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Antitussive, antispasmodic, bronchodilating and cardiac inotropic effects of the essential oil from *Blepharocalyx salicifolius* leaves

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Abstract

Ethopharmacology relevance

Blepharocalyx salicifolius (Kunth) O. Berg (Myrtaceae) is a tree native to Argentina and Uruguay that grows and is cultivated along the riverside of the Rio de la Plata. The leaves of this plant species, locally known as "anacahuita" are used in South America to prepare infusions for the empiric treatment of cough and bronchospasm, as well as diarrhoea and other intestinal disorders. Although previous phytochemical studies have been performed with the essential oil extracted from *Blepharocalyx salicifolius*, pharmacological evidence supporting its traditional use is still lacking.

Aim of the study: To experimentally evaluate the pharmacological properties of *Blepharocalyx salicifolius* based on its traditional use. The studies were performed with tincture (T-Bs) and essential oil (EO-Bs) prepared from its leaves, in isolated rat trachea, intestine and heart preparations.

Methods

The *ex-vivo* effects of T-Bs and EO-Bs were evaluated with the agonists carbachol (CCh) and calcium chloride (Ca^{2+}) in the contractile concentration-response curves (CRC) of the isolated intestine. The muscle relaxant effect of EO-Bs was evaluated in the isolated trachea and compared with the effect achieved with papaverine as a positive control. The T-Bs and EO-Bs cardiac effects were analysed by perfusion of an isolated rat heart before a period of ischemia/reperfusion (stunning model). The antitussive effect of both T-Bs and EO-Bs was evaluated in mice exposed to ammonia using codeine as a positive control.

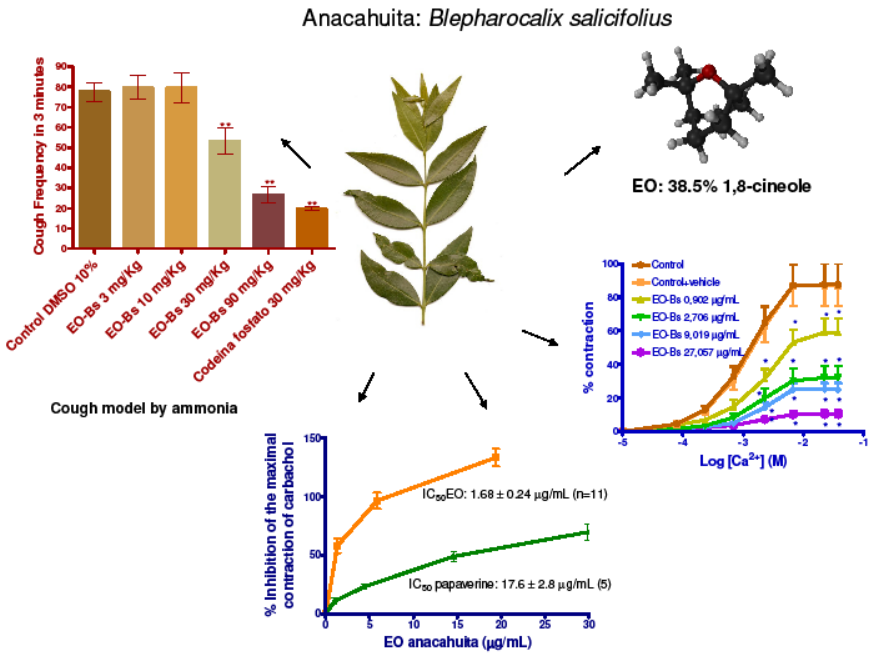
Results

Both T-Bs and EO-Bs induced a non-competitive inhibition of the CCh-CRC in the rat intestine, with IC_{50} values of 170.3 ± 48.5 µg T-Bs/mL (*n* = 6) and 5.9 ± 1.6 µg EO-Bs/mL (*n*= 6), respectively. EO-Bs also inhibited non-competitively the Ca^{2+}-CRC, with IC_{50} value of 1.8 ± 0.3 µg EO-Bs/mL (*n*= 8). A similar effect was obtained with the main active component of the EO-Bs 1,8-cineole. In isolated trachea, EO-Bs induced the relaxation of the CCh-contracted tissue (1.7 ± 0.2 µg EO-Bs/mL, *n* = 11) up to a maximal relaxation that was 1.9 times higher than that of papaverine. In the isolated heart, EO-Bs induced a poor negative inotropic response, and did not improve the contractile and energetic recovery after ischemia and reperfusion. In the mouse cough model, EO-Bs (90 mg/Kg) was as effective as codeine (30 mg/Kg) in reducing cough frequency.

Conclusions

The results indicate that the preparations from *Blepharocalyx salicifolius* leaves were effective as central antitussive, bronchodilating and antispasmodic agents, suggestive of a mechanism associated with the inhibition of Ca^{2+} influx into smooth muscle. The EO-Bs displayed only a poor ability to reduce cardiac inotropism, and was devoid of any cardioprotective properties. Thus, the present study validates the traditional use of this South American plant for asthma, cough and bronchospasm, shedding new light into its potency and putative mechanism of action.

Graphical abstract



Abbreviations

Ca^{2+}: ionic calcium; CCh: carbachol; CRC: concentration response curve; EO-Bs: essential oil of *Blepharocalyx salicifolius ;* T-Bs: tincture of *Blepharocalyx salicifolius;* IC_{50}: 50% inhibitory concentration ; EC_{50}: 50% effective concentration ; GC-FID-MS: gas chromatography with flame ionization and mass spectrometry detectors.

Key words

Blepharocalyx salicifolius; antitussive; bronchodilator; antispasmodic; essential oil

1. Introduction

The aromatic tree *Blepharocalyx salicifolius* (Kunth) O. Berg (syn. *B. tweediei* (Hook. et Arn.) Berg, Myrtaceae) is a plant native to South America that grows near the riverside of the Rio de la Plata and its marginal jungle in Argentina, on the hills of Uruguay and southern Brazil, and even in sandy soils of these South American countries (Lahitte et al. 1999). The plant is known as "anacahuita", which is a name derived from the Mexican nahuatl word "anatlquahuitl", meaning "paper tree" assigned by similarity to a Mexican tree that is also used for the treatment of respiratory diseases (Lahitte et al. 1999). The leaves of *Blepharocalyx salicifolius* are used to alleviate cough, sore throat, bronchospasm, intestinal diarrhoea as well as other intestinal disorders (Ratera and Ratera, 1980). In Uruguay, either an infusion or an alcoholic extract prepared with the leaves and fruits of this plant are used as tonic to alleviate the symptoms of indigestion and cough (Alonso and Desmarchelier, 2005). In southern Brazil, this plant is also used as external astringent in baths for the treatment of diarrhoea, leucorrhoea, urethritis and rectal prolapse (Mors et al. 2000), as antirheumatic and hypoglycemic agent (Alice et al. 1991), as diuretic and to treat stomach diseases (Moreira et al. 1999).

The composition of the *Blepharocalyx salicifolius* essential oil (EO-Bs) varies among the regions where this species grows (Dellacasa et al. 1997; Tucker et

al. 1993; Moreira et al. 1999; Limberger et al. 2001; Garneau et al. 2013), but its main active component has recently been identified as 1,8-cineole (Garneau et al. 2013). The active ingredient 1,8-cineole is also characteristic of the essential oil obtained from the leaves and fruits of *Eucalyptus globulus*; which is known to confer antiseptic and bronchodilating properties (Salari et al. 2006; Mulyaningsih et al. 2010). The bronchodilating effect of 1,8-cineole is the basis of the efficacy in the cough treatment (Juergens et al. 2003; Coelho-de-Souza et al. 2005; Nascimento et al. 2009).

Although a phytochemical characterization of the *Blepharocalyx salicifolius* essential oil has previously been carried out (Garneau et al. 2013), there are no reports describing the effects this plant has on respiratory and intestinal smooth muscle. The aim of this work was to evaluate the pharmacological effects of both the ethanolic extract or tincture (T-Bs) and the EO-Bs obtained from specimens growing in this region of the Country, on isolated rat trachea and small bowel preparations. Moreover, both the antitussive effect in mice, and the inotropic effect on isolated rat hearts of EO-Bs were also assessed and mechanisms of action determined.

2. Materials and Methods

2.1. Plant material and extraction

Leaves of *Blepharocalyx salicifolius* (Kunth) O. Berg (Myrtaceae) were collected around the field of the Facultad de Agricultura y Ciencias Forestales de la Universidad Nacional de La Plata (34º 52' S, 57° 54´ W) on December 2013, allowed to dry at room temperature for 4 weeks and identified as NDBAYON 1643 by MSc Agr Ing Marta Colares in the Herbarium de la Universidad Nacional de La Plata, according to Zuloaga et al. (2014). The EO-Bs was obtained by hydro-distillation using a Clevenger type apparatus according to Argentinean National Pharmacopoeia. For the biological studies, the EO-Bs was diluted in dimethylsulfoxide (DMSO). In some biological preparations, the tincture (T-Bs) prepared by maceration of leaves at 10% in ethanol 70° (yield 26.3% w/w after evaporation of tincture to residue) was tested. The T-Bs was diluted in Tyrode solution on the day of the experiment, and its concentration was expressed as µg residue/mL.

2.2. Analysis of the essential oil

The analysis of EO-Bs was performed by gas chromatography with flame ionization detector and coupled to a mass spectrometry detector GC-FID-MS. A gas chromatograph Agilent 7890A series (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector (FID), split / split-less (split ratio 1:100) was used and interconnected to a mass detector 5975C (Agilent Technologies, Santa Clara, CA, USA). Data acquisition, processing and instrument control were performed using the Agilent ChemStation (Agilent Technologies, Santa Clara, CA,

USA) software. The injector temperature was 250°C and FID temperature was 260°C. A capillary column HP-WAX, 60 m x 0.25 mm ID and 0.25 µm thick film was used for the FID and a nonpolar capillary column HP-1, with the same characteristics for the detector mass (70 eV), they were used for quantization and determination of linear retention indices. The oven temperature was programmed identically for both columns, starting at 100°C to 240°C at 2°C/min and kept constant for 15 min Helium was used as gas carrier at a constant flow of 1.8 mL/min, injection volume was 0.3 µL, hydrogen (30 mL/min) and air (300 mL/min) were used in the FID with nitrogen (30 mL/min) as auxiliary gas. Temperatures for the transfer line and the ion source were set at 280ºC and 230°C, respectively. The mass range (*m/z*) was 40-500 Da. Compound identification was conducted by analyzing the lineal retention indexes (relative to C_8–C_{24} *n*-alkanes) obtained in both columns and compared with those of either reference compounds, or compounds identified in chemically well-known essential oils and/or from bibliography (Babushok et al., 2011). Additionally, each mass spectra obtained was compared to those from the literature libraries (Adams, 2007; Wiley/NIST, 2008) and mass spectra obtained from reference compounds. Relative percentage contribution of the compounds was calculated from the FID responses by a computerized integration assuming all of the response factors were 1.

The density of EO-Bs was measured by means of a pycnometer at 20°C (AOAC, 1990).

2.3. Pharmacological studies

2.3.1. Animals

The research was conducted on both adult male and female Sprague-Dawley rats (200-250 g) as well as 2-3 month-old Swiss albino female mice (25-30 g), following internationally accepted principles of laboratory animal use and care as established by US guidelines (NIH publication 85-23 revised in 1985) and principles in the Declaration of Helsinski. The protocols and procedures were approved by an ethical local committee of Facultad de Ciencias Exactas de la Universidad Nacional de La Plata by numbers 015-05-15 and D-01-15-16.

2.3.2. Isolation of smooth muscles preparations and contractile measurements

Sprague-Dawley rats (200-250 g) were subjected to a 12 h fasting with free access to water before experimentation. Animals were anesthetized by urethane (doses of 1.5 g/Kg) and then quickly sacrificed by opening the thorax. Depending on the experiment, either the duodenum and ileum (about 2 cm long), and/or trachea were excised. Intestinal portions were individually mounted in organ baths containing constantly oxygenated Tyrode solution (20 mL, pH 8.2) at 37ºC (Emendorfer et al. 2005; Ragone et al. 2007; Chen et al. 2012; Matera et al. 2016). The trachea was dissected into 0.5 cm length rings, individually mounted in an organ bath containing oxygenated 20 mL Krebs solution. All preparations were equilibrated for at least 45 min at 1g of pre-load. Intestinal tissues were connected to isometric transducers WPI (USA) and the signals from four preparations were simultaneously amplified by a 4-channel preamplifier (WPI, USA). Data were stored in a personal computer with Eagle software (USA). The contractility of two tracheal rings were simultaneously detected by PanLab MLT0210/A isometric transducers

and recorded by a PowerLab 2/26 system with LabChart A/D program (AD Instruments, Australia).

2.3.3. Concentration-response curves with carbachol in small bowel

Concentration-response curves (CRC) of contractility to carbachol (CCh-CRC) were conducted in each rat duodenum or ileum. Previous tests in our laboratory had demonstrated that the CRC of either intestinal portion behave similarly (data not shown). Tissues were stabilized for 45 min in Tyrode's solution. Several CRC were conducted in the same preparation by cumulatively adding to the bath CCh (range 0.01 to 10 μg/mL) up to maximal contraction, first in the absence (control condition), and then in the presence of vehicle (control-vehicle with DMSO at 0.1% for comparing the EO-Bs series, or ethanol 70° at 1% for comparing the T-Bs series, respectively), followed by CCh with a sole sequentially increasing concentration of either EO-Bs, T-Bs or 1,8-cineole. The contractile effect of each CCh concentration was expressed as percentage of the maximal contractile response of the tissue obtained with the control of CCh-CRC. As positive control, the present CRC were compared with those obtained with verapamil assessed in our laboratory and previously reported (Blanco et al. 2013).

2.3.4. Concentration-response curves with $CaCl_2$ in small bowel

Several CRC were conducted in each organ after stabilization and removal of external Ca^{2+}. Then, Ca^{2+} CRC were done in a depolarizing solution of Tyrode-0Ca^{2+}-40 mmol/L K^{+}, first in the absence and then in the presence of each EO-Bs

concentration. The contractile effect was expressed as percentage of the maximal response obtained in the control Ca^{2+}-CRC. Each EO-Bs concentration was added 5 min before depolarization with the high-K^+ in Tyrode's-0Ca^{2+} that remained throughout the CRC. As a positive control, a previously reported Ca^{2+}-CRC in the presence of verapamil was used (Blanco et al. 2013). Also, the effect of 1,8-cineole, the main EO-Bs compound, was evaluated in independent Ca^{2+}-CRC experiments.

2.3.5. Pharmacological parameters of CRC

From the CRC performed with CCh and Ca^{2+}, the pEC_{50} (as –log EC_{50}, in mol/L) of the agonist was calculated. Since tincture and essential oil were a mix of compounds, the affinity ($pK_{B'}$) could not be calculated (Kenakin, 2014). The 50% inhibitory concentration (IC_{50}) was calculated by interpolation at 50% effect of the individual inhibitory curves, that is, those obtained for each biological preparation by plotting the maximal agonist effects in the absence and the presence of the subsequent concentrations of the antagonist (T-Bs, EO-Bs or 1,8-cineole), as previously described (Consolini et al. 2011; Matera et al. 2012). IC_{50} values of EO-Bs or 1,8-cineole were expressed as µg/mL after considering the respective density of the natural product. Similarly, the IC_{50} T-Bs values were expressed as µg residue/mL.

2.3.6. Experiments on isolated trachea

Rat tracheal rings were kept in chambers with 20 mL Krebs solution bubbled with 95% O_2 and 5% CO_2 at 37ºC, as described (Nascimento el al. 2009). EO-Bs

concentration-relaxation curves were conducted on CCh 10 µg/mL pre-contracted tissues, and values compared with those obtained with papaverine as a positive control.

2.3.7. Experiments on isolated rat hearts and the model of ischemia-reperfusion

Adult Sprague-Dawley rats (200 a 250 g weight) of either sex were heparinised (non-fractioned heparine 2000 IU) before being anesthetized with 25% urethane (1.5 grams/Kg, via i.p.). Hearts were rapidly excised and perfused through the coronary arteries by the Langendorff method, as previously described (Consolini et al. 2007; Ragone and Consolini 2009). Control Krebs (C) was perfused with a peristaltic pump (Gilson Minipuls 3, France), at a constant flow rate of 7 $ml.min^{-1}.g^{-1}$. This flow was sufficient to develop an optimal maximal pressure development (P) without significant edema, as previously described (Ragone et al. 2015; Colareda et al. 2016). Atria were removed and spontaneous beating was stopped by applying pressure on the focus in the interventricular septum. A latex balloon was introduced in the left ventricle, connected to a Bentley DEL900 pressure transducer by a flexible cannula. While continuously perfused, the heart was introduced into the chamber of a custom-made flow calorimeter (Ponce-Hornos et al. 1982), which was closed and submerged in a bath kept at controlled temperature of 37.0 ± 0.01ºC. Rat hearts were electrically stimulated with 5 V-5 ms at 3 Hz, by means of two electrodes connected to an electrical stimulator (Letica LE12406, Spain). The isovolumic left intraventricular pressure (LVP) at optimal volume and the total heat rate (Ht) were simultaneously and continuously recorded in a PowerLab 2/26 two-channel digital acquisition system (AD Instruments, Australia). The maximal pressure developed

over the diastolic level (P) during contractions, as well as the changes in diastolic pressure over the preischemic condition in Krebs-C (ΔLVEDP), were both calculated and expressed in mmHg.

The calorimeter used in the present study has been previously described (Ponce-Hornos et al. 1982: Ponce-Hornos et al. 1995; Consolini et al. 2007). Briefly, the internal chamber has two ceramic modules with 127 thermosensitive units (Melchor Thermoelectrics) each, which detect changes in temperature between the inside (heart) and the outside (bath). Calibration details (in mW/mV) with a constant electrical power on the muscle at the end of the experiment and the calorimetrical base lines can be seen in previous publications (Consolini et al. 2001; Consolini et al. 2007; Ragone et al. 2015). The calorimeter was submerged in a temperature-controlled water bath, connected with two other baths, one used for regulation (Lauda, Germany) and the other one for heating the perfusates, thus buffering the temperature. The calorimetrical signal was measured before and after introducing the heart, in the presence and absence of perfusion, in order to obtain a baseline of the total heat signal in the presence of the heart. The total heat rate (Ht) was calculated from that difference between both signals, expressed in mW/g wet weight.

The cardio-protective effect of EO-Bs was evaluated before exposing the heart to stunning by ischemia and reperfusion. An "initial control value" of P and Ht was recorded after an approximately 40 min stabilization period with Krebs-C solution, after which the heart was pretreated with or without (control group) T-Bs or EO-Bs. The heart was then exposed to a 20 min period of no-flow ischemia (I) followed by a 45 min reperfusion period (R) with Krebs solution without drugs. Heart contractility and heat release during I/R, were obtained, and expressed as percentage of either P or Ht steady initial value, respectively. Finally, muscle

economy was calculated as the P/Ht ratio, as previously described (Ragone et al. 2015; Colareda et al. 2016).

In order to determine whether EO-Bs had an effect on sarcoplasmic reticulum Ca^{2+} release, a group of hearts was treated with 3 µmol/L ryanodine (Rya) as a selective blocker before EO-Bs perfusion. Further, in order to evaluate the role of the mitochondrial Na/Ca-exchanger (mNCX), before and during EO-Bs perfusion hearts were also perfused with the selective inhibitor clonazepam (Clzp, 10 µmol/L) as previously reported (Consolini et al. 2007; Ragone et al. 2013).

2.3.8. Antitussive effect

The antitussive effect of EO-Bs was investigated with a model of cough induced in live mice by ammonia liquor (Shang et al. 2010; Wang et al. 2012). Briefly, 30 min after intraperitoneal (i.p.) administration of EO-Bs, each mouse was placed in a 1000 mL special glass chamber and exposed to vaporization of 25% NH_4OH (0.1 mL) for 60 seconds. Each mouse was continuously monitored by a trained observer during a 3 min period with the help of a stethoscope over the glass chamber. Cough was detected as a contraction of thoracic and abdominal muscles followed by the mouth opening with a coughing sound and jerking of the front body of the mouse. The frequency (number of coughing episodes in 3 min) and the latency time were counted. Groups of 8 mice each were assessed, including a negative control (10% DMSO), test solutions (EO-Bs at doses of 3, 10, 30 and 90 mg/Kg) and a positive control with codeine phosphate (30 mg/Kg). All doses were administrated via i.p in 0.1 mL/30 g body weight.

2.3.9. Solutions and drugs

The Tyrode solution used in intestinal tissue preparations had the following composition (in mmol/L): 150 NaCl, 2.7 KCl, 2 $MgCl_2$, 12 $NaHCO_3$, 0.4 PO_4H_2Na, 1.8 $CaCl_2$ (pH 8.2). The Ca-free (Tyrode's-0Ca^{2+}) was obtained by eliminating $CaCl_2$ from the Tyrode solution and the Tyrode-0Ca^{2+}-40 mmol/L K^+ by addition of 10% KCl (0.6 mL) to 20 mL Tyrode's 0Ca^{2+}.

The Krebs solution used for the tracheal rings, had the following composition (in mmol/L): 118.2 NaCl, 25 $NaHCO_3$, 11.7 dextrose, 4.7 KCl, 2.5 $CaCl_2$, 0.4 KH_2PO_4, 1.2 $MgSO_4$.7 H_2O, bubbled with 95% O_2-5% CO_2.

The Krebs-C solution used with the isolated heart preparation, had the following composition (in mmol/L): 1 $MgCl_2$, 125 NaCl, 0.5 NaH_2PO_4, 7 KCl, 2 $CaCl_2$, 25 $NaHCO_3$, and 6 dextrose, bubbled with 95% O_2-5% CO_2.

The natural products obtained by EO-Bs hydro-distillation (density 0.9019 g/mL) and the 1,8-cineole compound (density 0.9240 g/mL) were diluted in DMSO to 0.01% (90.19 µg/mL EO-Bs and 92.4 µg/mL 1,8-cineole) and then in water to reach the respective concentrations for addition to either the Tyrode or Krebs solutions. The effect of the DMSO (0.1% in Tyrode as maximal concentration) was also assessed in the CRC as a control-vehicle. Drugs employed in biological tests included: Carbamylcholine chloride (carbachol, CCh, Sigma, USA), papaverine chloride (Sigma, USA), ryanodine (Rya, Sigma, USA), clonazepam (Clzp, Saporiti, Argentina). CCh, papaverine, and Clzp were dissolved in water, and Rya, in DMSO.

The CCh-CRC was obtained by cumulative addition of increasing concentrations of CCh between 0.01 and 10 µg/mL. The Ca^{+2}-CRC was obtained by cumulative addition of increasing concentrations of $CaCl_2$ to reach values ranging from 0.0195 to 17.5 mmol/L in the 40 mmol/L K^+-0Ca^{2+} Tyrode´s solution. The EO-Bs concentrations used were 0.1, 0.3, 1, 3 and 10 µL EO-Bs/mL in Tyrode's solution, later expressed as µg EO-Bs/mL after considering the density.

2.3.10. Statistical analysis

All results are expressed as the mean ± SEM (*n* = number of tissue preparations in the *ex vivo* experiments, and of animals in the *in vivo* protocols). Statistical multiple comparisons were done by two-way ANOVA , as considering the two variables in the effect, namely the treatment and the x-axis variable, that is, the agonist concentration (as pCCh or pCa, in the CRC) or the time (in the cardiac I/R experiments). After ANOVA, the Bonferroni´s *post hoc test* was applied for comparing the effects of a given treatment with the respective effect in the control condition at each x-axis value. The one-way ANOVA was used for comparing more than two values of one variable (Figure 6), followed by Tukey´s *post hoc* tests. The Student's *t*-test was also used whenever necessary for the comparison of the effect elicited by either two treatments. Statistical analyses were conducted with Graph Pad Prism 4.0 software. The statistical $p < 0.05$ was considered significant in all tests performed.

3- Results

3.1. Composition of the essential oil

The essential oil yield was 1.2% (v/w as dried material), and the relative percentage composition of the EO-Bs is shown in Table 1. The main components are 1,8-cineole (38.5%), limonene (13.1%), β-pinene (9.1%), α-pinene (5.2%), linalool (4.3%), terpinen-4-ol (2.6%), α-terpineol (2.9%) and β-caryophyllene (2.3%). The density of EO-Bs was 0.9019 g/mL.

3.2. Effects on carbachol CRC in small bowel

The T-Bs (yield 26.3% w/w) inhibited the contractions of the control CCh-CRC significantly at concentrations of 79 µg/mL T-Bs and higher (* $P < 0.05$ vs control, Fig. 1A), in a behavior typical of non-competitive antagonism, with an IC_{50} value of 170.3 ± 48.5 µg/mL T-Bs (n = 6 preparations from 2 animals). The EO-Bs also inhibited the control CCh-CRC as a non-competitive antagonist at concentrations of 0.9 µg/mL EO-Bs and higher (* $P < 0.05$ vs control, Fig. 1B), with an IC_{50} value of 5.9 ± 1.6 µg/mL EO-Bs (n = 6 preparations from 2 animals).

3.3. Effects on calcium CRC in small bowel

To evaluate whether in the EO-Bs effect the inhibition of L-type channel-mediated Ca^{2+} influx was implicated, both EO-Bs and its main component 1,8-

cineole were assessed on the Ca^{2+}-CRC in Tyrode`s-40 mmol/L K^+ solution. Both, EO-Bs and 1,8-cineole non-competitively blocked the Ca^{2+}-CRC (EO-Bs significantly inhibited the maximal Ca^{2+} contraction at concentrations of 0.902 µg/mL and higher. and 1,8-cineole did it at concentrations of 0.924 µg/mL and higher. * $P < 0.05$ vs control in Fig. 2 A and B, respectively). The IC_{50} values were 1.77 ± 0.34 µg/mL (n = 8) for EO-Bs and 23.7 ± 5.5 µg/mL (n = 6) for 1,8-cineole, respectively.

3.4. Effects on the isolated trachea

The EO-Bs reduced the CCh-induced tonic contracture, with an IC_{50} value of 1.68 ± 0.24 µg EO-Bs/mL (n = 11). As Figure 3 shows, the maximal relaxation was 1.9 times higher than that obtained with papaverine, and the IC_{50} value was 10.5 times lower than that observed with this drug (17.6 ± 2.8 µg/mL, n = 5).

3.5. Effects on isolated hearts

Sequential perfusion of T-Bs diluted to 0.002, 0.02 and 0.2 % w/v (respectively 5.26, 52.6 and 526 µg extract/mL) did not significantly change the isolated hearts inotropism (P) (Figure 4A). The subsequent exposure to 20 min of ischemia in the presence of 0.2% T-Bs and 45 min of reperfusion without T-Bs (model of stunning due to I/R) did not modify the post-ischemic contractile recovery (PICR as % of initial P, Figure 4A, 2-way ANOVA P = 0.86), but slightly increased the energetic output measured as heat flow (% of initial Ht, Figure 4B, 2-way ANOVA P = 0.06), and significantly reduced the total muscle economy (P/Ht)

before and after I (Figure 4C, 2-way ANOVA $P = 0.005$). This loss of economy was in line with the increase in the diastolic tone during I/R (+49.8 ± 11.6 vs. +7.7 ± 1.9 mmHg of treated vs. control hearts at 5 minutes R, and +41.3 ± 12.5 vs. +1.7 ± 0.6 mmHg of treated vs. control groups at 45 minutes R, both $n = 7$ and $P < 0.001$ by unpaired t-test). A similar result was found when the preparation was perfused only with the lowest concentration of 0.002% T-Bs before I/R (results not shown).

EO-Bs perfusion at 0.0001% v/v (0.90 µg EO-Bs/mL) induced a marginal negative inotropism before I and slightly reduced the PICR (* $P < 0.05$ by post-test vs. control, Figure 5 A) without any change in Ht, so that it reduced the post-ischemic contractile economy (P/Ht) (* $P < 0.05$ by post-test vs. control, Figure 5B). EO-Bs also induced an increase in the diastolic contracture (ΔLVEDP) during the entire I/R period, when compared with the control group (* $P < 0.05$ by post-test vs. control, Figure 5C).

To assess whether the diastolic contracture was due to Ca^{2+} lost from mitochondria, hearts were pretreated with clonazepam (Clzp, 10 µmol/L) an inhibitor of the Ca^{2+} extrusion by the mitochondrial Na/Ca exchanger (mNCX), before and during perfusion with EO-Bs. Figure 5 shows that Clzp improved PICR and P/Ht respect to the EO-Bs effect alone (# $P < 0.05$ by post-test vs. EO-Bs, Figures 5A-B) during R, but it did not significantly reduce the increase in LVEDP elicited by EO-Bs (* $P < 0.05$ by post-test vs. control, Figure 5C).

To evaluate whether the effect of EO-Bs on diastolic contracture was caused by Ca^{2+} release from sarcoplasmic reticulum (SR), the effect of ryanodine (Rya, 3 µmol/L), a selective blocker of the ryanodine receptor type 2 (RyR2), was tested before and during the perfusion of EO-Bs and exposure to I/R. As expected, Rya

reduced P before I/R as well as the PICR and the recovery of P/Ht (* $P < 0.01$ by post-test vs. control and vs. EO-Bs, Figure 5A-B). However, Rya did not avoid diastolic contracture (* $P < 0.05$ by post-test vs. control, NS vs EO-Bs, Figure 5C), suggesting that the EO-Bs-induced contracture was independent of SR Ca^{2+} loss.

3.6. Antitussive effects of EO-Bs in mice

The i.p. administration of EO-Bs at doses of 3 and 10 mg/Kg did not induce any antitussive effects. Higher doses of 30 and 90 mg/Kg, however reduced the number of coughing episodes, being the highest dosage equipotent with the effect of codeine phosphate 30 mg/Kg (* $P < 0.01$ by post-test vs. control, Figure 6A). EO-Bs also had a tendency to increase the coughing latency time, although less than codeine (* $P < 0.05$ by post-test vs. control, Figure 6B).

4- Discussion

The present study describes the pharmacological effects of tincture (T) and essential oil (EO) obtained from dried leaves of *Blepharocalyx salicifolius* ("anacahuita"). Characterization of the essential oil of this plant species has already been determined elsewhere (Talenti et al. 1984; Tucker et al. 1993). Therefore, our aim was to expand on its bioactivity and possible mechanism(s) of action. To find out whether our products were in line with those characterized in previous studies, the composition of the volatile fraction used in the present pharmacological tests was first determined. The EO-Bs displayed a similar chromatographic profile than that reported by Talenti et al. (1984) and Tucker et al. (1993), who also worked with plant material obtained from Argentina, and found limonene and 1,8-cineole as the main constituents. Analyses carried out with plant material from other sources (e.g. Uruguay and Brazil) however, differ from our results (Dellacassa et al. 1997; Limberger et al. 2001; Furlán et al. 2002; Garneau et al. 2013; da Costa et al. 2014; Godinho et al. 2014). The differences in composition could be attributed to geographic and climatic factors prevailing in the places where the plant materials were obtained (Benomari et al. 2016). For these reasons, the chemical composition of the volatile fraction used in the pharmacological studies has always to be specified (Bandoni et al. 2009).

The present results demonstrate that the traditional use of *Blepharocalyx salicifolius* to alleviate cough, bronchospasm and diarrhoea is based on the bronchodilator, antitussive and intestinal antispasmodic effects, respectively. We further determined that this species exerted a slight negative inotropic effect; although, no cardioprotective properties against ischemia and reperfusion were found.

In the intestinal smooth muscle preparation, both T-Bs and EO-Bs inhibited contractility during the CCh-CRC in a non-competitive manner, with an IC_{50} value of about 170 µg T-Bs/mL and 5.9 µg EO-Bs/mL, respectively. To further study the mechanism implicated in the latter effect, EO-Bs was assessed on the Ca^{2+} CRC, and again found it as a non-competitive inhibitor (Fig. 2A). This effect may be mainly due to the presence of 1,8-cineole in EO-Bs, because a similar response was obtained with this purified compound (Fig. 2B). The IC_{50} value of EO-Bs (about 1.8 µg/mL) on Ca^{2+} CRC, was lower than that of 1,8-cineole (about 23.7 µg/mL), suggesting that other components could act synergistically with 1,8-cineole. This is in agreement with one of the phytopharmacological findings,in which complex mixtures such as an EO could be more potent than the isolated components (Williamson, 2001; Mulyaningsih et al. 2010). The pattern of the Ca^{2+} CRC non-competitive inhibition was found to be similar to that obtained with verapamil, a well known Ca^{2+} channels blocker, which had an IC_{50} value of 0.25 mg/mL in the Ca^{2+}-CRC done in isolated intestine (Blanco et al. 2013). Verapamil is known to block L-type Ca^{2+} channels from the inside of the cell, in a site different from the Ca^{2+} binding one (Spedding, 1985) thus explaining the non-competitive inhibition pattern. Both 1,8-cineole and EO-Bs showed the same typical behavior as non-competitive L-type Ca^{2+} channel blockers, with about 10 and 140 times higher potency than verapamil, respectively. This mechanism may also explain the muscle relaxant effect of 1,8-cineole in other smooth muscles such as bronchus (Nascimento et al. 2009).

On rat isolated trachea, the results showed the relaxant effect of EO-Bs on respiratory smooth muscle, which may support the traditional use of the plant extracts on both asthma and bronchospasm. EO-Bs displayed a relaxant activity that was about twice more relaxant and 10 times more potent than papaverine, a well

known phosphodiesterase inhibitor (Kaneda et al. 1998). The great efficacy found in the tracheal tissue was in line by the antitussive effect demonstrated in the *in vivo* coughing test in mice. The irritation test with ammonia vaporization showed that EO-Bs at 90 mg/Kg acts as a central antitussive, almost equivalent to 30 mg/Kg codeine phosphate.

The fact that EO-Bs inhibited Ca^{2+} CRC is consistent with a potential effect on calcium influx to the smooth muscle, and it suggested that this EO could have beneficial properties for the heart. The results in the moderate stunning isolated rat heart model showed that the perfusion of T-Bs at concentrations between 5.26 and 526 µg/mL and EO-Bs at 0.90 µg/mL induced a slight negative inotropic effect before ischemia. Considering that 1,8-cineole is the most abundant component in EO-Bs, the results are in agreement with the work of Soares et al. (2005), in which 1,8-cineole was found to induce negative inotropism in rat cardiac papillary muscle, in that case attributed to inhibition of Ca^{2+} influx. However, those properties were not responsible of cardioprotection in our model of moderate stunning, since neither T-Bs nor EO-Bs improved the contractile recovery during reperfusion. Cardiac energetic output, as assessed by heat release, remained unchanged after tissue treatment with EO-Bs; however, the total muscle economy (P/Ht) was reduced by both T-Bs and EO-Bs (Figures 4 and 5). Moreover, both T-Bs and EO-Bs induced diastolic contracture, considered as a caffeine-like effect (Bers 2001), which may account for the reduced muscle economy. To understand the cellular mechanism of this effect, the transporters that generally lead to a Ca^{2+} rise within the cytosol were selectively blocked, and the effects on the I/R event were evaluated. The SR Ca^{2+} release was blocked by ryanodine (Bers, 2001), and the mitochondrial Ca^{2+} extrusion through the mNCX was inhibited by clonazepam (Clzp) (Cox and Matlib, 1993;

Consolini et al. 2007). Despite Clzp partially improved contractility and economy (P/Ht) it did not reduce ΔLVEDP, suggesting that diastolic contracture was not due to mitochondrial Ca^{2+} extrusion through the mNCX. The increase in PICR could be explained by the consequently higher mitochondrial Ca^{2+} concentration, which activates metabolism enzymes and thus ATP and PCr synthesis (Gunter and Sheu, 2009). On the other hand, we assessed whether the effects of T-Bs and EO-Bs (diastolic contracture with reduced economy) were due to activation of the RyR2 and hence Ca^{2+} release from the SR, as it was observed with caffeine (Bers, 2001). The effects of caffeine include a high energy consumption associated with Ca^{2+} removal (NCX) (Bonazzola et al. 1992) as well as those seen with EO-Bs. Nevertheless, the diastolic contracture elicited by the EO-Bs was not affected by the SERCA blocker ryanodine (Figure 5 C). Rya strongly reduced P, Ht and P/Ht, as a consequence of the low SR Ca^{2+} storage. It can be concluded that plant extracts from *Blepharocalyx salicifolius* did not induce Ca^{2+} release through the RyR2 as caffeine does. The results suggest that EO-Bs could be directly activating the actin-myosin interaction at myofilaments level.

Conclusion

This is the first pharmacological study which supports the traditional uses of leaves from *Blepharocalyx salicifolius* for respiratory diseases. Our results demonstrated that the EO-Bs is effective in the treatment of cough and bronchospasm. It was found to be more potent than papaverine in the bronchodilating effect, and as good an antitussive agent as codeine. The mechanism is at least in part likely associated with the inhibition of Ca^{2+} influx to the smooth

muscle, a process in which the EO-Bs was also more potent than verapamil as an intestinal antispasmodic. Moreover, data from the present study provides evidences suggesting poor negative heart inotropism; which does not imply cardioprotection upon ischemia and reperfusion. Regarding the composition, the assessed EO-Bs had the same main components than those described for other samples of essential oil of Argentinean origin, that is 38% of 1,8-cineole and 13% of limonene. The compound 1,8-cineole showed an antispasmodic effect in the intestinal smooth muscle and a similar mechanism than that of the EO-Bs, suggesting that it is the main compound responsible of the observed pharmacological effects.

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Figure 1: Effects of tincture (T-Bs, A) and essential oil (EO-Bs, B) from *Blepharocalyx salicifolius* on the CRC of carbachol in small intestine. Results are shown as media and SEM (n = 6 in A and B). Two-way ANOVA´s: by treatment: $P < 0.0001$; and by log [CCh]: $P < 0.0001$ in both (A) and (B). Post-tests: * $P < 0.05$ vs. control.

Figure 2: Effects of EO-Bs (A) and 1,8-cineole (B) on the Ca^{2+}-CRC obtained in isolated rat intestine in a depolarizing media (both n = 8). Results are shown as media and SEM. Two-way ANOVA´s: by treatment: $P < 0.0001$ and by log [Ca^{2+}]: $P < 0.0001$ in both, (A) and (B). Post-tests: *$P < 0.001$ vs. control.

Figure 3: Concentration–response curves of EO-Bs (IC_{50} = 1.68 ± 0.24 µg/mL, n = 11) and papaverine (IC_{50} = 17.6 ± 2.8 µg/mL, n = 5) on isolated rat trachea pre-contracted by 10 µg/mL carbachol (CCh). Relaxation was calculated as percentage of the maximal CCh contraction. Results are shown as media and SEM. Note that IC_{50} value (at 50% of the maximal CCh contraction) of EO-Bs was about 10.5 times lower and that maximal relaxation of EO-Bs was 1.9 times bigger than those of papaverine.

Figure 4: Changes in maximal pressure development of contraction (P as % of initial) (A), total heat rate (Ht as %) (B) and muscle economy (P/Ht) (C) of isolated

rat hearts exposed to perfusion of the *Blepharocalyx salicifolius* tincture (T-Bs) sequentially at 0.002%, 0.02% and 0.2% before the ischemia and reperfusion (I/R) periods (n = 7) in comparison with the non treated hearts (control, n = 7) in the model of stunning due to I/R. Results are shown as media and SEM. Two-way ANOVA: by treatment: $P = 0.38$ (A), $P = 0.065$ (B) and $P = 0.005$ (C); by time: all $P < 0.0001$.

Figure 5: Changes in contractility as maximal pressure development P (as % of initial) (A), in muscle economy P/Ht (B) and in diastolic contracture evoked over the initial, ΔLVEDP in mmHg (C) of isolated rat hearts before and during I/R (control, n = 7) and after perfusion of the essential oil (EO-Bs at 0.0001% or 0.9 µg/mL, n = 5) in the absence and the presence of 10 µmol/L clonazepam (Clzp, n = 6) or 3 µmol/L ryanodine (Rya, n = 6). Results are shown as media and SEM. Two-way ANOVA: by treatment: all $P < 0.0001$; by time: all $P < 0.0001$. Post-tests: *$P < 0.001$ vs. control, # $P < 0.05$ vs. EO-Bs.

Figure 6: Effects of EO-Bs 3, 10, 30 and 90 mg/Kg on the number of cough episodes within 3 minutes (A) and on the latency time for cough (B) after misting ammonia liquor on mice. Control group mice were treated with 10% DMSO, and codeine phosphate (30 mg/Kg) was injected via i.p. for the positive control group (n = 7-9). Results are shown as media and SEM. One-way ANOVA: $P < 0.05$ in A and B. Post-tests: *$P < 0.05$, **$P < 0.01$, compared with control.

Table 1. Relative percentage composition of the essential oils obtained from leaves of *Blepharocalyx salicifolius*.

Compound	LRI HP-1	LRI[a]	LRI HP-WAX	LRI[b]	Relative content (%)
(E)-2-Hexenal#	826	827*	1240	1216*	Tr
Amyl acetate#	873	895**	1167	1177**	Tr
Bornilene ##	911	908**	1017	-	Tr
α-Thujene	926	926*	1036	1027*	0.1
α-Pinene #	935	935*	1043	1025*	5.2
α-Fenchene	950	945*	1089	1061*	0.2
Camphene #	951	947*	1100	1069*	0.5
Myrcene #	974	983*	1170	1161*	0.6
β-Pinene #	979	973*	1133	1110*	9.1
Isoamyl isobutanoate	996	1000**	1194	1190**	Tr
2-Methylbutyl isobutanoate	1001	1001**	1198	1203**	Tr
α-Phellandrene #	1005	999*	1191	1168*	0.3
δ-3-Carene	1011	1007*	1175	1147*	Tr

α-Terpinene #	1012	1011*	1206	1176*	0.5
p-Cymene #	1018	1015*	1286	1270*	1.3
(Z)-β-Ocimene	1020	1029*	1235	1235*	0.1
1,8-Cineole #	1022	1022*	1234	1211*	38.5
Limonene #	1024	1024*	1221	1198*	13.1
Acetophenone #	1036	1042*	1668	1648*	0.1
γ-Terpinene #	1047	1050*	1264	1245*	1.1
cis-Oxide linalool (Furanoid)	1056	1065*	1481	1446*	0.1
trans-Oxide linalool (Furanoid)	1072	1072*	1452	1454*	0.1
Linalool #	1081	1086*	1549	1543*	4.3
Terpinolene	1082	1079*	1305	1282*	0.5
2-Methylbutyl 2-methyl butanoate	1086	1090**	1280	1286**	0.1
α-endo-Fenchol	1103	1101*	1591	1570*	0.3
Camphor #	1125	1125*	1541	1515*	Tr
trans Sabinol	1131	1131*	1727	1717*	Tr
Camphene hydrate	1138	1136*	1605	1602*	0.1
Borneol #	1150	1153*	1711	1700*	0.8

Terpinen-4-ol #	1164	1165*	1614	1601*	2.6
α-Terpineol #	1172	1176*	1705	1694*	2.9
Myrtenol	1181	1194*	1796	1790*	0.1
cis-Carveol	1208	1206*	1869	1854*	Tr
Carvone #	1215	1218*	1745	1734*	Tr
Geraniol #	1231	1239*	1852	1839*	Tr
bornyl acetate	1271	1270*	1601	1579*	0.3
Alcohol Perilla	1281	1282*	2007	2007*	Tr
Thymol sym	1288	1304***	2288	2287**	0.1
exo-2-Hidroxycineole acetate ##	1324	-	?	-	0.3
Neryl acetate #	1340	1344*	1729	1718*	0.2
Geranyl acetate #	1359	1361*	1760	1751*	0.3
Methyl Eugenol #	1368	1376*	2019	2006*	0.1
α-Ylangene	1373	1370*	1500	1484*	0.1
α-Copaene	1376	1376*	1511	1491*	0.1
β-Elemene	1387	1388*	1603	1591*	0.1
α-Gurjunene	1410	1406*	1548	1529*	0.1

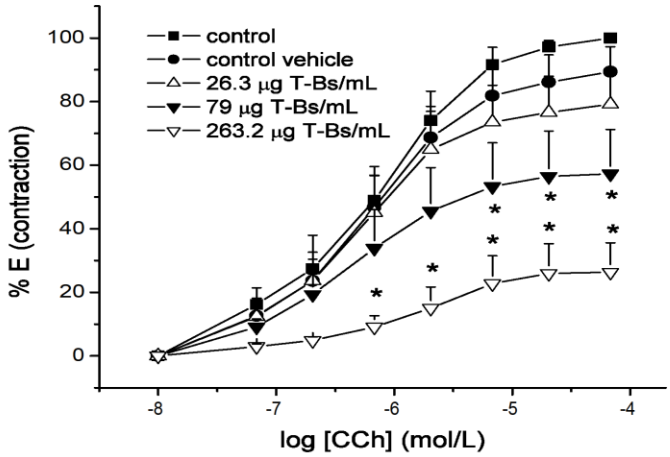
β-Caryophyllene #	1417	1419*	1614	1599*	2.3
Aromandendrene	1437	1439*	1622	1620*	0.2
α-Humulene	1449	1449*	1683	1667*	0.7
Alloaromadendrene	1456	1459*	1658	1649*	0.1
cis-Cadina-1(6),4-diene ##	1467	-	?	-	0.1
γ-Muurolene	1468	1473*	1699	1690*	0.3
α-amorphene	1473	1466*	1700	1693*	0.2
trans-Cadina-1(6),4-diene ##	1473	-	?	-	0.1
β-Selinene	1480	1481*	1732	1717*	0.5
α-Selinene	1489	1490*	1736	1725*	0.5
α-Muurolene	1492	1491*	1730	1723*	0.2
γ-Cadinene	1504	1506*	1766	1763*	0.2
cis-Calamenene	1508	1510*	1841	1835*	0.1
δ-Cadinene	1512	1514*	1763	1755*	0.9
α-Calacorene	1525	1530*	1922	1921*	0.2
α-Cadinene	1528	1527*	1798	1769*	0.1
Selina-3,7(11)-diene	1535	1538*	1789	1783*	0.8

Germacrene B	1550	1535*	1836	1824*	0.1
Spathulenol	1558	1566*	2125	2127*	0.2
Caryophyllene oxide iso ##	1563	-	1977	2000**	tr
Caryophyllene oxide	1565	1570*	1989	1986*	0.5
Guaiol	1581	1589*	2090	2089*	0.1
Rosifoliol	1593	1599**	2123	2144**	tr
1-10-*diepi*-Cubenol	1602	1606*	2056	2074*	0.1
1-*epi*-Cubenol	1614	1614*	2064	2088*	0.2
γ-Eudesmol	1614	1617*	2170	2176*	0.2
τ-Cadinol	1620	1626*	2171	2170*	0.6
β-Eudesmol	1629	1634*	2230	2238*	0.6
Cubenol	1632	1620*	2056	2068*	tr
α-Eudesmol	1636	1641*	2222	2223*	0.5
TOTAL					**94.9**
Aliphatic hydrocarbons		**0.1 %**			
Non oxigenated monoterpenes		**32.6 %**			
Oxygenated monoterpenes		**50.9 %**			

Non oxygenated sesquiterpenes	**8.0 %**
Oxygenated sesquiterpenes	**3.0 %**
Others	**0.3 %**

#: identified by two LRI, MS and standard comparison. ##: tentatively identified by one LRI and MS. All the other constituents were identified by two LRI and MS. LRI HP-1: experimental linear retention indices in the non-polar column. LRI HP-WAX: experimental linear retention indices in the polar column. tr: less than 0.05%. Compounds listed in order of elution in the non polar column. [a]: LRI in similar non polar column from bibliography (*: Babushok et al., 2011; **: Wiley/NIST, 2008; ***: Benomari et al., 2016). [b]: LRI in similar polar column from bibliography (the same references) as above).

A



B

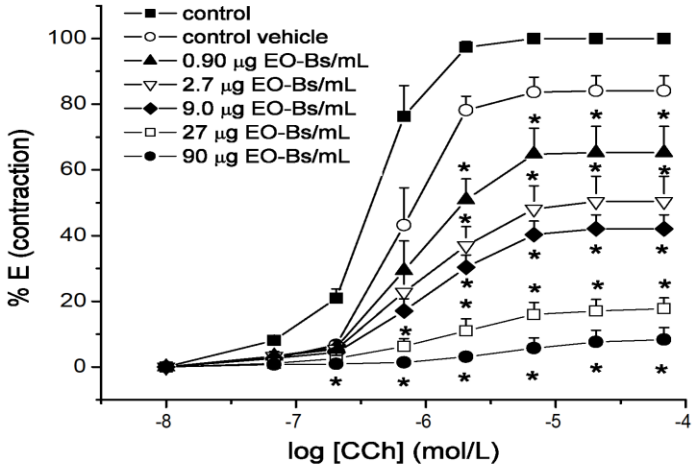
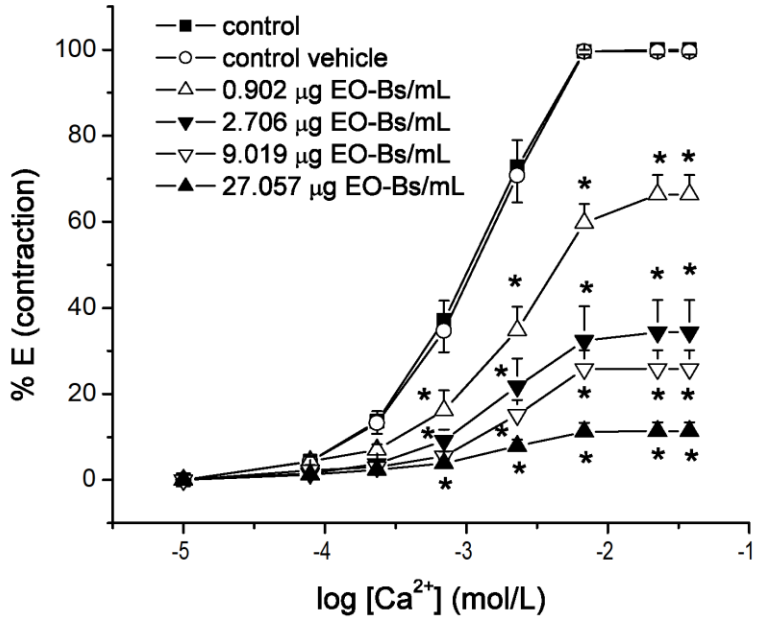


Figure 1

A


control
control vehicle
0.902 μg EO-Bs/mL
2.706 μg EO-Bs/mL
9.019 μg EO-Bs/mL
27.057 μg EO-Bs/mL
% E (contraction)
log [Ca2+] (mol/L)

B

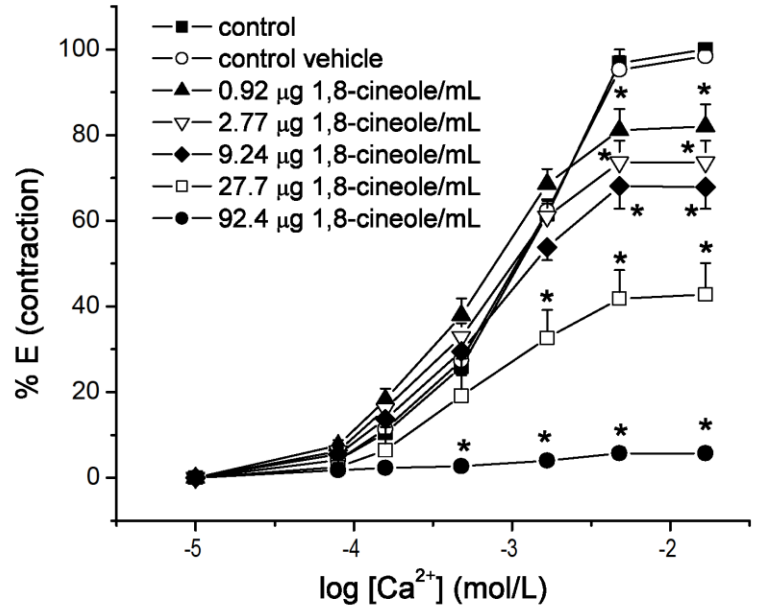

control
control vehicle
0.92 μg 1,8-cineole/mL
2.77 μg 1,8-cineole/mL
9.24 μg 1,8-cineole/mL
27.7 μg 1,8-cineole/mL
92.4 μg 1,8-cineole/mL
% E (contraction)
log [Ca2+] (mol/L)

Figure 2

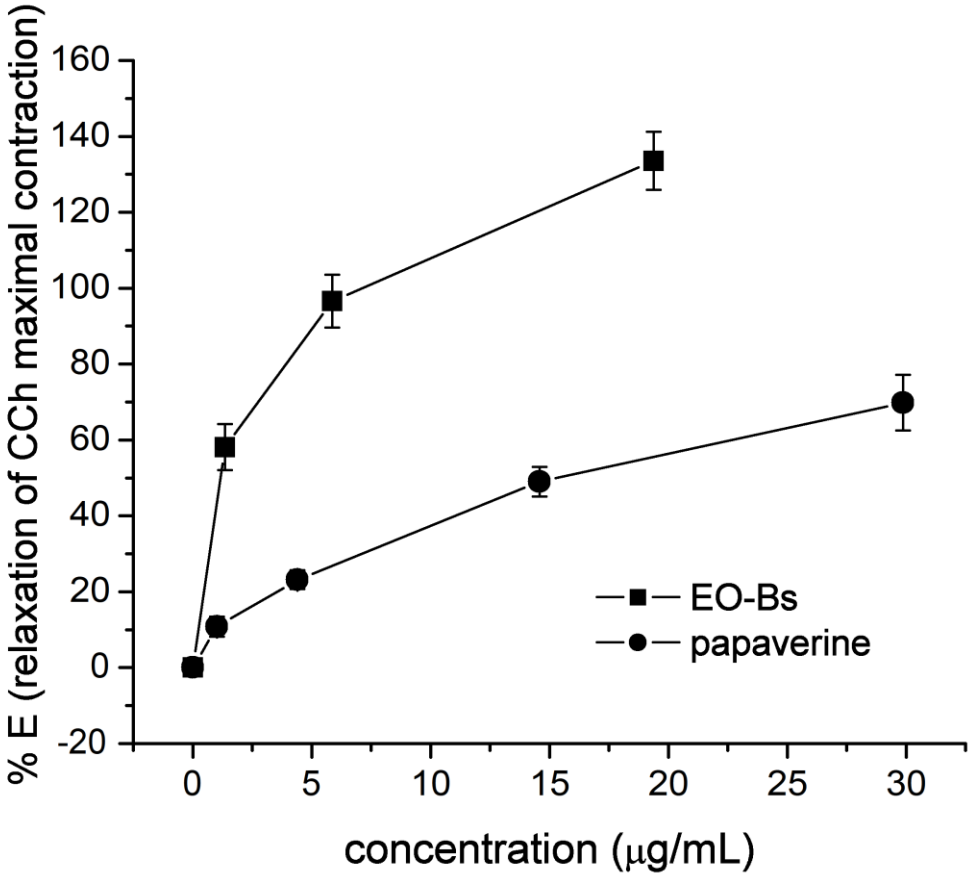


Figure 3

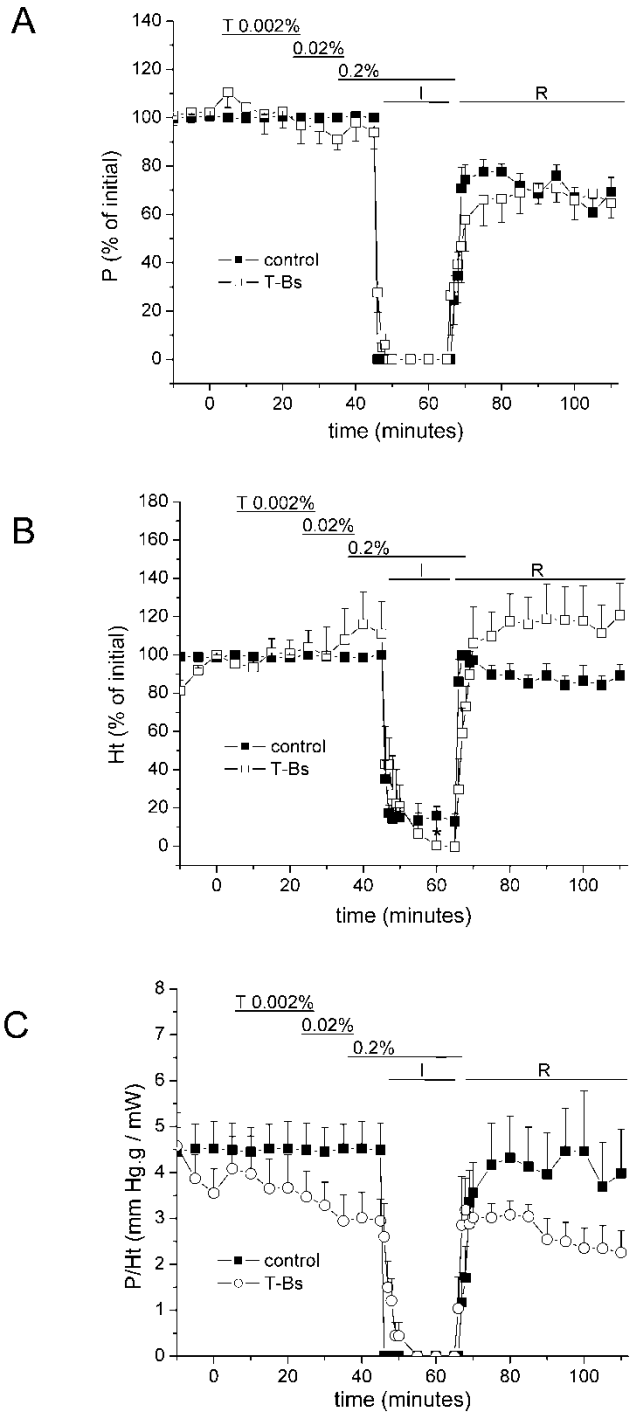
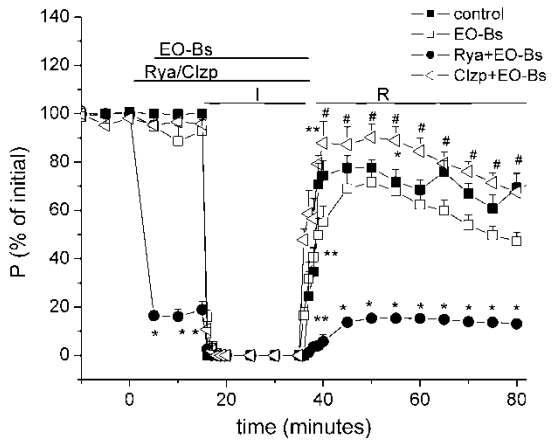
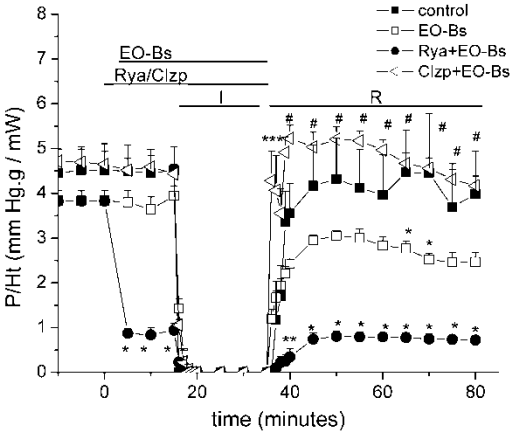

A
T 0.002%
0.02%
0.2%
I
R
P (% of initial)
control
T-Bs
time (minutes)
B
T 0.002%
0.02%
0.2%
I
R
Ht (% of initial)
control
T-Bs
time (minutes)
C
T 0.002%
0.02%
0.2%
I
R
P/Ht (mm Hg.g / mW)
control
T-Bs
time (minutes)

Figure 4

A



B



C

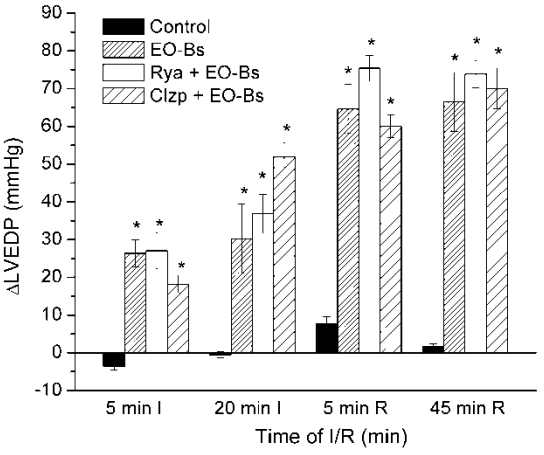


Figure 5

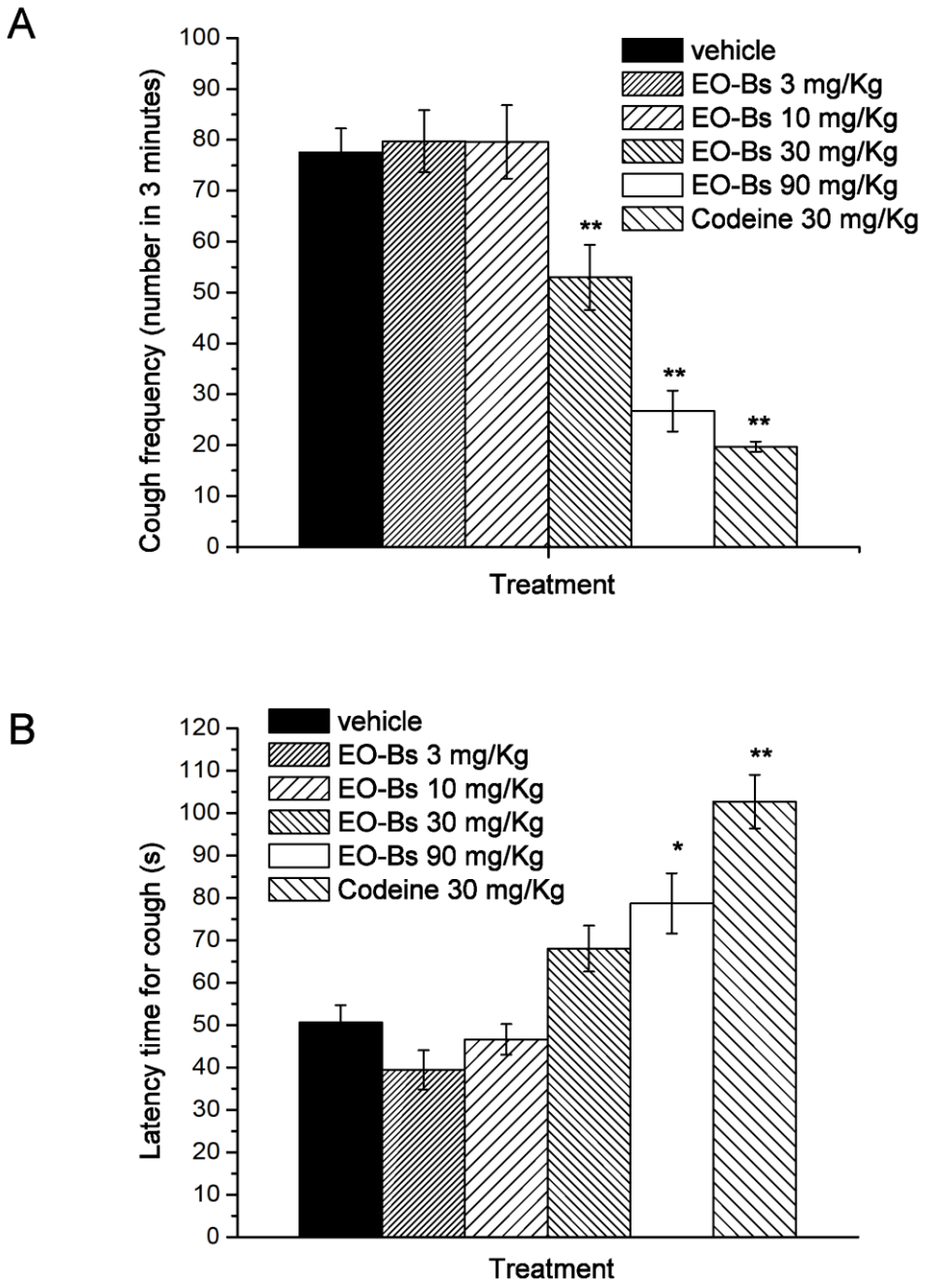

A
Cough frequency (number in 3 minutes)
Treatment
vehicle
EO-Bs 3 mg/Kg
EO-Bs 10 mg/Kg
EO-Bs 30 mg/Kg
EO-Bs 90 mg/Kg
Codeine 30 mg/Kg
**
**
**
B
Latency time for cough (s)
Treatment
vehicle
EO-Bs 3 mg/Kg
EO-Bs 10 mg/Kg
EO-Bs 30 mg/Kg
EO-Bs 90 mg/Kg
Codeine 30 mg/Kg
*
**

Figure 6

Table A.1: Results of 2-way ANOVA obtained from data in Figure 1, parts A and B. The results of Bonferroni´s "a posteriori" tests are shown in the respective figures.

Variables of 2-way ANOVA	Fig. 1 A T-Bs	Fig. 1 B EO-Bs
By treatment	F = 32.93 DFn = 4 P <0.0001	F = 186.5 DFn = 6 P < 0.0001
By log [CCh]	F = 53.09 DFn = 7 P < 0.0001	F = 202.7 DFn = 7 P < 0.0001
Interaction	F= 1.873 DFn = 28 P=0.0066	F= 13.49 DFn= 42 P < 0.0001
DF residual	240	280

Table A.2: Results of 2-way ANOVA obtained from data in Figure 2, parts A and B. The results of Bonferroni´s "a posteriori" tests are shown in the respective figures.

Variables of 2-way ANOVA	Fig. 2 A EO-Bs	Fig. 2 B 1,8-cineole
By treatment	F = 70.45 DFn = 5 P <0.0001	F = 153 DFn = 6 P < 0.0001
By log [Ca^{2+}]	F = 81.58 DFn = 7 P < 0.0001	F = 571 DFn = 6 P < 0.0001
Interaction	F= 6.066 DFn = 35 P < 0.0001	F= 21.67 DFn= 36 P < 0.0001
DF residual	336	245

Table A.4: Results of 2-way ANOVA obtained from data in Figure 4, parts A, B and C. The results of Bonferroni´s "a posteriori" tests are shown in the respective figures.

Variables of 2-way ANOVA	Fig. 4A %P	Fig. 4B %*Ht*	Fig. 4C P/Ht
By treatment	F = 0.0498 DFn = 1 P = 0.8235	F = 3.405 DFn = 1 P = 0.065	F = 7.968 DFn = 1 P = 0.005

By time	F = 83.04	F = 26.26	F = 13.71
	DFn = 32	DFn = 32	DFn = 32
	P < 0.0001	P < 0.0001	P<0.0001
Interaction	F= 1.297	F= 2.306	F= 1.964
	DFn = 32	DFn= 32	DFn= 32
	P=0.135	P= 0.0001	P= 0.0017
DF residual	363	429	396

Table A.5: Results of 2-way ANOVA obtained from data in Figure 5, parts A, B and C. The results of Bonferroni´s "a posteriori" tests are shown in the respective figures.

Variables of 2-way ANOVA	Fig. 5A %P	Fig. 5B P/Ht	Fig. 5C ΔLVEDP
By treatment	F = 568.5	F = 170.2	F = 137.8
	DFn = 3	DFn = 3	DFn = 3
	P < 0.0001	P < 0.0001	P < 0.0001
By time	F = 303.6	F = 51.48	F = 71.16
	DFn = 26	DFn = 26	DFn = 3
	P < 0.0001	P < 0.0001	P < 0.0001

Interaction	F= 19.3 DFn = 78 P < 0.0001	F= 4.872 DFn= 78 P < 0.0001	F= 7.208 DFn= 9 P < 0.0001
DF residual	540	540	80

Table A.6: Results of 1-way ANOVA obtained from data in Figure 6. part A and B. The results of Tukey´s "a posteriori" tests are shown in the respective figures.

Variables of 1-way ANOVA	Fig. 6 A Number of cough episodes	Fig. 6 B Latency time
By treatment	F = 21.39 DF = 5 P <0.0001	F = 17.1 DF = 5 P < 0.0001

DF residual	40	40