Antispasmodic effects of the essential oil of *Croton zehnteneri*, anethole, and estragole, on tracheal smooth muscle

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1. Introduction

*Croton* species are often associated with popular drugs for cancer, gastrointestinal ulcers and anti-inflammatory therapies [1, 2]. They are also reported to have a wide range of pharmacological properties such as: mutagenic, apoptotic, antioxidant, cytotoxic, and anti-proliferative activity [1, 2, 3, 4, 5, 6]. Amongst the *Crotons*, used in folk medicine it stands out *C. zehntneri* [7]. In the northeastern flora, it is plentiful *C. zehntneri* and it is dispersed in the “cerrados”, mainly in the northeast “caatinga” [7]. *C. zehntneri* leaves are popularly used as food, beverages and liqueurs flavoring. Extracts are derived from small bark and leaves and are widely used in folk medicine in Brazil. It is used in tea, decoctions or infusions for the treatment of several ailments, including anxiety, anorexia or relief of gastrointestinal and respiratory disorders [7, 8].

Essential oil of *Croton zehnteneri* (EOCz) has well-described pharmacological and biological activities, such as antielmintic [9], bactericidal [10], antifungal [11] and larvicidal [12]. It has a low acute and subacute toxicity [13]. Among the main terpenoid compounds present in EOCz are anethole (trans-anethole) (trans-1-methoxy-4-(1-propenyl)benzene, a colorless anise-flavored liquid 10 times sweeter than sugar and estragol (1-allyl-4-methoxybenzene), an isomer of anethole [14]. EOCz and its major constituents, anethole and estragole (Figure 1), have demonstrated antispasmodic [9, 10], cardiovascular [11], gastroprotective [15] and potent anti-inflammatory effect [16]. EOCz and anethole have healing activity [17]. Estragole blocks the compound action potential recorded from the sciatic nerve [18] as well as being able to block the excitability of afferent axons of the dorsal root ganglia by a local anesthetic mechanism [19]. Anethole promotes survival and activation of ovarian primordial follicles and improves in vitro production of bovine embryos [20, 21]. It has recently been reported that anethole and estragole...
promote relaxation of rat corpora cavernosa with mechanism predominantly dependent on release of autacoids (NO and prostanoids) [22]. Also in rats, EOCz and anethole were able to block sciatic nerve compound action potentials with similar pharmacological potency [23].

Because the leaves and small bark of C. zehntneri, are rich in essential oil content, and are widely used for treatment of respiratory problems, including asthma, we investigated the effect of EOCz on a model of acute respiratory anaphylaxis induced by ovalbumin (OVA) in animals previously sensitized [24]. These experiments demonstrated that EOCz protected against the anaphylactic derangement induced by OVA [24]. This protection included functionally significant and important attenuation of anaphylactic changes in several variables of respiratory mechanics. These include OVA-induced increase in Newtonian resistance, which represents resistance of respiratory conduits to air flow, plus increase of oxidative stress and of inflammatory cells in the pulmonary parenchyma [24]. Our research group has shown that antispasmodic action appears to be a common pharmacological effect of various essential oils and their constituents on the smooth musculature of various systems [22, 25, 26, 27, 28, 29, 30]. Accordingly, we hypothesized that this decrease in Newtonian resistance could be due to antispasmodic effect, and probably an anti-inflammatory activity. The trachealis muscle, however, has peculiarities that prevents direct transference of conclusions drawn from other smooth muscles. This led us to investigate the antispasmodic effect of EOCz and it is mechanism of action directly on the trachealis muscle. We also included an examination of the effects of OECz main constituents, anethole and estragole, on the airway smooth muscle contractility.

2. Materials and methods

2.1. Solutions and substances

Smooth muscle recordings were performed using modified Krebs-Henseleit’s solution (KHS) with the following composition (in mM): 118.0 NaCl, 4.8 KCl, 25.0 NaHCO₃, 2.5 CaCl₂-2H₂O, 1.2 KH₂PO₄, 1.2, MgSO₄-7H₂O, 11.0 glucose, and pH adjusted to 7.40 with HCl/NaOH, gased with O₂/CO₂ (95%/5%) mixture, at 37 °C. The EOCz, anethole and estragole were dissolved in dimethyl sulfoxide (DMSO) at a final DMSO concentration equal to or less than 0.05% (v/v, final working solution); stock solutions were prepared daily. In experiments requiring Ca²⁺-free solutions, ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (2 mM) was added to the KHS in those experiments using Ba²⁺ to induce contraction. For electrophysiological experiments, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), tetraethylammonium chloride (TEA), Mg-ATP, Li-GTP, papain, bovine serum albumin (BSA), dithiothreitol (DTT), collagenase type II, hyaluronidase, and salts were purchased from Sigma Chemical Company (St. Louis, MO, USA) or Reagen (Rio de Janeiro, RJ, Brazil). The agonist ACh and Bay-K 8644 were first dissolved in distilled water to make stock solutions. PDBu and nifedipine were first diluted in DMSO (final concentration ≤0.05%). All stock solutions were sonicated immediately before addition to the experimental chamber to attain the final desired concentrations. Unless otherwise stated, the EOCz, anethole and estragole concentrations used were: 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10, 30, 100, 1000, 2000, 3000 and 5000 μg/mL. Experiments were carried out at room temperature (22 ± 2 °C), and all reagents, anethole (99% purity), estragole (99%), nifedipine (≥98%), ACh (99 %), PDBu (99%), and Bay-K 8644 (≥98%) were analytical grade and purchased from Sigma-Aldrich or Reagen.

2.2. Plant material, extraction and chemical analysis

The leaves of C. zehntneri were collected in Viçosa do Ceará city (lat. 3°33’48” S; long. 41° 5’ 41” W., Ceará, Brazil). Plant identification was confirmed by Dr. FJ Abreu Matos (Laboratory of Natural Products, Federal University of Ceará, Fortaleza, CE, Brazil). A voucher specimen (No. 277477) was deposited at the herbarium Prisco Viana of the Federal University of Ceará. The EOCz was extracted and isolated by steam distillation from leaves cut into small pieces and analyzed chemically as previously described [17, 23].

The analysis of the chemical composition of the essential oil was made at the Parque de Desenvolvimento Tecnológico do Ceará (PADETEC) of the Federal University of Ceará (UFCE), CE, Brazil. The identification of the major components of the essential oil of the leaves of C. zehntneri was obtained through gas chromatography coupled to mass spectrometry (GC/MS), in a Hewlett-Packard model 5971 spectrometer, operating with 70 eV ionization energy (Figure 2). Capillary column of fused silica DB-5 (30 m × 0.25 mm d.i., 0.25 μm of film thickness) and helium gas carrier with a flow of 1 mL/min with split were used. The injector and detector temperatures were programmed at 250 °C and 200 °C, respectively. The column temperature was determined from 35 °C to 180 °C to 4°C/min, and then from 180 °C to 280 °C to 10°C/min. Mass spectra were done from 30 to 450 m/z. Individual components were identified by matching their mass spectra with those in the database, as well as by visual comparison of the standard fragmentation with those reported in the literature [31].

2.3. Animals and tissue preparation

Male Wistar rats (Rattus norvegicus) weighting 170–250g, were used. Animals were kept in a vivarium at State University of Ceará under controlled temperature (23–25 °C) and humidity (50–60%), with 12 h light/12 h dark cycle and free access to water and food. All animals were handled according to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication 85-23, revised 1996; http://www.nap.edu/readingroom/books/labrats/index.html), using methods that minimize animal suffering. The animals were euthanized by CO₂ inhalation. All procedures and protocols were reviewed and approved by the Animal Ethics Committee of the State University of Ceará with following protocol number: 06379067-0.

2.4. Measurement of tracheal muscle contraction

Tissue preparation and isometric tension recording procedures were performed as previously described [30]. After animal sacrifice, the trachea was dissected and connective tissues were removed. To study the muscular contraction, the trachea was sectioned in 3–4 cylindrical segments of cartilage rings, approximately 4–5 mm in length. These

Figure 1. Molecular structure of Anethole and Estragole, the main constituents of EOCz.
segments were mounted in a 5 mL organ bath chambers containing KHS solution and maintained at 37°C. The tracheal segments were mounted under tension of 1.0 g-force (gF) and allowed to stabilize for a period of 1 h before the addition of any substance. In order to verify the viability and integrity of the preparation, we added hypertonic KCl (3.0 M) to increase chamber K⁺ concentration to 60 mM. The contractions were recorded with an isometric force-displacement transducer (Grass, FT-03, Quincy, MA, USA) connected to an integrated recording system (amplifier (PM1000, DATAQ instruments), analog to digital converter (DI-200, DATAQ instruments), acquisition and analysis software (WinDaq, version 1.65, DATAQ Instruments, Akron, OH, USA) and computer).

After assessing tissue viability, all experimental series measuring force of contraction were performed using the compounds in parallel form, ie, from the four channels of the recording systems, one channel was used for external control, and one channel for each of the three agents: EOCz, anethole and estragole. In some experiments, each of these agents was added to the organ chamber in increasing concentrations (0.01–5000 μg/mL) and their effects on the resting tone of tracheal rings were evaluated. In other experiments, in order to investigate the effect of EOCz and its main constituents, anethole, and estragole, on [K⁺]-induced contraction (electromechanical excitation-contraction coupling (ECC)), a given concentration of one of each of these three agents was added to the organ bath chamber five minutes prior to increase in [K⁺] to 60 mM. This allowed drug effect on [K⁺]-induced contractions to reach the steady state. Subsequently to these contractions, the chamber solutions were washed out to allow recovery before the evaluation of a newer (0.1–300 μg/mL) concentration of agents (non-cumulative increase in concentration protocol). Similar non-cumulative protocols were used to investigate the effect of the three agents (0.1–5000 μg/mL) on the ACh-induced contraction (pharmacomechanical ECC). In other experimental series, the effects of EOCz, anethole or estragole (200 and 1000 μg/mL) were investigated on the contractions induced by 1 μM PDBu (primed with 30 mM KCl). We also studied the inhibitory effects of OECz (300 and 1000 μg/mL), anethole (100 and 300 μg/mL) and estragole (100, 300 and 1000 μg/mL) on the contractile response of tracheal rings to BaCl₂ (0.01–30 mM) in Ca²⁺-free medium. Relaxant effects of increasing cumulative concentrations of nifedipine on the sustained contractile response to 30 mM KCl or 30 mM KCl plus PDBu 1 μM, a PKC activator [32], was investigated.

2.5. Dissociation of tracheal smooth muscle cells and ionic current recordings

Dissociation of tracheal smooth muscle and ionic current recording were performed as previously described [30]. The trachea was immediately dissected, attached tissues and cartilage removed, and placed in a physiological saline solution (PSS) containing low concentration of Ca²⁺ (PSS-Ca²⁺ 0.05 mM). The tracheal smooth muscle was transferred to a falcon tube with an enzymatic solution containing high-Ca²⁺ concentration (PSS-Ca²⁺ 2.6 mM) with the following composition: 137 NaCl, 5.6 KCl, 0.44 NaHPO₄, 0.42 Na₂HPO₄, 4.17 NaHCO₃, 1 MgCl₂, 2.6 CaCl₂, 10.0 Hepes and 5.0 glucose, papain (0.9 mg/mL), BSA (1 mg/mL), and DTT (0.9 mg/mL). The falcon tube with tracheal smooth muscle strip were maintained 40 min in water bath at 37°C. The tracheal smooth muscle strips were transferred to another falcon tube containing 3 mL of PSS-Ca²⁺ 0.05 mM whose composition was (in mM): 137 NaCl, 5.6 KCl, 0.44 NaHPO₄, 0.42 Na₂HPO₄, 4.17 NaHCO₃, 3.55 MgCl₂, 0.05 CaCl₂, 10.0 Hepes, and 5.0 glucose with collagenase type II (1.0 mg/mL), BSA (1.0 mg/mL), and hyaluronidase (0.9 mg/mL). The falcon tube was placed in a water bath at 37°C during 18 min.

The dissociated cells was centrifuged at 1.500 r.p.m, washed, and re-suspended between each centrifugation with PSS-Ca²⁺ 0.05 mM whose composition was (in mM): 137 NaCl, 5.6 KCl, 0.44 NaHPO₄, 0.42 Na₂HPO₄, 4.17 NaHCO₃, 3.55 MgCl₂, 0.05 CaCl₂, 10.0 Hepes, and 5.0 glucose with collagenase type II (1.0 mg/mL), BSA (1.0 mg/mL), and hyaluronidase (0.9 mg/mL). The falcon tube was placed in a water bath at 37°C during 18 min.

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Because calcium currents from voltage dependent calcium channel (VDCC) in smooth muscle have small amplitudes, we used Bay-K 8644.
(10 μM), a calcium channel agonist [33]. The pipettes for the recording of ionic currents were constructed from glass capillaries (Perfecta, São Paulo, SP, Brazil, 75 mm length, 1.0 mm internal diameter, and 1.5 mm external diameter) with a micropipette puller (P-97 model, Sutter Instruments, San Francisco, CA, USA). The pipettes had resistances of 3–6 MΩ and were filled with a pipette solution with the following composition (in mM): 110 CsCl, 30 TEA-Cl, 20 EGTA, 4 MgATP, 10.0 Hepes, and 0.1 Li2-GTP and pH adjusted to 7.20 with CsOH. An Ag–AgCl electrode was used as a reference electrode. The ICa, in the whole-cell configuration, were recorded by an Axopatch 200B amplifier (Molecular Devices, USA) and controlled by the Clampex program (Molecular Devices, USA, version 10.2) for data acquisition. Capacitive currents were electronically compensated and a P/4 protocol was used to subtract linear leakage and residual capacitance [34]. The ICa were filtered with a low-pass filter (Bessel 4 poles), cutoff frequency of 5 kHz, and sampled at 10 kHz. Patch-clamp experiments were performed using an inverted microscope (Axiovert 200, Carl Zeiss, Germany). Cells were continuously perfused at 0.3–0.4 mL/min with solutions containing anethole (anethole þ DMSO þ PSS-Ca2+ 2.6 mM + 10 μM Bay-K 8644) or control (DMSO þ PSS-Ca2+ 2.6 mM + 10 μM Bay-K 8644). Solutions were added to the recording chamber via a three-way valve. K+ currents were minimized by the use of K+ channel blockers such as TEA and Cs+, present in the pipette, and PSS-Ca2+ 2.6 mM in the external solutions. All patch-clamp experiments were performed at room temperature (20–24 °C). The experimental protocol was organized in an experimental series detailed below:

### 2.5.1. Blockade of ICa by anethole

A test pulse from −60 mV (holding potential) to +10 mV, 500 ms duration, and 15 s pulse-to-pulse interval was applied. Since calcium currents display a rundown effect [35] we monitored current amplitude, duration, and 15 s pulse-to-pulse interval was applied. Since calcium currents were constructed from glass capillaries (Perfecta, São Paulo, SP, Brazil, 75 mm length, 1.0 mm internal diameter, and 1.5 mm external diameter) with a micropipette puller (P-97 model, Sutter Instruments, San Francisco, CA, USA). The pipettes had resistances of 3–6 MΩ and were filled with a pipette solution with the following composition (in mM): 110 CsCl, 30 TEA-Cl, 20 EGTA, 4 MgATP, 10.0 Hepes, and 0.1 Li2-GTP and pH adjusted to 7.20 with CsOH. An Ag–AgCl electrode was used as a reference electrode. The ICa, in the whole-cell configuration, were recorded by an Axopatch 200B amplifier (Molecular Devices, USA) and controlled by the Clampex program (Molecular Devices, USA, version 10.2) for data acquisition. Capacitive currents were electronically compensated and a P/4 protocol was used to subtract linear leakage and residual capacitance [34]. The ICa were filtered with a low-pass filter (Bessel 4 poles), cutoff frequency of 5 kHz, and sampled at 10 kHz. Patch-clamp experiments were performed using an inverted microscope (Axiovert 200, Carl Zeiss, Germany). Cells were continuously perfused at 0.3–0.4 mL/min with solutions containing anethole (anethole þ DMSO þ PSS-Ca2+ 2.6 mM + 10 μM Bay-K 8644) or control (DMSO þ PSS-Ca2+ 2.6 mM + 10 μM Bay-K 8644). Solutions were added to the recording chamber via a three-way valve. K+ currents were minimized by the use of K+ channel blockers such as TEA and Cs+, present in the pipette, and PSS-Ca2+ 2.6 mM in the external solutions. All patch-clamp experiments were performed at room temperature (20–24 °C). The experimental protocol was organized in an experimental series detailed below:

### 2.6. Statistical analysis

Data are expressed as mean ± SEM (n), where SEM is standard error of the mean, and n indicates the number of the tracheal rings. Concentration-response curve was determined and IC50 values (concentration at which 50% of maximal response is observed) for EOCz, anethole or estragole were calculated and adjusted with the Hill equation as follow:

\[
% \text{ of agonist concentration} = \min + \left( \frac{\max - \min}{1 + \left( \frac{\text{agonist}}{\text{IC}50} \right)^n} \right)
\]

where min. and max. are the minimum and maximum contraction fraction values, respectively, [agonist] is the concentration of agonist and k is the slope of Hill. The significance for the statistical tests was accepted when p ≤ 0.05 for occurrence of null hypothesis. For comparison of data from more than two different groups one-way or two-way ANOVA was used, as appropriate, followed by the Holm-Sidak post hoc test, for parametric data; for nonparametric data, Rank’s ANOVA followed by Dunn’s test was used.

### 3. Results

#### 3.1. Chemical composition of essential oil

The chemical composition of the EOCz sample used in this study was evaluated, by GC/MS, is shown in Table 1 and Figure 2. The two major constituents of EOCz, in descending order of concentration are: 80.46% trans-anethole and 9.53% estragole.

#### 3.2. Effect of EOCz, anethole, and estragole on resting tonus of rat tracheal rings

Initially, we examined the effect of EOCz, anethole, and estragole on the tracheal resting tonus. For the preparations equilibrated with 1.0 gF resting distension force, cumulative concentrations (1–5000 μg/mL) of EOCz, anethole or estragole were considered not to alter the resting tonus. This is because after one hour of equilibration, these agents promoted only very minor and not statistically significant (p > 0.05, ANOVA) alterations of the resting tonus, from 1.00 ± 0.006, 0.999 ± 0.007 or 0.999 ± 0.002 gF, respectively.

#### 3.3. Relaxant effect of EOCz, anethole, estragole, and nifedipine on the sustained contraction induced by KCl (electromechanical ECC) in rat tracheal rings

In order to investigate the effect of EOCz and its main constituents, anethole, and estragole, on K+–induced contraction, we studied the effects of these three agents administered non-cumulatively (see methods). Contractions were induced with 60 mM [K+] and nifedipine of

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### Table 1. Main constituents of the essential oil of the Croton zehntneri leaves.

| Compound          | Retention time/min | Percentage area % |
|-------------------|--------------------|-------------------|
| Alpha-pinene      | 6.642              | 0.20              |
| Sabineol          | 8.075              | 0.15              |
| Myrcene           | 8.808              | 1.65              |
| Alpha-phetlandrene| 9.450              | 0.17              |
| 1,8-Cineole       | 10.617             | 0.69              |
| Trans-beta-ocimene| 11.325             | 0.49              |
| Estragole         | 19.475             | 9.53              |
| Trans-anethole    | 25.292             | 80.46             |
| Not identified    | 28.983             | 0.19              |
| Beta elemene      | 29.458             | 0.18              |
| Trans-caryophyllene| 30.925             | 0.80              |
| Germacrone-D      | 34.067             | 0.89              |
| Bicyclogermacrene | 34.908             | 3.98              |
| Spathulenol       | 38.742             | 0.46              |
| Not identified    | 48.033             | 0.15              |

* Experimental retention indices.

* Relative proportions of essential oil constituents were expressed as percentages.
anethole (n = 8), or estragole (n = 12) induced a significant concentration-dependent blockade of 60 mM [K+]−-induced contraction (p < 0.05, two-way ANOVA followed by Holm-Sidak test); IC50 values were 145.8, 89.9 (corresponding to 0.61 mM), and 181.0 (1.22 mM) µg/mL, respectively (Figure 3A and Table 2). Maximum pharmacodynamic efficacy (100% blockade) was observed with anethole and estragole; for OECz, maximum arithmetic value of average blockade was 89 ± 8.9%, which was not considered different from 100%, since the 95% confidence limit was [62.8–115.2] % and these limits include 100 %.

For comparison, we studied the pharmacodynamic parameters of the inhibition of the K+−-induced contraction (60 mM) by nifedipine (Figure 3B), a specific L type VDCC blocker. IC50 values of nifedipine-induced blockade was 27.7 ± 3.46 µg/mL (0.08 ± 0.01 µM (n = 7)).

3.4. Inhibitory effect of OECz, anethole, and estragole on the contraction evoked by ACh (pharmacomechanical ECC) in rat tracheal rings

The effects of OECz, anethole and estragole on ACh-induced contraction were investigated. Concentration-dependently, OECz and its estragole constituent (0.1–5000 µg/mL) inhibited the non-cumulatively evoked contraction induced by ACh (10 µM) (Figure 4A); the IC50 were 606.1 µg/mL and 358.6 µg/mL (2.42 mM), respectively (Figure 4B and Table 2). Anethole (0.1–5000 µg/mL), on the other hand, concentration-dependently affected the contraction evoked by ACh in a biphasic manner. At lower concentrations (<30 µg/mL), anethole from 1 µg/mL, monotonically and significantly (p < 0.001, one-way ANOVA test, followed by Holm-Sidak post hoc test) increased tone, reaching 38% increase at 30 µg/mL. At higher concentrations, anethole inhibited tone (IC50 = 160.5 ± 33.0 µg/mL (1.05 ± 0.22 mM) (n = 8)); total contraction blockade occurred at the concentration of 300 µg/mL.

3.5. Inhibitory effects of OECz, anethole, and estragole on the contractions induced by Ba2+

In tracheal rings pre-incubated in Ca2+-free solution in the presence of high K+ (60 mM) solution, cumulatively increasing concentrations of Ba2+ (0.3, 1.0, 3.0, 10.0, 20.0, 30.0 mM) evoked concentration-dependent contractions (Figure 5), which at a maximal contraction amplitude, corresponded to about 88% of the contraction induced by 60 mM K+.

In preparations pre-exposed (5 min) to either of the three agents, Ba2+-induced contractions were blocked. With respect to OECz
3.6. Inhibitory effects of increasing concentrations of EOCz, anethole, and estragole on the contraction evoked by PDBu

Tracheal rings were pre-incubated in K+ (30 mM) and afterwards, Phorbol 12,13-Dibutyrate (PDBu) (1 μM), a PKC activator, was added. PDBu induced a sustained contraction and when the contraction reached steady state, non-cumulative addition of concentrations (200 or 1000 μg/mL) of EOCz, anethole or estragole was applied. EOCz, anethole, or estragole, at 1000 μg/ml (but not at 200 μg/ml) significantly (p < 0.001, two-way ANOVA on Rank’s followed by Dunn’s test) greatly reduced the PDBu-induced contractions (Figure 6C, D).

The pharmacodynamic parameters of the inhibitory activity of nifedipine on the 30 mM K+-induced contraction with and without PDBu (1.0 μM) in the KHS was investigated. In absence and in presence of PDBu, nifedipine showed maximal efficacy. The IC50 for nifedipine, however, was significantly increased from 4.5·10^{-9} ± 9.0·10^{-11} M, in absence, to 5.9·10^{-9} ± 2.7·10^{-10} M (n = 6), in presence of PDBu (p < 0.005, non-paired t-test) (Figure 6A, B).

3.7. Inhibitory effect of anethole on Ca2+- current through VDCC

Each of the three agents showed an IC50 for blockade of 60 mM (K+)‐induced contraction significantly smaller than for inhibition of ACh-induced contraction. This suggests an effect on VDCC as a major mechanism of antispasmodic effect. We thus directly investigated the activity of the major constituent of EOCz, anethole, on the inward Ca2+ current through L type VDCC.

In myocytes isolated from trachealis muscle, in presence of Bay K 8644, and with the patch clamp technique in whole cell mode, at end of 180 s of preparation exposure to 100 μg/ml (0.67 mM) anethole, peak inward ICa decreased to 64.27 ± 6.32% (n = 8) of control value (-106.1 ± 7.3 pA). (Figure 7).

4. Discussion

The major findings of this investigation is that EOCz, anethole and estragole whilst not affecting the spontaneous tonus of the trachealis muscle, acts as an antispasmodic agent on the respiratory smooth muscle, inhibiting the increase in tonus promoted by 60 mM [K+], ACh, Ba2+, and PDBu. Anethole, the major constituent of EOCz, also blocked the ICa through VDCC. Anethole and estragole, major constituents of EOCz, inhibited the major mechanism starts with activation of muscarinic cholinergic receptors; it then includes activation of receptor dependent calcium channels (RDCC) with inward Ca2+ flow and IP3 mediated Ca2+ release from intracellular stores [36, 39]. Amongst several contravet responses, each with difference in the mechanism of action, 60 mM K+-induced contraction was that one in which EOCz showed the greatest pharmacodynamic potency. This suggested that the major mechanism of action of the contraction inhibition by EOCz was blockade of activation of L type VDCC. This hypothesis was strengthened.
by two facts: i) the effect of EOCz on the Ba^{2+}-induced contraction; ii) the significantly greater IC_{50} for blockade of ACh-induced response, a contraction which mechanism includes RDCC activation and intracellular components not present in the contraction promoted by 60 mM [K^+] (Table 2).

Regarding the Ba^{2+}-induced contraction, although the protocol for this later type of contraction does not allow a rigorous comparison of pharmacodynamic parameters with those of the 60 mM K^{+}-induced contraction, some comparisons may be appropriate. Figure 5A demonstrated that the inhibition of the Ba^{2+}-induced contraction starts at concentration near to 300 and reaches total blockade at 1000 μg/mL EOCz. This concentration range for progressive increase in blockade intensity is similar to that of EOCz inhibitory effect on 60 mM K^{+} (Figure 3A). Thus, one can conclude that on Ba^{2+}, and on 60 mM K^{+}-induced contraction, EOCz acted with similar pharmacodynamic potency. This similarity is relevant to elucidation the mechanism of action because, in order to promote smooth muscle contraction, Ba^{2+} flows specifically inwardly through VDCC of the L type [30, 38].

Concerning the significantly greater IC_{50} for blockade of ACh-induced response, if EOCz inhibitory activity of contraction were done on a step of the contraction biochemical cascade common to both type of contraction, then the pharmacodynamics potency were expected to be the same on both types of contraction. Since they are different, then, on ACh contraction, EOCz is expected to act either on a single mechanism not present in KCl-induced contraction, or on a limiting step of multiple serial mechanism, which (not present in KCl-induced contraction) needs higher EOCz concentration for blockade. The major difference from ACh-induced to KCl-induced contraction is the major dependence of KCl-induced contraction on VKCC blockade [36, 39]. This fact, together with the greater pharmacodynamics potency on KCl-induced contraction, EOCz is expected to act either on a single mechanism not present in KCl-induced contraction, or on a limiting step of multiple serial mechanism, justify the suggestion that EOCz blockade of VKCC is the major mechanism of inhibition of KCl-induced contraction.

This conclusions of the discussion regarding EOCz mechanism of action applies to anethole and estragole. Both substances showed a significantly smaller IC_{50} on the 60 mM [K^+] than on ACh-induced contractions (Table 2). Thus, the data suggest that, similar to EOCz, anethole and estragole likely evoke inhibition of K^+ and ACh-induced contraction through different mechanisms of action, with activity on VDCC being the most important mechanism of action of these three agents.

Since our data suggest VDCC blockade as an important component of EOCz, anethole and estragole mechanism of action, direct elucidation of the involvement of calcium channels seemed important to us. We studied anethole because it is the major constituent of EOCz and because it may be representative of the other two agents EOCz and
Anethole and estragole are liposoluble terpenoids with low molecular weight, and very similar chemical molecular structure; both are position isomer. As expected, they have predominantly similar effects, but they also showed unexpected differences of effects and of pharmacodynamic characteristics. These differences likely contributed to make the effect of EOcz in some aspects different from those of both constituents, although predominantly more similar to those of estragole.

C. zehntneri leaves and small branches have received an intense and widespread use in Northeast of Brazil [7]. This use includes not only C. zehntneri as medicament in folk medicines, as teas, infusions and beverages, but also culinary uses as spicy ingredients for foods and beverages. Leaves and small branches of C. zehntneri have relatively high content of the EOcz (2–4 % dry weight) [43]. Due to this high content and the intense use of C. zehntneri in folk medicine, it has been suggested that this plant is likely effective in, at least, some effects sought in folk medicine and that EOcz is the active principle or that contributes to the effects [9, 44]. In this regard, the present study not only adds the respiratory system, and in particular the trachealis muscle, to the list of organs where EOcz and its major constituents have pharmacological antispasmodic efficacy, but also it adds an extensive study of concentration-effect relationship.

Several uses of C. zehntneri in folk medicine have been pharmacologically investigated. Among these studies are anti-inflammatory [17], antinociceptive [44] and intestinal antispasmodic properties [15, 26]. The present study adds to that list of pharmacological properties. We demonstrated that EOcz (and constituents) possess pharmacological antispasmodic activity with efficacy on the trachealis muscle. This demonstration is important because this effects on respiratory smooth muscle could not be easily anticipated from effects of C. zehntneri in other smooth muscles. In several important aspects respiratory smooth muscle differs from the intestinal and other smooth muscles [36, 45]. Our data is also important because EOcz-induced prevention of increase in respiratory Newtonian resistance in anaphylaxis [24] does not necessarily lead to conclusion of antispasmodic effect, since the increase in Newtonian resistance could largely result from increase in airway secretions. It is noteworthy that EOcz and anethole are documented to have very low toxicity [13, 16, 46] which makes these agents potential candidates for therapeutic use.

From a therapeutic point of view the use of EOcz as antispasmodic and muscle relaxant, is likely more relevant than anethole. EOcz induced no potentiation of contractant effects of an endogenous neurotransmitter, like ACh, whilst anethole did. It is important to emphasize in this case, the superiority of a mixture prepared by nature, when comparing EOcz, to a single constituent, anethole no matter the overwhelming majority of this constituent in the composition of the essential oil.

5. Conclusion

In conclusion, we have here demonstrated that EOcz, anethole and estragole act as antispasmodic agents on trachealis muscle, very probably via a blockade of L type VDCC as the most important mechanism of action. This represents a contribution to the ever growing list of pharmacologically relevant effects of this essential oil and its two major constituents. We also showed that, despite the fact that anethole is the overwhelmingly major constituent, EOcz is likely to have a better potential for therapeutic use, since it did not show pro-contractant effect at any concentration investigated. We thus believe our data have contributed to the scientific knowledge of the effects of EOcz related to uses of C. zehntneri in folk medicine.

Declarations

Author contribution statement

C.C. Lima and Á. Pereira-Gonçalves: performed the experiments; analyzed and interpreted the data; wrote the paper.
R.O.S. Fontenelle, S.M. Morais, E.H.S. Brito, R.S.N. Brilhante, R.A. Cordeiro, M.G.A. De Lima, I.C.C. Maia, B.D. De Sousa, S.M. De Morais, S.M. Freitas, Effect of C.C. Lima et al. Heliyon 6 (2020) e05445
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Additional information
No additional information is available for this paper.

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