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Chemical compositions and properties of *Schinus areira* L. essential oil on airway inflammation and cardiovascular system of mice and rabbits

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ABSTRACT

The main purpose was to investigate the effects of essential plant-oil of *Schinus areira* L. on hemodynamic functions in rabbits, as well as myocardial contractile strength and airways inflammation associated to bacterial endotoxin lipopolysaccharide (LPS) in mice.

This study shows the important properties of the essential oil (EO) of *S. areira* studied and these actions on lung with significant inhibition associated to LPS, all of which was assessed in mice bronchoalveolar lavage fluid and evidenced by stability of the percentage of alveolar macrophages, infiltration of polymorphonuclear leukocytes and tumor necrosis factor-α concentration, and without pathway modifications in conjugated dienes activity. Clinical status (morbidity or mortality), macroscopic morphology and lung/body weight index were unaffected by the administration of the EO *S. areira*.

Furthermore, the *ex vivo* analysis of isolated hearts demonstrated the negative inotropic action of the EO of *S. areira* in a mice model, and in rabbits changes in the hemodynamic parameters, such as a reduction of systolic blood pressure. We conclude that EO *S. areira* could be responsible for modifications on the cardiovascular and/or airway parameters.

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1. Introduction

Essential oils (EOs) are aromatic components obtained from different plant parts such as flower, buds, seed, leaves and fruits, and are composed of secondary metabolites such as terpenes. Terpenes act as a mechanism of defense against pathogen attack (viruses, herbivores, microbes or competing plants), which, despite extraction and distillation of the plants, do not lose their characteristics and composition. They have been employed for a long time in different industries, mainly in perfumes (fragrances and aftershaves), food (as flavoring and preservatives), pharmaceuticals (therapeutic action) and for centuries in traditional medicine (Bakkali et al., 2008).

Aromatic plants contain volatile oils that exhibit a variety of biological properties, that can release chemical compounds that interact with volcanic activity, forest fires, natural gas leaks, wood drying, etc. involving many substances such as methane, carbon dioxide, carbon monoxide, and other volatile compounds that are released in the air as a result of these biological processes and the EOs can have a profound impact on homeostasis of the human (Jia et al., 2010; Kabesch and Roger, 2004; Park et al., 2010; Weschler et al., 2006).

Aromatic components and particulate air pollutants together with their soluble components, may enter the lung and produce local inflammation and cardiac autonomic alteration, modifying the signal transduction of the myocardial adrenergic system, changing the intracellular levels of Ca^{2+} or by altering the sensitivity of certain proteins that regulate this ion and some of these mechanisms might be modulated by the terpenes present in the EO (Menezes et al., 2010; Nielsen et al., 2005).

The contamination with lipopolysaccharide (LPS) an endotoxin from the cell wall of gram-negative bacteria, it is present as a contaminant on airborne particles, including organic dust and cigarette smoke and several authors described that LPS instillation

Abbreviations: EO, essential oil; TNF-α, tumor necrosis factor-α; AMs, alveolar macrophages; PMNs, polymorphonuclear leukocytes; BALF, bronchoalveolar lavage fluid; LPS, lipopolysaccharide; CDs, conjugated dienes; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure.

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in mice results in a pulmonary inflammation (Rahman and MacNee, 1998).

The respiratory tract penetration is principally dependent on water solubility of the volatile organic compounds. Moreover, these volatile organic compounds are able to penetrate deeply into the lungs reaching the lower areas, like the alveoli, bronchioles or bronchia, producing a rise of allergic diseases (atopic rhinoconjunctivitis, atopic dermatitis, and asthma) and can even spread into the systemic circulation or to environment (Viegi et al., 2004; Zahed et al., 2010).

The anti-inflammatory activity of EOs has been investigated in inflammatory diseases such as allergy, rheumatism, arthritis and bronchitis (Passos et al., 2007; Süntar et al., 2012). They tend to have low mammalian toxicity, less environmental effects and wide public acceptance (Koroch et al., 2007).

Schinus areira L. [synonymous: *Schinus molle* L. var. a*reira* (L.) DC.] commonly called "peppertree", "molle" and "aguaribay" is a native plant from South America and nowadays it is distributed through Argentina, south-eastern of Brazil, Perú, Colombia, Ecuador, Uruguay and widely distributed in México, Central and Southern of California and West Texas, United States. It is a tree belonging to the Anacardiaceae family (Zunino et al., 2003).

All parts of this tree are used in traditional medicine as antibacterial, antifungal, antirheumatic, anti-conjunctivitis, tuberculosis, bronchitis, cough, etc. (Molina-Salinas et al., 2007; Zunino et al., 2003). A review of the EO composition of this plant was described by Zygadlo and Juliani (2002). This EO possesses antimicrobial activity, fungitoxic activity, antiradical power (Dikshit et al., 1986; Guala et al., 2009; Hayouni et al., 2008; Murray et al., 2009; Simionatto et al., 2011; Zahed et al., 2010).

A mice model of LPS-induced acute lung injury was used to evaluate anti-inflammatory activity of the EO of *S. areira* in lung inflammation and the effects of the EO of *S. areira* in cardiovascular system has not been fully investigated yet and due to the widespread domestic use of this plant, it is necessary to study at cardiorespiratory level.

Therefore, this study was designed to elucidate the response to different concentrations of EO on lung inflammation previously induced by intranasally administration of LPS, in addition to the local expression of TNF-α, lipid-conjugated dienes (CD) levels and cellular migration in bronchoalveolar lavage fluid (BALF). Finally, we analyzed the correlation of EO administration with the myocardial contractile strength (inotropic effects) in mice and with the hemodynamic functions in rabbits.

2. Material and methods

2.1. Collection of plant material

Aerial parts (leaves and flowers) of *S. areira* L. (Anacardacea) were collected from Mendiolaza, Córdoba, Argentina (31°14′ S; 64°15′ W).

The voucher specimens have been deposited in the Herbarium of the Museo Botánico de Córdoba, Argentina (M. P. Zunino No. 1341 CORD).

2.2. Essential oils extraction

Samples of at least 200 g of dried plant were hydrodistilled for 1 h using a Clevenger-type apparatus. The EO was dried over anhydrous sodium sulfate and stored at −20 °C to analyzes (Zunino et al., 2000).

2.3. Essential oils gas chromatography and mass spectrometry analyzes

For the quantification of individual components, EO was analyzed using a Perkin–Elmer Clarus 500 gas chromatograph equipped with a flame ionization detector (GC-FID). A capillary column DB-5 (30 m × 0.25 mm i.d. and 0.25 μm coating thickness) was used for the separation of individual components of the EO. Helium was employed as the carrier gas with a flow rate of 1 mL/min. The temperature program was 60 °C for 4 min, from 60 to 240 at 5 °C/min, with a final hold time of 10 min. The injector and detector were maintained at 260 and 280 °C, respectively. The sample was diluted 1:100 in n-hexane, 0.2 μL was injected with a 1:100 split ratio.

For the determination of the composition, EO samples were diluted with hexane. The injection volume was 1 μL. The identification of the terpenes of EO from *S. areira* was realized by GC–MS. A Perkin-Elmer Q700 GC–MS coupled with an ion trap mass detector was employed for the identification. A capillary column DB-5 (60 m × 0.25 mm i.d. and 0.25 μm coating thickness) was used for the separation of the components. Helium was used as carrier gas with a flow rate of 0.9 mL/min. The temperature program for the oven and injector was the same as that for the GC-FID. Ionization was realized by electron impact at 70 eV. Mass spectral data were acquired in the scan mode in the m/z range 35–450. Retention indices (RI) of the sample components were determined on the basis of homologous n-alkane hydrocarbons under the same conditions. The compounds were identified by comparing their retention indices and mass spectra with published data (Adams, 2007) and libraries NIST and Adams. The main components were further identified by coinjection of authentic standards (SIGMA, USA). The quantitative composition was obtained by peak area normalization, and the response factor for each component was considered to equal 1 (Zunino et al., 2003).

2.4. Animal preparation

Adult male Swiss albino mice (25–30 g) were randomly distributed in 9 groups and placed in small standard polycarbonate cages (30 × 20 × 15 cm, 6 animals per group). Separate groups of animals were used to obtain BALF for cellular counts and CD analysis and enzyme-linked immunosorbent assay (ELISA).

The animals were housed in appropriate facilities and maintained at 24–26 °C with 55–75% humidity and a 12/12-h light/dark cycle with continuous access to standard food and water *ad libitum*.

Experiments with adult male White Argentine rabbits (21 animals per group; 2.5–2.7 kg wt) were obtained from breeding grounds from Córdoba City and caged individually.

The animals were treated gently with regards to all abbreviation of distress. All efforts were made to avoid any unnecessary suffering and the experimental procedure were carried out in strict compliance with the U.S. National Institutes of Health guidelines for the experimental use of animals (Institute of Laboratory Animal Resources, 1996) and all animals studies were approved by a local animal use committee.

2.5. Study protocol

BALF samples were obtained from lung tissues subjected to the following treatments: Vehicle: instilled only with the phosphate buffered saline (PBS: 140 mM NaCl; 70 mM NaH_2PO_4; 3 mM KCl; 1.5 mM KH_2PO_4 at 37 °C), or with only one concentration of EO group during 3 h (EO groups: EO 5, EO 30 and EO 300 mg/kg; IP). The others procedures commenced with the administration of LPS followed by the different therapies: LPS (lipopolysaccharide of *Pseudomonas aeruginosa,* 100 μg LPS/kg of mouse body mass; serotype 10, ATCC27316, N° L9147, Sigma Aldrich, USA) during 2 h and were subsequently treated with only one doses from EO group concentration, or Dexamethasone (DX, 2.5 mg/kg; IP) during 3 h. Glucocorticoids such as DX, are widely used as anti-inflammatory agent in various inflammatory lung pathology and DX was used as positive controls dissolved in PBS. In accordance with Davicino et al., (2010), we used the IP injection because the majority of short-term protocols of toxicology effects have been conducted with IP or gavage administration.

2.5.1. Instillation in mice

BALF were obtained as previously reported (Roque et al., 2009). Animals were anesthetized with ether with a short lasting inhalation and acute lung inflammation was induced intranasally (i.t.) with LPS in 60 μl of PBS solutions. During the experimental anesthetic procedure, the mice were placed under a warming light and the temperature was maintained close to 37 °C. After i.t. through a polyethylene tube, mice were placed in vertical position to insure that the fluid was evenly distributed in both lungs.

After treatments, mice were sacrificed by CO_2 inhalation and immediately after, a mid-line incision was performed in the neck to isolate the trachea. A sterile cannula (22 GA Insyte, Becton Dickinson) was inserted into the trachea and 1-ml of ice-cold PBS was infused and the fluid was collected by aspiration. This procedure was repeated three times by flushing, resulting in a retrieved average volume greater than 90% of the amount previously instilled; these volumes did not differ among treatments. BALF samples were stored in ice until centrifuged at 300 *g* for 10 min. The supernatant was then discarded, leaving a 0.1–0.15 mL cell pellet. This pellet was re-suspended in 1-ml buffer and a 10-μl aliquot was used for cell counting using Trypan blue exclusion (0.4%) to determine cell viability; for differential cell counts, May-Grünewald Giemsa staining was used before air-drying and cover slipping. More than two hundred cells obtained by BALF, using morphologic criteria, were counted under the microscope to obtain the percentages of macrophages (MA) or polymorphonuclear neutrophils (PMNs) (Borron et al., 2000). After BALF procedure, the BALF were stored at −80 °C for CD and ELISA.

2.5.2. TNF-α in bronchoalveolar lavage fluid

TNF-α in BALF was quantified by ELISA protocol, according to the manufacturer's instructions (BD Biosciences). The range of detection of these kits was 35–2.240 pg/mL.

2.5.3. Detection of lipid peroxidation products in bronchoalveolar lavage fluid: conjugated dienes

The detection of CD (early oxidation products) levels of the lipids oxidation was extracted from BALF following the procedure by Ogura et al. (1994) with slight modifications. These modifications include: (i) the maximization of the precipitation efficiency, (ii) all experiments were performed twice in triplicate, (iii) 300 μl of BALF were extracted with 1.6 ml of n-hexane and the CD were measured spectrophotometrically by monitoring the absorbance at 233 nm at room temperature (e = 25000 $M-1\ cm^{-1}$).

2.6. Cardiac contractility in ex vivo mice

We used *ex vivo* isolated heart to evaluated the myocardial function. A single dose of EO was administered during 3hs (300 mg/kg; IP) and *ex vivo* cardiac contractility was determined. The heart of mice was removed quickly in toto and hung immediately in the Perspex chamber and perfused with 5 mL/min Krebs Ringer Bicarbonate medium (pH 7.4) gassed with 95% O_2 and 5% CO_2; they were kept at a temperature of 32 °C and subjected to increasing concentrations of norepinephrine (μM): 0.01, 0.1, 0.5, 1, 5 and 10 (Lo Presti et al., 2009).

2.7. Determination of arterial blood pressure in rabbits

Awaken animals were used to avoid the influences the baroreceptor reflex or sympathetic nerve activity intensely influenced by anesthesia and post-surgical. Therefore during the day of the experiment, experienced personnel collaborated to maintain the animals relatively immobile, calm, and tranquil (Matsumura et al., 2001; Menezes et al., 2010).

The effect of EO on the following hemodynamic parameters was evaluated: systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate.

The catheters were inserted into the central ear artery and taped to the ear of each animal, which had previously received local anesthesia with 1% lidocaine. Without restraining the rabbits, needles were intermittently connected to pressure transducer and after hemodynamics parameters had stabilized and the parameters were recorded Monitor (Multipar Feas SA, No. 36851, Argentina) as described: Vehicle group rabbits were studied using PBS infused during 45–50 min, and following the administration of dose of the EO (300 mg/kg; intravenous) using the same animal with the same time periods.

As described above, following the Vehicle administration, using different animals, one single dose of nifedipine 10-mg (Adalat retard; Bayer Argentina) was placed on the tongue of each animal with a syringe-type device; the mouths were kept closed until swallowing was confirmed. Nifedine is a Ca^{2+} channel antagonist widely used for the treatment of hypertension and nifedine was used as positive controls (Olivari et al., 1979).

2.8. Statistical analysis

The data were confirmed to be normally distributed by the Kolmogorov–Smirnov test. They were expressed as Means ± Standard Errors of the Means (SEM). The statistical significance of the differences between treatments and Vehicle was determined by factorial analysis of variance (ANOVA) followed by Duncan's multiple-range test. Differences were considered statistically significant when p values were lower than 0.05. Statistical analyzes were performed using Info-Stat software (Córdoba, Argentina, 2010).

3. Results

3.1. Chemical compositions of essential oil of S. areira

The separation of the EO components revealed that the chromatographic profile was predominantly composed by monoterpenes compounds (66.4%); the main components were α-pinene (13.80%), limonene (12.81%) and camphene (12.62%). Among minor components, we can mention sesquiterpenic compounds (32.95%) with β-caryophyllene (11.88%) and germacrene D (8.95%) as the main components in this fraction (Table 1).

3.2. Macroscopic evaluation, relative organ weight and clinical observations

The EO did not produce any deleterious effects on the overall performance of mice or rabbits, either on ambulation or in their clinical status during the procedure. No particular macroscopic lesions were found in the necropsies of lungs, liver and kidneys and none of the animals died (data not shown). Different treatments with EO of *S. areira* did not modify the relative organ weight of mice lungs compared to the vehicle group or between treatments groups (Vehicle:1,16 ± 0,16, n:5; LPS:1,07 ± 0,06, n:6; LPS + DX:0,98 ± 0,14, n:6; EO 5:0,95 ± 0,07, n:7; EO 30:1,02 ± 0,04, n:7; EO 300:1,10 ± 0,31, n:7; LPS + EO 5:0,92 ± 0,15, n:6; LPS + EO 30: 1,11 ± 0,22, n:7; LPS + EO 300:1,16 ± 0,16, n:6.

3.3. Effects of the essential oil of S. areira on BALF-cells

As shown in Table 2, the analysis of BALF-cells revealed that the exposure to LPS and LPS + EO 5 induced a significant decrease in viable cell counts in mice, compared to Vehicle. Also, the EO group was significantly different in terms of LPS only ($p < 0.05$).

In the EO group, LPS, LPS + DX and LPS + EO 5 ($p < 0.05$) induced an increase of PMNs compared to Vehicle. Moreover, LPS + EO 30 and 300 were the only treatments that yielded significant differences of PMNs compared to LPS in BALF.

As shown in Table 2, we evaluated the presence of AMs obtained from BALF samples of mice under different therapies. Vehicle, EO, LPS + DX and LPS + EO groups had significantly higher values than the LPS group ($p < 0.05$). Furthermore, the percentage of AMs in the EO, LPS + DX, LPS + EO 5 groups ($p < 0.05$) were significantly lower than Vehicle ($p < 0.05$).

3.4. Effects of essential oil of S. areira on TNF-α detection

Only the EO groups was detected a dose–response in TNF-α concentration (Table 3). However, the EO 5 and EO 30, LPS and LPS + EO groups the TNF-α in BALF were significantly higher than Vehicle, whereas in animals that received EO 30 and 300, LPS + DX and LPS + EO 5, the values of TNF-α were significantly lower than those from the LPS group ($p < 0.05$). No statistical differences were found in EO 300, LPS + DX versus Vehicle; as well as in EO 300, LPS + DX, LPS + EO 30 and LPS + EO 300 versus LPS group.

3.5. Effects of the essential oil of S. areira on stress oxidative

There is no significant difference in the dose- response relationship between EO group and vehicle in the detection of CD, as shown in Table. 3. CDs were determined in different experimental groups in BALF and unequal sample sizes due to sample losses.

In LPS and LPS + DX, they were significantly lower than Vehicle. In the EO and LPS + EO groups, they were significantly higher than LPS ($p < 0.05$). The Vehicle versus EO groups and LPS + EO groups showed no significant differences.

3.6. Effects of essential oil of S. areira on cardiac contractility

We evaluated the *ex vivo* activity of mice hearts pretreated for three hours with EO 300 mg/kg. Hearts were removed and subjected to increasing concentrations of norepinephrine. As shown in Fig. 1, the Basal group yielded significantly higher values in all points tested, in response to norepinephrine concentrations.

3.7. Effects of the essential oil of S. areira on cardiovascular parameters

Finally, we determined the *in vivo* SBP, DBP and MAP of rabbits pretreated with only EO 300 mg/kg body wt. This study had the

Table 1
Percentage composition of essential oil of *S. areira* L., determined by gas chromatography coupled to mass spectrometry.

Compounds[a]	Percentage	Retention index	Method of identification [b]
Hydrocarbon monoterpenes (65.65%)			
Tricyclene	1.99	925	GCMS
α-Pinene	13.80	937	GCMS-Co
Camphene	12.62	954	GCMS
Sabinene	1.59	975	GCMS-Co
β-Pinene	5.80	979	GCMS-Co
β-Myrcene	5.83	990	GCMS
α-Phellandrene	6.76	1003	GCMS
p-Cymene	1.92	1025	GCMS-Co
Limonene	12.81	1033	GCMS-Co
β-Phellandrene	2.53	1036	GCMS
Oxygenated monoterpenes (0.71%)			
1,8-Cineole	0.44	1037	GCMS
Bornyl acetate	0.27	1285	GCMS
Hydrocarbon sesquiterpenes (30.61%)			
α-Copaene	0.34	1376	GCMS
β-Bourbonene	tr [c]	1388	GCMS
β-Elemene	0.39	1394	GCMS
α-Gurjunene	0.39	1403	GCMS
β-Caryophyllene	11.88	1419	GCMS
α-Humulene	1.06	1455	GCMS
Allo aromadendrene	0.19	1460	GCMS
γ-Muurolene	0.20	1480	GCMS
Germacrene D	8.95	1485	GCMS
β-Selinene	0.17	1489	GCMS
Bicyclogermacrene	5.16	1494	GCMS
γ-Cadinene	0.22	1513	GCMS
δ-Cadinene	1.53	1524	GCMS
Germacrene B	0.13	1555	GCMS
Oxigenated sesquiterpenes (2.34%)			
Germacrene-D-4-ol	1.27	1576	GCMS
Spathulenol	0.50	1578	GCMS
τ-Cadinol	0.25	1640	GCMS
α-Cadinol	0.32	1653	GCMS
Total	99.31		

[a] In elution order from a DB-5 column.
[b] GCMS, peak identifications are based on MS comparison with file spectra; Co, peak identification are based on standard comparison with relative retention time.
[c] tr, trace (<0.05%).

benefit that the tests were conducted on conscious animals and induced only moderate and transient decrease of SBP.

The SBP decreased after treatment with EO or nifedipine at 5, 15 min ($p < 0.05$) and 10 min ($p < 0.01$) compared to Vehicle group (Table 4). Moreover, nifedipine decreased the SBP, DBP ($p < 0.01$) and MAP ($p < 0.001$) at 5, 10 and 15 min, and increased the heart rate in comparison to Vehicles ($p < 0.05$) and returning to basal values within the following 20–35 min.

4. Discussion

The separation of the EO of *S. areira* components in the chromatographic profile was principally composed by hydrocarbon monoterpenes compounds. A difference in composition (percentage of the same terpenes) of the oil was observed between the same specie at different locations (Díaz et al., 2008; Marongiu et al., 2004; Murray et al., 2009; Rouibi et al., 2010). However all the oils presented high percentage of sesquiterpene and monoterpene hydrocarbons. Marongiu et al. (2004) and Murray et al. (2009) have described limonene, α-pinene and β-phellandrene as the largest components of EO of *S. areira*. These slight differences in the quality of the oils could be probably due to changes of the constituents caused by different environmental factors related to soil, humidity, sun exposure, and other external influences (Edris, 2007).

Table 2
Percentage of viable cells (VCs), alveolar macrophages (AMs) and polymorphous neutrophils (PMNs) in BALF from LPS-treated mice.

Treatments	*n*	Percentage of cells in total BALF		
		VCs	PMNs	AMs
Vehicle	6	89.15 ± 4.38	3.42 ± 1.22	96.58 ± 1.22
EO 5	6	91.25 ± 1.64$^{\#}$	28.73 ± 4.78$^{*,\#}$	71.27 ± 4.78$^{*,\#}$
EO 30	6	90.37 ± 2.36$^{\#}$	34.16 ± 8.67$^{*,\#}$	77.23 ± 9.05$^{*,\#}$
EO 300	5	83.55 ± 3.97$^{\#}$	12.31 ± 4.8$^{*,\#}$	79.64 ± 8.88$^{*,\#}$
LPS	5	79.29 ± 4.19*	78.80 ± 4.8*	21.20 ± 4.8*
LPS + DX	5	87.90 ± 3.15	24.03 ± 4.68*	75.97 ± 4.6$^{*,\#}$
LPS + EO 5	6	69.95 ± 2.72*	29.46 ± 2.65*	70.54 ± 2.65$^{*,\#}$
LPS + EO 30	5	84.61 ± 4.38	13.86 ± 6.01$^{\#}$	86.14 ± 6.0$^{\#}$
LPS + EO 300	6	77.08 ± 5.70	15.39 ± 4. 83$^{\#}$	84.61 ± 4.83$^{\#}$

* $p < 0.05$ vs. vehicle group.
$^{\#}$ $p < 0.05$ vs. LPS in the vehicle group. Mean ± SEM; *n*, number of animals.

Table 3
Conjugated dienes (CDs) and TNF-α concentration in BALF from LPS-treated mice.

Treatments	*n*	BALF	
		CDs (μM/mL)	TNF-α (pg/mL)
Vehicle	5	12.72 ± 1.93	ND
EO 5	5	12.98 ± 1.60$^{\#}$	481.44 ± 167.90**
EO 30	6	9.75 ± 2.03$^{\#}$	92.46 ± 43.08$^{*,\#}$
EO 300	5	13.10 ± 1.55$^{\#}$	ND$^{\#}$
LPS	6	5.81 ± 0.31*	1159.98 ± 321.64**
LPS + DX	6	7.62 ± 0.79*	ND†
LPS + EO 5	6	12.75 ± 1.76$^{\#}$	101.58 ± 15.58*,†
LPS + EO 30	6	13.57 ± 1.44$^{\#}$	246.51 ± 98.40**
LPS + EO 300	6	12.56 ± 1.76$^{\#}$	186.59 ± 53.95*

ND, not detected.
Mean ± SEM; *n*, number of animals.
* $p < 0.05$ vs. vehicle group.
** $p < 0.01$ vs. vehicle group.
$^{\#}$ $p < 0.05$ vs. LPS.
† $p < 0.01$ vs. LPS.

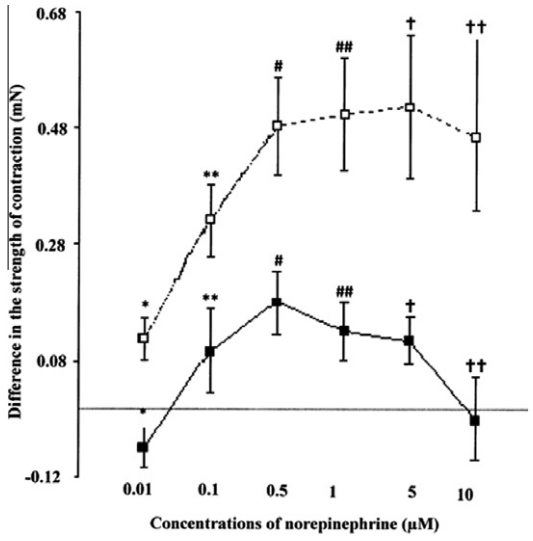

Fig. 1. Determination of cardiac contractility (mN, milli Newton) in *ex vivo* hearts of Swiss Albino mice, placed in the Perspex chamber and perfused with Krebs buffer, at 5 mL/min and subjected to increasing concentrations of norepinephrine (μM): 0.01, 0.1, 0.5, 1, 5 and 10. □ Basal: untreated animals and animals treated with ■ essential oil 300 mg/kg IP for 3 h prior to determination of cardiac contractility. Data are expressed as mean ± SEM ($n = 6$). All error bars represent SEM. ∗; ∗∗; #: $p \leqslant 0.05$; ##; †; ††: $p \leqslant 0.01$.

Table 4
Arterial blood pressure and *in vivo* heart-rate of rabbits.

Treatments	*n*	Arterial blood pressure (mm/Hg)			Heart rate (beats/min)
		S.B.P.	D.B.P.	M.A.P.	
Vehicle	13	96.82 ± 3.06	77.69 ± 2.57	83.46 ± 3.14	195.71 ± 18.08
EO 5 min	6	86.25 ± 1.49*	72.75 ± 3.10	79.08 ± 1.52	239.50 ± 21.18*
EO 10 min	6	83.11 ± 3.50#	71.78 ± 3.63	78.53 ± 2.01	227.00 ± 12.87
EO 15 min	6	86.88 ± 1.37*	73.27 ± 3.38	79.34 ± 2.14	224.07 ± 12.89
Nife 5 min	7	73.30 ± 7.54#	59.40 ± 4.06#	66.35 ± 5.58†	238.00 ± 12.01*
Nife 10 min	6	72.71 ± 6.68#	58.04 ± 3.45#	65.38 ± 4.84†	225.00 ± 37.00
Nife 15 min	6	73.40 ± 5.90#	59.57 ± 3.54#	66.61 ± 4.49†	209.67 ± 12.10

S.B.P., systolic blood pressure; D.B.P., diastolic blood pressure; M.A.P., mean arterial pressure; Nife, nifedipine.
Mean ± SEM; *n*, number of animals.
* $p < 0.05$.
# $p < 0.01$.
† $p < 0.001$.

Adverse effects of the EO of *S. areira* on morphometric parameters were quantified. In this context, the relationship between total body weight and lung weight was similar to vehicle and no macroscopic changes were observed in lung tissues of animals that received a single dose of EO of *S. areira*. Moreover, we did not detect any changes in the ambulation or clinical status of the animals while they were under the effects of LPS, EO of *S. areira* alone or a combination of both. However, Barrachina et al. (1997) described acute toxicity in rats that received a dose of hexane-dichloromethane extract obtained from *S. areira* producing total inhibition of motor activities and decreased pain threshold induced by chemical stimuli. Furthermore, Vargas Correa et al. (1991) have reported cross-reactivity produced by hypersensitivity to pollen of mango (*Magnifera indica*) with *S. molle* (synonymous variant of *S. areira*).

In the analysis of stimulation produced by LPS, a 3 h treatment with EO of *S. areira* at 30 or 300 mg/kg had the ability to maintain the levels of AMs within the normal range. This is due to the fact that these cells are found in the alveoli in high proportions, which probably reflects the activation of AMs into foam cells as response of acute inflammation (Borron et al., 2000).

The presence of PMNs and AMs in the airways after exposure to LPS is consistent with our previous studies (Roque et al., 2010). On the other hand, in our study we found a significant increase of PMNs was also detected in lungs of rodents exposed to LPS, considering that the EO of *S. areira* could have an active role in the activation of AMs in mice lungs.

The percentage of PMNs in BALF decreased after the administration of LPS + DX and LPS + EO 30 and 300 to levels similar to those of Vehicle. PMNs are involved in processes like phagocytosis, release of proteolytic enzymes, generation of active free radicals and synthesis of cytokines producing inflammation. In addition, the PMNs act as a significant source of TNF-α in the tissues during the acute lung inflammation elicited by LPS. However, the group of EO 5, 30 and 300 showed significant increase of the percentage of PMNs.

This phenomenon is probably due to the action of enantiomers from the terpenes present in the EO of *S. areira*, which may be developing biological effects or with putative synergism, antagonism or zero-interaction with the terpenes present in the EO of *S. areira* or with any step of the inflammation pathway (Menezes et al., 2010).

Monoterpenes are released as gas during the milling and processing of fresh wood. This poses a potential health hazard on workers of sawmills, causing skin rash as well as eye and mucous irritation (Hedenstierna et al., 1983). Even though we did not analyze each individual terpene included in our study, the (+) or (−) α-pinene, the main volatile (−) monoterpene or (+) or (−) limonene produced different responses on the respiratory airways of mice (Larsen et al., 2000; Nielsen et al., 2005).

The presence of these enantiomers could be responsible for the nonspecific response of PMNs, AMs or TNF-α. Also, a feasible explanation for the lack of a dose–response curve is for the limited range of EO of *S. areira* dosages studied. Davicino et al. (2010) have described that according to the extraction, preparation and mode of administration *S. areira* has pro-inflammatory or anti-inflammatory effects.

Additionally, in our experimental model, the secretion of cytokines TNF-α was significantly attenuated by EO of *S. areira* administration, but only at the dose of 5 mg/kg or DX upon stimulation by endotoxin LPS. TNF-α is a key regulatory cytokine involved in inflammatory processes and its biosynthesis and release is a complex process regulated by many pathways; in addition, the EO of *S. areira* could block or regulate the interaction of LPS with the immune system.

Also, in Table 2, EO 5 and 30 induced a significant increase of TNF-α; this rise was associated to the response of PMNs since these phagocytic cells, crucial in specific and non-specific immune responses, synthesize a variety of biological mediators, including pro- and anti-inflammatory cytokines. In inflammatory lung diseases, they have been associated with increased circulating levels of TNF-α. These effects could be due to the fact that small amounts of EO of *S. areira* can induce greater immune responses than higher concentrations of EO of *S. areira* (Neher et al., 2008).

One possible physiological explanation for the above mentioned data, Olafsson et al. (1997) involves the action of triterpenes from *S. areira* on the inhibition of the angiotensin converting enzyme (ACE). The ACE inhibitors produce a diminution of TNF-α concentrations, although it may be possible that there is some kind of relationship between neurohormones (norepinephrine, angiotensin) and pro-inflammatory cytokines (Fukuzawa et al., 1997). Recent data suggest that the renin-angiotensin system could play an important role in the pathogenesis of pulmonary inflammation, as well as on an increased production of free radicals (Wösten-van Asperen et al., 2010).

In addition, α-pinene develops anti-inflammatory activity by inhibiting the enzyme cyclooxygenase (Zandi and Breitner, 2001). Although other mechanisms could not be entirely discarded, its effects appear to involve an inhibitory action of the activity of the enzyme acetylcholinesterase (Murray et al., 2009). Nevertheless, the mechanisms postulated for the EO of *S. areira* should be confirmed with future research studies and remain necessary to fully understand the molecular mechanisms and should be directed to establish the steps of signal transduction.

The generation of oxygen-free radicals is a well understood mechanism of the inflammation process and in recent years, the balance between the ratios of oxidants/antioxidants in lung pathogenesis has taken special relevance (Till et al., 1985). Therefore, this ratio plays a clear role on body functions and an imbalance

of this delicate equilibrium directs to biochemical and cellular changes that may lead to significant pathological conditions within the environment complex (Cross et al., 1994).

In CDs assessed in BALF from mice lungs, LPS demonstrated to induce a significant decrease compared to the untreated group. This phenomenon can be attributed to changes in the non-physiological conditions induced by LPS; the concentration of CDs decreased significantly induced by the obligatory passage of conjugated dienic lipid hydroperoxides to peroxyl radicals (isoprostanes and decomposition products: *e.g.* malondialdehyde) but not to the CDs pathway (Dotan et al., 2004). In this context, the presence of EO 5, 30 or 300 of *S. areira* attenuated the progress to another pathway reaction that occurs during oxidative stress. Also, in small animals with faster metabolism, the rate of inflammation progress and the susceptibility to oxidative stress is higher. It should be noted that in mice lungs, the ratio oxidation–antioxidation is lower than in other tissues because they have slower metabolism and therefore, less exposure to oxidative damage (Richter, 1987).

Numerous studies have demonstrated the antioxidant properties of natural products, which are similar to those obtained in our study (Bakkali et al., 2008; Galvez Ranilla et al., 2010; Guala et al., 2009; Shen et al., 2010). The EO of *S. areira* could prevent oxidative damage by using physiological mechanisms similar to the endogenous antioxidant system, inhibiting or preventing the generation of free radicals, enhancing antioxidant enzymes activity or by blocking the amplification of oxidative damage (Graßmann, 2005). Nevertheless, the EO of *S. areira* could produce free radicals which oxidize and damage lipids, proteins and DNA in high concentrations (Bakkali et al., 2008). However, the exact mechanism of EO on the oxidative stress in BALF remains unknown.

In this context, increasing doses of norepinephrine in *ex vivo* hearts of mice pretreated with only doses of EO of *S. areira* tested, proving to have negative inotropic effects in mice; in addition, in non-anaesthetized normotensive rabbits, EO of *S. areira* significantly decreased the SBP at 5, 10 and 15 min post treatment in *in vivo* rabbits and increased their heart rate, but only at 5-min post EO. Moreover, the cardiovascular activity of EO of *S. areira* has been reported, and in agreement with other authors, in our data of the main chemical constituent of the EO of *S. areira* is the terpene β-caryophyllene, present in 11.8%, has been described as a Ca^{2+} channel blocker (Sensch et al., 2000). Based on this study, it can be suggested that the negative inotropic effect could induce significant hypotension mediated by the regulation of Ca^{2+} channels or to the presence of different compounds in the EO of *S. areira* alone (α- or β- pinene, 13.8% and 5.8% respectively in our study) or in combination, and/or to the association of different compounds present in EO of *S. areira* (Menezes et al., 2010). However, the hypotensive reaction could also be due to a decrease of the total peripheral resistance related with cardiac parasympathetic stimulation, which might in turn decrease cardiac output and consequently decline the arterial blood pressure.

In conclusions, our data presented here demonstrate that the EO of *S. areira* can mitigate, initiate or release small concentrations of pro or anti-inflammatory mediators in the lungs; in addition, some of the terpenes present from EO of *S. areira* might produce homeostatic changes of unknown clear origin in many people and short-term exposure to EO of *S. areira* has been significantly associated to cardiac autonomic dysfunction, as demonstrated by the decrease of SBP and contractility. Additional studies to refute or confirm the effects of this EO of *S. areira* on the respiratory and cardiovascular systems are necessary to investigate the possible mechanisms involved.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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