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Relaxant effects of *Peperomia hispidula* (Sw.) A. Dietr. on isolated rat tracheal rings

[Efecto relajante de *Peperomia hispidula* (Sw.) A. Dietr. sobre anillos aislados de tráquea de rata]

Jesús Arrieta[1], Yaraset López-Lorenzo[1], Mayra Beatriz Gómez-Patiño[2], Yolanda Sánchez-Mendoza[3] & María Elena Sánchez-Mendoza[1]

[1]*Escuela Superior de Medicina, Instituto Politécnico Nacional, Ciudad de México, México*
[2]*Instituto Politécnico Nacional-CNMN, Ciudad de México, México*
[3]*Unidad de Medicina Familiar No. 49, Instituto Mexicano del Seguro Social, Ciudad de México, México*
Contactos | Contacts: **María Elena SÁNCHEZ-MENDOZA** - ***E-mail address:*** **mesmendoza@hotmail.com**

Abstract: *Peperomia hispidula* (Sw.) A. Dietr. is used in Mexican traditional medicine for treating respiratory illnesses such as asthma. The latter disorder results from an excessive and inappropriate constriction of airway smooth muscle. The aim of the present study was to evaluate the relaxant activity of *P. hispidula* on isolated rat tracheal rings contracted with carbachol. The methyleugenol was identified as the main active constituent in the dichloromethane extract. To explore the possible mechanism of action, concentration-response curves were constructed in the presence and absence of propranolol (3 µM), indomethacin (10 µM), glibenclamide (1 µM), and L-NAME (300 µM), finding that neither reduced methyleugenol-induced smooth muscle relaxation. In conclusion, *P. hispidula* herein displayed relaxant activity on rat tracheal rings. The effect of methyleugenol, was probably not related to the activation of β_2-adrenoceptors, prostaglandins, K^+_{ATP} channels or nitric oxide.

Keywords: *Peperomia hispidula*; Medicinal plant; Rat trachea; Relaxant effect; Asthma

Resumen: *Peperomia hispidula* (Sw.) A. Dietr. es utilizada en la medicina tradicional mexicana para tratar enfermedades respiratorias como el asma. Este último trastorno es el resultado de una contracción excesiva e inapropiada del músculo liso de las vías respiratorias. El objetivo del presente estudio fue evaluar la actividad relajante de *P. hispidula* sobre anillos aislados de tráquea de rata contraídos con carbacol. El metileugenol fue identificado como el principal constituyente activo en el extracto de diclorometano. Para explorar el posible mecanismo de acción, se construyeron curvas concentración-respuesta en presencia y ausencia de propranolol (3 µM), indometacina (10 µM), glibenclamida (1 µM), y L-NAME (300 µM), encontrando que ninguno redujo la relajación del músculo liso inducida por metileugenol. En conclusión, *P. hispidula* muestra actividad relajante en anillos de tráquea de rata. El efecto de metileugenol, al parecer no está implicado con la activación de los receptores β_2-adrenérgicos, prostaglandinas, canales de K^+_{ATP} u óxido nítrico.

Palabras clave*: Peperomia hispidula*; Plantas medicinales; Tráquea de rata; Efecto relajante; Asma

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INTRODUCTION

Asthma is a chronic disease with a steadily increasing prevalence (Nagai, 2012), now affecting almost 235 million people in the world according to the World Health Organization (WHO). It is characterized by recurrent attacks of breathlessness and wheezing (WHO, 2018). An airflow limitation occurs during such attacks due to contractions of airway smooth muscle, inflammatory processes and mucus hypersecretion (Nagai, 2012).

Although several guidelines exist for the treatment of asthma (Nagai, 2012), its management falls into two general categories. The first involves direct inhibition of airway smooth muscle contraction or the stimulation of bronchorelaxation (Pera & Penn, 2016), which provides symptomatic relief for air flow limitation (Nagai, 2012). The second consists of suppressing airway inflammation to diminish the stimuli responsible for airway smooth muscle contraction (Pera & Penn, 2016). Most therapies combine drugs of these two types (Nagai, 2012).

Despite the existence of effective medications, asthma is poorly controlled by patients, who therefore present frequent symptoms and exacerbations (Barnes, 2017). As a result, many people with this disorder use complementary and/or alternative therapy. Herbal medicine represents the third most popular choice (Huntley & Ernst, 2000). It is known that some medicinal plants are able to relax airway smooth muscle and thus improve airflow (Águila *et al.*, 2015).

In parts of the State of Chiapas, Mexico, plants are commonly used to prepare infusions for the treatment of respiratory diseases such as asthma and coughing. *Peperomia hispidula* (Sw.) A. Dietr. (Piperaceae), locally called "lenteja" (lentil), is a case in point. To our knowledge, no pharmacological exploration of this practice has yet been carried out. The aim of the present study was to evaluate the relaxant activity of *P. hispidula* on isolated rat trachea rings contracted with carbachol.

MATERIAL AND METHODS

Plant material

P. hispidula was collected in the Ejido Zaragoza Nueva Alemania, Municipality of Tapachula, State of Chiapas, Mexico (longitude -92° 15' 16.0''W and latitude 14° 57' 06.5''N), during August of 2016. Specimens (voucher #1429) are available in the HERITH Herbarium of Instituto Tecnológico de Huejutla in the State of Hidalgo, Mexico.

Extraction and isolation of methyleugenol

The complete plant of *P. hispidula* was dried at room temperature (22 ± 2° C) in the shade and then pulverized before extracting 3 kg of plant material with hexane (6 L), dichloromethane (6 L) and finally methanol (6 L). Extraction was carried out three times during 3 days for each solvent by employing the maceration method. The resulting solutions were each filtered and concentrated in a rotary evaporator, and then the solvents were evaporated under vacuum to furnish 37, 94 and 203 g of extract, respectively.

The dichloromethane extract showed the best relaxant effect. Thus, this extract (55 g) was subjected to percolation over a silica gel column and four fractions were obtained using the next step gradient: F1 (9.5 g) from hexane/EtOAc (9:1, v:v, 2 L), F2 (16 g) from hexane/EtOAc (7:3, v:v, 2 L), F3 (12 g) from hexane/EtOAc (1:1, v:v, 2 L) and F4 (14 g) from EtOAc (2 L). F1 and F2 fractions were the most active. The bioassay-guided study continued with the F2 fraction because it was obtained in a higher yield. Hence, 11 g of this fraction were chromatographed on a silica gel column by using a step gradient of hexane, mixtures of hexane/EtOAc and EtOAc, resulting in methyleugenol (96% of purity) as the principal compound, which is consistent with the finding previously reported by our group (Sánchez-Mendoza *et al*., 2015). To determine whether methyleugenol is present in the other fractions, ultra-high-performance liquid chromatography and mass spectrometry (UHPLC-MS) were conducted with the methodology previously described by our group (Sánchez-Mendoza *et al*., 2015).

Pharmacological experiments

Drugs

Acetylcholine chloride, carbachol chloride, isoprenaline, DL-propranolol hydrochloride, indomethacin and glibenclamide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Just prior to the biological assay, the corresponding substance (the extracts, fractions, methyleugenol or glibenclamide) was suspended in water with traces of Tween 80, and indomethacin was dissolved in ethanol. The final concentration of Tween 80 (0.05%) or ethanol (1%) did not significantly affect the tracheal response. The other compounds were dissolved in water.

Preparation of rat trachea

Adult male rats (180-220 g, 63 rats) were purchased from the Bioterium of the Universidad Autónoma Metropolitana, Xochimilco campus, in Mexico City. The animals were maintained under standard conditions, having free access to food and water. The experimental procedures were carried out in accordance with the Mexican Official Norm for Lab Animal Care and Handling (NOM-062-ZOO-1999, Especificaciones Técnicas para la Producción, Cuidado y Uso de Animales de Laboratorio) and international rules on the care and use of lab animals. The study was approved by the Internal Committee for the Care and Use of Lab Animals (CICUAL, according to the initials in Spanish) of the Escuela Superior de Medicina, Instituto Politécnico Nacional, with registration number CICUAL-08/4-12-2017.

Following the euthanization of rats by intraperitoneal injection of sodium pentobarbital (75 mg kg^{-1}), the trachea was dissected and the connective and adipose tissues were removed. The trachea from each rat was cut into seven rings about 2 mm long and mounted between two hooks inserted into the lumen, with one side fixed and the other connected to a force transducer Biopac TSD 125C (Santa Barbara, CA, USA) to continuously register isometric tension on a Biopac System polygraph MP150. The rings were washed in an isolated organ bath by applying Krebs solution (in mM: 118 NaCl, 4.7 KCl, 1.2 NaH_2PO_4, 1.2 $MgSO_4 \cdot 7H_2O$, 2.5 $CaCl_2 \cdot 2H_2O$, 25 $NaHCO_3$ and 11.1 glucose) at 15-min intervals. The solution was maintained at 37°C under constant bubbling with 5% CO_2 and 95% O_2. The values found were digitalized and analyzed by means of software for data acquisition (Acknowledge 4.0) (Santa Barbara, CA, USA). An initial tension of 2 g was applied and allowed to reach equilibration during 60 min. Three μM of acetylcholine chloride were added to verify the integrity of the tracheal rings, which were subsequently washed with fresh Krebs solution (Sánchez-Mendoza *et al*., 2008).

Effects of the extracts, fractions, methyleugenol or isoprenaline on carbachol-induced tracheal ring contraction

Thirty minutes after stimulation with acetylcholine chloride, the rings were contracted with carbachol (3 μM). When the plateau was reached, increasing concentrations of the extract or fractions (17.7, 31, 56, 100, 177, or 316 μg/mL), methyleugenol (1 x 10^{-5} to 1 x 10^{-3} M) or isoprenaline (1 x 10^{-9} to 1 x 10^{-2} M, used as reference drug) were cumulatively added to the organ bath every 6 min.

Effect of methyleugenol on tracheal ring contraction induced by potassium chloride

In another assay, the rings were contracted with KCl (60 mM) 30 minutes after stimulation with acetylcholine chloride. When the plateau was reached, methyleugenol was added cumulatively (1 x 10^{-5} to 1 x 10^{-3} M) every 6 min.

Effect of propranolol on the relaxant activity of methyleugenol

To assess the participation of β-adrenoceptors in the tracheal relaxation produced by methyleugenol, the isolated tracheal rings were preincubated with 3 μM of propranolol for 15 min, and then carbachol (3 μM) was added. When the plateau of the contraction was reached, methyleugenol was cumulatively added (1 x 10^{-5} to 1 x 10^{-3} M) every 6 min. Preincubation with propranolol was omitted for the control.

Effect of indomethacin on the relaxant activity of methyleugenol

To assess the possible role of prostaglandins in the methyleugenol-induced tracheal relaxation, the tissues were preincubated with 10 μM of indomethacin for 15 min before the application of carbachol (3 μM). When the plateau of the contraction was reached, methyleugenol was cumulatively added (1 x 10^{-5} to 1 x 10^{-3} M) every 6 min. Preincubation with indomethacin was omitted for the control.

Effect of glibenclamide on the relaxant activity of methyleugenol

To determine whether the relaxation of tracheal smooth muscle presently induced by methyleugenol is related to a blockade of the ATP-sensitive potassium channel, the isolated tracheal rings were preincubated with 1 μM of glibenclamide for 15 min prior to the addition of carbachol (3 μM). When the plateau of the contraction was reached, methyleugenol was cumulatively added (1 x 10^{-5} to 1 x 10^{-3} M) every 6 min. Preincubation with glibenclamide was omitted for the control.

Effect of L-NAME on the relaxant activity of methyleugenol

To test the involvement of nitric oxide in the methyleugenol-induced relaxation, the isolated

tracheal rings were pretreated with 300 μM of L - NAME for 15 min before the application of carbachol (3 μM). When the plateau of the contraction was reached, methyleugenol was cumulatively added (1 x 10^{-5} to 1 x 10^{-3} M) every 6 min. The use of L-NAME was omitted for the control.

Statistical analysis

Data are expressed as the mean ± S.E.M. of at least three experiments. The EC_{50} values were calculated by linear regression (Talladira, 2000). The Student's t-test was utilized to compare the EC_{50} values of methyleugenol in the presence and absence of the corresponding inhibitors, and one-way analysis of variance followed by Dunnett's Multiple Comparison Test was employed to determine the significant difference between several groups against a control group. A p-value <0.05 was considered statistically significant.

RESULTS

Effect of the extracts and fractions of P. hispidula on carbachol-induced tracheal ring contraction

Cumulative concentrations of the hexane, dichloromethane and methanol extracts of *P. hispidula* (17.7 to 316 µg/mL) reduced the tracheal contraction evoked by carbachol (Figure No. 1) in a concentration-dependent manner. The dichloro-methane extract was the most active, reaching 60.87 ± 4.3% of the maximum relaxant effect at a concentration of 316 µg/mL. The fractionation of the dichloromethane extract afforded four fractions, of which F1 and F2 were the most active (Figure No. 2). Their concentration-dependent relaxant activity reached a maximum of 81.53 ± 3.88 and 84.02 ± 5.07%, respectively, at 316 µg/mL. The EC_{50} values obtained for F1 and F2 were 153.47 ± 3.91 and 128.47 ± 6.23 µg/mL, respectively.

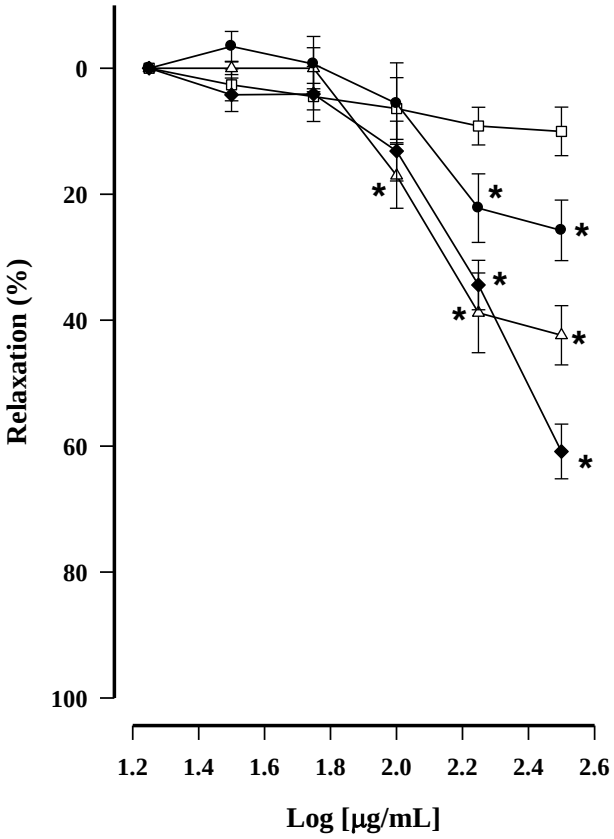
UHPLC-MS analysis

The UHPLC-MS quantitative analysis, using the extracted ion chromatogram (EIC) for the specific ion m/z 201.08, showed that methyleugenol constitutes 28.4, 31.2, 8.8 and 9.5% of F1, F2, F3 and F4, respectively (Figure No. 3). These data coincide with the biological activity of the four fractions. Fractions F1 and F2 exhibited the greatest effect, while fractions F3 and F4 had a more limited activity (Figure No. 2).

Effect of methyleugenol and isoprenaline on carbachol induced tracheal ring contraction

Methyleugenol (Figure No. 4) was obtained from silica gel column chromatography of F2. In relation to the contraction caused by carbachol (3 µM), this compound displayed a maximum relaxant activity at 1 x 10^{-3} M (114.90 ± 2.46%) and an EC_{50} of 108 ± 6.3 µM (19.24 µg/mL). Isoprenaline, the reference drug, provided a maximum effect at 3.16 x 10^{-3} M (108.43 ± 6.3%, data not showed).

Figure No. 1

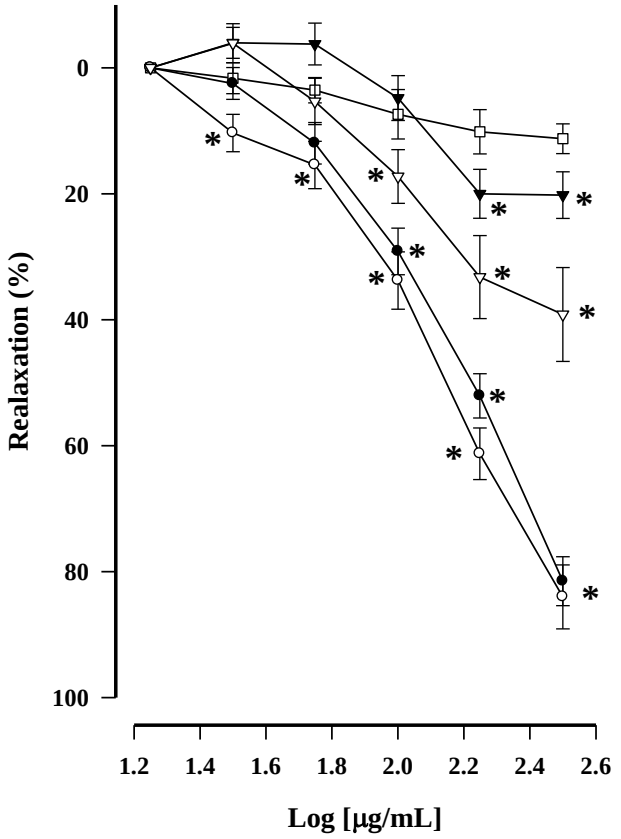


The relaxant effects of the □ vehicle, △ hexane, ◆ dichloromethane and ● methanol extracts of *P. hispidula* (17.7 to 316 µg/mL) on rat tracheal rings contracted with carbachol (3 µM). Results are expressed as the mean ± S.E.M., (n=3), *p<0.05

Effect of methyleugenol on KCl-induced tracheal ring contraction

Methyleugenol produced a concentration-dependent relaxation of the tracheal rings contracted with KCl (60 mM). A maximum relaxant effect of 114.83 ± 5.57% was caused by 1 x 10^{-3} M of methyleugenol, which showed an EC_{50}=73.14 ± 5.6 μM.

Figure No. 2



The relaxant effects of the □ vehicle, ● F1, ○ F2, ▼ F3 and ▽ F4 **fractions of the dichloromethane extract (17.7 to 316 μg/mL) on rat tracheal rings contracted with carbachol (3 μM). Results are expressed as the mean ± S.E.M., (n=3), *p<0.05.**

Mechanisms of the relaxant effect of methyleugenol on tracheal rings

The relaxant activity of methyleugenol was not modified by propranolol, indomethacin, glibenclamide, or L-NAME. Therefore, the mechanisms of action of methyleugenol are not related to β_2-adrenoceptors, prostaglandin E_2, ATP-sensitive potassium channels and nitric oxide (Table No. 1).

DISCUSSION

According to the results of the bioassay-guided fractionation of *P. hispidula*, the dichloromethane extract is the most active, followed by the extracts from hexane and methanol. This suggests that *P. hispidula* has more than one active compound. The two most active fractions obtained from the dichloromethane extract were F1 and F2, and both exhibited the same efficacy.

In a previous report by our group, all the fractions from the dichloromethane extract of *P. hispidula* were found to contain methyleugenol (Sánchez-Mendoza *et al*., 2015). Therefore, the percentage of methyleugenol in each of these fractions was presently examined. Through a UHPLC-MS analysis, similar percentages of methyleugenol were detected in F1 and F2 which explain both of them have the same efficacy. Along the same line, the F3 and F4 fractions had a lower percentage of methyleugenol and elicited a more limited relaxant effect on carbachol-contracted tracheal rings.

The main compound from F2 was methyleugenol, which relaxed the contraction produced by carbachol in concentration-dependent manner. It is likely that methyleugenol is the only responsible for the activity of F2, since this fraction has an EC_{50} of 128.47 μg/mL compared to the value of 19.24 μg/mL for methyleugenol.

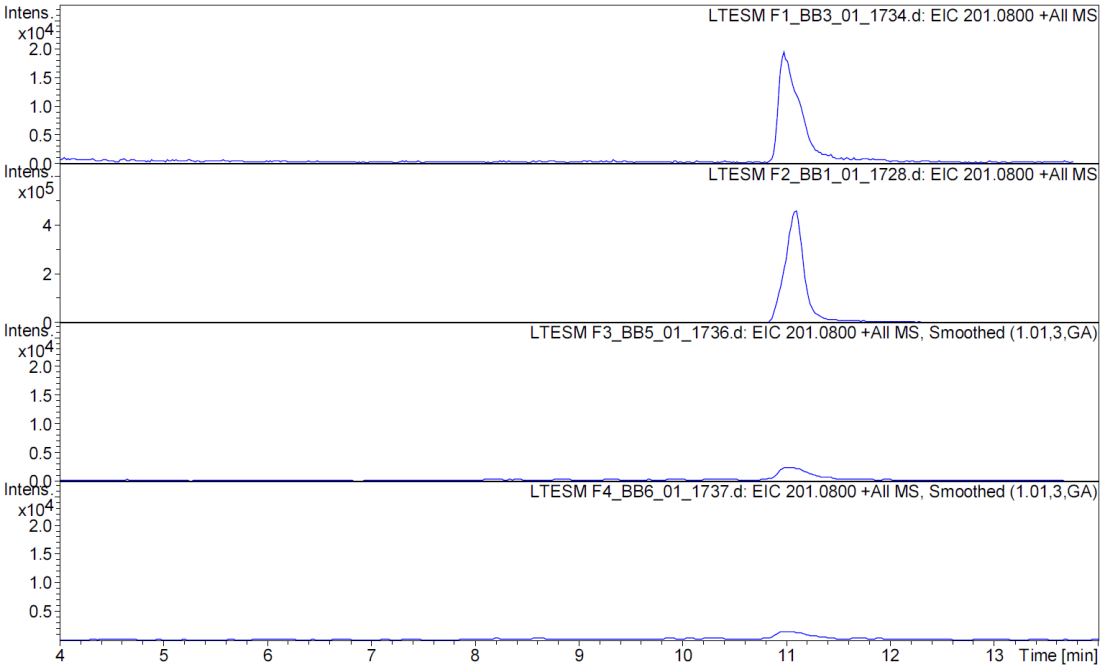
By showing that methyleugenol can relax tracheal rat smooth muscle, the current results provide support for the popular practice of treating asthma and other respiratory disorders with *P. hispidula*. To our knowledge, this is the first report of the relaxant activity of methyleugenol in tracheal smooth muscle tissue. The same compound produces relaxation in the ileum of guinea pigs (Magalhaes *et al*., 1998; Lima *et al*., 2000), and is known for its antidepressive (Norte *et al*., 2005), antinociceptive (Yano *et al*., 2006) and gastroprotective (Sánchez-Mendoza *et al*., 2015) activity. Moreover, methyleugenol is a constituent of the essential oils of many aromatic plants (Lima *et al*., 2000), and is widely used as a supplemental agent in food and a fragrance in cosmetics (Ding *et al*., 2014).

Regarding its mechanism of action, methyleugenol was able to relax tracheal rat smooth muscle contracted with carbachol and KCl, yielding

the same maximum relaxing effect in both cases. However, it was more potent in the tissue contracted with KCl, which contracted through a receptor-independent mechanism by inducing depolarization (Soder & Petkov, 2011; Águila *et al.*, 2015).

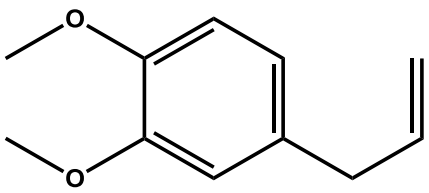
Apart from Ca^{2+}, Na^{+} has proven to be relevant in airway smooth muscle contraction (Sommer *et al.*, 2017) because it can contribute to membrane depolarization through voltage-dependent Na^{+} channels (Sommer *et al.*, 2016). Studies have shown that the voltage-gated Na^{+} channel is expressed in rabbit and human bronchial smooth muscle cells, in the latter mainly is present the Nav1.7 channel. These channels may participate in airway remodeling with asthma (Bradley *et al.*, 2013; Sommer *et al.*, 2017; Nakajima *et al.*, 2008). Interestingly, a methyleugenol-induced inhibition of Nav1.7 channels has been found by employing the technique of whole-cell patch clamp recording (Wang *et al.*, 2015). Further research is needed to explore the activity of methyleugenol in tracheal smooth muscle cells.

Figure No. 3



Extracted ion chromatograms for m/z 201.08 of fractions F1, F2, F3 and F4

Figure No. 4



Methyleugenol

Table 1
Effects of methyleugenol on carbachol-induced contractions of rat tracheal rings, in the presence and absence of different inhibitors

Treatment	EC_{50} ± SEM (µM)	Maximal response ± SEM (%)
Methyleugenol (ME)	**108 ± 6.3**	**114.90 ± 2.4**
Propranolol + ME	**99 ± 5.6**	**111.2 ± 3.4**
Indomethacin + ME	**115 ± 4.9**	**113.15 ± 6.14**
Glibenclamide + ME	**93 ± 14**	**115.43 ± 2.5**
L-NAME + ME	**92 ± 7.3**	**114.40 ± 4.27**

EC_{50}, concentration that caused 50% of their maximum effect

Isoprenaline, on the other hand, a non-specific β-agonist (herein used as a reference drug), also produced a concentration-dependent relaxation of the carbachol-induced contraction of tracheal rings. However, with a high concentration of this drug we obtained the maximum relaxant effect, what is due to the low β_2 receptor density in rat airway smooth muscle (Yousif & Thulesius, 1999). These receptors are responsible for the relaxant effect exerted by this kind of drug on smooth muscle (Barisione *et al*., 2010).

Several pathways of G-protein-coupled receptors (GPCRs) and non-GPCRs have been studied to attempt to understand airway smooth muscle relaxation. One such pathway, that of Gs-coupled GPCRs, elicits an increase in the secondary messenger cAMP, (which is important for bronchodilation) when are activated the β_2 adrenoceptors or EP_2 and EP_4 receptors. The two latter receptors can be activated by PGE_2 (Prakash, 2016). This pathway was examined by pretreatment with propranolol and indomethacin. However, they were herein unable to modify the activity of methyleugenol. Consequently, β_2-receptors and prostaglandins are not involved in the mechanism of action of methyleugenol.

Other possible targets on airway smooth muscle cells are the various types of ion channels that they express. For example, the opening of potassium channels, such K^+_{ATP} channels, can cause a relaxant response (Pelaia *et al*., 2002; Fitzgerald *et al*., 2014). Additionally, NO is a ligand of soluble guanylyl cyclase, an enzyme that enhances the production of cGMP. The latter is responsible for initiating downstream signaling leading to the relaxation of the trachea (Dupont *et al*., 2014). The possible role of these pathways in the effect of methyleugenol was explored by pretreatment with glibenclamide and L-NAME, which were unable to modify the relaxant activity. This indicates that ATP-sensitive potassium channels and nitric oxide do not participate in the mechanism of relaxation of smooth muscle tissue exerted by methyleugenol.

Methyleugenol is known to act as an agonist of $GABA_A$ receptors, and these receptors are Cl^- permeable ion channels (Ding *et al*., 2014), which in turn serve to hyperpolarize the cell and limit depolarization (Pera & Penn, 2016). The $GABA_A$ receptors in airway smooth muscle cells, which appear to be potent bronchodilators (Mizuta *et al*., 2008; Prakash, 2016), are expressed on airway smooth muscle cells in cultures of human and guinea pig tracheal rings (Mizuta *et al*., 2008). A selective $GABA_A$ agonist reportedly attenuates airway constriction induced by tachykinin, histamine (Mizuta *et al*., 2008), acetylcholine or electric vagal nerve stimulation in guinea pigs (Gleason *et al*., 2009). Although GABA reportedly does not have any effect on contractile responses to acetylcholine in rats, it has been shown to inhibit tracheal contractions evoked by electrical field stimulation (Özdem *et al*., 2000). Hence, it would be interesting to examine the

possible participation of $GABA_A$ receptors, as well as other mechanisms for methyleugenol, in airway smooth muscle relaxation in rats.

CONCLUSIONS

In conclusion, the present bioassay-guided study is the first report of relaxant activity induced by *P. hispidula* on rat tracheal ring smooth muscle. Methyleugenol was identified as the main compound involved in this muscle relaxation, an effect that was not related to β_2-adrenoceptors, prostaglandins, ATP-sensitive potassium channels or nitric oxide. Although further research is needed in regard to the mechanisms responsible for the relaxant activity of methyleugenol, the current results offer pharmacological evidence in support of using this plant to treat respiratory diseases.

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