

# Relaxant Effect of the Extracts of *Crataegus mexicana* on Guinea Pig Tracheal Smooth Muscle

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## ABSTRACT

*Crataegus* sp. has been used in the traditional medicine of Mexico as well as other countries for the treatment of several respiratory diseases, such as flu, cough and asthma. The tracheal relaxant effect of the leaves of *C. mexicana* is investigated here for the first time, through a bioassay-guided study by using isolated tracheal rings of guinea-pig as an experimental model. The hexane extract was the most active compared to dichloromethane and methanol. An active fraction was obtained from the hexane extract. Assays by HPLC-MS reveal that at least 14 compounds may exist in it. In addition, the results suggest that relaxant effect of the effective fraction was in part related to the activity of  $\alpha$ -adrenergic receptors and not to  $K^+_{ATP}$  channels. This study represents the first in which the relaxant effect of leaves of *C. mexicana* on tracheal rings of guinea pig was clearly demonstrated. More studies are required to correctly identify the bioactive compounds that contribute to the relaxant effects of *Crataegus mexicana*, and to know the mechanisms of action of these compounds.

**Key words:** Rosaceae, hawthorn, bronchodilator, respiratory diseases, traditional medicine.

## INTRODUCTION

The *Crataegus* genus (Rosaceae) comprises approximately 280 species and is found in northern temperate regions of East Asia, Europe, and Eastern North America.<sup>[1]</sup> The common name for the *Crataegus* species is hawthorn, and in Mexico is known as Tejocote.<sup>[2]</sup> Fruits, leaves, and flowers of the *Crataegus* sp. contain a number of chemical compounds, such as flavonoids, oligomeric proanthocyanidins, phenolic acids, triterpene acids, organic acids, sterols and trace amounts of cardioactive amines.<sup>[1, 3]</sup>

Several biological activities for that genus, such as cardioprotective, hypolipidemic, antioxidant, anti-inflammatory,

antispasmodic, diuretic, digestive and others, have been reported.<sup>[1,4,5]</sup> Throughout Europe numerous preparations with the fruit, leaf and flower are currently available alone and in combination with other herbal extracts.<sup>[3]</sup> In addition, the *Crataegus* sp. has been used in the traditional medicine of Mexico as well as other countries for the treatment of several respiratory diseases, such as flu, cough, cold, bronchitis and asthma.<sup>[6,7]</sup>

In spite of the widespread use of the *Crataegus* plant species for purposes of medical treatment, no studies exist regarding its usefulness in respiratory diseases. Therefore, we decided to investigate the tracheal relaxant effect of the leaves of *Crataegus mexicana* Moc. & Sessé ex DC. (Rosaceae) by using isolated tracheal rings of guinea-pig as an experimental model.

## MATERIAL AND METHODS

### Plant material

The leaves of *C. mexicana* were collected in Chapingo, in the state of Mexico, during February of 2009. The identification was performed by Ernestina Cedillo, from the Herbarium of the Division of Forestry Sciences, Chapingo Autonomous University, with the voucher number 62654.

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### Extraction and bioassay-guided fractionation

The leaves of *C. mexicana* were dried at room temperature ( $22 \pm 2^\circ\text{C}$ ) in the shade. After grinding 3.2 kg of leaves, they were successively extracted by maceration at room temperature ( $22 \pm 2^\circ\text{C}$ ) for 3 days, first with hexane (12 L  $\times$  3), then dichloromethane (12 L  $\times$  3) and finally methanol (12 L  $\times$  3). Evaporation of the solvents in vacuum yielded 91.0, 49.5 and 592.8 g of syrupy residues, respectively. In accordance with the bioassay-guided study of the extracts, the hexane extract (77 g) was subjected to separation over a silica gel column (0.063-0.200 mm, 770 g) by using a step gradient of hexane (2 L, F1), hexane/EtOAc (9:1, 2 L, F2), hexane/EtOAc (7:3, 2 L, F3), hexane/EtOAc (1:1, 2 L, F4), EtOAc (2 L, F5) and MeOH (2 L, F6). The F2 and F3 fractions were the most active. The F2 fraction (16 g) was chromatographed on a silica gel column (320 g), obtaining three fractions, neither one of which presented activity (data not shown). F3 fraction (11.8 g) was chromatographed on a silica gel column (220 g) and was eluted with hexane, hexane/EtOAc mixtures, EtOAc and MeOH, obtaining eight fractions, of which the fraction F3' was the most active. In order to identify the

chemical compounds of F3', a sample was analyzed by the HPLC-MS technique (Figure 1).

### Animals

All the experiments were performed with adult male guinea pigs (350-450 g) obtained from the animal house of the Superior Medicine School (IPN). Procedures involving animals and their care were conducted in conformity with the Mexican Official Norm for Animal Care and Handling (NOM-062-ZOO-1999) and in compliance with international rules on care and use of laboratory animals. The guinea pigs were housed under standard conditions, food and water being available *ad libitum*.

### Drugs

Acetylcholine chloride, histamine dihydrochloride, carbachol chloride, propranolol hydrochloride, salbutamol, glibenclamide and KCl were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The other reagents used were of analytical grade. Glibenclamide was dissolved in dimethyl sulfoxide (DMSO) and diluted with water. The other drugs

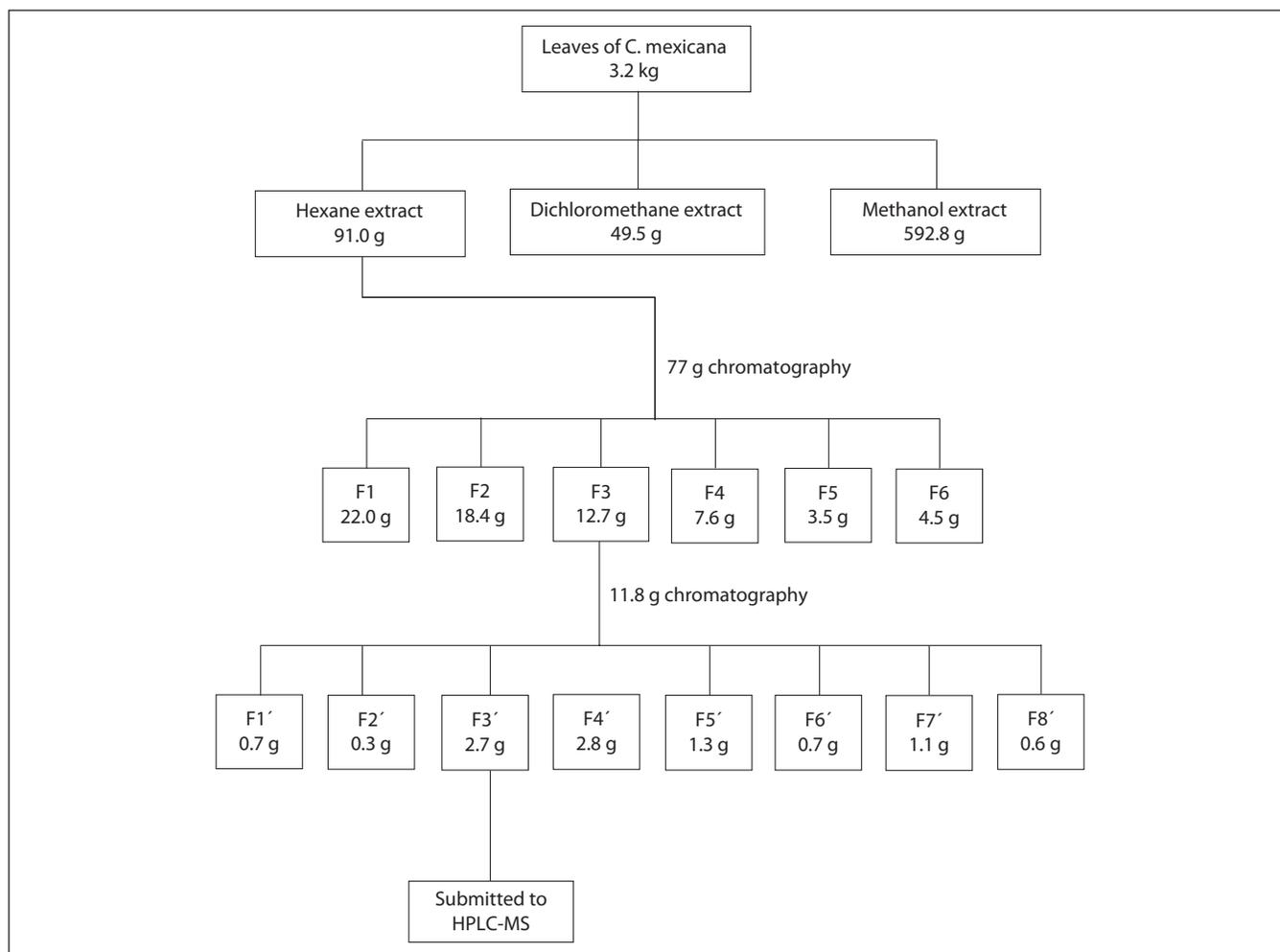


Figure 1: Scheme of bioassay-guided fractionation of the hexane extract from *Crataegus mexicana*.

were dissolved in distilled water. The extracts and fractions were suspended in distilled water with traces of Tween 80. The final concentration of DMSO or Tween 80 was less than 0.1% and did not significantly affect the tracheal response.

### Preparation of guinea pig trachea

The animals were euthanized by intraperitoneal injection with an overdose of sodium pentobarbital (75 mg kg<sup>-1</sup>), the trachea was dissected and the connective tissue was cleaned off. Twelve tracheal rings about 2 mm in length, containing two to three cartilages each, were obtained from each guinea pig. Each tracheal ring was hung between two hooks inserted into the lumen, and placed in a 10 mL organ bath containing Krebs solution with the following composition (mM): NaCl 118.0, KCl 4.7, NaH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub>·7H<sub>2</sub>O 1.2, CaCl<sub>2</sub>·2H<sub>2</sub>O 2.5, NaHCO<sub>3</sub> 25.0, glucose 11.1. This solution was maintained at 37 ± 0.1°C and bubbled with 5% CO<sub>2</sub>-95% O<sub>2</sub> mixture. Isometric tension was recorded through a twelve-channel Biopack System polygraph MP100 via a Biopac TSD 125C force transducer (Santa Barbara, USA). The data were digitalized and analyzed by means of software for data acquisition (Acknowledge 3.8.1) (Santa Barbara, USA). Tissues were placed under a resting tension of 1.5 g and allowed to stabilize for 60 min. They were washed with fresh Krebs solution at 15 min intervals before starting the experiments. After the stabilization period the tracheal rings were submitted to pre-stimulation with acetylcholine chloride (3 μM) two times at 30 min intervals, and after this stimulation they were washed with fresh Krebs solution.<sup>[8]</sup>

### Effect of extracts and different fractions on the pre-contracted guinea pig trachea

Thirty minutes after stimulation with acetylcholine, the rings were contracted with carbachol (3 μM). When the plateau of the contraction was reached, 31.6, 56.2, 100.0, 133.3, 177.8, 237.1, 316.2, 421.6 or 562.3 μg/mL of the test extracts or fractions were cumulatively injected every 5 min. Then, their effective concentration thirty or fifty (EC<sub>30</sub> or EC<sub>50</sub>) was calculated.

### Effect of the hexane extract on the histamine or KCl pre-contracted guinea pig trachea

Thirty minutes after stimulation with acetylcholine, the tracheal rings were contracted with histamine (30 μM) or KCl 40 (mM) in independent experiments. When the plateau of the contraction was reached, concentrations from 31.6 to 562.3 μg/mL of hexane extract were cumulatively injected every 5 min. Then, the percentages of the relaxation provoked in each treatment were calculated as well as the respective EC<sub>50</sub>. As a relaxant standard drug we used salbutamol.

### Effect of propranolol on the relaxant activity of the F3' fraction

The relaxant activity of the F3' fraction was studied on the carbachol chloride contracted guinea pig tracheal rings in the absence or presence of propranolol. The relaxant activity of salbutamol was used as a reference. When the plateau of the contraction was reached, 3 μM of propranolol was injected into the organ bath, and then every 5 min a concentration from 31.6 to 562.3 μg/mL of the F3' fraction, or from 10<sup>-9</sup> to 10<sup>-7</sup> M of salbutamol, were cumulatively injected.<sup>[8]</sup> The percentage of the relaxation induced by each treatment was calculated as well as the respective EC<sub>50</sub>.

### Effect of blocking the ATP-sensitive potassium channel on the relaxant effect of the F3' fraction

The relaxant effect of F3' on the carbachol contracted guinea pig tracheal ring was studied in the absence or the presence of glibenclamide. For this purpose, 10 μM glibenclamide was injected in the organ bath after the plateau of the contraction was reached, and then 31.6 to 562.3 μg/mL of the F3' fraction were cumulatively injected every 5 min<sup>[8]</sup> followed by the calculation of the EC<sub>50</sub> for each treatment.

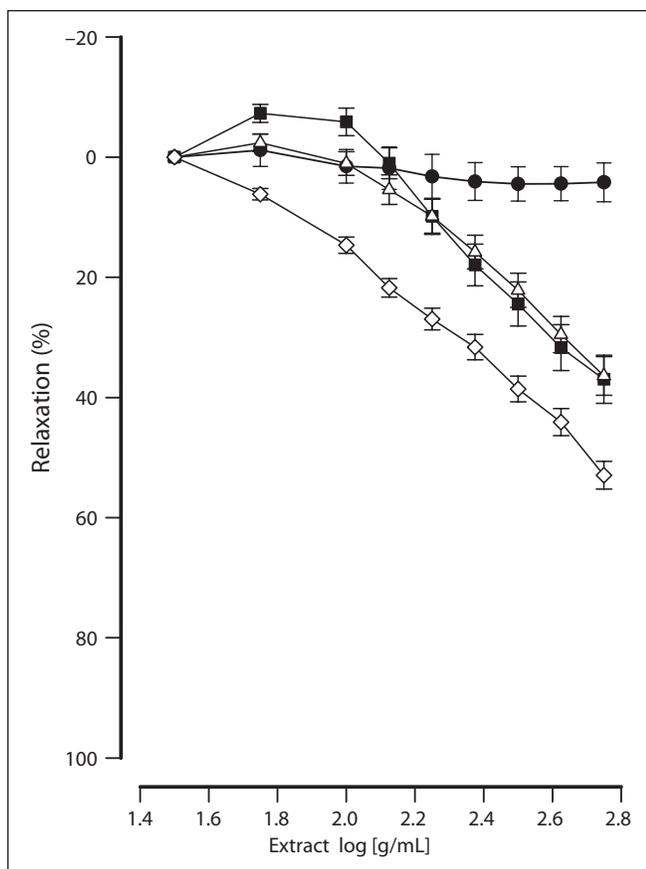
### Data analysis

Values of EC<sub>30</sub> and EC<sub>50</sub> were calculated by linear regression.<sup>[9]</sup> All values are shown as the mean ± SEM of at least six experiments. The differences among these values were statistically calculated by one-way analysis of variance (ANOVA), and then determined by Dunnett's test. The differences were considered statistically significant if the p-value was less than 0.05.

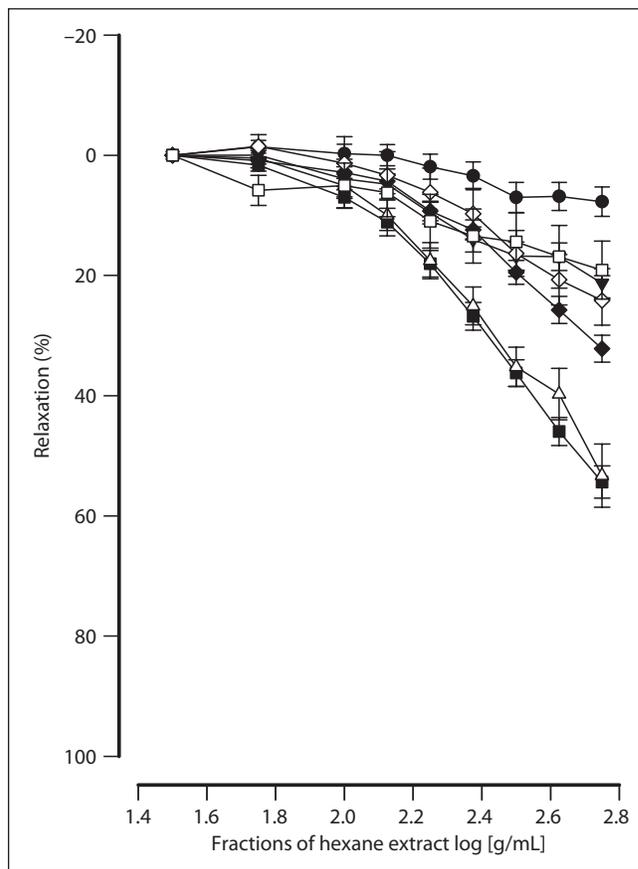
## RESULTS

### Bioassay-guided fractionation

The relaxing activities of different extracts of *C. mexicana* on carbachol (3 μM) pre-contracted tracheal rings are given in Figure 2. The hexane extract was more potent (EC<sub>30</sub> = 224.9 ± 8.8 μg/mL) and more active, (its maximum effect value (E<sub>max</sub>) was 52.9 ± 2.3%) than the dichloromethane and methanol extracts. The E<sub>max</sub> values of dichloromethane (E<sub>max</sub> = 36.4 ± 3.2%) and methanol (E<sub>max</sub> = 36.9 ± 4.0%) were significantly different (p<0.05) than the hexane extract. Of the six (F1 to F6) fractions obtained from silica gel separation of hexane extract, F2 and F3 were found to be the most active fractions (Figure 3), with the EC<sub>30</sub> = 246.2 ± 6.7 μg/mL and 253.0 ± 5.6 μg/mL, respectively. These values are not significantly different (p<0.05). Then F2 and F3 were each separated by silica gel column. Only sufficient quantities of three fractions were obtained from F2. However, when evaluated no activity was observed (data no shown). From F3 we obtained eight fractions (F1' to



**Figure 2:** The relaxant effect of the ( $\diamond$ ) hexane, ( $\Delta$ ) dichloromethane and ( $\blacksquare$ ) methanol extracts of *Crataegus mexicana* (31.62 to 562.34  $\mu\text{g/mL}$ ) or ( $\bullet$ ) control (vehicle) on carbachol (3  $\mu\text{M}$ ) induced precontraction in guinea pig tracheal rings. Each point value represents the mean  $\pm$  S.E.M. (n = 6)



**Figure 3:** The relaxant effects of ( $\blacksquare$ ) F1, ( $\blacksquare$ ) F2, ( $\Delta$ ) F3, ( $\blacklozenge$ ) F4, ( $\diamond$ ) F5 and ( $\square$ ) F6 fractions of the hexane extract (31.62 to 562.34  $\mu\text{g/mL}$ ) or ( $\bullet$ ) control (vehicle) on carbachol (3  $\mu\text{M}$ ) induced precontraction in guinea pig tracheal rings. Each point value represents the mean  $\pm$  S.E.M. (n = 6).

F8'), fraction F3' being the most active, with a maximum relaxant effect of  $97.5 \pm 3.8\%$  (Table 1) and obtained a value of  $EC_{50} = 285.9 \pm 6.2 \mu\text{g/mL}$ . Assays by HPLC-MS of fraction F3' reveal that at least 14 compounds may exist in it (Figure 4).

### Effect of the hexane extract on the histamine or KCl pre-contracted guinea pig trachea ring

When 30  $\mu\text{M}$  of histamine was used to contract the tracheal rings, the hexane extract produced a concentration-dependent relaxation on the pre-contracted organ (Figure 5A), with an  $E_{\text{max}} = 118.2 \pm 6.7\%$  and an  $EC_{50} = 144.0 \pm 9.3 \mu\text{g/mL}$ . In the same way, when 40 mM of KCl was used to induce the contraction of the rings, the hexane extract produced a concentration-dependent relaxation (Figure 5), with an  $E_{\text{max}} = 88.5 \pm 4.7\%$  and an  $EC_{50} = 303.5 \pm 8.2 \mu\text{g/mL}$ . Salbutamol used as relaxant standard drug was able to relax the histamine, KCl and carbachol induced pre-contraction (data not shown). The  $E_{\text{max}}$  values of all these evaluations were significantly different from those of the control (vehicles).

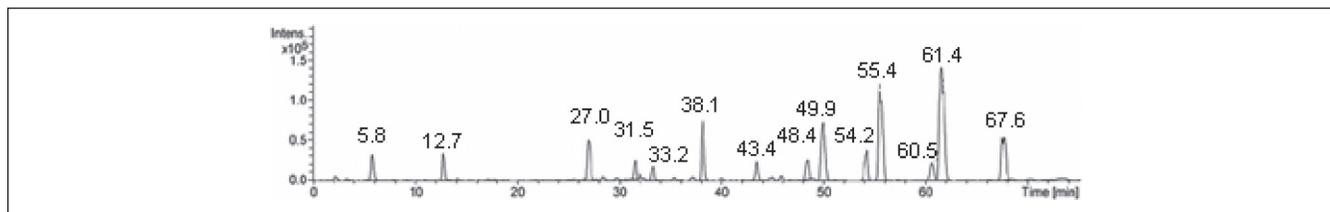
**Table 1.  $E_{\text{max}}$  (%) values of the second fractionation against carbachol (3  $\mu\text{M}$ ) induced precontractions.**

Fraction (562 $\mu\text{g/mL}$ )	$E_{\text{max}}$ (%)
Control	$26.2 \pm 4.3$
F1'	$50.4 \pm 4.7'$
F2'	$69.3 \pm 3.8'$
F3'	$97.5 \pm 3.8'$
F4'	$53.0 \pm 4.0'$
F5'	$45.5 \pm 3.2'$
F6'	$56.5 \pm 3.6'$
F7'	$29.7 \pm 2.6$
F8'	$36.0 \pm 3.9'$

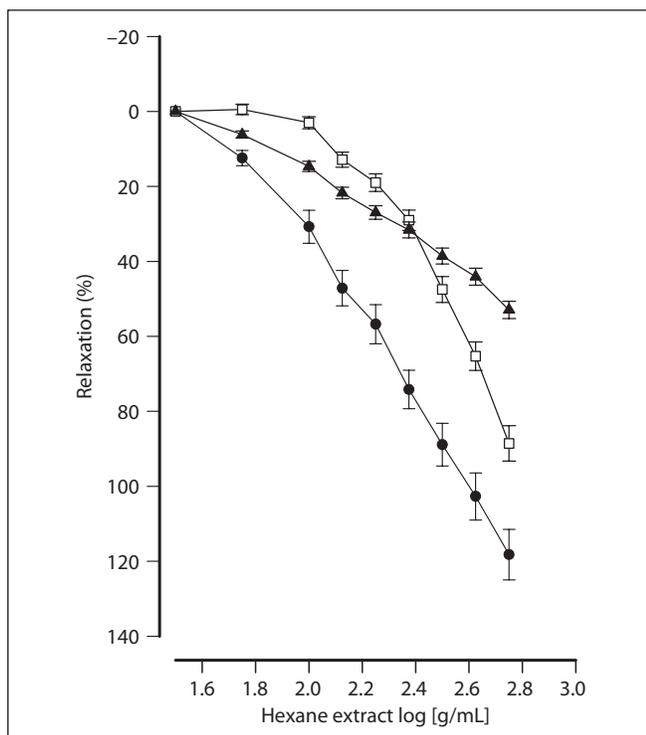
Values are presented as the mean  $\pm$  SEM (n=6, n being the number of experiments). \* $p < 0.05$  ANOVA followed by the Dunnett's test.

### Effect of propranolol and glibenclamide on the relaxant effect of the F3' fraction

The effect of the F3' fraction was modified significantly ( $p < 0.05$ ) by pretreatment with the  $\beta$ -adrenoceptor antagonist, propranolol, at 3  $\mu\text{M}$  (Figure 6A). The  $EC_{50}$  values were  $229.8 \pm 9.0$  and  $278.2 \pm 8.7 \mu\text{g/mL}$  in the presence or absence of propranolol, respectively. Propranolol completely blocked



**Figure 4:** HPLC spectra of F3' of *Crataegus mexicana*. Retention times of major peaks are indicated.



**Figure 5:** The relaxant effect of the hexane extract (31.62 to 562.34 µg/mL) on the (●) histamine (30 µM), (□) KCl (40 mM) or (▲) Carbachol (3 µM) induced precontraction in guinea pig tracheal rings. Each point value represents the mean ± S.E.M. (n = 6).

the salbutamol (as a relaxant standard drug) relaxant effect after the carbachol induced contraction (data are not shown). Also, glibenclamide (10 µM), an ATP-sensitive potassium channel blocker, did not affect the log concentration-response curves of the F3' fraction (Figure 6B).

## DISCUSSION

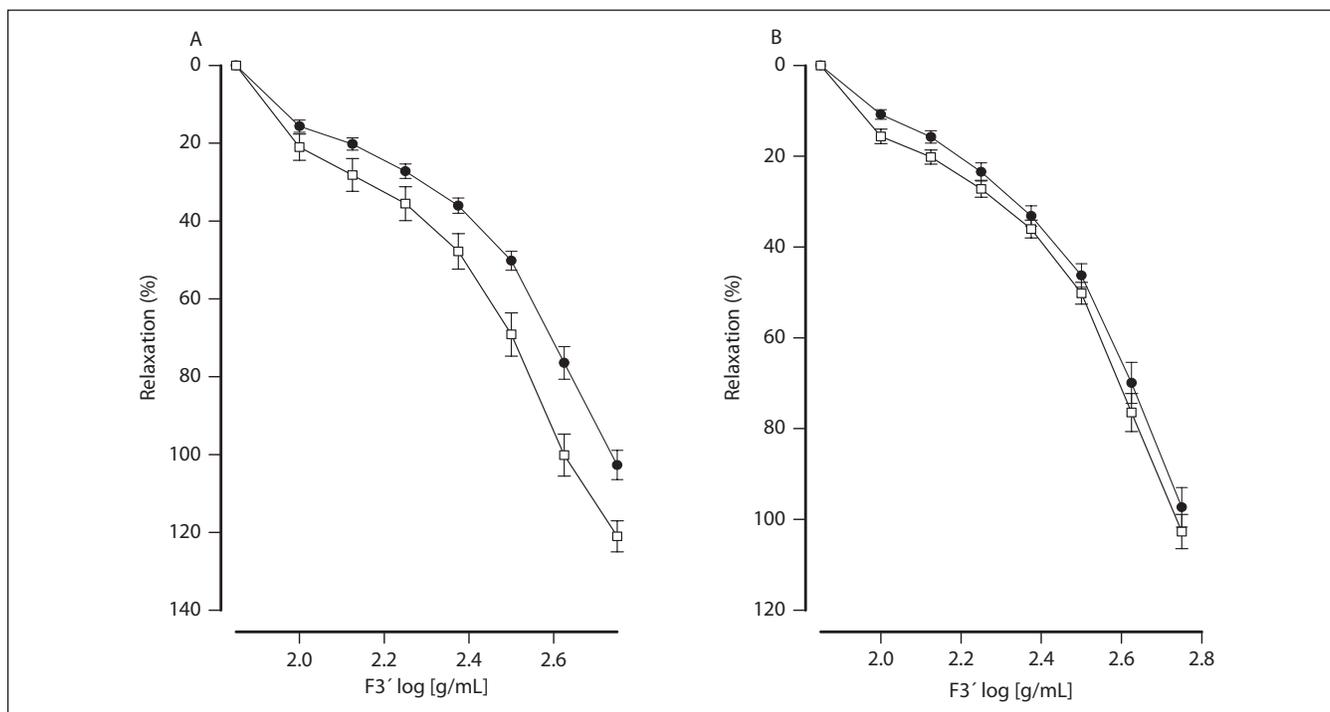
In this study we provide preliminary scientific support to the popular practice of employing *Crataegus mexicana* in the treatment of respiratory diseases. It was found that extracts obtained from the leaves of this plant have a mild relaxant effect in the tracheal smooth muscle of the guinea pig model in preparations precontracted with carbachol. The hexane extract was the most active (Figure 2), but interestingly its effect was even greater with the tracheal muscle precontracted with other contractile agents,

such as histamine or KCl, than with carbachol (Figure 5). As has been reported, the contractions induced by histamine and KCl are mainly dependent on  $Ca^{2+}$  from the extracellular medium, specifically an increased  $Ca^{2+}$  influx across the membrane,<sup>[10]</sup> and can be eliminated by voltage-operated calcium channels blockers.<sup>[11]</sup> Considering the aforementioned, in the relaxant effect of the hexane extract a reduction of the  $Ca^{2+}$  influx through calcium channels could possibly be implicated. However, further studies are needed to corroborate this idea.

The bioassay-guided study was performed following the relaxation of tissues precontracted with carbachol. F2 and F3 obtained from the first fractionation of the hexane extract were active, presenting a  $54.3 \pm 2.6$  and  $53.3 \pm 5.2\%$  maximum relaxant effect, respectively (Figure 3). However, the similar relaxant effect of F2, F3 and the hexane extract (Figure 2) suggest that more than one of the compounds of the plant contribute to the relaxant effect.

On the other hand, neither of the three fractions obtained from F2 were able to produce a relaxant effect alone (data not shown), suggesting that the compounds present in F2 need to be together in order to produce the relaxant effect.

Some authors consider that the action of the *Crataegus* extract is attributable to a complex of active compounds, which can be termed the synergic effect.<sup>[12]</sup> In fact, many studies have been conducted to ascertain if hawthorn extracts can exert any therapeutic benefits in the treatment of cardiovascular disease or delay its onset. Frequently whole plant extracts and/or flavonoid combinations have been used as opposed to specific isolated classes of phytochemicals. It has been found that the separation of phenolic extracts of *Crataegus* into individual compounds does not appear to be beneficial regarding their anti-oxidant effects, as mixtures of compounds tend to exert stronger effects than those of individual compounds at the same concentrations.<sup>[12]</sup> Interestingly, when F3 was separated in the second fractionation, we found that F3' presented the maximum relaxant effect (Table 1). The HPLC-MS (Figure 4) results showed that this active fraction contained a highly complex mixture. Further separation of F3' will certainly be necessary in order to correctly identify bioactive compounds that contribute to the relaxant effect



**Figure 6:** The relaxant effect of F3' (31.62 to 562.34  $\mu\text{g/mL}$ ) on carbachol (3  $\mu\text{M}$ ) induced precontraction A) in the (□) absence or (●) presence of propranolol. B) in the (□) absence or (●) presence of glibenclamide. Each point value represents the mean  $\pm$  S.E.M. ( $n = 6$ ).

of *Crataegus mexicana*. Nonetheless, the results described in the present study provide a starting point for further investigation of multiple relaxant compounds in the *Crataegus* species.

In an attempt to provide information about the mechanism of the relaxant effect of F3', the participation of the  $\beta$ -adrenergic receptor was evaluated by pretreating the rings with propranolol (a  $\beta$ -adrenoceptor antagonist). The results show that the  $\text{EC}_{50}$  of F3' increased by the pretreatment with propranolol, which suggests that the effect is in part related to the activation of  $\beta_2$ -adrenergic receptors (Figure 6A). Since the pretreatment with glibenclamide did not affect the relaxant effect of F3' (Figure 6B), the participation of ATP-sensitive  $\text{K}^+$  channels can be discarded.

## CONCLUSION

This study represents the first in which the relaxant effect of leaves of *C. mexicana* on tracheal rings of guinea pig was clearly demonstrated. Of the extracts of *C. mexicana*, hexane was the most active, its relaxant effect being independent of the contractile agent used. In the bioassay guided study, F3' was identified as the main relaxing fraction, with an activity related to a mild  $\beta$ -adrenergic participation and unrelated to  $\text{K}^+$  channels. More studies are required to correctly identify the bioactive compounds that contribute

to the relaxant effects of *Crataegus mexicana*, and to know the mechanisms of action of these compounds.

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