Spasmolytic activity of essential oil from *Lippia microphylla* Cham. (Verbenaceae) is mediated by modulation of Ca²⁺ signaling on animal and cellular models

A atividade espasmolítica do óleo essencial obtido de *Lippia microphylla* Cham. (Verbenaceae) é mediada pela modulação da sinalização do cálcio em modelos animais e celulares

La actividad espasmolítica del aceite esencial obtenido de *Lippia microphylla* Cham. (Verbenaceae) está mediada por la modulación de la señalización del calcio en modelos animales y celulares

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Abstract

Lippia species (Verbenaceae) are widely used on folk medicine to treat respiratory and gastrointestinal disorders. In this work, we evaluated the relaxant effect of the essential oil from *Lippia microphylla* Cham. (LM-EO) and its major components on guinea pig ileum as well as elucidate the involved mechanisms. LM-EO inhibited phasic contractions as well as relaxed tonic contractions induced by several contractile agents on guinea pig ileum. Similar results were observed in tests using thymol and carvacrol, the major constituents of LM-EO. Voltage-dependent calcium channel (Ca$_V$) blockade was suggested since LM-EO shifted to the right non parallelly CaCl$_2$-induced cumulative contractions and confirmed when LM-EO relaxed the ileum pre contracted by S-(-)-Bay K8644, a selective Ca$_V$ agonist. On circular layer, LM-EO inhibited phasic contractions, being equipotent to the effect observed in the intact ileum. In the myocytes from ileum longitudinal layer, LM-EO reduced fluorescence intensity induced by histamine, similarly to verapamil (Ca$_V$

blocker), indicating a decrease in cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_c$). In conclusion, LM-EO presents a spasmolytic activity on guinea pig ileum by Ca_V blockade and $[Ca^{2+}]_c$ reduction. These results present a rationale for the use of *L. microphylla* as a potential product to intestinal disorders.

Keywords: *Lippia microphylla* Cham; Verbenaceae; Essential oil; Thymol; Carvacrol; Intestinal smooth muscle.

Resumo

As espécies do gênero *Lippia* (Verbenaceae) são amplamente usadas na medicina tradicional no tratamento de desordens respiratórias e gastrointestinais. Neste estudo, avaliou-se o efeito relaxante do óleo essencial obtido de *Lippia microphylla* Cham. (LM-EO) e seus componentes majoritários em íleo isolado de cobaias bem como elucidou-se os mecanismos envolvidos. LM-EO inibiu as contrações fásicas e relaxou as tônicas induzidas por vários agentes contráteis em íleo de cobaia. Resultados semelhantes foram observados nos protocolos utilizando-se timol e carvacrol, os componentes majoritários de LM-EO. O bloqueio dos canais de cálcio dependentes de voltagem (Ca_V) foi sugerido uma vez que LM-EO deslocou para a direita e de maneira não paralela as contrações cumulativas induzidas por $CaCl_2$ e confirmou-se quando LM-EO relaxou o íleo pré-contraído por S-(-)-Bay K8644, um agonista seletivo dos Ca_V. Na camada muscular circular do íleo, LM-EO inibiu as contrações fásicas, sendo equipotente em relação aos efeitos no íleo íntegro. No miócitos isolados da camada longitudinal do íleo, LM-EO reduziu a intensidade da fluorescência induzida por histamina, semelhantemente ao verapamil (bloqueador Ca_V), indicando uma redução na concentração citosólica de cálcio ($[Ca^{2+}]_c$). Dessa forma, LM-EO apresenta uma atividade espasmolítica em íleo de cobaia pelo bloqueio dos Ca_V e consequente redução da $[Ca^{2+}]_c$. Esses resultados demostram-se como uma justificativa para o uso de *L. microphylla* como um produto terapêutico nas desordens intestinais.

Palavras-chave: *Lippia microphylla* Cham; Verbenaceae; Óleo essencial; Timol; Carvacrol; Músculo liso intestinal.

Resumen

Las especies del género *Lippia* (Verbenaceae) se utilizan ampliamente en la medicina tradicional para tratar desórdenes respiratorios y gastrointestinales. En este estudio, el efecto relajante del aceite esencial obtenido de *Lippia microphylla* Cham. (LM-EO) y sus componentes principales en el íleon de cobaya, así como los mecanismos implicados. LM-EO inhibió las contracciones fásicas y relajó la tonicidad inducida por varios agentes contráctiles en el íleon de cobaya. Se observaron resultados similares en los protocolos que utilizan timol y carvacrol, los componentes principales de LM-EO. Se sugirió el bloqueo de los canales de calcio dependientes de voltaje (Ca_V) ya que LM-EO se desplazó hacia la derecha y de manera no paralela las contracciones acumulativas inducidas por $CaCl_2$ y se confirmó cuando LM-EO relajó el íleon precontraído por S-(-)-Bay K8644, un agonista selectivo de Ca_V. En la capa muscular circular del íleon, LM-EO inhibió las contracciones fásicas, siendo equipotente en relación a los efectos sobre el íleon intacto. En miocitos aislados de la capa de íleon longitudinal, LM-EO redujo la intensidad de la fluorescencia inducida por histamina, de manera similar al verapamilo (bloqueador de Ca_V), lo que indica una reducción en la concentración de calcio citosólico ($[Ca^{2+}]_c$). Por tanto, LM-EO presenta actividad espasmolítica en el íleon de cobaya al bloquear el Ca_V y la consiguiente reducción de $[Ca^{2+}]_c$. Estos resultados se muestran como una justificación para el uso de *L. microphylla* como producto terapéutico en trastornos intestinales.

Palabras clave: *Lippia microphylla* Cham; Verbenaceae; Aceite essencial; Timol; Carvacrol; Músculo liso intestinal.

1. Introduction

Medicinal plants usage presents a respectable position in folk medicine (Agra et al., 2008) and natural products isolated from those plants are reported to be useful in a wide range of cosmetic and therapeutic conditions including digestive disorders, gynecological problems, dyspnea, spasmodic cough and flatulent colic (Tepe; Tepe, 2015).

Verbenaceae presents approximately 35 genus and 1000 species that thrives in tropical and subtropical regions, represents one of the centers of highest diversity in South America (Marx et al., 2010), in Brazil are found 17 genus and 250 species (Santos et al., 2009). Many species of *Lippia* are used on folk medicine as important remedies in the treatment of respiratory and gastrointestinal disorders, and cited as antimalarial, antiviral and cytostatic agents (Soraru; Bandoni, 1978; Pascual et al., 2001; Scarpa, 2004; Alonso; Desmarchelier, 2015). Several pharmacological activities have been demonstrated in studies with species from this genus, such as anticancer (Lemos et al., 2007), antiradical, acetylcholinesterase inhibitor (Morais et al., 2013), antibacterial (Rivero-Cruz et al., 2011) and spasmolytic (Rivero-Cruz et al., 2011; Görnemann et al., 2008; Blanco et al., 2013; Silva et al., 2016).

Lippia microphylla Cham. is an aromatic and medicinal shrub, branchy and crumbly found only in Guyana and Brazil (Pascual et al., 2001) popularly known as "alecrim-do-mato", "alecrim-de-tabuleiro" and "alecrim-pimenta", and used as

antiseptic or in the treatment of respiratory diseases as colds, flu, bronchitis, cough and asthma (Agra et al., 2008; Pascual et al., 2001). From aerial parts of *L. microphylla* was obtained the essential oil that showed to be bactericidal against *Shigella flexneri*, *Escherichia coli, Streptococcus pyogenes* and *Staphylococcus aureus* (Rodrigues et al., 2011), antifungal (De Souza et al., 2005), synergic with antibacterial as gentamicin and norfloxacin (Coutinho et al., 2011) and antitumor (Xavier et al., 2015).

Two previous reports have shown the chemical composition of the essential oil from *L. microphylla* (LM-EO): Pascual and cols presented the following components: 1.8-cineol, α-terpineol, terpinen-4-ol, methylthymol, sabinene, γ-terpinene and thymol; and the sesquiterpenes β-cariophylene e α-humulene (Pascual et al., 2001). On the other hand, Costa and cols presented as main compounds of LM-EO 1.8-cineol, thymol and α-pinene (Costa et al., 2005). In this study, we used a sample of LM-EO from leaves of Northeast Brazil that had its chemical characterization carried out and twenty-six compounds representing 99.9% of LM-EO were identified. The main chemical components were identified as thymol (46.5%), carvacrol (31.7%), *p*-cymene (9.0%) and γ-terpinene (2.9%) (Xavier et al., 2015).

Thus, the present study aimed to evaluate the potential uses of the essential oil obtained of the leaves from *L. microphylla* (LM-EO) in the treatment of intestinal disorders, besides to compare the LM-EO main isolated components in the same experimental models. In addition, we investigated the involved mechanisms on the LM-EO spasmolytic action.

2. Methodology

2.1 Botanic Material

Leaves of *L. microphylla* were collected in June in Serra Branca municipality, Paraíba State, Brazil. The plant material was identified by Maria de Fátima Agra (PhD) of the Botany Sector of Universidade Federal da Paraíba (UFPB). A voucher specimen (Agra 6118) is deposited at the Herbarium Prof. Lauro Pires Xavier (JPB) of UFPB. The essential oil was extracted and chemical constituents identified by Xavier and cols (Xavier et al., 2015).

2.2 Animals

Adult guinea pigs (*Cavia porcellus*) of both sexes from Bioterium Prof. Thomas George of UFPB weighing 300-500 g were used. The animals had free access to food and water, were kept in rooms at 21 ± 1 °C submitted to a 12 h light-dark cycle and fasted for 18 h before the experiments. Actions on reducing pain, stress and any suffering were taken in accordance with the Guidelines for the ethical use of animals in applied ethology studies and previously approved and performed in accordance with the Ethic Committee on Animal Use CEUA/UFPB (protocol 0504/12).

2.3 Chemicals

Potassium chloride (KCl), calcium chloride bihydrate (CaCl$_2$) were obtained from Vetec (Duque de Caxias, RJ, Brazil), histamine dihydrochloride, carbamylcholine hydrochloride (CCh) were obtained from Merck (Rio de Janeiro, RJ, Brazil). Cesium chloride (CsCl), Cremophor EL®, S-(-)-Bay K8644, verapamil, carvacrol and thymol were purchased from Sigma-Aldrich (Duque de Caxias, RJ, Brazil). All substances were dissolved and diluted in distilled water, except S-(-)-Bay K8644, which were dissolved in ethanol. LM-EO (10 mg/mL), carvacrol and thymol (10^{-2} M) were solubilized in Cremophor EL® (3%) and diluted in distilled water. Cremophor EL® never exceeding 0.01% (v/v) in the organ baths, resulting in no observable solvent effects on ileum muscle tone (unpublished data). Thymol/carvacrol mixture (1.46:1) were prepared and used at concentrations equivalent to those found in LM-EO. Thus, to prepare 1 mL of the thymol/carvacrol mixture 10^{-2} M, the solutions (10^{-2} M) of the constituents (595 µL of thymol and 405 µL of carvacrol) were mixed.

Hanks' Balanced Salt Solution (HBSS), Dulbecco's Modified Eagle Medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin, trypsin/EDTA solution (1:250), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), fetal bovine serum (FBS) and L-glutamine were acquired from Cultilab (Campinas, SP, Brazil) and Fluo-4 NW dye mix was obtained from Invitrogen (Carlsbad, CA, USA).

2.4 Guinea pig ileum preparation and measurement of contractile response

Animals were euthanized by guillotine decapitation. Ileum was immediately removed, cleaned of fat and connective tissue, immersed in physiological solution at room temperature and bubbled with carbogen (95% O_2 and 5% CO_2). Ileum segments (2-3 cm) were suspended in organ baths under arresting tension of 1.0 g at 37 °C. Tissues were allowed to stabilize for 30 min (Daniel et al., 2001). The physiological solution was a modified Krebs solution (mM): NaCl (117.0), KCl (4.7), $MgSO_4$ (1.3), NaH_2PO_4 (1.2), $CaCl_2$ (2.5), glucose (11.0) and $NaHCO_3$ (25.0) (Sun; Benishin, 1994) and a depolarizing solution (KCl 70 mM) nominally Ca^{2+}-free (mM): NaCl (51.7), KCl (70.0), $MgSO_4$ (1.3), NaH_2PO_4 (1.2), glucose (11.0) and $NaHCO_3$ (25.0) (Van Rossum, 1963). All solutions had their pH adjusted to 7.4 with 1M HCl or NaOH. Concentrations are given as final concentrations in the bath solution. Isotonic contractions were recorded using isotonic levers coupled to kymographs and smoked drums (DTF, Brazil). Isometric transducers (TIM 05) coupled to an amplifier (AECAD04F) connected to digital acquisition system AQCAD 2.1.6 from AVS Projetos (São Paulo, SP, Brazil) were used to record isometric contractions. The reversal of LM-EO, carvacrol, thymol or thymol/carvacrol mixture, relaxant effect was analyzed by its removal of organ bath, then physiological solution was added, after 60 min a new contraction was induced and we observed that the organs responsiveness was not altered (data not shown).

2.5 Cell culture

Guinea pig ileum was collected as described above. The longitudinal smooth muscle layer was carefully stripped off and the pieces placed in warmed physiological solution. Slices from the longitudinal smooth muscle layer of the guinea pig ileum were prepared as previously described (Shimuta et al., 1990). Fragments of longitudinal smooth muscle were used to prepare cell cultures (Claro et al., 2007). Cultures were kept in Dulbecco's Modified Eagle Medium supplemented with heat inactivated bovine serum fetal 10%, penicillin 100 U/mL, streptomycin 100 μg/mL and glutamine 0.02 M. Confluent cells in the mono layer were separated with trypsin 0.25% at 37 °C and cultured in DMEM. Then, supernatant was aspirated, the pellets suspended in 5 mL of HBSS and centrifuged for 5 min. The supernatant was discarded and the pellets formed were used in the experiments. All procedures were performed in aseptic environment using laminar flow cabinet.

2.6 Statistical analysis

Data are expressed as means and standard error of the mean (S.E.M.). Concentration values of a drug that produces 50% of its maximum effect (EC_{50}) or reduces the response to an agonist by 50% (IC_{50}) were determined with non-linear regression (Neubig et al., 2003). Differences between means were statistically compared using Student's *t*-test and/or one-way variance analysis (ANOVA) followed by Bonferroni's post-test correction when appropriate. The null hypothesis was rejected when $p < 0.05$. Data were analyzed using GraphPad Prism (GraphPad Software Inc., San Diego CA, USA).

2.7 Experimental protocols
2.7.1 Effect of LM-EO and/or its isolated compounds in the phasic contractions on guinea pig ileum

Guinea pig ileum was obtained as described above. After the stabilization period, to evaluate the phasic component of the ileum, two phasic contractions induced by CCh or histamine 10^{-6} M were obtained and used as control (100%). LM-EO, or

its compounds, was added in the organ baths in different concentrations in distinct experiments and afterwards a third contraction was obtained in products presence. IC_{50} values were expressed as mean and S.E.M. and assessed by non-linear regression.

2.7.2 Effect of LM-EO and/or its isolated compounds in the tonic contractions on guinea pig ileum

To evaluate the tonic component of the ileum contraction, tonic contractions were obtained by addition of KCl 40 mM, CCh 10^{-5} M or histamine 10^{-6} M, and LM-EO was cumulatively added, in different preparations, to observe a possible relaxation curve. Relaxant potency was expressed as mean and S.E.M. of EC_{50} individual values assessed through non-linear regression. Similarly, carvacrol, thymol or thymol/carvacrol mixture were added cumulatively, in different preparations, to observe its effects on guinea pig ileum pre-contracted by KCl 40 mM.

2.7.3 Investigation of LM-EO mechanism of action on guinea pig ileum
2.7.3.1 Effect of LM-EO on carbachol-induced tonic contractions in absence and presence of CsCl

Guinea pig ileum was obtained as described above. After the stabilization period, was pre-incubated with CsCl 5 mM, a non-selective potassium channel blocker, for 20 min (Cecchi et al., 1987). Then, a contraction was induced with CCh 10^{-5} M and on the tonic component of this contraction, LM-EO was added in cumulative concentrations. Relaxant efficacy and potency were expressed as mean and S.E.M. of EC_{50} individual values assessed through non-linear regression, and compared in the absence and presence of the blocker.

2.7.3.2 Effect of LM-EO on CaCl₂-induced cumulative contractions in depolarizing medium nominally Ca²⁺-free

After the tissue stabilization period, modified Krebs solution was replaced by a depolarizing solution (KCl 70 mM) nominally Ca^{2+}-free for 45 min. Two similar $CaCl_2$ cumulative concentration-response curves were obtained (control) and LM-EO incubated, in different concentrations, in the absence of $CaCl_2$ for 15 min and a third $CaCl_2$ cumulative curve was obtained in the presence LM-EO. Each preparation was exposed to a single essential oil concentration (Van Rossum, 1963).

2.7.3.3 Effect of LM-EO on S-(-)-Bay K8644-induced tonic contractions

After the tissue stabilization period, the ileum was partially depolarized with KCl 15 mM (Usowicz et al., 1995) and a contraction was induced by S-(-)-Bay K8644 3×10^{-7} M, a selective voltage-dependent calcium channel (Ca_V) agonist to L-type or Ca_V1 (Ferrante et al., 1989). In the tonic contraction, LM-EO was added cumulatively in order to obtain a relaxation curve. EC_{50} and maximum effect (E_{max}) were used to analyze relative potency and efficacy, respectively. Verapamil, a Ca_V blocker, was used as a positive control.

2.7.3.4 Effect of LM-EO on phasic contractions on circular layer

Guinea pig ileum was obtained as described above, and to obtain the circular layer, the mucosal layer was mechanically removed by friction with cotton soaked in modified Krebs solution on the inner wall of the organ. A ring-shaped segment of about 5 mm were suspended in organ baths under arresting tension of 0.5 g at 37 °C, and stabilized for 60 min (Cheng; Shinokuza, 1987). After the stabilization period, to evaluate the phasic component of the circular layer, two phasic contractions induced by CCh 10^{-6} M was obtained and used as control (100%). LM-EO was added in the organ baths in different concentrations in distinct experiments, for 15 min, and afterwards a third contraction was obtained in essential oil presence. IC_{50} values were expressed as mean and S.E.M. and assessed by non-linear regression.

2.7.3.5 Effect of LM-EO on longitudinal layer myocytes of guinea pig ileum

2.7.3.5.1 Effect of LM-EO on ileum longitudinal myocyte viability

Cellular viability was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] assay for investigating a possible LM-EO cytotoxicity on myocytes from ileum longitudinal layer (Denizot; Lang, 1986). Briefly, ileal myocytes were seeded on 96-well plates and cultured in DMEM at 37 °C and 5% CO_2 for 2 or 24 h. After the cell adherence period, the supernatant was removed and LM-EO (81 μg/mL) diluted in DMEM was added in the wells and then MTT 5 mg/mL was added to each well for 6 h. Water-insoluble dark blue formazan crystals formed in viable cells were solubilized in DMSO, and the absorbance was measured at 540 nm using a microplate reader (FlexStation 3). The number of viable cells is correlated with the intensity of absorbance of the wells of the microplate. The control was given only the DMEM culture medium without incubation of LM-EO. All experiments were performed in triplicate.

2.7.3.5.2 Effect of LM-EO on cytosolic Ca^{2+} concentration of ileum longitudinal myocytes

Homogenized pellets with culture medium were cultured in black 96-well microplates, approximately 40,000 cells per well, and stabilized in incubator for 24 h for cell adhesion. After the adherence period, the culture medium of each well was discarded and 50 μL fluo-4 Direct Calcium Assay Kit, according to the manufacturer's instructions (Molecular Probes/Invitrogen, USA), was added and let rest for 40 min at 37 °C in CO_2 incubator, protected from light. Fluo-4 is a high-affinity calcium indicator that fluoresces when excited at 488 nm and, even in low concentrations, can almost double the fluorescence of other dyes, which is valuable in low density plated cell lines (Paredes et al., 2008). After the period of fluorophore incorporation, the fluorescence was quantified in a FlexStation 3 microplate reader using the Soft Max Pro software (Molecular Devices, USA). Fluo-4 was excited at 490 nm, and light emission was detected at 524 nm. Records were obtained without interruption for 4 min. Fluorescence intensity was increased by adding histamine 10^{-6} M and after LM-EO (9 μg/mL) was added, in different experiments, to assess the essential oil action in modify the cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_c$). Verapamil (10^{-6} M), Ca_V standard blocker, was used as a positive control.

3. Results

3.1 Effect of LM-EO and/or its isolated compounds in the phasic contractions on guinea pig ileum

On guinea pig ileum, LM-EO (3-81 μg/mL, n = 5) antagonized concentration-dependently the phasic contractions induced by CCh or histamine 10^{-6} M, (IC_{50} = 24.4 ± 2.9 and 15.8 ± 2.3 μg/mL, respectively, Figure 1A and B), being more potent in inhibit the last one. In a similar manner, thymol, carvacrol and thymol/carvacrol mixture antagonized CCh- (IC_{50} = 21.3 ± 3.8; 16.0 ± 2.6 and 27.9 ± 4.8 μg/mL, respectively, n = 5, Fig. 1C, E and G) or histamine- (IC_{50} = 14.2 ± 1.6; 13.6 ± 1.3 and 13.0 ± 2.1 μg/mL, respectively, n = 5, Fig. 1D, F and H) induced phasic contractions. Compared with LM-EO, in neither case was no statistical difference.

Figure 1 - Effect of LM-EO, thymol, carvacrol or thymol/carvacrol mixture in the phasic contractions induced by CCh 10^{-6} M (**A**, **C**, **E** and **G**) or histamine 10^{-6} M (**B**, **D**, **F** and **H**) on guinea pig ileum. Symbols and vertical bars represent the mean and S.E.M., respectively, (n = 5). One-way ANOVA followed by Bonferroni's post-test, *p < 0.05 and ***p < 0.001 (control *vs.* LM-EO).
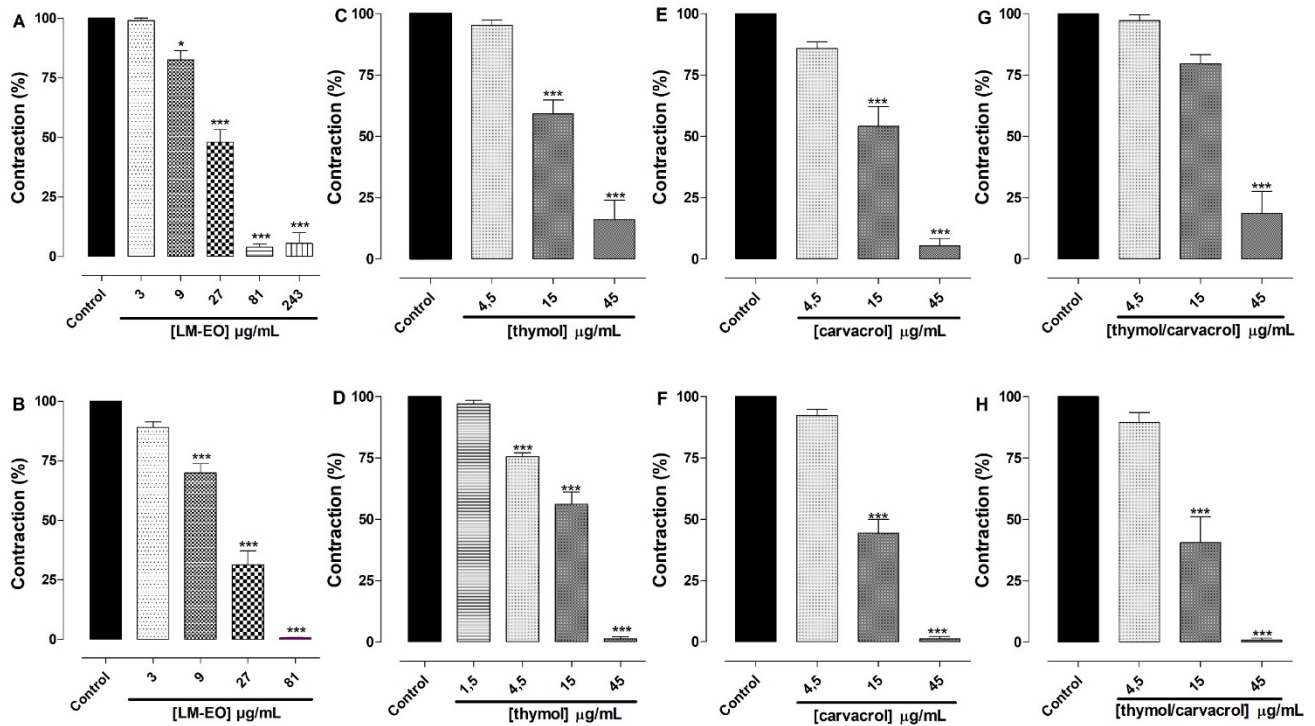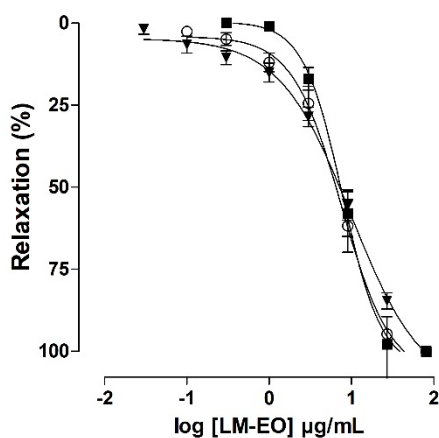

Source: Authors.

3.2 Effect of LM-EO and/or its isolated compounds in the tonic contractions on guinea pig ileum

In the same way, LM-EO relaxed pre-contracted organ by KCl 40 mM, CCh 10^{-5} M or histamine 10^{-6} M (EC$_{50}$ = 7.6 ± 0.8; 7.2 ± 1.3 and 6.8 ± 0.6 μg/mL, respectively, Figure 2), being equipotent in the three situations. Further, we evaluated the effect of the 2 major constituents of LM-EO on the tonic contraction induced by KCl 40 mM in isolated experiments or as a mixture. We found that thymol, carvacrol and thymol/carvacrol mixture relaxed the ileum in a concentration-dependent manner (EC$_{50}$ = 5.1 ± 1.1; 11.5 ± 1 and 5.1 ± 1.1 μg/mL, respectively, Figure 3), being carvacrol the least potent among the three, they did not show statistic difference when compared to LM-EO.

Figure 2 - Effect of LM-EO in the tonic contractions induced by CCh 10^{-5} M ($\bigcirc$), histamine 10^{-6} M ($\blacktriangledown$) or KCl 40 mM ($\blacksquare$) on guinea pig ileum. Symbols and vertical bars represent the mean and S.E.M., respectively, (n = 5).

Source: Authors.

Figure 3 - Effect of thymol ($\bullet$), carvacrol ($\bigcirc$) or thymol/carvacrol mixture ($\square$) in the tonic contractions induced by KCl 40 mM on guinea pig ileum. Symbols and vertical bars represent the mean and S.E.M., respectively, (n = 5).
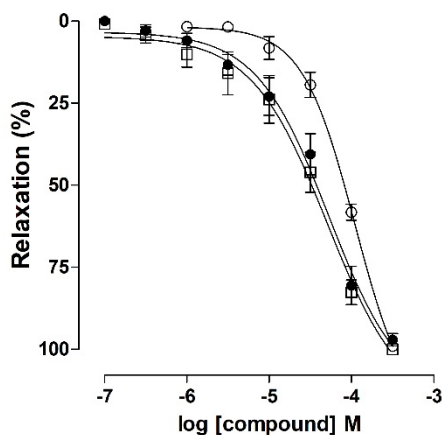
Source: Authors.

3.3 Investigation of LM-EO mechanism of action on guinea pig ileum

3.3.1 Effect of LM-EO on carbachol-induced tonic contractions in absence and presence of CsCl

LM-EO (0.03-81 µg/mL, n = 3) relaxation curve was not altered in the presence ($EC_{50} = 6.0 \pm 0.8$ µg/mL) of CsCl 5 mM, a non-selective potassium channel blocker, since the EC_{50} values did not differ in relation to control ($EC_{50} = 7.2 \pm 1.3$ µg/mL). LM-EO E_{max} was reached at 81 µg/mL, in both the absence and presence this blocker (Figure 4).

Figure 4 - Effect of LM-EO in the tonic contractions induced by CCh 10^{-5} M in the absence ($\bigcirc$) and presence of CsCl ($\blacklozenge$) on guinea pig ileum. Symbols and vertical bars represent the mean and S.E.M., respectively, (n = 3).
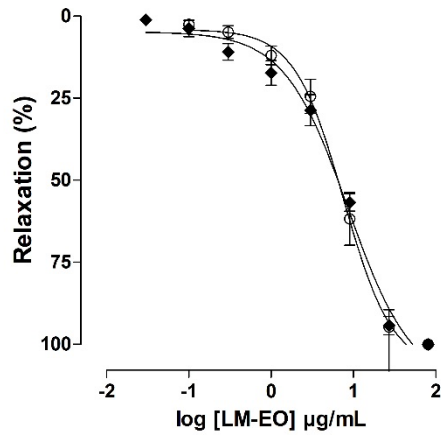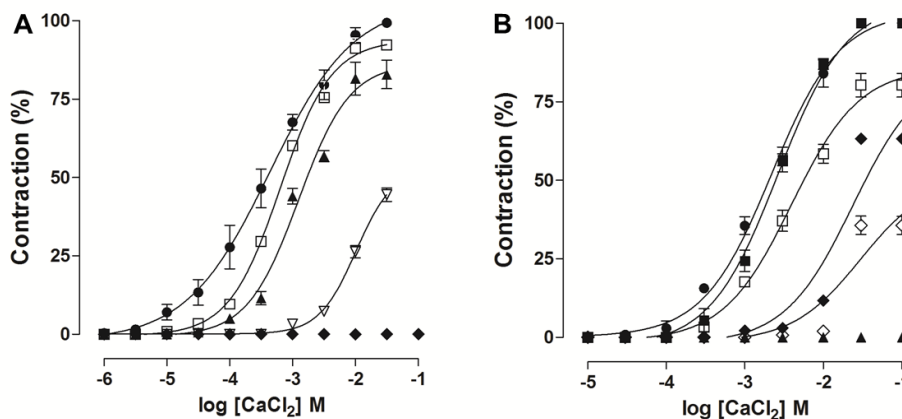

Source: Authors.

3.3.2 Effect of LM-EO on CaCl₂-induced cumulative contractions in depolarizing medium nominally Ca²⁺-free

LM-EO (3-81 µg/mL, n = 5) concentration-dependently inhibited Ca^{2+}-induced cumulative contractions in a depolarizing medium (KCl 70 mM) nominally Ca^{2+}-free (Figure 5A). CaCl₂ cumulative concentration-response curves were nonparallel shifted to the right, with E_{max} reduced from 100% (control) to 90.0 ± 2.3, 84.3 ± 4.3, 45.5 ± 2.1 and 0%. EC_{50} values were altered from $4.1 \pm 0.8 \times 10^{-4}$ M (control) to $6.3 \pm 0.5 \times 10^{-4}$, $11.9 \pm 0.8 \times 10^{-4}$ and $77.1 \pm 5.1 \times 10^{-4}$ M in the presence of 3, 9, 27 and 81 µg/mL of LM-EO. Similar results were observed using verapamil (3×10^{-8}–3×10^{-6} M), a classical Ca_V blocker, on CaCl₂ cumulative concentration-response curves (Figure 5B).

Figure 5 - Cumulative concentration-response curves to CaCl₂ in a depolarizing medium nominally Ca²⁺-free in the absence ($\bullet$) and presence of LM-EO (**A**): 3 ($\square$), 9 ($\blacktriangle$), 27 ($\triangle$) and 81 µg/mL ($\blacklozenge$) or verapamil (**B**): 3×10^{-8} ($\blacksquare$), 10^{-7} ($\square$), 3×10^{-7} ($\blacklozenge$), 10^{-6} ($\diamondsuit$) and 3×10^{-6} M ($\blacktriangle$). Symbols and vertical bars represent the mean and S.E.M., respectively, (n = 5).
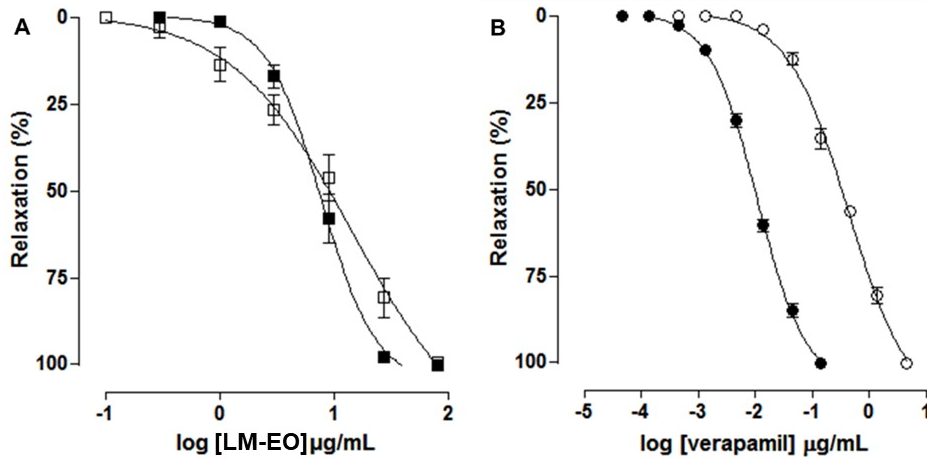

Source: Authors.

3.3.3 Effect of LM-EO on S-(-)-Bay K8644-induced tonic contractions

Cumulative addition of LM-EO (0.1-81 µg/mL, n = 5) to the tonic contraction elicited by S-(-)-Bay K8644 3×10^{-7} M induced a relaxation on guinea pig ileum in a concentration-dependent manner ($EC_{50} = 8.5 \pm 1.5$ µg/mL, Figure 6A). This effect was similar when the contractions were elicited with KCl 40 mM, CCh 10^{-5} M or histamine 10^{-6} M ($EC_{50} = 7.6 \pm 0.8$; 7.2 ± 1.3

and 6.8 ± 0.6 μg/mL, respectively) and similar results were observed using verapamil, a Cav blocker, on tonic contractions induced by KCl 40 mM or S-(-)-Bay K8644 3×10^{-7} M (Figure 6B).

Figure 6 - Effect of LM-EO (**A**) or verapamil (**B**) in the tonic contractions induced by KCl 40 mM (■,●) or S-(-)-Bay K8644 3×10^{-7} M (□,○). Symbols and vertical bars represent the mean and S.E.M., respectively, (n = 5).
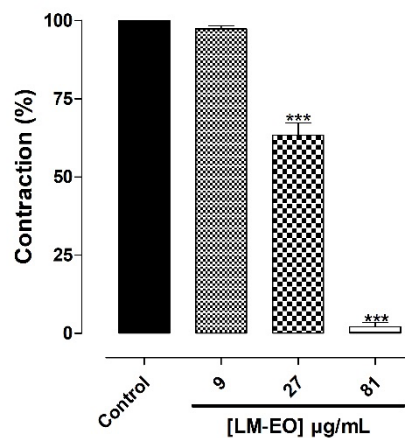

Source: Authors.

3.3.4 Effect of LM-EO on phasic contractions on circular layer of guinea pig ileum

On circular layer of guinea pig ileum, LM-EO (9-81 μg/mL, n = 5) inhibited concentration-dependently the phasic contractions induced by CCh 10^{-6} M, ($IC_{50} = 30.1 \pm 1.5$ μg/mL, Figure 7). EC_{50} values did not differ in relation to intact ileum ($EC_{50} = 24.4 \pm 2.9$ μg/mL).

Figure 7 - Effect of LM-EO in the phasic contractions induced by CCh 10^{-6} M on circular layer of guinea pig ileum. Symbols and vertical bars represent the mean and S.E.M., respectively, (n = 5). One-way ANOVA followed by Bonferroni's post-test, ***$p < 0.001$ (control *vs*. LM-EO).
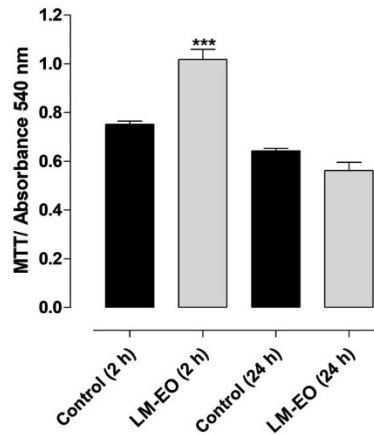

Source: authors

3.3.5 Effect of LM-EO on longitudinal layer myocytes of guinea pig ileum

3.3.5.1 Effect of LM-EO on ileum longitudinal myocyte viability

LM-EO (81 µg/mL, n = 3) did not induce cellular death in the myocytes from ileum longitudinal layer during 2 or 24 h of exposure was observed at Figure 8.

Figure 8. Effect of LM-EO on cell viability of myocytes from the longitudinal layer of guinea pig ileum. Student's *t*-test, ***$p < 0.001$ (control *vs.* LM-EO).
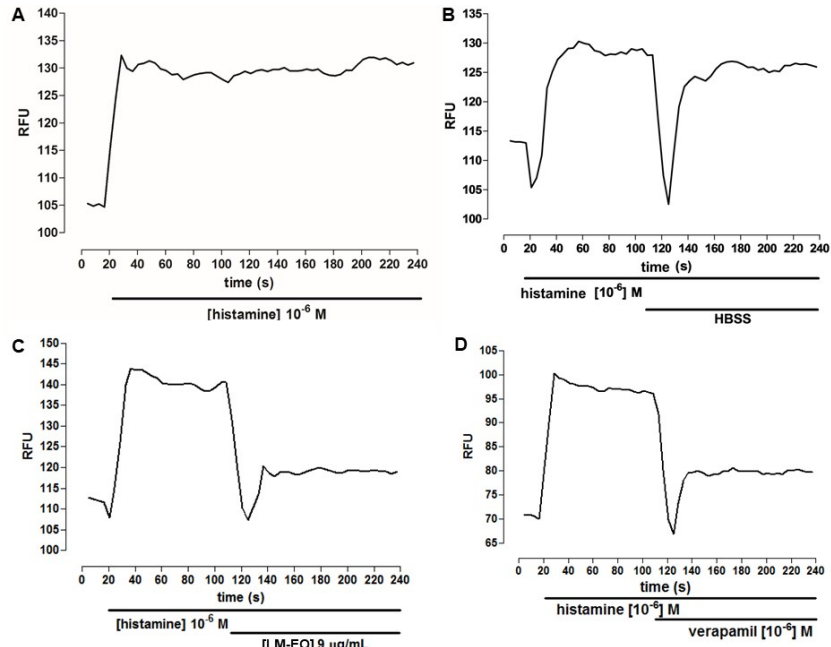

Source: Authors.

3.3.5.2 Effect of LM-EO on cytosolic Ca^{2+} concentration of ileum longitudinal myocytes

An increase in the [Ca^{2+}]$_c$ in myocytes from the ileum longitudinal layer loaded with calcium fluorophore fluo-4 was induced by histamine (10^{-6} M) and the fluorescence peak occurred 20 s after histamine addition and remained stable throughout the stimulation (220 s, Figure 9A). Then, LM-EO (9 µg/mL) was added, reducing the fluorescence intensity (30.4 ± 16.8%) in the cells exposed to the essential oil, throughout the observation period, which indicates a significant [Ca^{2+}]$_c$ decrease (Figure 9C and 10). A similar reduction of 34.5 ± 5.9% on [Ca^{2+}]$_c$ was obtained with verapamil 10^{-6} M (Figure 9D and 10). The observed fluorescence decline in the initial 20 s after LM-EO addition is an artifact since the same modification was observed after HBSS addition to the preparations (Figure 9B).

Figure 9. Representative records of control (**A**), HBSS (**B**), LM-EO (9 µg/mL) (**C**) and verapamil (10^{-6} M) (**D**) effects under the sign of calcium in myocytes from the guinea pig ileum longitudinal layer stimulated with histamine and loaded with fluo-4. RFU = relative fluorescence units.

Source: Authors.

Figure 10. Effect of LM-EO or verapamil on the fluorescence induced by histamine on guinea pig ileum myocytes. Columns and vertical bars represent the mean and S.E.M., respectively, (n = 3). Student's *t*-test, **$p < 0.01$ (histamine *vs.* LM-EO or verapamil).
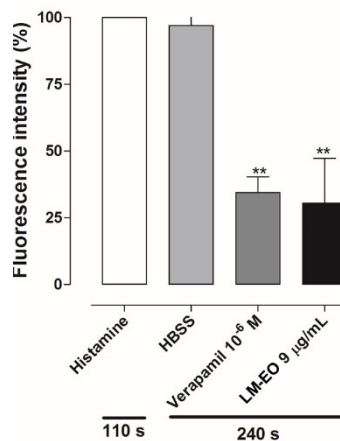
Source: Authors.

4. Discussion

Initially, we demonstrated the relaxant effect of *Lippia microphylla* Cham. essential oil, as well as of the carvacrol, thymol and thymol/carvacrol mixture (in the same proportion found in essential oil – 1.46:1, respectively) on guinea pig ileum. Additionally, we characterized the mechanism involved in the relaxant action of LM-EO that occurs due to a Ca$_V$1 inactivation and attenuation of Ca^{2+} influx, reducing the [Ca^{2+}]$_c$ in intestinal smooth muscles.

Ileal smooth muscle is known to present biphasic contraction where, in the first phase, the muscle exhibits a fast and transient contraction (phasic component) followed by a long-lasting second phase characterized by the maintained tonic contraction (tonic component) (Horie et al., 2005; Tanahashi et al., 2009). However, both phasic and tonic agonist-induced contractions depend on extracellular Ca^{2+} since both are inhibited in its absence (Honda; Takano; Kamiya, 1996).

LM-EO inhibited phasic contractions induced by CCh or histamine on guinea pig ileum, being more potent against the contractions induced by histamine. Since the major compounds of LM-EO are carvacrol and thymol, we decided to investigate if the LM-EO was more or less effective than its isolated compounds. We observed that, although carvacrol and thymol are already described as spasmolytic drugs in literature (Santos et al., 2011), its effects on guinea pig ileum are similar to LM-EO. Interesting when we use the thymol/carvacrol mixture, it shows the same behavior than LM-EO, *i.e.*, both LM-EO and mixed thymol/carvacrol were more potent in inhibiting contractions induced by histamine, but showed no difference among themselves (Fig. 1). These results suggest that it is safe to expect from LM-EO the same action than its isolated compounds.

Similarly, LM-EO relaxed guinea pig ileum pre-contracted by KCl (electromechanical coupling) and CCh or histamine (pharmaco- and electromechanical coupling, Figure 2). Since LM-EO acted on both contraction components, we hypothesized that the essential oil is acting in a common pathway related to the cascade of events elicited by these agents that leads to smooth muscle contraction: Ca^{2+} influx through Ca_V. As we find out that carvacrol, thymol or the thymol/carvacrol mixture present similar effect than LM-EO, we decided to verify its effect on guinea pig ileum pre-contracted by KCl 40 mM. The results shows that both thymol and the thymol/carvacrol mixture are more potent than carvacrol alone (Figure 3), but they are equipotent when compared to the essential oil. These results reinforce the hypothesis that the use of essential oil by the population are in agreement with the pharmacological findings for its compounds isolated and indicates LM-EO as a relaxant agent on intestinal smooth muscle.

It is reported that potassium channels play a key role in the regulation of membrane potential and cellular excitability, being smooth muscle contraction depends on a balance between increasing K^+ conductance, leading to repolarization/hyperpolarization, and decreasing it, leading to depolarization (Knot; Brayden; Nelson, 1996). Their activity can be regulated by voltage, Ca^{2+} or neurotransmitters, thus stimulating their signaling pathways (Alexander et al., 2017), because K^+ flux through its channels regulates Ca^{2+} influx (Thorneloe; Nelson, 2005). The equipotency of LM-EO against CCh-, histamine- and KCl-induced contractions indicates that the membrane depolarization induced by the elevation of extracellular K^+ concentration hinders the relaxation caused by LM-EO, suggesting is possibly activation of K^+ channels, leading to membrane hyperpolarizing and subsequently preventing Ca^{2+} influx through Ca_V1. However, the relaxant potency of LM-EO was not altered in the presence of CsCl, a non-selective K^+ channel blocker (Figure 4), discarding the involvement of these channels.

The common pathway related to the cascade of events elicited by contractile agents used in this work that leads to smooth muscle contraction is the Ca^{2+} influx through Ca_V. Thus, $CaCl_2$-induced contractions in a depolarizing medium nominally Ca^{2+}-free were used to assess the possible Ca_V blockade. This protocol is based on the fact that contraction will be obtained almost exclusively by Ca^{2+} from extracellular medium, since depolarization promoted by elevated extracellular K^+ concentrations leads to Ca_V opening (Rembold, 1992). LM-EO inhibited $CaCl_2$-induced contractions (Figure 5) reinforcing the hypothesis of calcium influx blockade in its relaxant effect on guinea pig ileum. Since the main chemical components of LM-EO, thymol presented relaxant activity on rat ileum (Begrow et al., 2010) and vasorelaxant action due to Ca_V blockade and carvacrol showed vasorelaxant activity on rat aorta (Peixoto-Neves et al., 2010) both constituents could be contributing to the relaxant activity of LM-EO on guinea pig ileum, but further studies are necessary to confirm this hypothesis.

In smooth muscle, Ca_V1 are the main responsible to calcium influx. These channels are subdivided as $Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$ and $Ca_V1.4$ and are sensitive to dihydropyridine and high voltage (Alexander et al., 2017). Thus, the next step was to confirm and identify the Ca_V subtype involved on LM-EO relaxant activity. Therefore, tonic contractions were obtained using

S-(-)-Bay K8644, a agonist for Ca$_V$1, that opens directly these channels, but not by depolarization (Spedding; Paoletti, 1992). LM-EO relaxed the guinea pig ileum pre-contracted by S-(-)-Bay K8644 in a similar manner to the relaxant effect on KCl- CCh- or histamine-contracted ileum, suggests that LM-EO attenuated Ca^{2+} influx by an indirect mechanism. Additionally verapamil relaxed the ileum pre-contracted by S-(-)-Bay K8644 and KCl similarly to LM-EO, that reinforce a Ca$_V$ blockade (Figure 6). Furthermore, as the more expressive Ca$_V$ subtype in ileum is Ca$_V$1.2 (Catterall, 2011), we confirm that the Ca^{2+} influx blockade through Ca$_V$1.2 is implicated in the mechanism underlying the LM-EO relaxant action on guinea pig ileum.

Intestinal smooth muscle has two layers separated by a system of neurons, known as the myenteric plexus. In the longitudinal layer cells are arranged in parallel along the gut axis and the circular layer, innermost, the cells are in parallel with the intestinal circumference. These two cell types found in the gut show different signaling pathways by which can be contracted (Makhlouf; Murthy, 1997). In muscle from both layers, G protein coupled receptors agonists initiate contraction by an increase in [Ca^{2+}]$_c$. Initial contraction and increase of [Ca^{2+}]$_c$ were not affected by Ca^{2+} channel blockers or extracellular calcium withdrawal into smooth muscle cells of the circular layer, but were abolished in cells of the longitudinal layer. This indicates that the contraction in this layer is dependent of Ca^{2+} influx (Murthy, 2006). LM-EO inhibited phasic contractions induced by CCh on circular layer of guinea pig ileum (Figure 7), being equipotent to the effect observed in the intact ileum. This result reinforces the activity of essential oil on the calcium influx blockade.

In order to relax smooth muscle it is required a reduction of [Ca^{2+}]$_c$, since contraction trigger and part of the contraction maintenance depend on [Ca^{2+}]$_c$ increase (Somlyo; Somlyo, 1994; Webb, 2003). Nowadays, techniques that use fluorescent indicators allow us to measure the cytosolic Ca^{2+} concentration in several models of smooth muscles (Wray; Burdyga; Noble, 2005). All results presented until now, supposedly, indicate a reduction in the [Ca^{2+}]$_c$ availability through Ca$_V$ blockade. Thus, we evaluated LM-EO action in myocytes isolated from ileum longitudinal layer and so provide data about safety and [Ca^{2+}]$_c$ reduction induced by the essential oil. Cell viability was evaluated in two intervals using the LM-EO maximal concentration used in *in vitro* experiments, in 2 or 24 h. LM-EO exposure to the cells was not observed any cell death (Figure 8), this result shows that any reduction in the [Ca^{2+}]$_c$ is not due to cell death and attribute safety to LM-EO at cellular level. LM-EO reduced the fluorescence, around 60%, emitted in the presence of histamine, which indicates [Ca^{2+}]$_c$ reduction as well as verapamil (Figure 9 and 10). The results in cellular experiments support our findings in functional level and evidence that LM-EO reduces [Ca^{2+}]$_c$ to play its relaxant role on intestinal smooth muscle.

5. Conclusions

In the search for drugs with therapeutic potential for treating intestinal disorders, LM-EO is presented as a relaxant agent in intestinal tract. LM-EO and its main chemical constituents showed an equipotency that indicates the essential oil as a promising product to be used to improve the current pharmacotherapy to intestinal-related conditions, as diarrhea or cramps. Futhermore, LM-EO mechanism of action seems to involve the [Ca^{2+}]$_c$ reduction by blockade of Ca$_V$ on guinea pig ileum as observed in functional and cellular levels.

Acknowledgments

Conflict of Interest

The authors declare no competing financial interest.

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