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Vasorelaxant effect of the *Lippia alba* essential oil and its major constituent, citral, on the contractility of isolated rat aorta

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ABSTRACT

The *Lippia alba* (Mill.) N.E. Brown (Verbenaceae) species popularly known as lemon balm has sedative, analgesic and spasmolytic properties. This study aimed to evaluate the vasorelaxant effect of the *L. alba* essential oil (EOLa) and its major constituent, citral, rat on aorta. Isometric muscle contraction were induced by potassium (K 60 mM) or phenylephrine (PHE, 0.1 μM) in isolated aortic rings. EOLa and citral promoted a smooth muscle relaxant action, which was potentiated by the presence of the endothelium; PHE-induced contractions (0.1 μM) in aorta with endothelium, had EC_{50} values of 352.73 ± 19.39 μg/mL and 99.34 ± 7.2 μg/mL for EOLa and citral, respectively. In the presence of a nitric oxide synthase inhibitor, L-NAME, the EC_{50} values were 654.19 ± 10.46 μg/mL and 601.66 ± 10.922 μg/mL for EOLa and citral, respectively. EOLa and citral dose-dependently relaxed contractions induced by BAY-K 8644, a calcium channel agonist, and by Phorbol 12,13-dibutyrate an activator of protein kinase C. EOLa and citral produced a vasorelaxant effect in isolated aorta which was potentiated by the presence of endothelium. In summary, EOLa and citral, probably using several mechanisms of action, relaxed aortic smooth muscle with maximal pharmacologic efficacy.

1. Introduction

The genus Lippia (Verbenaceae) includes more than 200 species of herbs, shrubs and small trees that are mainly distributed throughout the South and Central America countries, and Tropical Africa territories. Their chemical profiling indicates common components exist in their essential oils namely limonene or citral [1]. Numerous papers have presented ethnopharmacological studies dealing with Lippia. Most of them are traditionally utilized as gastrointestinal and respiratory remedies [2]. Some Lippia species have shown antimicrobial [3], anti-hyperalgesic [4], bronchodilators [5], Antiinflammatory effects [6].

The *Lippia alba* (Mill.) N.E. Brown species, belonging to the Verbenaceae family, is found in all tropical and subtropical South American areas [2,7]. In Brazil, this species popularly known as "lemon balm", is widely cited for the treatment of diseases relating to respiratory, digestive and nervous system disorders in different regions of Brazil, as Cerrado and Carrasco areas [8–10].

Numerous papers have reported biological effect of *Lippia alba* using extracts or essential oil. The essential oil produced a variety of effects: antiparasitic activity [11], antimicrobial [12], insecticidal activity [13], sedative and anesthestic effects [14], depressor activity in sciatic nevers [15], anxiolytic and depressant activity [16] and antispasmodic effect over tracheal smooth muscle of rats [17].

Citral is a major constituent found in the essential oil of the Lippia genus [1,2]. Citral is a secondary metabolite resulting from the union of two chemical compounds, the geranial trans-isomer ((E)-3,7-dimethyl-2,6-octadienal) and the neral cis-isomer ((z)-3,7-dimethyl-2,6-octadienal) [18]. Studies have shown that citral has proven pharmacological actions, such as: antibacterial [19,20], anticonvulcivant [21], antifungal [22], anti-tumoral [23], anti-parasitic [24], sedative [25], insecticide [13], antinociceptive and inflammatory activity [26,27], antispasmogens activity [28] and vasorelaxant [29].

To date there are only few studies characterizing the vasorelaxant effect of the *Lippia alba* essential oil, as well as its major constituent citral. The present work aimed to investigate the action of these natural products on the smooth muscle of isolated rat aorta.

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2. Materials and methods

2.1. Botanic material

The essential oil extracted from a *Lippia alba* (Mill.) N. E. Brown sample was supplied by Dr. Sergio Horta (Federal University of Ceará experimental farm) and analyzed in the Natural Products Laboratory and Technological Development Park (PADETEC) of the Federal University of Ceará. The main components detected in the oil composition were citral 75.92% [geranial (41.81%) and neral (34.11%)], 1-limonene (9.85%), carvone (8.92%), gamma-terpinene (2.05%), benzene, 1-methyl-3-(1-methylethyl) (1.02%) and published by Sousa et al (2015).

Monitoring of the present constituents in the *Lippia alba* essential oil (EOLa) was performed through chromatographic techniques/GC-FID, analyzed at PADETEC.

2.2. Solutions and drugs

The modified Tyrode (TM) solution used had the following composition in mM: 136.0 NaCl; 5.0 KCl; 0.98 $MgCl_2$; 0.36 NaH_2PO_4; 11.9 $NaHCO_3$; 2.0 $CaCl_2$ and 5.5 Glucose, maintained at 37 °C and adjusted to a 7.4 pH using 1 M HCl and/or NaOH (1 M). The EOLa and citral were prepared as solutions diluted directly in Tyrode and tween; Bay-K8644 and phorbol 12,13-dibutyrate (PDB) was diluted in dimethyl sulfoxide (DMSO); nifedipine was diluted in ethanol; indomethacin was diluted in 5% bicarbonate, and the remaining drugs and their stocks were diluted in distilled water. The calcium-free solution or "zero calcium" (0 Ca^{2+}) was prepared by omitting of $CaCl_2$ from the Tyrode solution and the addition of 0.2 mM EGTA. All the salts and reagents used were of analytical grade and purity obtained from the Sigma-Aldrich Company (St. Louis, Missouri, USA).

2.3. Animals

Male Wistar rats (*Rattus norvegicus*) weighing between 200–300 grams were obtained from the Central Animal Facility of the Regional University of Cariri-URCA. The rats were housed under constant humidity and 23 ± 2 °C temperature, with a 12 h light/dark cycle. The rats had access to water and ration *ad libitum*, and were treated according to the Brazilian College of Animal Experimentation (COBEA), Brazil. Their use was approved by the Ethics Committee on the Use of Animals (CEUA) -URCA, registered under protocol number: 00084/2014.2.

2.4. Preparation of tissue

Animals were euthanized by decapitation, followed by dissection of the thoracic aorta, which was sectioned in circular transverse segments measuring 4–5 mm in length. The segments were kept in a 10 mL chamber containing Tyrode solution, which were aerated and maintained at 37 °C with a pH of 7.4. The tissue contractile activity was measured with a rod connected to the tissue and to a force transducer (TRI, 210 model, Panlab, Spain). The output of the transducer was connected to a differential amplifier (DATAQ, PM-1000 model, USA) and to a digital to analog converter board (DATAQ DI-200) installed on a computer. The collected data was stored in files through the WINDAQ software (DATAQ Instruments, Inc. USA). The isolated aortic rings were subjected to a 1 g tension, and maintained for a 1 h stabilization period. All protocols started with two subsequent contractions produced by the hypertonic addition of 60 mM KCl (K60) to the aorta rings. After reaching stable plateau value the response amplitude was considered a value in reference to which various other contractions were expressed. Experiments dealing with PDB-induced contractions were done in Ca^{2+}-free solutions prepared by adding 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and avoiding the addition of Ca^{2+} salts to Tyrode solution (0Ca^{2+} Tyrode) preparation. Only experiments with reproducible contractions were accepted for the experimental use. Endothelial functional integrity was verified by addition of ACh (10 μM) and Phenylephrine (PHE) (0.1 μM) which induced relaxation and contraction, respectively.

All experiments included a control preparation which was subjected to the same experimental protocols but without drugs. The control preparations received only the vehicle diluted in Tyrode's solution, at the same concentrations used in the experimental preparations.

3. Statistical analysis

Data are expressed as mean ± S.E.M. The Sigma Plot 11.0 software was used for statistical analysis and graphics production. Results considered statistically significant had a null hypothesis probability of less than 5% ($p < 0.05$). Student t tests and analysis of variance (one or two-way ANOVA) were used, followed by Bonferroni's *t*-test and Holm-Sidak multiple comparisons method, when appropriate. The EC_{50} values were determined as the concentration of the substance capable of producing 50% inhibition or maximum effect. A logarithmic interpolation was performed on calculations for each experiment. Where this was not possible, the linear relation between two points of the EC_{50} was performed.

4. Results

Increasing and cumulative concentrations (1–1000 μg/mL) of the EOLa were added to the intrinsic muscular tone of aortic rings with preserved and non-preserved endothelium. The presence or absence of the endothelium did not cause any statistically significant relaxant or contractile effect on the basal tone of the aortic ring preparations ($p > 0.05$, *one-way* ANOVA). The EOLa and citral influence on K^+ and PHE induced contractions was evaluated by adding increasing and cumulative concentrations of the EOLa (1–1000 μg/mL) and citral (1–1000 μg/mL) to the aorta preparations with and without endothelium that had been previously contracted with KCl (60 mM) or with PHE (0.1 μM).

In preparations pre-contracted with 60 mM KCl in the presence of a preserved endothelium (Fig. 1A), increasing concentrations of the EOLa and citral promoted concentration-dependent relaxations that was significant at concentrations ≥ 10 μg/mL for the EOLa and ≥ 30 μg/mL for citral. EOLa produced a EC_{50} (83.30 μg/mL) with endothelium and EC_{50} (356.20 μg/mL) without endothelium. Citral showed EC_{50} values 110.80 μg/mL and 487.20 μg/mL in presence and absence of endothelium, respectively. In non-preserved endothelial preparations, EOLa and citral relaxant effects were observed from 100 μg/mL and 300 μg/mL, respectively ($p < 0.05$, *one-way* ANOVA, followed by Holm-Sidak) (Fig. 1B). For aortic rings without endothelium EOLa and citral produced a decrease in smooth muscle relaxation which was statistically significant ($p < 0.05$, unpaired Student t test). These results indicate EOLa and citral may be acting in conjunction with endothelial mediators such as NO and cyclooxygenases, to enhance muscle relaxation.

In the aortic rings where contractions were evoked with PHE (0.1 μM), the substances were also able to cause significant relaxation in a concentration-dependent manner ($p < 0.001$, *one-way* ANOVA). In the rings with endothelium (Fig. 1C), the addition of the EOLa on citral caused total relaxation, with an EC_{50} of 352.73 μg/mL (EOLa) and 99.34 μg/mL (citral), respectively. The aortic preparations without endothelium (Fig. 1D) the EOLa showed a lower EC_{50} (566.06 μg/mL) than citral (915.94 μg/mL).

To evaluate the influence of endothelium mediators on the vasorelaxant effect, preparations were pre-incubated with the nitric oxide synthase inhibitor, L-NAME (100 μM), or indomethacin (10 μM), a non-selective cyclooxygenase inhibitor. Aortic rings with preserved endothelium were exposed for 20 min to L-NAME (100 μM), and