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Involvement of the PKA pathway and inhibition of voltage gated Ca2+ channels in antihyperalgesic activity of Lippia grata/β-cyclodextrin

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ABSTRACT

Neuropathic pain (NP) is a difficult condition to treat because of the modest efficacy of available drugs. New treatments are required. In the study we aimed to investigate the effects of the essential oil from *Lippia grata* alone or complexed in β-cyclodextrin (LG or LG-βCD) on persistent inflammatory and neuropathic pain in a mouse model. We also investigated Ca^{2+} currents in rat dorsal root ganglion (DRG) neurons. Male Swiss mice were treated with LG or LG/β-CD (24 mg/kg, *i.g.*) and their effect was evaluated using an acute inflammatory pleurisy model and nociception triggered by intraplantar injection of an agonist of the TRPs channels. We also tested their effect in chronic pain models: injection of Freund's Complete Adjuvant and partial sciatic nerve ligation (PSNL). In the pleurisy model, LG reduced the number of leukocytes and the levels of TNF-α and IL-1β. It also inhibited cinnamaldehyde and menthol-induced nociceptive behavior. The pain threshold in mechanical and thermal hyperalgesia was increased and paw edema was decreased in models of inflammatory and neuropathic pain. PSNL increased inflammatory protein contents and LG and LG-βCD restored the protein contents of TNF-α, NF-κB, and PKA, but not IL-1β and IL-10. LG inhibited voltage gated Ca^{2+} channels from DRG neurons. Our results suggested that LG or LG-βCD produce anti-hyperalgesic effect in chronic pain models through reductions in TNF-α levels and PKA, and inhibited voltage-gated calcium channels and may be innovative therapeutic agents for the management of NP.

1. Introduction

According to the World Health Organization (WHO), about 20% of the population live with some degree of chronic pain, which is more common in women, older individuals and people with relative deprivation [1]. Neuropathic pain (NP) is one of the most important types of chronic pain. It is triggered by lesions to the somatosensory nervous system that alter its structure and function [2], causing hyperalgesia,

Abbreviations: β-cyclodextrin, β-CD; Dorsal root ganglion, DGR; Essencial Oil, EO; Freund's Complete Adjuvant, FCA; Interleukin-10, IL-10; Interleukin-1β, IL-1β; *Lippia grata*, LG; *Lippia grata* complexed with β-cyclodextrin, LG/β-CD; factor nuclear kappa B, NF-kB; Neurophatic pain, NP; protein kinase A, PKA; partial sciatic nerve ligation, PSNL; tumor necrosis factor alpha, TNF-α; transient receptor potential Ankyrin 1, TRPA1; transient receptor potential melastatin 8, TRPM8; transient receptor potential vanilloid 1, TRPV 1; voltage gated calcium channel, VGCC

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allodynia and spontaneous pain [3]. Because NP does not have a role in protecting individuals against tissue damage it is not simply a symptom, but a disease in itself [4,5]. Moreover, NP is one of the most difficult types of chronic pain to treat, due to the limitations of the current therapeutic arsenal and to the lack of understanding of its neurophysiological basis.

Peripheral nerve injury induces the activation of spinal cord glial cells including astrocytes, microglia, and oligodendrocytes [6,7]. Once activated, glial cells release pro-inflammatory mediators (such as cytokines) that activate/sensitize nociceptors in the spinal cord, enhancing nociceptive neurotransmission. Cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL-1β), and IL-33, which induce nociceptor sensitization, play a key role in the production of pain commonly experienced by patients with NP [7,8]. This complex interaction between inflammatory mediators and neuronal cells leads to central sensitization, decreases peripheral sensitization and induces spontaneous firing of nociceptors [9,10].

Additionally, the voltage gated calcium channels (VGCCs) have a pivotal role in the ascending pain pathways, because they are required for the generation and transmission of the pain process, especially in primary afferent neurons [11]. N-type calcium channels are localized to synaptic nerve terminals in laminae 1 and 2 of the dorsal horn where their activation can generate an increase in the release of neurotransmitters (such as glutamate and substance P) that are essential in establishing pain and increasing pain sensitivity [12]. Moreover, calcium channel activation in DRG cells by pro-inflammatory cytokines (such as TNF-α and IL-1β) leads to increased cellular excitability with consequent activation of the ascending pain pathways [13]. Anti-TNF-α therapy has been shown to improve axonal regeneration and consequently reduce pain in experimental models of sciatic nerve ligation. Thus, new drugs that block or attenuate the action of VGCCs, or regulate of levels of pro-inflammatory cytokines (such as TNF-α) may be potentially useful for treating NP and may also help to induce neural regeneration in these patients [14–16].

The complexity and multiplicity of its underlying pathophysiological mechanisms has made it difficult to identify tractable targets with broad involvement in NP [5]. Thus, the first treatment of choice includes available analgesics - nonsteroidal anti-inflammatory drugs, amine reuptake inhibitors, antiepileptic drugs and opioids – which have varying, but typically low levels of analgesic efficacy, and are generally coupled with deleterious effects [5,17–19]. As current treatments lack efficacy, some researchers have turned their attention to medicinal plants as a source of new active compounds because they are already widely used, especially in relation to the treatment of pain and inflammation [20–24].

The plants belonging to the genus *Lippia* (Verbenaceae) comprise more than 250 species, which are widely distributed in most tropical and subtropical countries. This genus is constituted of aromatic plants, rich in essential oils (EO). Their pharmacological properties are attributed to the terpene compounds making up most of the EO which have proven clinical applicability, already being used in various drugs [16,22,25,26].

Recently, our research group demonstrated that the essential oil of the species *Lippia grata* (LG) complexed in β-cyclodextrin (β-CD), given orally, presented analgesic activity in orofacial pain models and a fibromyalgia-like mice model [27,28]. Despite its proven effect as an antinociceptive agent in acute pain, probably linked to some terpenoids such as camphor, borneol and β-caryophyllene, little is known about the effect of this essential oil on chronic pain such as NP. Thus, we decided to investigate whether the essential oil of *L. grata* leaves complexed in β-cyclodextrin (LG/β-CD) produces an anti-hyperalgesic effect in persistent neuropathic (partial sciatic nerve ligation [PSNL]) and inflammatory pain (intraplantar injection of Freund's Complete Adjuvant- CFA) in rodent pain models and also the possible mechanisms underlying any anti-hyperalgesic profile.

2. Material and methods

2.1. Plant material and essential oil

The fresh leaves of *L. grata* were collected in June 2011 in Capim Grosso, Bahia, in north-eastern Brazil (11°19′28.4″S, 40°09′08″W). L. grata was identified by Dr. Tania Silva (Herbarium of The State University of Feira de Santana, UEFS), where a voucher specimen has been deposited (HUEFS 169543). The essential oil was extracted by hydrodistillation in accordance with the methodologies previously described by our group [27]. All procedures for access to genetic patrimony and associated traditional knowledge were carried out and the project was registered in SISGEN (protocol A0FBC55).

2.2. Preparation of binary mixture of LG with βCD

Preparation and physical-chemical characterization of the *L. grata* (LG) leaf essential oil complexed with β-cyclodextrin (βCD) was carried out in accordance with [27] the methodologies previously described by our group [29,30].

2.3. Animals

Experimental protocols were performed using male Swiss mice (28–35 g) and adult male Wistar rats (180–220 g) obtained from the Animal Facilities of the Federal University of Sergipe (UFS/Brazil) and the Federal University of Minas Gerais (UFMG/Brazil) respectively. Animals were randomly housed in controlled-temperature rooms (22–24 °C), under a 12/12 h light-dark cycle, with access to water and food *ad libitum* until use. All behavioral protocols were performed between 9:00 a.m. and 3:00 p.m. Experimental protocols were approved by the Animal Care and Use Committee at UFS/Brazil (65/15) and UFMG/Brazil (CEUA 149/2015). Rats were used only for the electrophysiological recordings in the dorsal root ganglia (DRG) neurons. The behavioral experiments and animal treatment were performed with the examiner blinded to the group. We made all necessary efforts to minimize the number of rodents used and any discomfort to them.

2.4. Carrageenan-induced pleurisy

Pleurisy was induced in the mice by intrapleural administration of 100 µl of 1% (w/v) carrageenan suspension in sterile saline solution [31]. A specially adapted 13 × 5 needle was introduced into the right side of the thoracic cavity for injection of the carrageenan solution. The animals were pre-treated with LG or LG-βCD (24 mg/kg, gavage) or vehicle 60 min before the injection of the inflammatory agent. Four hours after induction of the pleurisy, the animals were euthanized, and the pleural inflammatory exudate was removed by aspiration through pleural lavage with 1 mL of sterile phosphate buffered saline (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). Total leukocytes were counted in a Neubauer chamber, using a light microscope [32]. The fluid obtained from the pleural cavity was collected for further determination of cytokine (TNF-α, IL-1β) levels.

2.5. Pain behavior induced by the intraplantar injection of TRPM8 and TRPA1 agonists

To evaluate the possible involvement of transient receptor potential cation channels (TRP), subfamily A member 1 (TRPA1) and melastatin 8 (TRPM8), on the antinociceptive effect of LG and LG/β-CD, mice were submitted to a test using either cinnamaldehyde or menthol, activators of these channels respectively, as previously described by Ref. [33]. The mice were pretreated with LG, LG/β-CD (24 mg/kg, *i.g.*) or vehicle (10 mL/kg, *i.g.*) 1 h prior to the injection of 20 µL of cinnamaldehyde

(an activator of the TRPA1 channel, 10 nmol/paw), menthol (an activator of the TRPM8 channel, 1.2μmol/paw) or corresponding vehicle in the ventral surface of the right hind paw. The animals were individually placed in an acrylic chamber (9 × 11 × 13 cm), and paw licking, or biting were recorded for 5 min (cinnamaldehyde), or 20 min (menthol). The time spent licking/biting the injected paw was considered indicative of nociception.

2.6. Complete Freund's adjuvant induced inflammation

Mice received 20 μL of complete Freund's adjuvant (CFA 1 mg/mL of heat killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monooleate; Sigma-Aldrich) in the plantar region of the right hind paw, according to a previously reported method [34]. The degree of inflammation was evaluated by measuring the volume of paw edema 2 and 4 h after the CFA injection and mechanical hyperalgesia 24 h after the CFA injection and then daily for 8 consecutive days.

2.7. Segmental spinal nerve ligation-induced neuropathic pain

Partial sciatic nerve ligation (PSNL) was performed according to the procedure previously described in mice [35]. Mice were anaesthetized with a premixed solution containing ketamine (80 mg/kg, *i.p.*) and xylazine (10 mg/kg, *i.p.*). Partial ligation of the right sciatic nerve was performed by ligating the distal one-third to one-half of the dorsal portion of the sciatic nerve. In sham-operated mice, the sciatic nerve was exposed without ligation. The wound was closed and covered with 10% povidone-iodine solution. The sham-operated mice received only vehicle (10 mL/kg, *i.g.*), and the PSNL-operated mice were randomly divided into control and treatment groups, which received vehicle (10 mL/kg, *i.g.*) or LG and LG/β-CD (24 mg/kg, *i.g.*), respectively, 7 days after surgery. The mechanical hyperalgesia response was recorded before surgery (B), immediately before treatment (0 h), and after treatment (1, 2, 3, 4, 6 and 8 h) to verify the time-course effect of the treatment as described below. To investigate the effects of repeated treatment on the mechanical hyperalgesia response, LG and LG/β-CD (24 mg/kg, *i.g.*) were administered once daily for 7 consecutive days (from the 8th to 14th day after PSNL), and the effects were examined 1–2 h after treatment. After 2 days without treatment, treatment was resumed on the 17th and 18th days after PSNL to assess the development of LG and LG/β-CD tolerance.

2.8. Plethysmometer test

The volume of each mouse paw was measured (mL) with a plethysmometer (EFF 361; Insight®, Brazil) before (Vo) and after (VT) the CFA injection, as described previously [98]. The amount of paw swelling was determined for each mouse and data were represented as paw volume variation (Δ, mL).

2.9. Measurement of mechanical hypersensitivity

The mice were acclimatized in individual transparent boxes on a wire-mesh platform to allow access to the ventral surface of the hind paws. Their sensitivity to mechanical stimulation generated by the gradual increase of pressure of a manual force transducer (electronic analgesimeter, model EFF-301, Insight®, Brazil), adapted with a polypropylene tip, was evaluated. This test indicates hyperalgesia by automatically recording the pressure intensity needed to evoke a flexion reflex of the hind paw, which is defined as a withdrawal of the paw followed by rapid movements, characterized as flinches [36].

2.10. Thermal hyperalgesia evaluation

For the hot plate test, the mice were placed in the hot plate apparatus (EFF 361; Insight®, Brazil) maintained at 55 °C. The first ipsilateral hind paw flexion reflex was considered the nociceptive endpoint. The response latency was recorded before the surgery and at the 10th day post-surgery. The maximum latency (cut-off) was set at 20s to avoid tissue damage [37,38]. To obtain data purely derived from the treatment, the inhibition values are presented as the difference between the basal values of vehicle or drug-treated animals and the respective controls.

2.11. Determination of cytokines levels

The levels of TNF-α, IL-1β or IL-10 in sciatic nerve or lumbar spinal cord segments (L4 and L6) were determined at 17 days after PLSN. The samples were homogenized in 150 μL of phosphate-buffered saline (PBS 50 mM, pH 7.4) containing EDTA (0.3%), nonidet P 40 (0.5%) and protease inhibitors. The samples were centrifuged at 6000 or 8000 rpm for 10 min at 4 °C, and the supernatant obtained was used to determine cytokines levels. The protein concentration was measured using the method of Bradford [39] with bovine serum albumin as the standard protein.

TNF-α, IL-1β and IL-10 levels were determined using enzyme-linked immunosorbent assay (ELISA) kits (eBioscience®) according to the manufacturer's instructions, and colorimetric measurements at 450 nm were made using a microplate reader (ASYS®). The concentration was obtained by interpolation from a standard curve. All results were expressed as picograms (pg) of cytokine per milligram (mg) of total protein.

2.12. Western blot assay

For the preparation of total protein homogenate, the mice were killed by cervical dislocation on the 17th day after PNSL, the spinal cord and the sciatic nerve were removed. For Western blot assay the tissues were homogenized in 150 μl of a lysis solution RIPA contend 1 mM EDTA, 20 mM Tris–HCl, pH 7,5, 150 mM NaCl, 1 mM EGTA, 1% sodium deoxycholate, 1% nonidet 40, containing proteases and phosphatases inhibitors. For the electrophoresis analysis, samples were dissolved in 25% (v/v) of solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris–HCl, pH 6.8 and boiled for 5 min. The protein concentration was determined by the method of Bradford [39] using BSA as the standard.

Tissue homogenates (50 μg) were separated by SDS-PAGE and transferred to PVDF membranes (BioRad) for 16 h at 25 V in transfer buffer (48 Mm Tris, 39 mM glycine, 20% methanol). The PVDF membranes were washed for 15 min in Tris–buffered saline with 0.1% Tween-20 (T-TBS; 0.5 M NaCl, 20 Mm Tris, 0.1% Tween-20, pH 7.5), followed by 1 h incubation in blocking solution (T-TBS plus 5% defatted dried milk). After incubation, the blots were washed three times for 15 min with T-TBS, and then incubated overnight at 4 °C in blocking solution containing the following antibodies: anti-PKA (Imuny IM0409) diluted 1:1000; anti PKAcα (Cell Signaling Technology), diluted 1:1000; anti-phospho-NFκB (Ser536) (93H1) (Cell Signaling Technology) diluted 1:1000; anti- NFκBp65 (D14E12) XP (Cell Signaling Technology) diluted 1:1000; and anti-β-actin (Cell Signaling Technology) diluted 1:1000. The blots were then washed three times for 15 min with T-TBS and incubated for 2 h in blocking solution containing peroxidase conjugated anti-rabbit IgG diluted 1:2000. The blots were washed twice again for 15 min with T-TBS. The blots were then developed using a chemiluminescence substrate. Blots were quantified, and optical density values were obtained for the studied proteins. All results were expressed as a relative ratio to β-actin.

2.13. Measurement of motor performance

2.13.1. Evaluation of muscle strength

The hindpaw grip strength evaluation, previously described by Burnes et al. [99], was used before the experiment as a baseline

measurement and after PLSN. The mice were pulled by the tail to measure the grip strength of the hindpaw. The animals were evaluated 3 times and the mean calculated to obtain the absolute strength (g). The mice then received the same pharmacological treatment as previously described.

2.13.2. Rotarod test

In order to discard possible non-specific muscle relaxant or sedative effects of essential oil of Lippia grata, motor performance was evaluated using the rota-rod test [100]. The apparatus consisted of a bar with a diameter of 2.5 cm, subdivided into six compartments by disks 25 cm in diameter (AVS Projetos, Ribeirão Preto, SP, Brasil). The bar rotated at a constant speed of 22 rotations per min. The animals were selected 24 h previously by eliminating those mice that did not remain on the bar for two consecutive periods of 120 s. Animals were treated with vehicle (saline) or LG or LG-βCD (24 mg/kg, *i.g.*), and testing was performed 1.5, 3.5 and 5.5 h after treatment. A cut-off time of 120s was used.

2.14. Voltage clamp

We recorded calcium currents (Ica + +) from adult male Wistar rats' dorsal root ganglia (DRG) neurons. We used the patch-clamp technique [40] on neurons that were perfused (1 ml/min) and dialyzed with Na^+,K^+-free solutions containing channel blockers to isolate calcium currents from other membrane currents. The bath solution contained (in mM): choline chloride (105), TEA chloride (30), $CaCl_2$ (2), $MgCl_2$ (4) and HEPES (10) and it was adjusted to pH 7.4 with tetraethylammonium hydrochloride. The pipette internal solution was (in mM): CsCl (126), MgATP (1), EDTA (1), EGTA (10), HEPES (10) and it was adjusted to pH 7.2 with CsOH. All experiments were performed at room temperature.

The cells were kept in a holding potential of −80 mV and at every 7 s we applied depolarizing pulses to 0 mV for 200 ms. The liquid junction potential was corrected to eliminate the difference of −6 mV between the pipette and bath solutions. The sampling rate was 10 kHz.

Pipettes were prepared from glass capillaries (World Precision Instruments, Sarasota, USA) pulled and heat-polished (PP 830 puller and MF-830 microforge, Narishige, Tokyo, Japan) to a tip resistance of 1–3 MΩ. Data were acquired using a patch-clamp amplifier (Multiclamp 700B, Axon Instruments, USA) and a digitizer (Digidata 1200 series, Axon Instruments, USA) controlled by the pClamp 8.2 software (Axon Instruments, USA).

The LG essential oil was diluted in DMSO (33% v/v) and added to the bath resulting in the following concentrations: 0.1, 0.3 or 1.0 μL/mL. As control, we used DMSO at its highest concentration.

We recorded calcium current (I_{Ca}) for 1–4 min, in order to estimate the current rundown. Once the current reached stability we calculated the percentage of inhibition using the last control and the first experimental currents. In some cells we performed multiple drug applications, washing it at different intervals.

2.15. Statistical analysis

Results were expressed as mean ± S.E.M. The differences between groups were analyzed using one-way or two-way ANOVA, followed by the Tukey test or Bonferroni test, respectively. Values of $p < 0.05$ were considered statistically significant. All statistical analyzes were performed using GraphPad Prism 5.0 software (GraphPad Prism Software Inc., San Diego, CA, USA). The dose response curves of Ica + + inhibition were fitted to a Hill equation, which we used to calculate IC50. Student's two-tailed T-test was used to analyze the paired data.

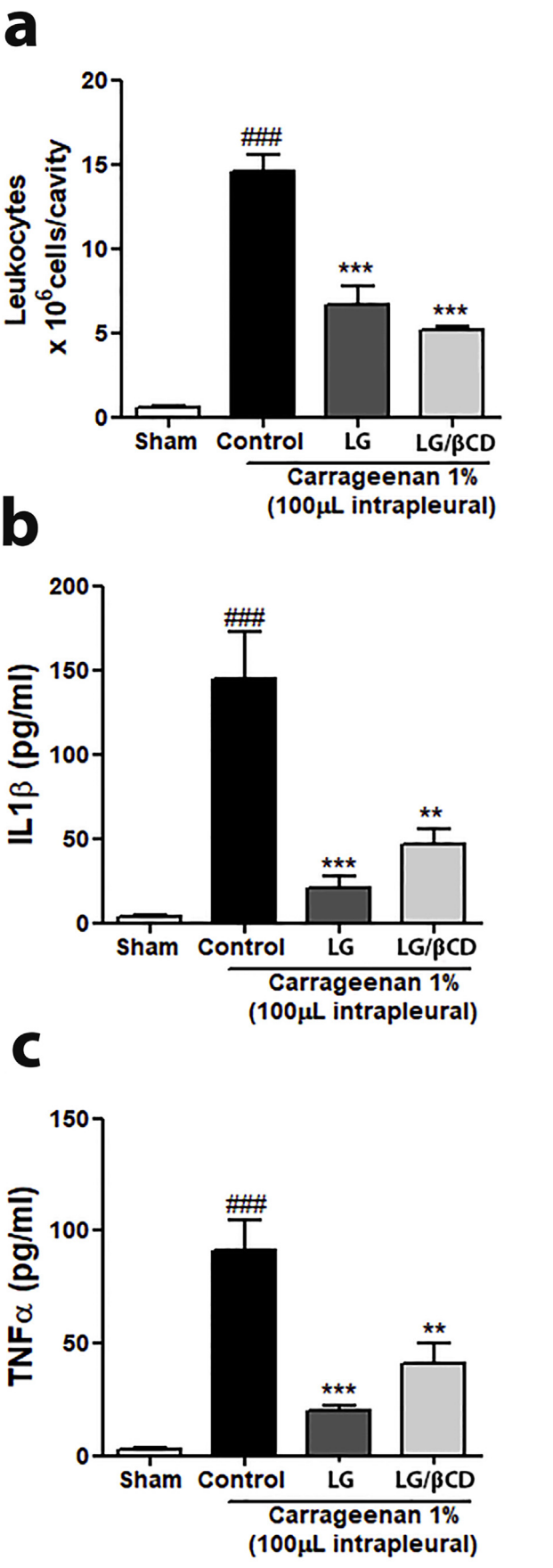

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Fig. 1. Effect of LG and LG/β-CD on the number of PMNs **(A)** and on the levels of pro-inflammatory cytokines TNF-α **(B)** and IL-1β **(C)**. The analyses were made from the exudates at 4 h after the induction of pleurisy by carragenan injection. Data are reported as means ± SEM of 8 animals. Statistical analysis was performed using a one-way ANOVA followed by the Tukey's test.$^{**}p < 0.01$, $^{***}p < 0.001$ vs control group; $^{\#\#\#}p < 0.01$ vs sham group.

3. Results

3.1. Carrageenan-induced pleurisy

All mice that had received carrageenan developed acute pleurisy, producing leukocyte migration (turbid exudate) (Fig. 1A). The administration of carrageenan into the pleural space of mice induced an inflammatory process with a significant increase in total leukocyte count compared with untreated-mice. Mice pretreated with LG as well as LG/β-CD showed a significant attenuation of the number of leukocytes within the exudate [$F(3,25) = 50.51$; $p = 0.0001$)]. In addition, the levels of TNF-α [$F(3,9) = 14.67$; $p = 0.0008$)] and IL-1β [$F(3,10) = 20.16$; $p = 0.0001$)] were significantly elevated in the exudate from mice at 4 h after carrageenan administration in the control group. In contrast, the levels of these cytokines were reduced in mice treated with LG and LG/β-CD (Fig. 1B and C) compared to the control group ($p < 0.001$).

3.2. Pain induced by TRPA1 and TRPM8 agonists

As shown in Fig. 2A, the LG and LG/β-CD (24 mg/kg, *i.g.*) inhibited the nociceptive behavior induced by cinnamaldehyde. Pretreatment with camphor (7.6 mg/kg, s.c.), a nonspecific TRP blocker, inhibited the cinnamaldehyde-induced nociception [$F(4,20) = 18.65$; $p = 0.0001$)]. Intraplantar injection of menthol produced a marked nociception in mice. Previous treatment with LG and LG/β-CD (24 mg/kg, *i.g.*) inhibited menthol-induced nociceptive behavior [$F(3,16) = 57.05$; $p = 0.0001$)] (Fig. 2B).

3.3. CFA-induced chronic inflammatory pain

To investigate the antinociceptive and anti-inflammatory activity of LG and LG/β-CD, a chronic inflammatory model was used. For this, mechanical hypersensitivity was evaluated from day 1 to day 8 after an intraplantar injection of CFA in the mice. As shown in Fig. 3A, CFA caused significant mechanical hypersensitivity characterized by a reduced paw withdrawal threshold compared to the control group ($p < 0.001$). Oral administration of LG and LG/β-CD was able to significantly reverse mechanical hypersensitivity, which lasted up to 6 h in the LG group ($p < 0.001$) and 8 h in LG/β-CD group ($p < 0.0001$)). This antihyperalgesic effect was maintained while LG and LG/β-CD was orally administered daily, until the 8th day posttreatment ($p = 0.0001$). When treatment was interrupted for 2 days, mechanical hypersensitivity in the treated group was reestablished. On the 8th day, the treatment was restarted and LG and LG/β-CD were able to reduce mechanical hypersensitivity with a profile similar to the 1st day. These results excluded the possibility of the development of a tolerance effect of LG and LG/β-CD. Paw edema was also evaluated after CFA induction. The administration of LG and LG/β-CD 60 min before CFA significantly reduced ($p < 0.01$) paw edema at 2 and 4 h after the CFA injection (Fig. 3B). The results obtained with control groups supported the effects of LG and LG/β-CD, since the vehicle (distilled water) had no activity.

3.4. Segmental spinal nerve ligation-induced neuropathic pain

Partial sciatic nerve ligation produced a marked and long-lasting development of hyperalgesia on the ipsilateral side eight days after PSNL in the control group, and in the experimental groups treated with

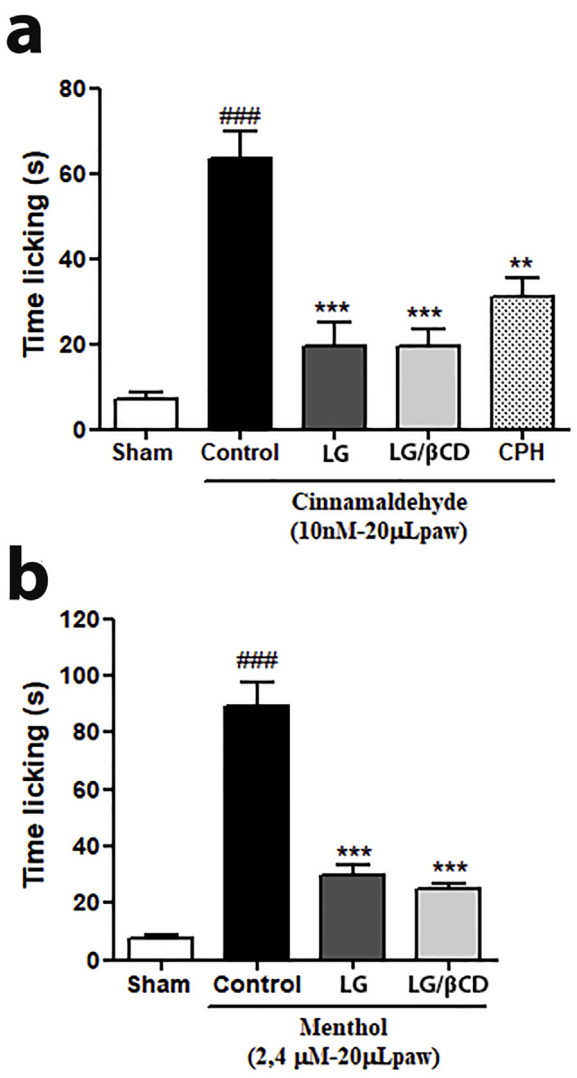

Fig. 2. Effect of LG and LG/β-CD on the time licking after the intraplantar injection of cinnamaldehyde 10 nM **(A)** and menthol 2,4 µM **(B).** Oral treatment was given 1 h prior to injection and the paw licking or biting was recorded for 5 min (cinnamaldehyde) and 20 min (menthol). Data expressed as means ± SEM of 8 animals. Statistical analysis was performed using a one-way ANOVA followed by the Tukey's test.$^{**}p < 0.01$, $^{***}p < 0.001$ vs control group; $^{\#\#\#}p < 0.01$ vs sham group.

LG and LG/β-CD (24 mg/kg, *i.g.*). The group that underwent sham surgery maintained the frequency response at baseline levels throughout the experiment (Fig. 4A). Acute oral treatment with the LG and LG/β-CD (24 mg/kg, *i.g.*) significantly decreased the paw withdrawal response, which lasted up to 6 h ($p < 0.001$). This antihyperalgesic effect was maintained while LG and LG/β-CD were orally administered daily until the 14th day posttreatment ($p < 0.001$). When treatment was interrupted for 2 days, mechanical hyperalgesia in the treated group was reestablished. On the 17th day the treatment was restarted, and LG and LG/β-CD were able to reduce mechanical hyperalgesia with an effect profile similar to the 1st day.

Regarding thermal hyperalgesia, the results presented in Fig. 4B show that PSNL induced a decrease in paw withdrawal latency to thermal stimulus (heat) compared to baseline. However, pretreatment with LG and LG/β-CD (24 mg/kg, *i.g.*) reduced the thermal hyperalgesia induced by PSNL and the response latency increased compared to the vehicle group ($p = 0.0001$).

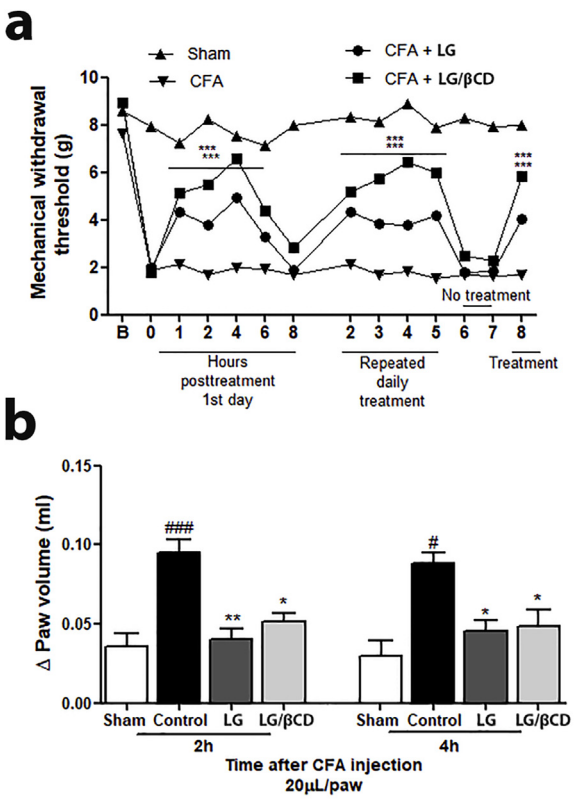

Fig. 3. Effect of LG and LG/β-CD on (**A**) mechanical hyperalgesia 24 h after the CFA injection and daily for 8 consecutive days; and on (**B**) paw edema 2 and 4 h after the CFA injection. Data are reported as means ± SEM of 8–10 animals. Statistically significant differences from sham and control group as determined by One-way ANOVA followed by Bonferroni (**A**) and Tukey's test (**B**). $^{*}p < 0.05$ $^{**}p < 0.01$, $^{***}p < 0.001$ vs control group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.01$ vs sham group.

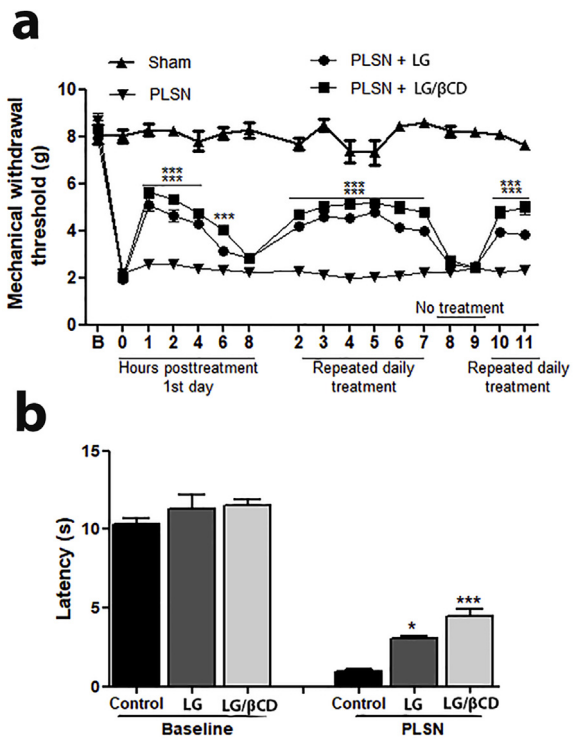

Fig. 4. Effect of LG and LG/β-CD on (**A**) mechanical hyperalgesia response after partial sciatic nerve ligation. The measures were recorded before surgery (B), immediately before treatment (0 h), after treatment (1, 2, 3, 4, 6 and 8 h) and daily for 8 days with a two-day break (eighth and ninth day); and on (**B**) the time of latency on the hot plate 1 h after the oral treatment. Data are reported as means ± SEM of 8–10 animals. Statistically significant differences from sham and control group as determined by One-way ANOVA followed by Bonferroni (**A**) and Tukey's test (**B**). $^{*}p < 0.05$, $^{***}p < 0.001$ vs control group.

3.5. Locomotor activity

Acute and prolonged intragastric treatment with LG and LG-βCD (24 mg/kg) did not alter the locomotor activity of animals in the rotarod and Grip Strength Meter tests, compared with the control group (data not shown).

3.6. Cytokine levels in sciatic nerve and spinal cord

The effects of LG and LG/β-CD (24 mg/kg, *i.g.*) were evaluated on TNF-α, IL-1β and IL-10 levels in the sciatic nerve and spinal cord 17 days after surgery. The results showed that PNSL increased TNF-α and IL-1β ($p < 0.05$) production in the sciatic nerve [$F(3,18) = 9.891$; $p = 0.0002$)] and spinal cord [$F(3,17) = 9.369$; $p = 0.0007$)]. In addition, LG and LG/β-CD were able to reduce the TNF-α levels in both structures ($p < 0.05$) when compared to the PNSL control group (Fig. 5A and D). No difference was found in IL-1β production between the treated-groups and the control group (Fig. 5).

3.7. NFκB and PKAc-α immunocontent in sciatic nerve and spinal cord

Fig. 6A summarizes the effect of LG and LG-βCD (24 mg/kg, *i.g*) on NFκB activation in sciatic nerve and spinal cord tissue. The results showed that PSNL increased phospho-NFκB (Ser536)/total NFκB immunocontent ratios compared to the sham group in the sciatic nerve [$F(3,16) = 11.86$; $p = 0.0002$)] and spinal cord [$F(3,17) = 4.466$; $p = 0.0173$)]. However, LG and LG-βCD presented unaltered phospho-NFκB (Ser536)/total NFκB immunocontent ratios in the sciatic nerve and spinal cord (Figures A, C).

Fig. 6B shows the PKAc-α immunocontent assessed by western blot. The results determined that LG and LG-βCD were able to reduce PKAc-α immunocontent compared to the control group in the sciatic nerve [$F(3,12) = 11.13$; $p = 0.0001$)] and spinal cord [$F(3,16) = 7.315$; $p = 0.0028$)] (Fig. 6B and C).

3.8. Inhibition of calcium current by LG

Our results show that the essential oil produced an inhibitory effect on calcium current (Ica) with fast onset (Fig. 7A and B). Perfusion of the *L. grata* at 0.1 μL/mL on DRG neurons decreased the peak of ICa by 24 ± 2% (mean ± SEM, n = 5; $p < 0.05$). The sustained component at the end of Ica was inhibited by 62 ± 8% (n = 5; $p < 0.05$). At an intermediate concentration (0.3 μL/mL), peak I_{Ca} decreased 71 ± 8% (n = 5; $p < 0.05$). The peak of I_{Ca} was essentially eliminated (inhibition of 94 ± 3%, n = 3; $p < 0.001$) by the essential oil at the highest concentration (1.0 μL/mL). The calculated IC50 was 0.21 μL/mL. The dose-response relationship is shown in Fig. 7C.

The inhibition caused by *L. grata* was reversible (Fig. 7B). After a 70-second wash, we observed a partial recovery of the calcium current.

4. Discussion

The International Association for the Study of Pain (IASP), defines neuropathic pain as “pain caused by a lesion or disease of the somatosensory system” [41]. The most dramatic symptom for NP patients is intense, persistent and often disabling chronic pain, therefore pain management is the main focus of new drugs for the control of NP.

There is strong evidence that both the peripheral nervous system and the central nervous system exert mechanisms that culminate in

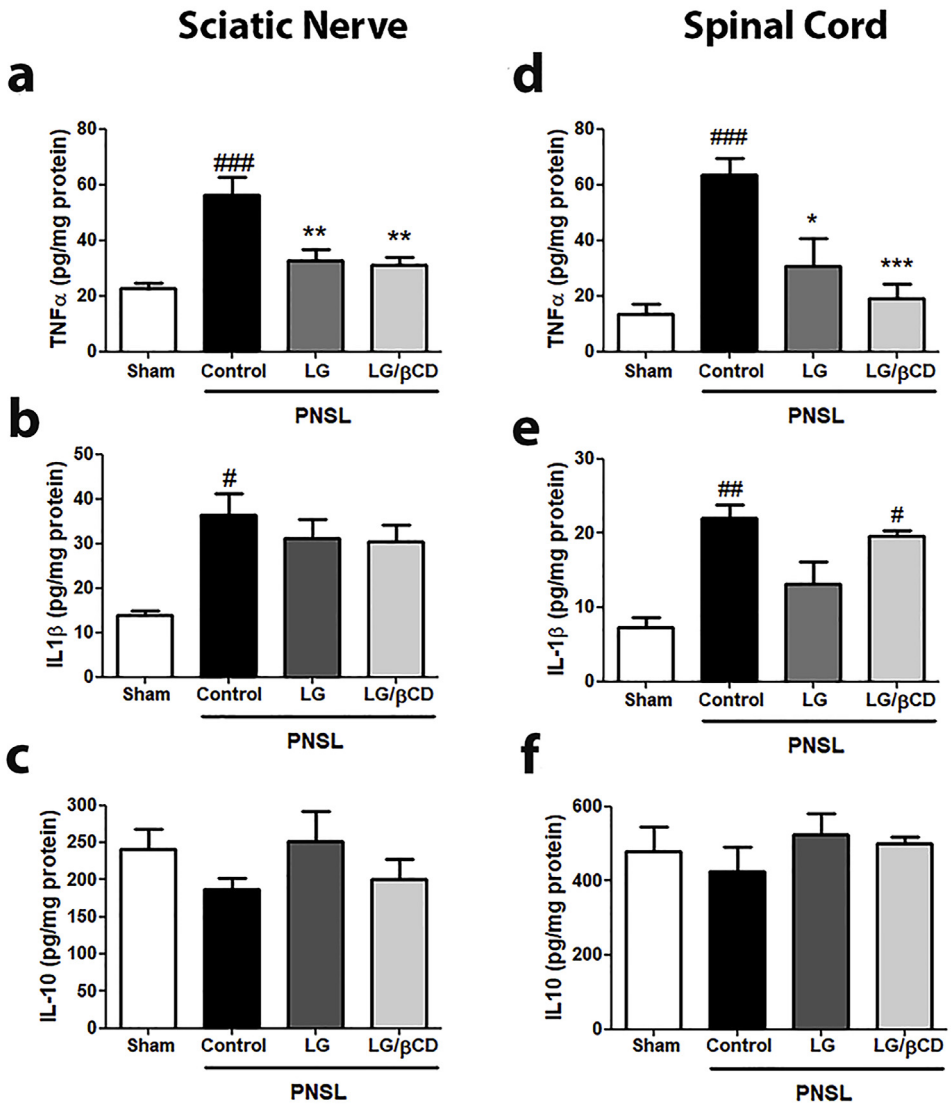

Fig. 5. Effect of LG and LG/β-CD on the levels of pro-inflammatory cytokines TNF-α (**A** and **D**) and IL-1β (**B** and **E**) and anti-inflammatory cytokine IL-10 (**C** and **F**) on the sciatic nerve and spinal cord, respectively. Data expressed as means ± SEM of 8–10 animals. Statistically significant differences from sham and control group as determined by One-way ANOVA followed by Tukey's test. $^{*}p < 0.05^{**}p < 0.01$, $^{***}p < 0.001$ vs control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.01$ vs sham group.

chronic pain, mainly NP. Tissue damage can result in activation of nociceptors through the release of several mediators, including excitatory amino acids, peptides, protons, lipids and cytokines, which bind to receptors and activate signaling pathways, among these are protein kinases A and C, calcium/calmodulin-dependent protein kinase, and mitogen-activated protein kinases (MAPKs) [42]. The essential oil of *L. grata* (rich in camphene, camphor, 1.8-cineole, borneol, β-caryophyllene and bicyclogermacrene) has shown interesting peripheral and central pharmacological activity, notably in the control of different types of pain. Siqueira-Lima et al. [27], showed that LG-βCD protected against orofacial nociception induced by formalin, capsaicin and glutamate in animal models. The authors also investigated the effects in a fibromyalgia-like animal model which seem to be related to the opioidergic and serotonergic pathways. In addition, LG-βCD treatment induced a significant decrease in Fos protein expression (Fos-positive cells) in the dorsal horn of the spinal cord, which suggests the possible involvement of descending pain-inhibitory mechanisms in its anti-hyperalgesic profile [28].

The present study aimed to understand the possible mechanisms by which the essential oil of *L. grata* leaves induces its analgesic effect in animal models. Our results demonstrated that essential LG and LG-βCD pretreatment reduces inflammatory pain by three main mechanisms: 1) inhibition of leukocyte migration; 2) inhibition of NF-kB activation and hyperalgesic cytokine production; and 3) involvement of the PKA pathway, which could sensitize ions channels such as TRPs (transient receptor potential channels).

We performed the carrageenan induced-pleurisy test to evaluate whether LG and LG/β-CD could produce some alteration in the production of pro-inflammatory cytokines because it is an acute protocol for the screening of possible anti-inflammatory drugs. There are no other reports in the literature of this type of approach to evaluate the effects of LG. Moreover, this test is characterized by leukocyte migration and the release of chemical mediators important in the inflammatory process, notably pro-inflammatory cytokines such as TNF-α and IL-1β [43]. As shown in Fig. 1, the pretreatment with LG or LG-βCD decreased the number of leucocytes recruitment to the pleural exudate and drastically reduced TNF-α and IL-1β levels. This result corroborated our initial hypothesis that LG has a possible anti-inflammatory effect as suggested by Siqueira-Lima et al. [27], and the inhibition of these two cytokines previously shown to be important in the establishment and severity of pain felt by NP which seems to be the most promising role of monoterpenes in modulating proinflammatory

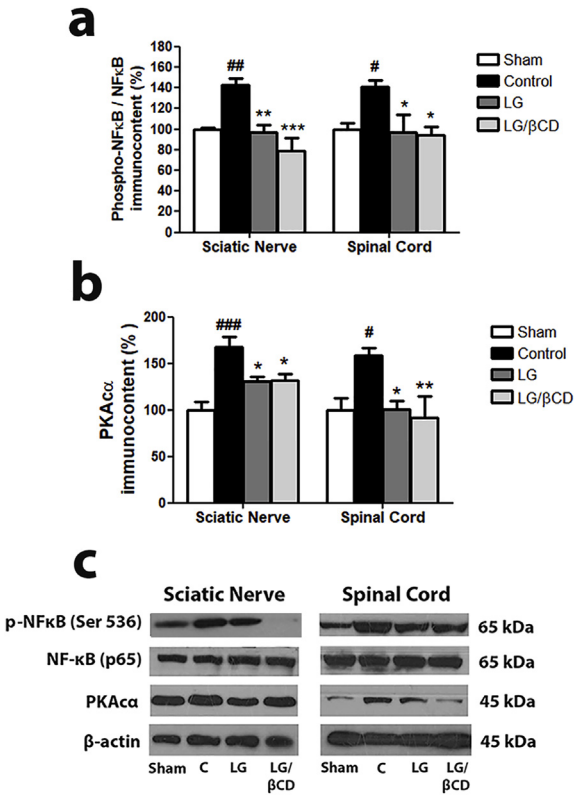

Fig. 6. Effect of LG and LG-βCD on phospho-NFκB/NFκB immunocontent ratio (A) and PKAcα immunocontent (B) in the sciatic nerve and spinal cord of mice submitted to PSNL. Representative blots are shown (C). Data are reported as means ± SEM of 5–6 animals and expressed as percent of sham value. Statistically significant differences from sham and control group as determined by One-way ANOVA followed by Tukey's test. $^{*}p < 0.05$ $^{**}p < 0.01$, $^{***}p < 0.001$ vs control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.01$ vs sham group.

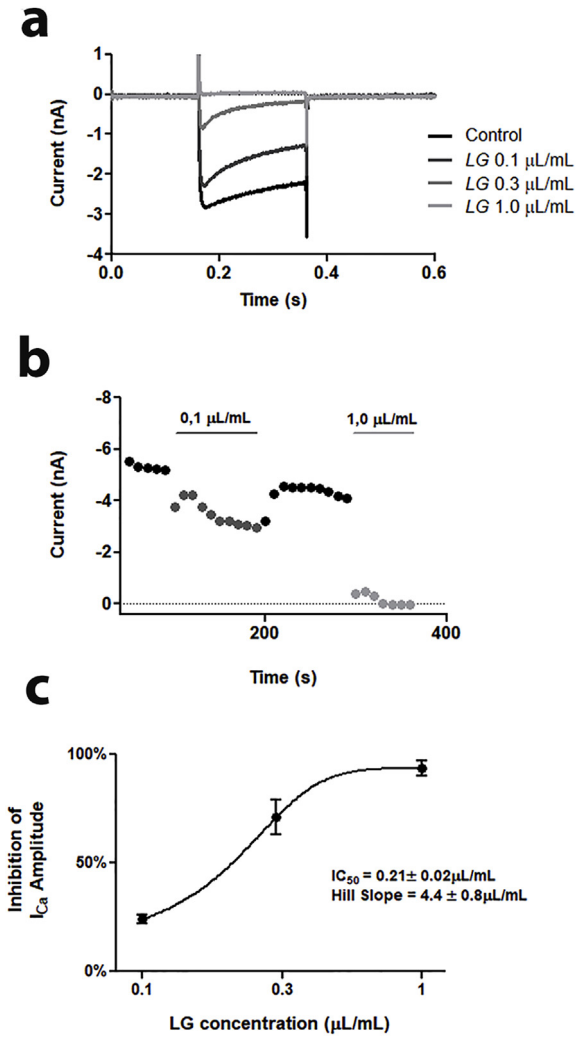

Fig. 7. Effect of essential oil of *Lippia grata* on amplitude and sustained component of Ca^{2+} current (I_{Ca}) in dorsal root ganglion neurons from *Wistar* rats. (a) Representative traces of I_{Ca} recorded before and after 7s of oil application at 0.1, 0.3 or 1.0 µL/mL in the same cell. **(b)** Time course of peak Ca^{+2} currents showing their inhibition and recovery after washout. **(d)** Dose-response relationship between the essential oil concentration and the inhibition of the Ca^{+2} current amplitude. IC50 was 0.21 ± 0.02 and the Hill slope was 4.4 ± 0.8.

cytokines as part of the mechanism of action [23,24,44,97]. Moreover, the inhibition by LG of these two cytokines previously shown to be important in NP pain is encouraging in terms of new treatment approaches.

The inhibition of pro-inflammatory cytokines is key to the management of inflammatory conditions which are common to chronic pain such as NP [45]. TNF-α is established as one of the most important cytokines in relation to cellular chemotaxis [46,47]. and inflammatory hyperalgesia [48,49], common processes in patients with NP. Some studies have suggested that TNF-α can act as a molecular marker of the pain process in NP patients and that reducing its levels or silencing its expression are promising targets for new therapeutic approaches [50–52]. Additionally, a recent study reported that overexpression of TNF-α in nociceptive neurons activates a cellular cascade, with subsequent TRPV1 phosphorylation and an increase in pain signaling which seems to be important in long lasting pain [53].

On the basis of these findings, we investigated the effect of LG or LG-βCD on edema and mechanical hyperalgesia induced by CFA. CFA causes a chronic inflammatory response, characterized by mechanical and thermal hyperalgesia and edema. This response is dependent on the severity of the tissue lesion and leads to the production and release of several inflammatory mediators [54,55]. We demonstrated that CFA induced edema and mechanical hyperalgesia was reduced by LG and LG/β-CD.

It is worth mentioning that the edema and hyperalgesia associated with inflammation are independent responses [56]. In addition, it is known that mechanical and thermal hyperalgesia observed in the CFA-induced inflammatory pain model were attenuated by TRPV1 gene deletion and activation of the opioid and adenosine A1 receptors [57]. This result is in line with the results of Siqueira-Lima et al. [27,28], who demonstrated a possible effect of LG/β-CD on TRPV1 and opioid receptors. Moreover, it is possible to infer that the reduction in edema by LG and LG/β-CD may be due to either decreased production or release of pro-inflammatory cytokines as researchers have shown that NSAIDs reduce edema in this way in a does dependent manner [58,59].

In an attempt to better understand TNF-α and IL-1β levels after pretreatment with LG or LG/β-CD, we assessed it in a partial sciatic nerve ligation of mice (an NP-animal model), since the inflammatory process is an important factor for pathogenesis of NP [60]. In peripheral nerve injury, glial cells support neuroinflammation and induce the release of pro-inflammatory mediators, such as cytokines (TNF-α, IL-1β and IL-6) [61–63]. TNF-α is the key cytokine released during neuroinflammation during NP. Due to its peripheral and central actions it is known as a “pro-neuropathic” cytokine and once released triggers the development of hypersensitivity after nerve injury [64–67]. We demonstrated that LG or LG/β-CD were effective in reducing thermal and mechanical hyperalgesia and also in decreasing TNF-α levels (but not

IL-1β levels) in the sciatic nerve and spinal cord of mice. This selective effect of modulating TNF-α levels in the injured nerve (which seems to be promising in terms of neuroregeneration) and in the spinal cord (which inhibits the activation of a cascade of factors that culminate in the usual hyperalgesic process in NP patients) is a very exciting finding. It highlights the importance of our results in relation to chronic pain management given that the literature (in animal models) suggests that treatments targeting control of TNF-α levels can partially alleviate NP. This could be a promising new approach in relation to the development of new painkillers to treat NP [68].

The inflammatory environment that sensitizes nociceptors and accompanies pain is transcriptionally regulated, such as with the nuclear factor NF-κB [69]. NF-κB is a nuclear transcription factor that regulates the expression of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α [70,71]. This factor plays a pivotal role in immune and inflammatory responses [72,73]. NF-κB activation in the spinal cord can be associated with inflammatory pain hypersensitivity [74] and induces the degradation of the inhibitor IκBα from the NF-κB-IκBα complex, followed by phosphorylation of NF-κB p65 and its translocation into the nucleus [75]. The decrease in phosphorylation of the NF-κB p65 subunit found in LG or LG/β-CD-treated animals indicates reduced NF-κB activation, leading to a decrease in cytokine production in the sciatic nerve and spinal cord. This finding agrees with the lower TNF-α levels found in the LG or LG-βCD treated groups when compared to control animals.

We also demonstrated the involvement of adenylyl cyclase and synthesis of cyclic adenosine monophosphate/protein kinase A (AMPc/PKA) in the LG or LG/β-CD treated animals in the NP-model. Studies have demonstrated that PKA acts downstream of inflammatory mediators and is implicated in the processing of nociception in NP, enhancing nociceptive hypersensitization [76–78]. PKA enhances the activity of sensory neurons, provoking sensitization at the peripheral C-fiber terminals resulting in a reduced nociceptive threshold or at the central terminals leading to increased nociceptive processing at the spinal cord level [78–80]. This enzyme is involved in cAMP responsive element binding prolonged synaptic plasticity during central sensitization, regulating nociception-related gene expression in nociceptive neurons, such as c-Fos, COX-2, NK-1 [81,82]. In addition, the PKA pathway sensitizes ion channels, such as TRPs, that promote neuronal and nociceptor excitability, leading to pain and hyperalgesia [77,83]. Thus, the inhibition of PKA activity induced by LG or LG/β-CD treatment could be causing TRP dephosphorylation and consequently ion channel desensitization, decreasing neuronal excitability and producing a painkiller effect. Therefore, PKA seems to be an important target for the anti-hyperalgesic effect of this essential oil.

Alterations in the normal neurophysiology of the spinal cord and dorsal root ganglion (DRG) play important roles in NP. Wang et al. [84], showed that the activation of cAMP/PKA in DRG neurons by bradykinin contributes to sensitizing TRPA1. Interestingly, TRPA1 acts an integrator molecule and can respond to the release of inflammatory mediators, allowing the amplification of the inflammatory and nociceptive processes [85]. In order to provide more direct evidence of the participation of TRPA1 in the effect of LG or LG-βCD, their antinociceptive effect was investigated in cinnamaldehyde induced-pain in the mice. LG or LG/β-CD significantly attenuated cinnamaldehyde-induced nociception, and this analgesic activity could be related to modulation and/or blockade of the TRP receptors (TRPV1 and TRPA1). Camphor, 1,8-cineole and β-caryophyllene are components of LG and are reported to exert analgesic effects, at least in part, through the inhibition of TRPA1 [86–88] and activation of TRPM8 [87,89]. Many drugs of natural origin, including essential oil compounds, interact with TRPM8 afferents (as activators or as inactivators) [90–92]. Our results are not clear enough to prove the involvement of LG and LG-CD with TRPM8, however, the compounds present in LG such as camphor and 1,8-cineole could possibly be acting on this receptor, thereby preventing its activation.

The expression of TNF-α in the spinal cord and DRG has been implicated in NP establishment, where the over expression of this cytokine is upregulated in satellite cells of the DRG after peripheral nerve injury [68]. The mechanical hyperalgesia induced by spinal nerve injury is associated with upregulation of satellite cells and TNF-α in the contralateral DRG [93,94]. Therefore, the overexpression of TNF-α in the sensory neurons increases the activity of TRPV1-dependent Ca^{2+} influx, resulting in inflammatory sensitization and pain [53]. The terpenes (camphor, 1.8-cineole and borneol) present in LG are compounds that commonly act on the TRPA1 receptors and also modulate TNF-α, thereby ameliorating pain [87,88]. Thus, the consistent inhibitory effect on the Ca^{2+} current of DRG neurons produced by LG reinforces the hypothesis that Ca^{2+} and TRP receptors are involved in the pharmacological mechanism of LG and that its major components may be acting synergistically in relation to this.

We compared the biologic effects of pure LG (non-βCD-complexed) and LG/β-CD for the first time in “in vivo” protocols and found a significant analgesic effect, although the mechanism of action is not yet clear. However, pure LG has low water solubility (data not shown) and showed reduced effectiveness in the animal models we used that could limit its clinical use, particularly in chronic conditions. However, cyclodextrins (CDs) have been demonstrated to be a useful tool to improve a number of chemical and pharmacological properties of non-polar compounds [30,95,96]. Our findings showed that the use of the inclusion complex improved the analgesic effect of LG, maintaining its action for 2 h longer than pure LG. Although LG and LG/β-CD were used at the same dose (24 mg/kg) it is noteworthy that LG/β-CD was prepared using a molar concentration of 1:1, so the effective dose of LG present in the β-CD-complex is lower when compared to LG alone. This fact is extremely important because it means the pharmacological effect was obtained with a smaller dose of LG.

5. Conclusion

The essential oil from *L. grata* leaf exerts an outstanding anti-hyperalgesic effect on pain of different origins and with equally diverse pharmacological mechanisms. These results are in line with the use of this medicinal plant in folk medicine for pain relief, and the complexation of its essential oil in βCD was shown to increase its effectiveness. The antihyperalgesic effect of LG is related to its anti-inflammatory profile (especially in mitigating pro-inflammatory cytokines), with the involvement of the PKA pathway and also the inhibition of voltage Ca^{2+} current. Moreover, the inhibition of TNF-α appears to be key to its analgesic profile. Thus, considering the limited number of drugs currently available for the treatment of NP, our results provide a rationale for the development of interventions targeting the study of LG as a potential new tool for chronic pain management in patients with NP, which could lead to better treatments for those affected by this chronic pain in the future.

Declaration of competing InterestCOI

The authors declare that they have no conflict of interest.

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