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Relaxant activity of *Hyptis suaveolens* on isolated guinea pig tracheal rings

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Jesús Arrieta Valencia[1] • jearrval@yahoo.com.mx
ORCID: 000-0003-2928-744X

Héctor Jonathan Reyes Cortés[2] • johnreyesct@gmail.com
ORCID: 0009-0009-7751-1216

Leticia Cruz Antonio[2] • letycruza@yahoo.com.mx
ORCID: 0000-0001-8812-9711

Yaraset López Lorenzo[3] • yarlop_2310@outlook.com
ORCID: 0000-0002-3886-2955

María Elena Sánchez Mendoza[1] • mesmendoza@hotmail.com
ORCID: 0000-0003-4689-6757

1 Escuela Superior de Medicina, Instituto Politécnico Nacional. Ciudad de México. México

2 Facultad de Estudios Superiores Zaragoza, Universidad Nacional Autónoma de México. Ciudad de México. México

3 Ingeniería en Tecnología Ambiental, Universidad Politécnica de Chiapas. Suchiapa, Chiapas.

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— Abstract—

Asthma is a chronic disease that leads to difficulty in breathing due to the restriction of airflow. This is attributed to the contraction of bronchial smooth muscle tissue by diverse inflammatory mediators. Since existing therapies have not achieved adequate control of the symptomology of most patients, new options are needed. *Hyptis suaveolens* is used in the State of Chiapas, Mexico, to treat asthma, but its activity has not been corroborated scientifically. The aim of the current contribution was to determine the muscle relaxant activity of *Hyptis suaveolens* in the model of rings isolated from guinea pig trachea. The hexane, dichloromethane, and methanol extracts of the plant were prepared by maceration. The extract with the greatest activity was separated by column chromatography to find the most active fraction. The participation of β adrenergic and muscarinic receptors was determined by constructing concentrationresponse curves from the following treatments: the active subfraction of *Hyptis suaveolens* (56177 µg/mL), isoproterenol (3170 µM) in the absence and presence of propranolol (0.3 µM), and carbachol ($1x10^{-16}$-0.01 M) in the absence and presence of the active subfraction (100 y 177 µg/mL) or of atropine (0.3 y 3 µM). The dichloromethane extract was the most active ($p<0.05$), and its most active subfraction (F4') showed an EC_{50} of 91.19 ± 1.83 µg/mL. The relaxant effect of F4' was not inhibited by propranolol. F4' behaved as a competitive antagonist of muscarinic receptors ($p<0.01$). In conclusion, *Hyptis suaveolens* exerts a muscle relaxant activity on rings isolated from guinea pig trachea. An active subfraction of the dichloromethane extract acted as a competitive antagonist on muscarinic receptors, but β_2 adrenergic receptors did not participate in its mechanism of action.

Keywords:

Hyptis suaveolens; asthma; relaxant activity; medicinal plants; guinea pig tracheal rings.

The number of people affected by asthma worldwide is approximately 300 million, which is estimated to rise to 400 million by 2025 (Taur and Patil, 2011). In Mexico, about 7.0% of the population (~8.5 million inhabitants) suffers from this chronic respiratory disease (Arteaga-Badillo *et al*., 2020). The most common symptoms of asthma are coughing, wheezing, chest tightness, and shortness of breath (dyspnea) (WHO, 2023).

The difficulty of asthma patients to breathe stems from the capacity of various inflammatory mediators to contract airway smooth muscle, thus restricting airflow and preventing an adequate gaseous exchange of O_2 and CO_2 in the blood (Arteaga-Badillo *et al*., 2020). Excessive narrowing of the airways is considered to be the main cause of the morbidity and mortality that results from this disease. The exacerbation of symptoms may lead to disability, a loss of workdays, and hospitalization (Dowell *et al*., 2014).

The drugs administered to manage asthma are anti-inflammatory agents (e.g., corticosteroids, antileukotrienes, and chromones) and bronchodilators (e.g., β_2 adrenergic agonists, anticholinergics, and methylxanthines) (Arteaga-Badillo *et al*., 2020). Bronchodilators achieve symptomatic improvement by relaxing airway smooth muscle tone. The different pharmacological regimens available to reduce symptoms are unsatisfactory for many patients because of producing adverse effects. As a consequence, patients often suspend treatment, leading to the reappearance of symptoms (Taur and Patil, 2011). Some studies indicate that over 50% of asthma patients are unable to control their symptoms (Arteaga-Badillo *et al*., 2020). Hence, many asthma patients seek complementary or alternative medicine (Taur and Patil, 2011), such as the use of immunomodulatory agents and herbal medicine (Arteaga-Badillo *et al*., 2020). Certain medicinal plants can improve airflow by relaxing airway smooth muscle tone (Águila *et al*., 2015), thus relieving the symptoms of patients.

In the state of Chiapas, Mexico, the *Hyptis suaveolens* (Lamiaceae) Poit. plant is used in traditional medicine to treat different diseases, including asthma. The plant is known locally as “chía”, “confitura”, and “hierba de burro”. Although experimental evidence exists for the anti-inflammatory, antinociceptive, anti-cancer, anti-hyperglycemic, and anti-ulcer activity of *Hyptis suaveolens* (Santos *et al*., 2007; Vera-Arzave *et al*., 2012; Bayala *et al*., 2020; Mishra *et al*., 2021), no scientific study has yet been published on the relaxant effects of this plant on bronchial smooth muscle for the treatment of asthma. Therefore, the aim of the current contribution was to determine the relaxant effect of *Hyptis suaveolens* on the smooth muscle of isolated guinea pig tracheal rings.

METHODOLOGY

Vegetable material

Hyptis suaveolens was collected in the municipality of Copainalá (Chiapas, Mexico) in August, 2018. A specimen of the collection was identified as *Hyptis suaveolens* by the CHIP herbarium (registration number 27939), which belongs to the Ministry of Environment, Housing, and Natural History of Tuxtla Gutiérrez, Chiapas.

Preparation of extracts and chromatographic fractionation

The aerial parts (stems and leaves) of *Hyptis suaveolens* were dried in the shade at room temperature and then pulverized. Subsequently, the steps that characterize a bioassay-guided study were followed (Cornejo-Báez *et al*., 2020), beginning with the maceration of 20 kilos of plant material in different solvents: hexane, dichloromethane, and methanol (in increasing order of polarity) (Sharma & Gupta, 2015). The plant material was in contact with each solvent for three days, after which time the solution was filtered and the plant material concentrated under reduced pressure in a rotary evaporator (Sharma & Gupta, 2015). The process was carried out three times with each solvent, yielding 2.5% of the hexane extract, 2.3% of the dichloromethane extract, and 5.6% of the methanol extract. The most active extract was dichloromethane (440 g), and therefore it was fractionated by column chromatography (Cornejo-Báez *et al*., 2020) in a column packed with silica gel. Mixtures of hexane and ethyl acetate in ratios of 9:1, 7:3, and 5:5 generated fractions F1, F2, and F3, respectively. Ethyl acetate and methanol were used for the mobile phase to provide fractions F4 and F5, respectively. The yields of these fractions were 22% (F1), 21% (F2), 17% (F3), 21% (F4), and 18% (F5). Subsequently, 80.5 g of the most active fraction (F4) was separated by silica gel column chromatography. The subfractions were obtained with mixtures of hexane and ethyl acetate (Sharma & Gupta, 2015) in increasing polarity as eluents: 9:1 (F1´), 8:2 (F2´), 7:3 (F3´), and 5:5 (F4´). The yields were 26% (F1´), 29% (F2´), 33% (F3´), and 1.7% (F4´).

Pharmacological experiments

Animals

Male guinea pigs of the Hartley strain (300-450 g) were used. All procedures complied with the national regulation for the care and experimentation with laboratory animals (SAGARPA.NOM-062-ZOO-1999). The protocol

was approved by the internal ethics committee (CICUAL) of the School of Medicine of the Instituto Politécnico Nacional (registration number: CICUAL-08/4-12-2017).

Drugs

Acetylcholine chloride, carbachol, isoproterenol, propranolol, and atropine were purchased from Sigma-Aldrich (Mexico). All solutions were prepared with distilled water on the same day that the biological tests were performed.

Extracts, fractions, and subfractions

The extracts, fractions, and subfractions were suspended in a solution of Tween 80 (0.05%) and distilled water, prepared on the same day they were evaluated (Arrieta *et al*., 2018).

Preparation of the tracheal rings

The guinea pigs were sacrificed by administering an overdose of pentobarbital (95 mg/kg) intraperitoneally. The trachea was immediately dissected, and the attached tissue was cleaned. The trachea was always maintained in a Krebs solution, which consisted of the following compounds (measured in mM): NaCl (118), $NaHCO_3$ (25.0), glucose (11), KCl (4.7), $CaCl_2{\cdot}2H_2O$ (2.2), KH_2PO_4 (1.2), and $MgSO_4{\cdot}7H_2O$ (1.2). The Krebs solution was kept at a pH of 7.4 and a temperature of 37 °C, and was constantly ventilated with a mixture of 95% O_2 and 5% CO_2. Eight rings were obtained from each guinea pig trachea, and these were placed in isolated organ chambers with a volume of 10 mL. The rings were clamped between two hooks inserted into the lumen. One hook was fixed to the chamber and the other to a Biopac TSD 125c force transducer that recorded isometric tension on a Biopac MP150 polygraph. Each ring was adjusted to a tension of 2 g and allowed to rest for 30 min. Upon completion of this time, the viability of the tissue was determined by adding a solution of 30 µM acetylcholine. After washing the tissue with Krebs solution every 15 min for 1 h, the experiments were performed (Arrieta *et al*., 2018). The values were digitized and analyzed with data acquisition software (Acknowledge 4.0).

Evaluation of the relaxant activity of *Hyptis suaveolens*

The tissue was contracted with carbachol (3 µM) and when a plateau was reached, the vehicle or one of the treatments was applied. The concentrations

of the active agents were applied in increasing order, one every 7 min. The concentrations were 17.7, 31.6, 56.2, 100, 177, and 316 µg/mL for the extracts, 75, 100, 133, 177, 237, and 316 µg/mL for the fractions, and 56-177 µg/mL for the subfractions (Arrieta *et al*., 2018). In all cases, the maximum contraction induced by carbachol alone was considered as 100%.

Assessment of the participation of β2 adrenergic receptors

The tracheal rings were incubated with a propranolol solution (0.3 µM) or the vehicle for 15 min. Subsequently, they were contracted with carbachol (3 µM) until reaching a plateau (15 min), and then increasing concentrations of the active subfraction F4' (56, 75, 100, 133, and 177 µg/mL) or isoproterenol (3-170 µM) were added, one concentration every 7 min. Isoproterenol served as a reference drug in an independent experiment (Arrieta *et al*., 2018). The maximum contraction induced by carbachol alone in the presence of the vehicle was considered as 100%.

Evaluation of the participation of muscarinic receptors

The rings were incubated with atropine (0.3 and 3 µM), F4' (the most active subfraction, at 100 and 177 µg/mL), or the vehicle for 15 min in independent experiments. Upon completion of this time, increasing concentrations of carbachol (1x10-16 to 0.01 M) were applied every 7 min. The maximum contraction achieved by carbachol in the presence of the vehicle was considered as 100% (Sánchez-Mendoza *et al*., 2008).

Statistical analysis

Data are expressed as the mean ± the standard error of the mean (SEM) of at least six assays. EC_{30} and EC_{50} values (the concentration that produces 30% and 50% of the maximum effect, respectively) were calculated by linear regression (Talladira, 2000). The Student's *t*-test was used to compare the difference between two groups, and Dunnett's test to compare the differences between several groups. Statistical significance was set at $p \leq 0.05$.

RESULTS

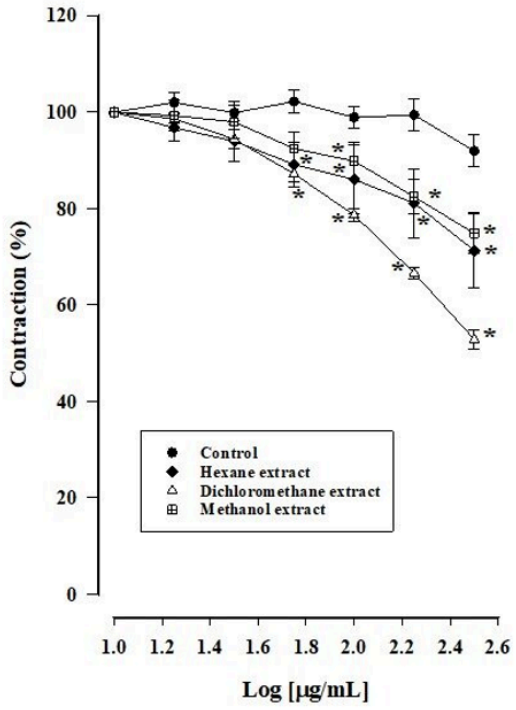
The relaxant effect generated by Hyptis suaveolens

The hexane, dichloromethane, and methanol extracts relaxed the smooth muscle of tracheal rings in a concentration-dependent manner (Figure 1), with the greatest effect produced by the dichloromethane extract. The latter

extract reached a maximum relaxation value of 47.16 ±1.93% at a concentration of 316 µg/mL and had an EC30 value of 138.07 µg/mL.

The fractionation of the dichloromethane extract by column chromatography provided five fractions. Whereas F1 was inactive, fractions F2-F5 showed a concentration-dependent relaxant effect, and F4 was clearly the most effective and potent (Table 1).

Fraction F4 (ethyl acetate) was then separated into four subfractions. F4´, obtained with a mixture of hexane/AcOEt (5:5), afforded the greatest relaxant effect. Both F4 and F4´ exhibited equal efficiency, furnishing an almost 100% relaxant effect (Figure 2), though at different concentrations.



Data are expressed as the mean ± SEM of six assays. * p≤0.05 versus the control, ANOVA followed by Dunnett's test.

Figure 1. The relaxant effect of *Hyptis suaveolens* extracts on the smooth muscle of guinea pig tracheal rings contracted with carbachol (3 µM).

F4´ was more potent than F4, as can be appreciated by the EC_{50} values (Table 2). To determine the complexity of the F4´ subfraction, thin layer chromatography was performed. Three compounds were detected, suggesting the participation of more than one compound in the relaxant activity of this subfraction.

Table 1

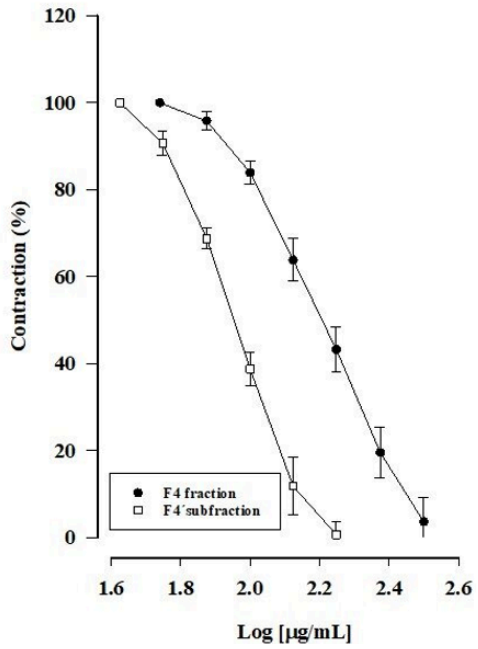
The maximum relaxant effect (Emax) and EC_{30} values of the fractions of the dichloromethane extract of Hyptis suaveolens when applied to the smooth muscle of guinea pig tracheal rings contracted with carbachol (3 µM)

Treatment	Emax (%) ± EEM	CE30 (µg/mL) ± EEM
Control	3.07 ± 3.98	-
F1	14.41 ± 5.13	-
F2	*64.17 ± 4.92	**191.66 ± 10.53
F3	*61.9 ± 4.44	**156.78 ± 4.93
F4	*96.37 ± 5.66	114.08 ± 3.44
F5	*50.49 ± 4.73	**225.01 ± 16.73

* p≤0.05 versus the control, ANOVA followed by Dunnett's test; ** p≤0.05 versus F4, ANOVA followed by Dunnett's test.

Evaluation of the participation of β_2 adrenergic receptors

The relaxant effect of F4´ was not inhibited by the presence of propranolol, indicating that the activation of β2 adrenergic receptors was not involved (Figure 3A). In contrast, the relaxant effect of the reference drug isoproterenol (a β-adrenergic receptor agonist) was entirely inhibited by the presence of propranolol (Figure 3B).



Data are expressed as the mean ± SEM of six assays. * p≤0.05 between groups, Student's *t*-test.

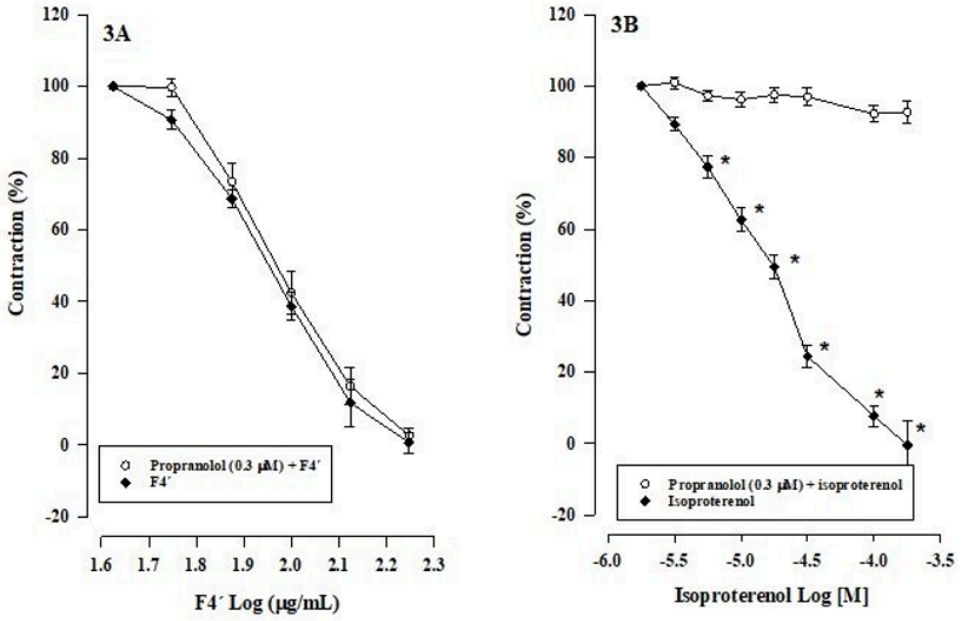
Figure 2. The relaxant effect of fraction F4 and subfraction F4´ on the smooth muscle of guinea pig tracheal rings contracted with carbachol (3 µM)

Table 2

The maximum relaxant effect (Emax) and EC50 values of fraction F4 and subfraction F4', tested on the smooth muscle of guinea pig tracheal rings contracted with carbachol (3 µM)

Fractions	Emax (%) ± EEM	CE_{50} (µg/mL) ± EEM
F4 (Ethyl acetate)	96.37 ± 5.66	151.47 ± 3.91
F4´ (Hexane/AcOEt 5:5)	99.33 ± 2.90	*91.19 ± 1.83

* p ≤ 0.001 between the two groups, Student's *t*-test.



Data are expressed as the mean ± SEM of six assays. * p≤0.05 between the two groups, Student's t-test.

Figure 3. The relaxant effect produced by subfraction F4´ (A) and isoproterenol (B) on the smooth muscle of guinea pig tracheal rings contracted with carbachol (3 µM), both in the absence and presence of propranolol pretreatment

Participation of muscarinic receptors

The concentration-response curves of carbachol were shifted to the right in the presence of F4´ (at 100 and 177µg/mL) (Figure 4). This decline in potency is reflected in the EC_{50} values (Table 3). On the other hand, the presence of F4´ did not modify the maximum contractile effect of carbachol (Figure 4). Thus, F4´ behaves like a competitive antagonist of muscarinic receptors. The reference drug atropine (a competitive antagonist of muscarinic receptors)

also caused a shift to the right in the concentration-contractile response curves of carbachol (Figure 5). This decreased potency is reflected in the EC_{50} values (Table 4).

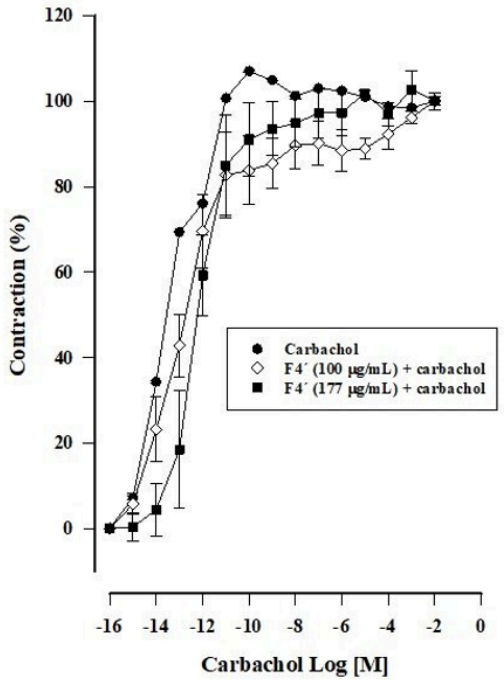


Figure 4. The effect of the F4´ subfraction on the smooth muscle of guinea pig tracheal rings subjected to carbachol-induced contractions. Data are expressed as the mean ± SEM of six assays

Table 3

The EC_{50} values of carbachol tested on the smooth muscle of guinea pig tracheal rings in the absence and presence of subfraction F4´ (100 and 177 µg/mL)

Treatment	CE_{50} (M) ± EEM
Carbachol	$2.27 \times 10^{-15} \pm 2.86 \times 10^{-15}$
F4´ (100 µg/mL) + Carbachol	$*2.29 \times 10^{-13} \pm 3.53 \times 10^{-13}$
F4´ (177 µg/mL) + Carbachol	$*1.92 \times 10^{-12} \pm 2.61 \times 10^{-12}$

* p≤0.01 compared to the carbachol-only group, ANOVA followed by Dunnett's test.

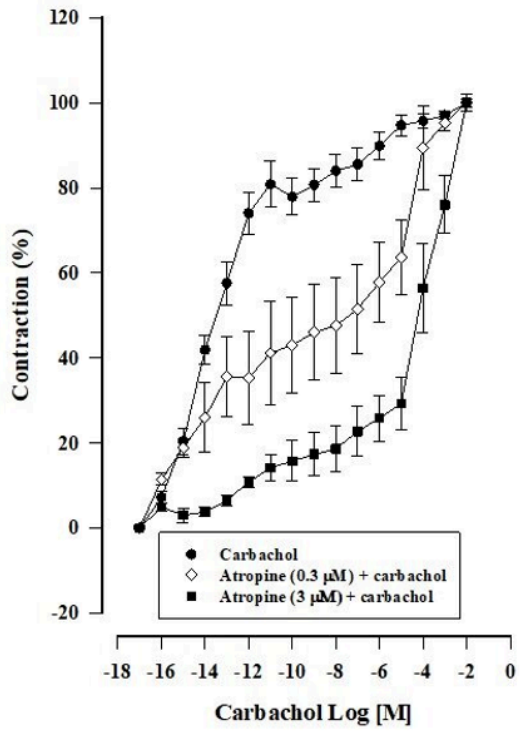


Figure 5. The effect of atropine on the smooth muscle of guinea pig tracheal rings subjected to carbachol-induced contractions.

Table 4

The EC_{50} values of carbachol when tested on the smooth muscle of guinea pig tracheal rings in the absence and presence of atropine

Treatment	CE_{50} (M) ± EEM
Carbachol	$2.79 \times 10^{-13} \pm 1.56 \times 10^{-13}$
Atropine 0.3 µM + Carbachol	$*1.050 \times 10^{-08} \pm 1.1 \times 10^{-08}$
Atropine 3 µM + Carbachol	*0.0078 ± 0.0136

* p≤0.01 compared to the carbachol-only group, ANOVA followed by Dunnett's test.

DISCUSSION

Scientific support for relaxant effect of *Hyptis suaveolens* on bronchial smooth muscle is herein provided for the first time. This plant has been reported to produce an anti-inflammatory effect in an *in vivo* model (Misrha, 2021), which is relevant because asthma patients have elevated levels of IgE, which trigger an inflammatory reaction through the release of mediators such as histamine, prostaglandins, and leukotrienes. These in turn lead to the contraction of airway smooth muscle (Taur & Patil, 2011). Hence,

Hyptis suaveolens could possibly contribute to the treatment of asthma through dual activity: the inhibition of inflammation and relaxation of bronchial smooth muscle.

In the bioassay-guided study of *Hyptis suaveolens*, the greatest relaxant activity on tracheal rings was found for the dichloromethane extract, which was separated into five fractions. Since four fractions were active (F2-F5), there is more than one compound with a relaxant activity in this extract. The most active fraction was F4.

From the fractionation of F4, four subfractions were obtained, with F4´ showing the greatest relaxant activity. Thin layer chromatography of F4´ suggests that it consists of at least three compounds. In future research, these compounds should be isolated and evaluated in order to determine the one or the combination capable of producing the best relaxant effect.

Regarding the possible mechanism of action of F4 and F4´, airway smooth muscle undergoes relaxation by directly activating relaxant mechanisms or inhibiting the effect of contractile agonists (e.g., acetylcholine and leukotrienes) (Thirstrup, 2000). Relaxant mechanisms are directly activated through the stimulation of AMPc and/or GMPc, the inhibition of cyclic nucleotide degradation, and the modulation of the activity of cell membrane ion channels. In the current study, an evaluation was made of the participation of β_2-adrenergic receptors and muscarinic receptors in the relaxant activity of subfraction F4´.

β_2-adrenergic receptors are coupled to guanine nucleotide-binding proteins (G proteins). When activated, they stimulate the enzyme adenylyl cyclase, which catalyzes the formation of the second messenger AMPc. The latter leads to the activation of protein kinase A (PKA), and this in turn phosphorylates myosin light chain kinases and several other proteins. The phosphorylation of myosin light chain kinases decreases their affinity for Ca^{2+}/calmodulin, which results in smooth muscle relaxation (Barisione *et al*., 2010; Alkawadri *et al*., 2022).

To assess the contribution of β_2-adrenergic receptors to the relaxant activity of F4´, relaxation curves were constructed for F4´ in the presence and absence of propranolol (a β-adrenergic receptor antagonist). Given that the relaxation curves were not modified, the participation of these receptors in the mechanism of action of F4´ was ruled out. The reference drug isoproterenol (a β-adrenergic agonist) was also examined in the presence and absence of propranolol. Its relaxant effect was inhibited by propranolol.

In regard to muscarinic receptors, they are involved in the control of airway smooth muscle tone and the diameter of the trachea. M_2 and M_3 receptors are located in smooth muscle. M_2 receptors have been reported to prevent the relaxation normally caused by β-adrenergic receptors by inhibiting the activity of the enzyme adenylyl cyclase. There is also evidence of the

M_2 receptor-induced potentiation of the contractile role of M_3 muscarinic receptors (Soukup *et al*., 2017; Alkawadri *et al*., 2022). M_3 receptors are the main subtypes involved in the contractile response in smooth muscle (Soukup *et al*., 2017). When an agonist binds to these receptors, they activate a Gq protein that binds to the enzyme phospholipase C (PLCβ), which generates the second messengers IP3 and DAG, known to contribute to smooth muscle contraction (Soukup *et al*., 2017).

According to the current results, carbachol and F4´ compete for the same binding site on M_3 muscarinic receptors (Blumenthal, 2019). In the presence and absence of F4´, carbachol was equally effective in contracting smooth muscle. In the presence of F4´, however, a greater concentration of carbachol was required to achieve the same contractile effect, indicating reduced potency. Hence, F4´ behaves like a competitive antagonist of M_3 receptors. In confirmation of this conclusion, the reference drug atropine (a competitive antagonist of muscarinic receptors) had the same effect on carbachol, causing a lower potency without affecting its efficiency.

Short-acting anticholinergics (ipratropium and oxitropium) and one long-acting anticholinergic (tiotropium) is currently used in the treatment of asthma. One approach now employed in the development of new anticholinergic drugs for asthma is to design compounds with prolonged action on muscarinic M_3 receptors and with less effect on M_2 receptors (Soukup *et al*., 2017). In this sense, future research is necessary on the compounds responsible for the activity of F4' in order to evaluate the relaxant effect and antagonistic behavior individually and in combinations. Additionally, it is important to continue to explore other mechanisms of action of *Hyptis suaveolens*.

CONCLUSIONS

The present bioassay-guided study of *Hyptis suaveolens* demonstrated that produces a relaxant effect on the smooth muscle of guinea pig tracheal rings, which could possibly be useful for the improvement of the symptoms of asthmatic patients. The most active was subfraction (F4´). According to the results of exploring the mechanism of action of F4´, muscarinic receptors but not β_2-adrenergic receptors are involved. F4´ acted as a competitive antagonist of muscarinic receptors. .

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