Relaxant effect of carvacrol, citronellal and p-cymene, monoterpenes present in *Thymus* and *Cymbopogon* species, in guinea-pig trachea: A comparative study

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Respiratory diseases are responsible for significant morbidity and mortality in worldwide. One of most important respiratory disease is asthma, which the current treatment is based on use of β₂-agonists combined to inhaled corticosteroids. However, in recent years, many *in vitro* studies have attempted to validate the use of aromatic medicinal plants and its chemical compounds, such as monoterpenes, as potential therapeutically alternatives. In this work it was demonstrated that three different monoterpenes (carvacrol, citronellal and p-cymene) has relaxant effect in guinea-pig trachea, somehow showing different profile regarding epithelium presence, potency and efficacy.

**Key words:** Monoterpenes, carvacrol, p-cymene, citronellal, guinea-pig trachea, relaxant effect.

**INTRODUCTION**

Respiratory diseases are responsible for significant morbidity and mortality in worldwide. Among all respiratory diseases, asthma and chronic obstructive pulmonary disease (COPD) are of the most common in the world (Ambrosino and Paggiaro, 2012). According to World Health Organization (WHO), 300 million people suffer from asthma and this number could reach 400 million by 2025, accounting for 200,000 deaths per year (Meetoo, 2008). Asthma is the main airway chronic disease that causes substantial morbidity among affected individuals, it has no cure, but could be controlled by treatment (Schatz et al., 2006) which currently consists of combating its two main pathophysiology components, the hyper-responsiveness of airways smooth muscle by...
bronchodilators and lung inflammation by glucocorticoids (Sarr et al., 2012; Camoretti-Mercado, 2009). Asthma treatment guidelines advocate the use of β2-agonists combined with inhaled corticosteroids (Miller-Larsson and Selroos, 2006). Although in recent years, many in vitro studies have attempted to validate the uses in traditional medicine of medicinal plants for treatment of diseases related to the respiratory system by demonstrating pharmacological effects of chemical constituents isolated from these plants (Sarret al., 2010).

Many medicinal plants such as *Eucalyptus citriodora*, *Eucalyptus globulus*, *Mentha piperita*, *Origanum syriacum*, *Salvia fruticosa* and *Rosmarinus officinalis* have been described to act directly on the respiratory tract, the coughing reflex and the airflow (McKay and Blumberg, 2006, Rakover, et al., 2008). Something common to these species is the fact that they all produce essential oils which are composed of volatile aromatic compounds bio-synthetized by them according to the growth stage, local climate, season, geographical distribution and others (Richter and Schellenberg, 2007). There are several chemical compounds present in the essential oils, however, monoterpenes, isoprene-related compounds, are the major components of these oils and the pharmacological profiles of various medicinal plants have been attributed to them (Quintans-Júnior et al., 2010).

Some monoterpenes have been demonstrated to have effects on the airways, such as thymol, menthol and camphor, including effects on airway smooth muscle (Abanses et al., 2009; Begrow et al., 2010; Burrow et al., 1983; Kawasuji et al., 2005; Millqvist et al., 2012; Turletti, 1951). Some other monoterpenes as eucalyptol, gentiopricrin and carvacrol have direct effect upon smooth muscle contractility (Boskabady and Jandaghi, 2003; Nascimento et al., 2009; Rojas et al., 2000). However, several monoterpenes which could potentially have application in respiratory disorders have not been studied through the pharmacological approach using in vitro models. Thus, the present study aimed to investigate and compare the effects of three different monoterpenes, 2-methyl-5-(1-methylethyl) phenol (carvacrol), 7-dimethyl-6-octene-1-al (citronellal) and 4-isopropyl-1-methylbenzene (p-cymene) on airway smooth muscle, using experimental model of the isolated guinea pig trachea (Figure 1).

**MATERIALS AND METHODS**

**Drugs**

All the salts used in Krebs solution were purchased from VETEC QuimicaFina LTDA (Rio de Janeiro, BR). The monoterpenes carvacrol, citronellal and p-cymene, carbachol and arachidonic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

**Animals**

Guinea pigs of both sexes weighing between 350 to 500 g were used in this study. The animals were kept in the Animal Care Facilities of the Federal University of São Francisco Valley (UNIVASF) at a controlled temperature (22 ± 0.2°C) and 12/12 h light/dark cycle (lights on 06:00 to 18:00 h) in appropriate cages containing food and water ad libitum. The animals were killed by concussion followed by exsanguination by cutting the cervical vessels and their tracheas were removed. Experimental protocols and procedures were approved by the Ethics Committee on Animal Experiments by UNIVASF (CEUA/UNIVASF #27091051).

**Protocols**

After removing the adhering fat and connective tissue the trachea was cut into rings (lengths of 4 to 5 mm) and were suspended in 10 ml organ baths containing Krebs solution (in mM: NaCl 118, KCl 4.6, KH₂PO₄·H₂O 1.1, MgSO₄·7H₂O 5.7, CaCl₂·2H₂O 2.5, NaHCO₃ 25 and D-glucose 11) at 37 ± 0.1°C and bubbled with 95% O₂ and 5% CO₂ carbogenic mixture. The tracheal rings were preloaded under an isometric tension of 1 g and allowed to incubate for at least 60 min with changes of Krebs solution every 15 min. Isometric...
contractions were monitored by force transducers TRO 015 (Panlab®, Barcelona, Spain) connected to an amplifier (Insight®, RibeirãoPreto, SP, Brazil) and data acquisition was performed through a PC running the software WinDaq (DATAQ Instruments®, Akron, OH, USA). After the resting period a first contraction was induced by carbachol (10⁻⁶ M). To evaluate the presence of epithelium when the contraction was stable (~10 min after carbachol addition) the arachidonic acid was added to organ bath (10⁻⁴ M). When the relaxation induced by arachidonic acid reached more than 50% of maximal effect, the tracheal ring was considered with epithelium. Rings without epithelium were obtained by causing friction carefully inside the tracheal tube and the absence of epithelium was confirmed by the absence of relaxation induced by arachidonic acid. Afterward, the organ bath was washed out and after 30 min a second contraction was induced by carbachol and as soon as it became stable (~10 min after carbachol addition), a cumulative concentration-response curve (10⁻⁷ to 10⁻³ M) was constructed for each monoterpene and its relaxant effect were registered.

Statistical analysis

The relaxing effect is represented by the inverse percentage of the contraction force obtained by adding carbachol. The relaxation was considered maximal (100%) when the base line reaches the resting tension level. Data in the graphs were expressed as mean ± standard error of mean (SEM). The half maximal effective concentration (EC₅₀) values were calculated through a non-linear regression equation from the values of relaxation obtained from each single dose-response curve for each monoterpene. Differences between means were considered statistically significant when p value calculated by unpaired Student’s t test was lower than 0.05. Statistical analyzes were performed using the Graph-Pad Prism® 5.0 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

The monoterpenes carvacrol, citronellal and p-cymene induced a relaxing effect in a concentration-dependent manner (range of 10⁻⁷ to 10⁻³ M) of the tracheal rings pre-contracted with carbachol (10⁻⁶ M) both in the presence (Figure 2) and in absence (Figure 3) of functional epithelium. The carvacrol had a greater efficacy both in the presence and absence of epithelium than the other monoterpenes, reaching an Emax of 100% of relaxing at 10⁻⁴ M while citronellal attained an Emax of 79.6 ± 10.2 and 90.2 ± 5.0% in the presence and absence of epithelium, respectively, and p-cymene an Emax of 69.0 ± 9.4 and 79.0 ± 4.4% in the presence and absence of epithelium, respectively. These isoprene compounds showed no statistical difference in their efficacy regarding the presence or absence of epithelium (Figures 2 and 3). The carvacrol was also the monoterpene among the three that has greatest potency to relax the guinea-pig trachea, showing an EC₅₀ value of 2.1 ± 0.6 × 10⁻⁵ and 2.3 ± 0.3 × 10⁻⁵ M in both the presence and absence of epithelium, respectively, with no significant difference between these values. The other two monoterpenes showed no significant differences between their potential for being equipotent in relaxing the trachea. However, citronellal showed EC₅₀ values of 1.4 ± 0.6 × 10⁻⁴ and 6.9 ± 1.4 × 10⁻⁵ M in the presence and absence of epithelium, respectively, with significant difference between these...
values while p-cymene presented EC50 values of $6.3 \pm 0.3 \times 10^{-4}$ and $1.7 \pm 0.3 \times 10^{-4}$ M with no difference between them (Figure 4).

**DISCUSSION**

Essential oils extracted from aromatic plants of many species have attracted interest from industry and researchers worldwide due to its fragrances as well as their applications in various fields of interest according to their biological activities (Lis-Balchin et al., 1997; Pauli and Knobloch, 1987; Perez-Alfonso et al., 2012). It is speculated that among the various chemical constituents present in these oils monoterpenes stand out as the main aromatic compounds and responsible for various biological activities (Bassole et al., 2003; Cimanga et al., 2002; Hadian et al., 2012; Martins et al., 1999).

Monoterpenes are mostly used in industry to mask odors (Osada et al., 2013), scents for all purposes, food additive (Yu et al., 2012), flavor adjuvant (Fukimoto et al., 2006; Heck, 2010), pharmaceutical adjuvants (Narishetty and Panchagnula, 2004) and as active ingredients in some ointments or topical creams used to relieve symptoms of chest congestion, cough and asthma (Abanses et al., 2009; Ando et al., 1993). Despite these applications, the monoterpenes have several biological activities but the microbial activity is highlighted for many of them, such as thymol (Perez-Alfonso et al., 2012), eugenol (Pei et al., 2009; Sleha et al., 2012; Veras et al., 2012), cinnamaldehyde (Du et al., 2012), alpha-pinene (Sartorelli et al., 2007), limonene (Di Pasqua et al., 2006), trans-cinnamaldehyde, perillaldehyde, citral, eugenol, citronellal (Sato et al., 2006; Sleha et al., 2012), p-cymene (Kisko and Roller, 2005; Rattanachaikunsopon and Phumkhachom, 2010) and carvacrol (Didry et al., 1993).

Indeed carvacrol is one of the most cited antimicrobial monoterpane in the literature. Moreover carvacrol has activity against many microorganisms, such as *Schistosoma mansoni* (Ait-Ouazzou et al., 2013), *Campylobacter jejuni* (van Alphen et al., 2012), *Mycoplasma hominis* (Sleha et al., 2012), *Penicillium digitatum* and *P. italicum* (Perez-Alfonso et al., 2012), *Staphylococcus aureus* and *Staphylococcus epidermidis* (Nostro et al., 2012), *Salmonella* ssp. (Inamuco et al., 2012; Luz Ida et al., 2012; Moschonas et al., 2012), *Escherichia coli* (Obaidat and Frank, 2009) and others (Nostro and Papalia, 2012). Furthermore, carvacrol has anticancer (Koparal and Zeytinoglu, 2003; Zeytinoglu et al., 2003), anti-hyperlipidemic (Aristatile et al., 2009), anti-inflammatory (Fachini-Queiroz et al., 2012; Lima et al., 2012), anti-ulcer (Silva et al., 2012), antinociceptive...
Besides the antimicrobial activity some monoterpenes, such as citronellal, has anti-inflammatory (Melo et al., 2011b), anti-nociceptive (Melo et al., 2010; Quintans-Junior et al., 2010), larvicidal (Barros et al., 2009) and anticancer activities (Osato, 1965; Osato et al., 1961; Osato et al., 1954), while p-cymene modulates cytokine production (Zhong et al., 2012) has anti-inflammatory (Xie et al., 2012) and antinociceptive activity (Bonjardim et al., 2012).

Now, we demonstrate, through this work, that citronellal, p-cymene and carvacrol have relaxing effect on guinea-pig trachea and carvacrol has greater efficacy and potency among the three monoterpenes tested. Although there were already reports that carvacrol has relaxant effect in guinea-pig trachea (Boskabady and Jandaghi, 2003; Van den Broucke and Lemli, 1982) for the first time is reported relaxing effect for citronellal and p-cymene in that organ. In terms of potency, citronellal and p-cymene were equipotent in relaxing the trachea, but citronellal was more effective than p-cymene in doing so, showing a higher value of E_max.

Some studies suggest that carvacrol has a putative mechanism of action for some of its biological effects. Some of these studies claim that carvacrol has in vitro acetyl cholinesterase inhibitory properties (Jukic et al., 2007), an antagonist-like effect on muscarinic (Boskabady et al., 2011a) and histamine H_1 receptors (Boskabady et al., 2012), and an agonist-like effect on β_2-adrenoceptors (Boskabady et al., 2011b). Additionally, previous studies have described the antioxidant profile of carvacrol (Guimarães et al., 2010) and its action to modulate the release of cytokines, as IL-10 (Lima et al., 2013). Therefore, these activities may be acting synergistically to produce relaxant activity on the guinea pig trachea.

Moreover, we demonstrate that carvacrol induced a relaxing effect on tracheal rings pre-contracted with carbachol, a muscarinic agonist insensitive acetylic holinesterase. Thus, the fact that the supposedly carvacrol possess inhibitory effect on acetyl cholinesterase or an antagonistic effect on H_1 receptors does not explain its relaxing effect in our experimental conditions. However, an anti-muscarinic effect or an agonist effect on beta-adrenergic receptors could explain its mechanism of action regarding our data. However, the lack of information specifically about the relaxing effect of p-cymene and citronellal associated lack of studies about their possible biochemical pathways, which would serve to explain some of its biological effects, not allowing us to speculate much about the mechanism of action of the relaxing effect for these two monoterpenes. It is widely known that the epithelium is described as a layer of cells whose function goes well beyond the lining of the airways and in fact it is responsible for produce and release many mediators of smooth muscle contractile function (Advenier et al., 1988; Filep et al., 1993; Hay et al., 1988; Holroyde, 1986; Schlemper and Calixto, 1995) and its removal modifies the response to drugs that somehow stimulate the production of the so-called epithelium-dependent relaxant factors (Conroy et al., 1995; Daffonchio et al., 1990; Fine et al., 1989; Shikada and Tanaka, 1995).

Our results show that relaxant effect of carvacrol and p-cymene on tracheal rings do not depend of presence of the epithelium. This fact is evidenced by the absence of difference between the EC_{50} and E_{max} values for both monoterpenes when comparing its effects in the presence and absence of epithelium. However, although no significant differences in the E_{max} was evidenced for citronellal in tracheal rings; with epithelium this monoterpenes was about two-fold less potent than in rings without epithelium, suggesting that the relaxing effect of citronellal is influenced by factors derived from the tracheal epithelium.

**Conclusion**

The carvacrol, p-cymene and citronellal have relaxant effect in smooth muscle of trachea. However, carvacrol and p-cymene have its effect irrespective of the tracheal epithelium while citronellal seems to depend of epithelium-derived relaxing factors to reach its effect. Moreover, the carvacrol was more effective and more potent in relaxing the trachea in this study.

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**Conflict of interest**

The authors report no declarations of interest.

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Silva et al. 887
