SUPPLEMENTARY MATERIAL

Essential oil from *Lippia microphylla* Cham. modulates nitric oxide pathway and calcium influx to exert a tocolytic effect in rat uterus

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Abstract

The essential oil of *Lippia microphylla* (LM-OE) presents several pharmacological activities. This work evaluates the tocolytic effect of LM-OE on rats. LM-OE inhibited phasic contractions and relaxed tonic contractions on rat uterus. Considering that nitric oxide (NO) pathway regulates uterine contraction, LM-OE potency was attenuated in the presence of NO synthase (NOS) inhibitor and this reduction was reversed in the presence of a NOS substrate. Similarly, the relaxant potency of LM-OE was reduced in the presence of soluble guanylyl cyclase (sGC) and protein kinase G (PKG) inhibitors. LM-OE also demonstrates a positive modulation of large and small conductance calcium-activated, voltage-gated and adenosine triphosphate-sensitive potassium channels and inhibited curves to $CaCl_2$ as well as relaxed the uterus pre-contracted by S-(-)-Bay K8644, suggesting voltage-gated calcium channels type-1 (Ca_V1) blockade. Thus, the tocolytic effect of LM-OE on rat involves positive modulation of NO/NOS/sGC/PKG/K^+-channels pathway and Ca^{2+} influx blockade through Ca_V1.

Keywords: *Lippia microphylla* Cham; tocolytic effect; nitric oxide pathway; potassium channels, calcium influx, rat uterus

1 Experimental

1.1 Chemicals

$CaCl_2$, $C_6H_{12}O_6$, $NaHCO_3$, $MgCl_2$, KCl, and NaCl were purchased from Química Moderna (São Paulo, Brazil). Cremophor EL®, diethylstilbestrol, oxytocin and S-(-)-1,4-dihydro-2,6dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-3pyridinecarboxylic acid methyl ester (S()Bay K8644), cesium chloride (CsCl), glibencalmide, tetraethylammonium ion (TEA^+), apamin, 4aminopyridine (4-AP), L-arginine hydrochloride, 1H-[1,2,4]-oxadiazolo-[4,3-a]-quinoxalin-1-one (ODQ) and N_ω-nitro-L-arginine methyl ester hydrochloride (L NAME) were obtained from Merck (São Paulo, Brazil). All substances were dissolved in distilled water, except for diethylstilbestrol and S()Bay K8644 which were solubilized in absolute ethanol P.A. obtained from Fmaia (São Paulo, Brazil). Carbogen mixture (95% O_2 and 5% CO_2) was purchased from White Martins (João Pessoa, Brazil).

1.2 Botanic material

Leaves of *Lippia microphylla* Cham. were collected in the Serra Branca municipality, Paraíba State, Brazil, and identified by Maria de Fátima Agra (PhD) from the Botany Sector of Universidade Federal da Paraíba (UFPB). A voucher specimen was deposited in the Herbarium Prof. Lauro Pires Xavier (JPB) under the identification code Agra 6118. The essential oil used in this work was extracted and chemical constituents identified by Xavier et al. (2015). LM-OE was solubilized in Cremophor EL® (3%), diluted in distilled water to a concentration of 10 mg/mL and re-diluted in distilled water as required for each experimental protocol. The final Cremophor EL® concentration never exceeded 0.01% (v / v), at that concentration, it is devoid of significant relaxing or contractile effect on rat uterus (data not shown).

1.3 Animals and tension measurement

Virgin female rats (*Rattus norvegicus*), weighing 150-250 g, estrogenized with diethylstilbestrol 1.0 mg/kg subcutaneously (s.c.) 24 h prior to the experiments, were used in this study. The animals had full access to food (Presence®, Brazil) and water, they were kept in rooms at 21 ± 1 °C and submitted to a 12 h light-dark cycle. All experimental procedures were approved by the Ethical Committee in Animal Use of UFPB (Protocol 1005/13). Animals were euthanized by decapitation in a guillotine. Uterine horns were removed, cleaned of adhering fat and connective tissue, and suspended by a cotton thread in organ baths (6 mL) containing the Locke-Ringer solution at pH 7.4 (in mM*:* NaCl 154.0; KCl 5.63; $CaCl_2$ 2.16; $MgCl_2$ 2.1; $NaHCO_3$ 5.95; and glucose 5.55), kept under temperature of 32 °C and continuously bubbled with a carbogen mixture, to stabilize for 40 min, in a resting load of 1.0 g (baseline) (Adapted of Revuelta et al. 1987). The isotonic contractions were recorded on the smoked drum through levers coupled to Kymographs DTF (São Paulo, Brazil). Organ baths were coupled to a pump thermostat model 12002 Polystat Cole-Palmer (Vernon Hills, USA) and isometric contractions were registered by isometric force transducers (TIM 05), coupled to an amplifier (AECAD04F) and a digital system software AQCAD version 2.4.1 for data acquisition and analysis for ANCAD. The system contained a thermostatic pump (BT-60) (AVS Projects, São Paulo, Brazil).

1.4 Effect of LM-OE on phasic and tonic contractions

After the stabilization period, two similar magnitude isotonic contractions were obtained with oxytocin (OXY) 10^{-2} IU/mL. LM-OE was added in different preparations and after 5 min of the incubation period, a third contraction was induced in the presence of essential oil. The maximum effect (E_{max}) and the negative logarithm to base 10 of concentration that reduces 50% of the response to an agonist (pIC_{50}) were calculated and compared to control, being pIC_{50} values calculated by nonlinear regression. In other protocol, isometric contractions were induced by KCl 60 mM or OXY 10^{-2} IU/mL and during the tonic phase, LM-OE was cumulatively added to obtain concentration-response curves in different preparations. The results were expressed as the reverse percentage of initial contraction elicited by the contractile agents. E_{max} and the

negative logarithm to base 10 of concentration that produces 50% of its maximal effect (pEC_{50}) were calculated and compared to both contractile agents.

1.5 Effect of LM-OE on the NO pathway

After the stabilization period, isometric contractions were induced by OXY 10^{-2} IU/mL and during the tonic phase, LM-OE was cumulatively added to obtain concentration-response curves in different preparations. In other experiments, L-NAME 10^{-4} M, a non-selective nitric oxide synthase (NOS) inhibitor (Munglue et al. 2012); L-NAME 10^{-4} M + L-arginine 10^{-3} M, substrate for NOS (Bulbul et al. 2007); L-arginine 10^{-3} M; ODQ 10^{-6} M, a soluble guanylyl cyclase (sGC) inhibitor (Parra et al. 2000) or Rp-8-Br-PET-cGMPS 10^{-6} M, a protein kinase G (PKG) inhibitor (Parra et al. 2000) were added to the organ bath for 20 min. After this period, a new contraction was induced by OXY 10^{-2} IU/mL, then, the LM-OE was cumulatively added to the tonic component of this contraction. The results were expressed as the reverse percentage of initial contraction elicited by the contractile agents. E_{max} and the pEC_{50} were calculated and compared in the absence and presence of each inhibitor.

1.6 Effect of LM-OE on K^+ channels

After the stabilization period, isometric contractions were induced by OXY 10^{-2} IU/mL and during the tonic phase, LM-OE was cumulatively added to obtain concentration-response curves in different preparations. In other experiments, CsCl 5 x 10^{-3} M, a non-selective K^+ channels blocker (Latorre et al. 1989); TEA^+ 10^{-3} M, apamin 10^{-7} M, 4-AP 3 x 10^{-3} M or glibenclamide 10^{-5} M, potassium channels selective blockers of large (BK_{Ca}) and small (SK_{Ca}) conductance calcium-activated; voltage-gated (Kv) adenosine triphosphate-sensitive (K_{ATP}), respectively (Piper et al. 1990; Hughest and Hollingsworth 1997; Tsai et al. 1998; Ayar et al. 2001; Aaronson et al. 2006), were added to the organ bath for 20 min. After this period, a new contraction was induced by OXY 10^{-2} IU/mL and, the LM-OE was cumulatively added to the tonic component of this contraction. The results were expressed as the reverse percentage of initial contraction elicited by the contractile agents. E_{max} and the pEC_{50} were calculated and compared in the absence and presence of each blocker.

1.7 Effect of LM-OE on calcium signalling

After the 30 min stabilization period, the Locke-Ringer solution was replaced by a nominally without Ca^{2+} Locke-Ringer solution. After 30 min, KCl 60 mM was added to produce smooth muscle depolarization and it remained in the bath until the end of the experiment. Ten minutes after KCl addition, $CaCl_2$ (10^{-6}-10^{-1} M) was added in cumulative concentrations, each being left to act the necessary amount of time to produce a maximum effect. When the maximal effect was achieved (100% contraction), the preparation was washed with Ca^{2+}free solution. A second concentration-response curve was obtained 90 min after washing in LM-OE presence, incubated for 15 min after the addition of KCl, in different preparations (Adapted of Revuelta et al. 1987). The inhibitory effect produced by LM-OE was evaluated based on the analysis of pEC_{50} and E_{max} values for $CaCl_2$ in the absence and presence of the essential oil.
In other protocol, the preparations were pre-contracted by KCl 60 mM or partially depolarized by KCl 15 mM for 10 min followed by a tonic contraction induced by S-(-)-Bay K8644 3 x 10^{-7} M (Calixto and Rae 1991), a type 1 Ca_V selective agonist (Spedding and Paoletti 1992), then, LM-OE was cumulatively added after the plateau phase to obtain the relaxation curve. The results were expressed as the reverse percentage of initial contraction elicited by contractile agents. E_{max} and pEC_{50} values were calculated as previously described.

1.8 Statistical analysis

The values were expressed as the mean and standard error of the mean (S.E.M.) and statistically analyzed by the Student's *t*-test or one-way variance analysis (ANOVA) followed by Tukey's test. The null hypothesis was rejected when $p < 0.05$. pIC_{50} or pEC_{50} were calculated by nonlinear regression (Neubig et al. 2003) and

E_{max} values were used as a measure of effectiveness. The data were analyzed by GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

Supplementary material

Inhibitors	E_{max} (%)	pEC_{50}
Absence	100	0.45 ± 0.07
L-NAME	100	1.39 ± 0.07*
L-arginine	100	$0.70 \pm 0.15^{\#}$
L-NAME + L-arginine	100	$0.82 \pm 0.08^{\#}$
ODQ	100	0.99 ± 0.05*
Rp-8-Br-PET-cGMPS	99.2 ± 0.8	0.94 ± 0.05*

Table S1. Values of E_{max} and pEC_{50} from LM-OE on the NO/sGC/PKG pathway on rat uterus. One-way ANOVA followed by Tukey's test (n = 5), *$p < 0.05$ (absence *vs*. L-NAME, ODQ and Rp-8-Br-PET-cGMPS) and $^{\#}p < 0.05$ (L-NAME *vs.* L-arginine and L-NAME + L-arginine).

Blockers	E_{max} (%)	pEC_{50}
Absence	100	0.45 ± 0.07
CsCl	100	1.05 ± 0.05*
TEA^{+}	100	1.02 ± 0.01*
Apamin	100	0.98 ± 0.05*
4-AP	100	1.07 ± 0.07*
Glibenclamide	100	0.98 ± 0.04*

Table S2. Values of E_{max} and pEC_{50} from LM-OE on K^{+} channels modulation on rat uterus. One-way ANOVA followed by Tukey's test (n = 5), *$p < 0.05$ (absence *vs*. blockers).

LM-OE (µg/mL)	E_{max} (%)	pEC_{50}
Absence	100	3.14 ± 0.03
9	87.5 ± 1.8	2.88 ± 0.12
27	68.1 ± 5.7*	2.65 ± 0.08
81	40.7 ± 3.0*	1.62 ± 0.09
243	7.9 ± 2.5*	1.26 ± 0.9*

Table S3. Values of E_{max} and pEC_{50} for $CaCl_2$ in the absence and presence of LM-OE on rat uterus. One-way ANOVA followed by Tukey's test (n = 5), *$p < 0.05$ (absence *vs*. LM-OE).

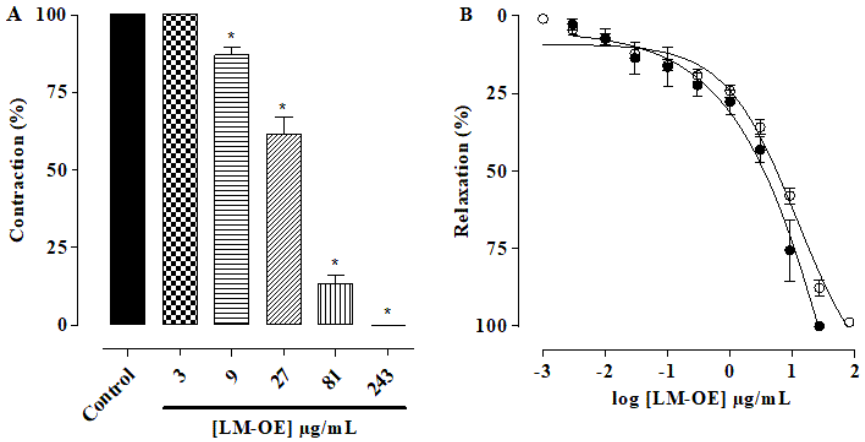

Figure 1. Tocolytic effect of LM-OE on phasic contractions induced by OXY 10^{-2} IU/mL (A) and tonic contractions induced by OXY 10^{-2} IU/mL (●) or KCl 60 mM (○) (B) on rat. Columns or symbols and vertical bars represent mean and S.E.M., respectively (n = 5). One-way ANOVA followed by Tukey's test, *$p < 0.05$ (control *vs*. LM-OE).

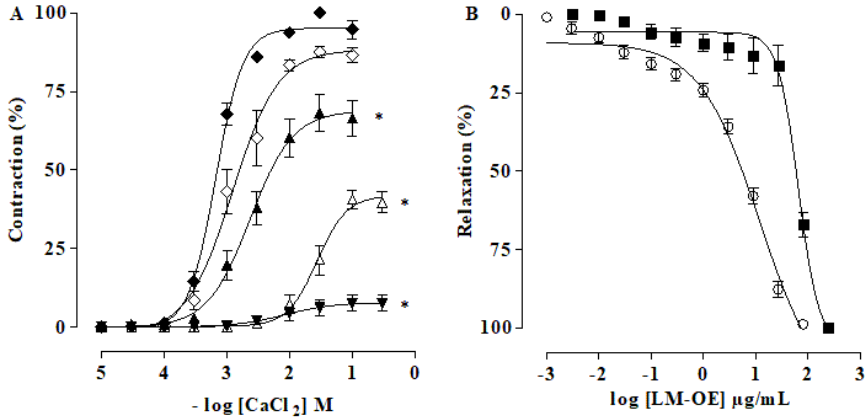

Figure 2. Tocolytic effect of LM-OE on cumulative concentration--response curves to $CaCl_2$ in depolarizing medium (KCl 60 mM) nominally without Ca^{2+} in the absence (◆) and presence of 9 (◇), 27 (▲), 81 (△) or 243 µg/mL (▼) (A) and tonic contractions induced by KCl 60 mM (○) or S-(-)-Bay K8644 3 x 10^{-7} M (■) (B) on rat. Symbols and vertical bars represent the mean and S.E.M., respectively (n = 5). One-way ANOVA followed by Tukey's test, *$p < 0.05$ (control *vs*. LM-OE).

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