneal cavity 4h after intraperitoneal administration of carrageenan. Mice were pretreated with vehicle, EOLp (200, 300 and 400 mg/kg, p.o.), and dexamethasone (DEXA, 2 mg/kg i.p.), 1h before the injection of a phlogistic stimulus. Each column represents mean ± S.E.M. (n = 6). Statistical analysis: ANOVA "one-way" followed by Dunnett's test. *****p*<0.01, ******p*<0.001 vs control group

DISCUSSION

Previous studies have suggested that central nervous system (CNS) depression and non-specific muscle relaxation effects can reduce motor response coordination and invalidate behavioral test results (de Almeida *et al*., 2004; Quintans Júnior *et al*., 2010). We therefore evaluated the effect of EOLp with a rota-rod apparatus and no significant alteration in mice motor coordination was observed. Thus, the activity observed for EOLp in the nociception tests cannot be attributed to inhibitory effects on the CNS or muscle relaxation.

In order to solubilize nonpolar molecules or complex mixtures such as essential oils, the formation of inclusion complexes with CDs has been extensively used (Santos *et al*., 2015). Besides protect physicochemical properties of the essential oils from the oxidation, high temperatures, light degradation and volatilization (Marreto *et al*., 2008), in natural products, β-CD complexation can improve pharmacological properties; whether analgesic or anti-inflammatory (Quintans-Júnior *et al*., 2013; Siqueira-Lima *et al*., 2014; Rodrigues *et al*., 2017). Therefore, whether β-CD complexation might improve the antinociceptive profile of the EOLp was evaluated. We initially performed the acetic acid-induced writhing test; a classical model of visceral pain used as a screening tool for evaluation of analgesic or anti-inflammatory drugs (Mohamad *et al*., 2010). When administered by intraperitoneal injection, acetic acid promotes activation of cationic channels expressed in peripheral fibers and also stimulates the release of inflammatory mediators like bradykinin, histamine cytokines, and eicosanoids (Deraedt *et al*., 1980). These mediators can increase vascular permeability, reduce the nociception threshold, and stimulate nociceptive fiber nerve terminals (Le Bars *et al*., 2001).

Our results indicated that EOLp (300 and 400/mg/kg, p.o.) reduced the number of writhes induced by acetic acid (Figure No. 2). Similar to species of the same genus such as *L. alba* (Costa *et al*., 1989; Viana *et al*., 1998), *L. multiflora* (Abena *et al*., 2003), *L. nodiflora* (Ahmed *et al*., 2004), and *L. gracilis* (Mendes *et al*., 2010; Guilhon *et al*., 2011; Guimarães *et al*., 2012). Rotundifolone, the major constituent of EOLp (71.7%) (Santos, *et al*., 2014), also presents efficacy equivalent to EOLp in acetic acid-induced writhings (Sousa *et al*., 2009). However, EOLp/β-CD presented no effect in any of the doses we tested.

It is already described in the literature the possibility of CDs to increase physicochemical properties of apolar substances, also pharmacological

properties in relation to nociception and inflammation, (de Oliveira *et al*., 2015; Brito *et al*., 2015; Lima *et al*., 2016; Santos *et al*., 2017). Oil extracts (EO) from *Lippia grata* (Siqueira-Lima *et al*., 2014), *Ocimum basilicum* (Nascimento *et al*., 2015) and *Hyptis pectinata* (Menezes *et al*., 2015), in their inclusion complex with CDs, have all exhibited superior antinociceptive effect when compared to their isolated form. Surprisingly, this was not found in our study; EOLp tested was more effective in reducing antinociceptive behavior. The results presented in this study clearly demonstrate that EOLp/β-CD lost its analgesic properties when compared with equivalent doses of EOLp alone. This is the first essential oil that presented minor analgesic profile when complexed with β-CD, which usually improves it (Nascimento *et al*., 2017). Araujo-Filho *et al*., (2017) recently demonstrated that β-CD improves the analgesic effect of D-limonene. Thus, the weak effect found for EOLp/β-CD may be due to stereo-chemical difficulties in rotundifolone cavity or surface complexing with β-CD (Lima *et al*., 2016; Menezes *et al*., 2018).

Proceeding with antinociceptive evaluation, yet due to the low specificity of the acetic acid-induced writhing test, it is not possible to us confirm if the antinociception produced was central or peripheral. We therefore used more specific nociception models, such as the formalin test, in order to more precisely characterize the antinociceptive activity exhibited. The formalin-induced nociception test it is a biphasic experimental pain model. The first phase (neurogenic) is related to the activation of TRPA1 receptors (McNamara, *et al*., 2007) at the nociceptive nerve endings, which promote pain by the release of substance P, glutamate, and bradykinin (Hunskaar & Hole, 1987; Tjølsen *et al*., 1992). After the first and before de second phase, there is a moment known as 'interphase' or 'quiescent period' which nociceptor excitability is decreased (Tjølsen *et al*., 1992). The second (inflammatory) phase occurs due to spinal cord stimulus, after sensitization of nociceptors, with the release of serotonin, histamine, prostaglandins (PGE2), nitric oxide (NO), glutamate, aspartate, and bradykinin (Henry *et al*., 1999; Silva *et al*., 2016). Diminished paw licking time in both phases of the formalin test is characteristic of drugs acting at central levels, such as opioid analgesics. However, peripheral non-steroidal anti-inflammatory and corticosteroid drugs cause inhibition only at the second phase (Mansouri *et al*., 2014).

Acute treatment with EOLp (300 and 400 mg/kg, p.o.) produces significant inhibition of nociceptive behavior in both phases of the formalin test (Figure No. 3A); similar to *L. gracilis* essential oil at the same dose (400 mg/kg, p.o.), but different from rotundifolone (200 mg/kg, p.o.) (Sousa *et al*., 2009) and R-(+)-limonene (50 mg/kg, i.p.) (do Amaral *et al*., 2007), which inhibited only the second phase; indicating that the antinociceptive effects of these drugs may originate in anti-inflammatory events. Similarly, we also evaluated orofacial nociception by upper lip injection of formalin (s.c.) to induce a biphasic nociceptive response with ipsilateral forepaw face-rubbing episodes, sometimes using the hind paw, directed to the perinasal area (Luccarini *et al*., 2006). The administration of EOLp (300 mg/kg, p.o.) decreased face-rubbing behavior during both phases of formalin-induced orofacial nociception (Table No. 1), potentially suggesting central antinociceptive activity.

It is already known that glutamate participates of the nociception signals from the nociceptive nerve ending to the dorsal horn of the spinal cord and the trigeminal sub nucleus caudalis. Blockage of glutamate reuptake has been indicated as a new method to treat chronic inflammatory pain (Yang *et al*., 2015). Glutamate injection evokes pronounced nociceptive response which is mediated by neuropeptides (Substance P) released from C fibers, and activation of glutamate ionotropic receptors such as methyl-D-aspartate (NMDA). This promote the production of NO, and pro-inflammatory cytokines, such as TNF-α and IL-1β, which act in neuronal excitability (Millan, 1999; Pereira *et al*., 2010). Since pretreatment with EOLp (300 mg/kg, *p*.o.) significantly protected against glutamate induced orofacial nociception (Table No. 1), we suggest that antinociceptive effect of EOLp may be related to glutamate receptors, probably NMDA, which can limit mediator production (Ribas *et al*., 2008; Bonjardim *et al*., 2011).

Sakurada *et al*. (2003) proposed using the capsaicin-induced nociception test for studying compounds which act on neurogenic pain. Extracted from the red pepper, capsaicin injected into animals, produces inflammation and increases the excitability of spinal and trigeminal nociceptive neurons (Pelissier *et al*., 2002), and in animals, promotes nociceptive behavior (Hu *et al*., 2005; Lam *et al*., 2009). Capsaicin activates the sensitive transient receptor potential of vaniloid 1 (TRPV1), which is an important receptor in pain transduction, increasing

the expression of TNF receptor 1 (TNFR1) (Waning *et al*., 2007; Ma *et al*., 2009), and the release of neuropeptides such as tachykinins (neurokinin A), substance P, calcitonin, glutamate, aspartate, oxide nitric, and other pro-inflammatory substances (Honda *et al*., 2008). As demonstrated in Table No. 1, pretreatment with EOLp (300 mg/kg, p.o.) produced a significant reduction in neurogenic inflammatory nociception when induced by capsaicin injection into the right upper lip (perinasal area). As this effect was similar to that of a glutamate model; recently Ro *et al*. (2014) showed that there is an interaction between NMDA and TRPV1 receptors in the trigeminal ganglion, and they concluded that TRPV1 is an integrator of glutamate receptor signaling in trigeminal muscle nociceptors (Damasceno *et al*., 2016); thus, we suggest that EOLp could antagonize the TRPV1 channel leading to glutamate release reductions.

In an attempt to confirm that EOLp presents central analgesic effects we performed hot plate testing, which is a behavioral model consisting of exposing the animal to a noxious thermal stimulus. The nociceptive parameters evaluated: the time to animal to jump or lick the hind leg represent integrated supra-spinal responses. Thermal stimulation is associated with activation of TRPV1 receptors at Aδ and C fibers, which transmit nociceptive signals to the dorsal horn of the spinal cord, and finally to the somatosensory cortex (Yi *et al*., 2014). Due to its activation of the CNS, this model is used to evaluate opioid, sedative, and hypnotic drug effects (Yeung *et al*., 1977). Our data showed (Figure No. 3B), that the animals pretreated with EOLp do not present increased hot plate response latency, confirming that the analgesic effects presented by EOLp are not mediated at CNS levels.

Since EOLp presented analgesic effect in the second phase of both formalin tests, and during glutamate and capsaicin induced orofacial nociception, it seems to involve anti-inflammatory events. Since mediators produced in inflammation sites can promote pain by activation or sensitization of nociceptors adjacent to the injured tissue, the anti-inflammatory activity of EOLp was therefore also investigated. IL-1β, IL-6, and TNF-α are pro-inflammatory cytokines found to increase the production of substance P and PGE_2 in a number of neuronal and glial cells, contributing to the development of pain behavior following a peripheral nerve injury (Carvalho *et al*., 2013; de Almeida *et al*., 2017).

In order to evaluate possible anti-inflammatory effect, carrageenan-induced peritonitis was promoted. Carrageenan is a linear sulfated polysaccharide derived from red algae with robust inflammatory properties, and is often used in models to screen for molecules with anti-inflammatory activity. Carrageenan-induced peritonitis techniques allow quantification of leukocytes migrating into the peritoneal cavity through the activity of chemotactic agents. After injection of carrageenan, EOLp (300 and 400 mg/kg) significantly prevented peritoneal cavity leukocyte recruitment. The results are consistent with a previous study with *Protium heptaphyllum* essential oil (Amaral *et al*., 2009) which contains limonene as one of the major compounds. Another study has also reported that (+)-limonene epoxide reduces cell migration (de Almeida *et al*., 2017). However, there are no reports in the literature concerning rotundifolone (a major constituent of EOLp) anti-inflammatory effect.

Peritonitis involves exudation and production of mediators such as nitric oxide and cytokines (IL-1β and TNF-α) (Lima *et al*., 2012). Thus, the effect of EOLp was evaluated from levels of IL-1β and TNF-α in the peritoneal exudate of mice with carrageenan-induced peritonitis. EOLp reduced concentrations of IL-1β, however TNF-α levels were not altered. The same has been reported for (+)-limonene epoxide (de Almeida *et al*., 2017). Upon cell injury, infection, and inflammation, IL-1β is released: primarily by monocytes, macrophages and mast cells, and as well as by non-immune cells, like neuronal and glial cells (Schwann cells, microglia, and astrocytes). Overproduction of IL-1β is implicated in the pathophysiological changes which occur in inflammatory diseases (Zhang & An, 2007; Ren & Torres, 2009).

CONCLUSION

The results of this paper allow us to conclude that EOLp has better antinociceptive effect in mice when compared to EOLp/β-CD complex. Thus, though CDs are efficient as drug delivery system with EOs inclusion complex, but not with EOs rich in peroxide substances. EOLp is a promising option for treating inflammation and pain-related disorders, significantly inhibiting acetic acid-induced writhing in both phases of formalin-induced nociception in mice, and in orofacial nociceptive behavior induced by formalin, glutamate, and capsaicin. Additionally, it reduced leucocyte migration to the peritoneal cavity, while

reducing IL-1β levels, which together confirm its anti-inflammatory effect.

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