Full Length Research Paper

Differential inhibition of agonists-induced tracheal contraction after *in vitro* enriched-extracts treatment

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In our previous studies, pharmacological actions of Senegalese plants on the modulation of tracheal contractile responses induced by acetylcholine were demonstrated. Here, we aim to demonstrate whether the pharmacological effects previously observed, could be obtained with other contractile agonists, such as histamine and potassium chloride (KCl), in the isolated trachea after treatment with the same extracts. For this purpose, changes in contractility of isolated trachea from male Wistar rats were assessed in organ chambers. Rings were first exposed to methanolic plants extracts or solvent. After a short-term incubation period, they were contracted with acetylcholine, histamine or KCl in a cumulative manner. *Salvadora persica* crude extract did not alter the contractile responses of tracheal smooth muscle. Those of *Hymenocardia acida* persistently and significantly attenuate the contractile responses of the three agonists. *Cymbopogon giganteus* crude extract, in contrast, significantly potentiates the contractile responses to histamine and KCl and has no effect on contractions induced by acetylcholine. The responses obtained with the extract of *Gossypium barbadense* depend on the agonist used. Similarly, *Guiera senegalensis* crude extract significantly increases the contractile responses to low concentrations of KCl and histamine and significantly reduces those induced by acetylcholine. Altogether, these results clearly indicate that the potential benefit of medicinal plants depends on the asthmatic disease component, especially mediated by contractile agonists for which the efficacy of bioactive compounds varies considerably.

Key words: Agonists, smooth muscle, trachea, asthma, medicinal plants.

INTRODUCTION

Airway regulation is relatively complex. It involves cholinergic and adrenergic mechanisms, known for many years (Barnes, 1992; Douglas, 1990; Karlsson, 1986; Robuschi, 1988; Freitag et al., 1996). However, a non-adrenergic non-cholinergic (NANC) system has been reported (Lei et al., 1993; Ingenito et al., 1995; Ito and Fujisawa, 1998; Widdicombe, 1998). The cholinergic and adrenergic systems neurotransmitter have also been well identified, namely acetylcholine and adrenaline, respectively. For the NANC system, a large number of peptides

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appear to be involved, such as vasoactive intestinal peptide (VIP), substance P, tachykinins, calcitonin gene related peptide (CGRP) and neuropeptide Y (NPY) (Kneussl, 1986; Douglas, 1990; Joos, 1989; Frossard and Fajac, 1995).

During respiratory diseases such as asthma, bronchoconstriction due to cholinergic stimuli is generally observed (Jongejan et al., 1991; Joos et al., 2000; Pauli, 1982; Ritz et al., 2009; Rodriguez de la Vega, 1986). Exaggeration of cholinergic reflexes would be responsible for the abnormal response observed in asthmatics known as bronchial hyper-responsiveness (BHR). Subjects with bronchial hyper-reactivity in response to various physical, chemical or pharmacological stimuli exhibit excessive and acute bronchial obstruction as compared to normal subjects. It has been shown that several cell populations are involved in the pathophysiology of BHR, especially inflammatory cells (Roquet et al., 1996; Bavbek et al., 1996; Hoogsteden et al., 1999; Van Schoor et al., 2000; Ward et al., 2002). Among the inflammatory mediators, histamine plays an important role. Indeed, it acts both as a bronchoconstrictor agent, but also as a mucus production inducer and thus significantly contributes to bronchial obstruction (Nickel et al., 2002; Shioya et al., 2004). Treatment of asthma results from its pathophysio-logy and importance is given to bronchodilators, including beta 2-agonists, which are defined by their ability to quickly correct the airway obstruction and are used in acute treatment. Similarly, the synthetic anticholinergic, which most often are muscarinic receptor antagonists, are also regarded as bronchodilators, but less powerful than beta 2-agonists (Hogan and Markos, 2007). Never-theless, these two treatment strategies do not prevent airway hyper-responsiveness. However, the various phar-macopoeias, particularly African traditional medicines, contain significant therapeutic potential, but whose effectiveness is to be proven. Characterization of their role in preventing tracheal constriction is a major theme of our research laboratory. It would be interesting, there-fore, in addition to demonstrating their preventive therapy effectiveness, to study specifically their differential effect on tracheal tone in terms of agonists involved.

MATERIALS AND METHODS

Acetylcholine and histamine were purchased from Sigma Chemical Co (Saint Quentin-Fallavier, France). Potassium chloride (KCl) was purchased from Scharlauchemie SA (Gato Perez, Pol Ind Mas D En Cisa). Adrenaline was a generous gift from Pharmacie National d'Approvisionnement, Dakar, Senegal. All reagent and extract were diluted in Krebs bicarbonate solution before use.

Preparation of enriched extracts

Leaves of *Guiera senegalensis, Melaleuca leucodendron, Hymenocardia acida, Cymbopogon giganteus* and *Gossypium barbadense*, roots of *Salvadora persica* and seed of *Cassia occidentalis* were prepared as previously described (Sar al., 2010).

In brief, powdered material was subjected to extraction for 2 h using a 60% methanol/water solution and macerated extract was then filtered in vacuum conditions and evaporated on a rotary evaporator.

Tracheal reactivity studies

Six-week-old male Wistar rats were procured from local Institute, Dakar, Senegal. Animals were kept on a 12:12-h light-darkness cycle, singly caged and fed on standard rat feed and given free access to water. All animal procedures were approved and conducted in accordance with the Guide for the Care and Use of Laboratory Animals as promulgated by the Senegalese Academic Bioethic Committee (Décret n° 2009-729). Immediately before each experiment, tracheas were removed from adults rats weighing 150 to 200 g after anaesthesia with pentobarbital (60 mg/kg, i.p.), cleaned of connective tissue and cut into rings (3 to 4 mm in length). Rings were suspended in organ baths (Panlab-TRI 202P) containing oxygenated (95% O_2; 5% CO_2) Krebs bicarbonate solution (mM: NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.18, $CaCl_2$ 1.25, $NaHCO_3$ 25 and D-glucose 11, pH 7.4, 37°C) for the deter-mination of changes in isometric tension. Following equilibration for 60 min under a resting tension of 2 g, rings were contracted with acetylcholine (1 µM) and the relaxation to phenylephrine (1 µM) was determined. After washout and a 30 min equilibration period, rings were incubated with methanolic plants extract (10^{-1} mg/ml) or solvent for 30 min before acetylcholine (10^{-9} to 10^{-3} M), histamine (10^{-9} to 10^{-5} M) or KCl (10^{-2} to 3.10^{-1}M) was administered in a cumulative manner and a concentration-contraction curve constructed.

Statistical analysis

Values are expressed as mean ± standard error of mean (SEM). Statistical evaluation was performed with Student's t test for paired data (when it involves two levels of contraction in 2 different conditions and induced by the same concentration of agonist) or analysis of variance (ANOVA; for the comparison of dose-response curves for two treatment conditions). Values of $p < 0.05$ were considered statistically significant.

RESULTS

In order to characterize the preventive effect of plant extracts on tracheal hyper-reactivity, we proceeded to study the contractile response to various agonists when compared with those already obtained with acetylcholine (Sar al., 2010). Preventive treatment of tracheal rings with extracts showed different patterns of response (Table 1).

S. persica extract does not alter rat tracheal motor function

On contractile responses to KCl (Figure 1A), histamine (Figure 1B) or acetylcholine (Figure 1C), no significant modifications were observed following treatment with *S. persica*. Indeed, contractile responses of treated-tracheal rings with *S. persica* extract are not significantly different with those obtained with untreated controls, regardless of

the agonist used.

C. giganteus extract only modifies responses to histamine on rat trachea

On contractile responses to KCl (Figure 1D) or acetyl-choline (Figure 1F), no significant modifications were observed following treatment with *C. giganteus*. It was the same with the overall response to histamine (Figure 1E) for which no significant difference was observed between treated rings as compared to controls. However, to intermediate concentrations of histamine (between 10^{-8} and 10^{-7} M), the plant extract significantly increased the contractile responses.

G. senegalensis extract effects on tracheal contractile response are agonist-dependent

Responses obtained with *G. senegalensis* extract are rather contradictory. If no difference with KCl (Figure 2A) or histamine (Figure 2B) was observed between treated and control rings, such was not the case with acetylcholine, for which a significant inhibitory effect was noted (Figure 2C). This suggests that the effects of *G. senegalensis* depend on the type of agonist involved. However, it is important to note a significant increase in the contractile response to intermediate concentrations of KCl following treatment with *G. senegalensis* extract (Figure 2A), although the maximum contractile responses (Table 1) were not significantly different for treated rings (E_{max}: 4.87 ± 1.23 g) as compared to untreated controls (E_{max}: 5.40 ± 0.30 g). Indeed, on the basis of EC_{50} (Table 1), the concentrations values of KCl after treatment with *G. senegalensis* extract (EC_{50}: 7.10^{-3} ± 0.2 M) were significantly lower than those necessary without treatment (EC_{50}: 98.10^{-3} ± 6 M).

G. barbadense extract potentiates the tracheal contractile responses

Compared to contractile responses obtained with untreated tracheal rings, treatment with *G. barbadense* extract significantly increased contractile responses induced by KCl (Figure 2D) and acetylcholine (Figure 2F). By contrast, no significant change in contractile responses to histamine was observed (Figure 2E).

Beneficial preventive effect of *H. acida* on tracheal hyper-reactivity

The results obtained with *H. acida* are more interesting. Indeed, methanol extract of this plant significantly reduced reduced the contractions induced by KCl (Figure 3A), for

which there are variation of maximal effect (E_{max}) from 5.40 ± 0.30 g for untreated rings (control) to 3.01 ± 1.68 g for exposed tracheal rings. These changes are accompanied by a significant increase in EC_{50} ($1.17.10^{-6}$ ± 0.15 M for tracheal treated-rings versus $4.58.10^{-7}$ ± 1.92 for controls) and a reducing effect of about 45% of the contraction induced by KCl after exposition to the plant extracts (Table 1 and Figure 4). Compared to acetyl-choline, similar results were observed, but with a more pronounced inhibitory effect of about 80% (Figure 4). Inhibitory responses were also obtained with histamine (Figure 3B) and acetylcholine (Figure 3C). However, the percentage inhibition obtained (44.24 ± 10% for KCl; 73.33 ± 24% for histamine) is always lower than that obtained with acetylcholine (81.89 ± 16%), as shown in Figure 4.

DISCUSSION

Interesting results obtained in this study, allowed us to have a better understanding of the effectiveness of medicinal plants extracts to prevent tracheal contractile response induced by different agonists.

The choice of the trachea is related to difficulties in handling the rat bronchi in our isolated organ system. Indeed, the diameter of rat bronchi is generally small and is a source of additional difficulty during experiments. So it is easier to work with the trachea, which is very acces-sible and whose smooth muscle, although less important, has the same contractile properties than other parts of the airways. It is then possible to extrapolate the results of tracheal motor function to those of bronchial motility.

One major finding of this study is the ability of *H. acida* extracts to significantly reduce the contractile responses induced by KCl, histamine and acetylcholine. For the other extracts, the results are highly variable: from the lack of effect on the contractile response (*S. persica* and *C. giganteus*) to potentiation (*G. barbadense*).

The choice of the main agonists used was guided by their direct involvement in airways smooth muscle cells contractility or those of their signaling pathways in the mechanisms of the pathophysiology of respiratory diseases, particularly asthma. Indeed, many studies have already demonstrated the involvement of KCl (Bai and Sanderson, 2009; Chen et al., 2008; Estrada-Soto et al., 2012 ; Evangelista et al., 2007; Ghayur and Gilani, 2006; Gonzalez and Santacana, 2001), histamine (Liu et al., 2006; Joos et al., 1997; Yamahara et al., 1995; Kai et al., 1992; Norris and Eyre, 1982; Eyre and Besner, 1979) or acetylcholine (Matsunaga et al., 2009; Gupta and Fahim, 2007; Chu et al., 2007; Gonzalez and Santacana, 2000; Preuss and Goldie, 1999) in preventive or curative studies by herbal extracts. The mechanisms involved in the inhibitory or potentiating effects of the tracheal tone are correlated to their interaction with different signaling pathway.

Table 1. Maximal response (E_{max}) and required agonists concentration for 50% of this effect (EC_{50}) after isolated rat trachea impregnation or not by different extracts.

Condition	E_{max} (g)			EC_{50} (M)			Interpretation
	KCl	Hist	ACh	KCl	Hist	ACh	
Control	5.40 ± 0.30	0.90 ± 0.13	3.70 ± 0.56	$98 \times 10^{-3} \pm 6$	$4.58.10^{-7} \pm 1.92$	$1.79 \times 10^{-6} \pm 0.066$	-
Gossypium barbadense	6.09 ± 1.27^{ns}	0.67 ± 0.20^{ns}	$6.08 \pm 0.75^{**}$	$5 \times 10^{-3} \pm 0.8^{*}$	$2.78.10^{-6} \pm 0.06^{*}$	$8.76 \times 10^{-7} \pm 3.09^{*}$	(+) KCl, Hist and (-) ACh
Guiera senegalensis	4.87 ± 1.23^{ns}	0.64 ± 0.30^{ns}	$1.75 \pm 0.40^{*}$	$7 \times 10^{-3} \pm 0.2^{*}$	$9.16.10^{-11} \pm 3.26^{**}$	$6.28 \times 10^{-6} \pm 1.31^{*}$	(+) KCl, Hist and (-) ACh
Salvadora persica	5.29 ± 0.59^{ns}	0.74 ± 0.17^{ns}	3.51 ± 0.58^{ns}	$78 \times 10^{-3} \pm 9^{ns}$	$5.01.10^{-6} \pm 1.004^{*}$	$1.92 \times 10^{-6} \pm 0.076^{ns}$	(0) KCl, Hist and (+) ACh
Hymenocardia acida	$3.01 \pm 1.68^{*}$	$0.24 \pm 0.08^{*}$	$0.67 \pm 0.19^{**}$	$200.7 \times 10^{-3} \pm 27^{*}$	$1.17.10^{-6} \pm 0.15^{**}$	$7.37 \times 10^{-6} \pm 2.22^{*}$	(-) KCl, Hist and ACh
Cymbopogon giganteus	4.65 ± 0.25^{ns}	0.81 ± 0.15^{ns}	4.18 ± 0.12^{ns}	$39 \times 10^{-3} \pm 2^{ns}$	$1.03.10^{-8} \pm 0.038^{*}$	$1.20 \times 10^{-6} \pm 0.41^{ns}$	(+) Hist

(+) Potentiation; (0) Without effect; (-) Inhibition; ns: not significant; *$p < 0.05$ and **$p < 0.01$. Hist: histamine; ACh: acetylcholine.

Thus, K+-depolarization (KCl) of smooth muscle has long been known to cause Ca^{2+}-dependent contraction (Clelland et al., 2010; Kaneda et al., 2006, 2009; Bordallo et al., 2008). However, multiple Ca^{2+} oscillations in the presence of KCl, reported by numerous studies (Perez and Sanderson, 2005; Lamboley et al., 2003) indicate a more complex mechanism than a simple elevation of Ca^{2+} and alternative mechanism of KCl action must be considered. KCl-induced depolarization may release neurotransmitters or protein signalling to stimulate contractions (Kaneda et al., 2006; Shore et al., 1983; Mbikou et al., 2011; Janssen et al., 2004; Gosens et al., 2004). KCl does not only elevates intracellular free Ca^{2+}, myosin light chain kinase activity, but also can inhibit myosin light chain phosphatase activity by activation of rhoA kinase (ROCK) (Ratz et al., 2009; Nakanishi et al., 2009). It should be noted, but not demonstrated in this study, that all these different mechanisms may be involved in potentiating or inhibitory effects of studied extracts for KCl-induced contraction. Finally, we cannot exclude a potassium channel opening effect for these plants, which may contribute on its inhibitory effect.

Histamine, produced by mast cells, is also a known mediator involved in BHR in asthma by activation of histamine receptors H1 causing bronchoconstriction and numerous authors have also shown that H1-receptor antagonists reversed histamine-induced contraction in a dose-dependent manner (Jacob et al., 2007; Roumestan et al., 2008; Bavbek et al., 1996). However, histamine remained a weak agonist (E_{max} does not exceed 1 g of tension) for tracheal smooth muscle segments contraction. Thus, the role of histamine in constricting the bronchial muscle may be negligible.

Airway hyper-reactivity is also a consequence of acetylcholine effects in respiratory diseases (Chen et al., 2008; Franova et al., 2007; Islami et al., 2004). Indeed, the cholinergic innervation of parasympathetic system is the most important bronchoconstrictor in humans and animals (Pendry, 1993; Widdicombe, 1986). Its role in the regulation of bronchial tone is important and is linked to multiple mechanisms. In animals, the bronchoconstriction induced by cholinergic agonists is observed in the trachea and larger sizes bronchioles and it involves M3-receptors (Rogers, 2001). In the cholinergic system, acetylcholine release is massively from storage vesicles resulting in a depolarization that requires the presence of Ca^{2+}. Although *G. senegalensis* extracts had no effect on responses to histamine, it was able to significantly inhibit the responses to acetylcholine and increase those of KCl. As for *G. barbadense,* it significantly increases the responses to KCl and acetylcholine while it has no significant effect on histamine contractility. *G. barbadense* seems not to be effective on modulating tracheal contractility in spite of its large use for cough and respiratory illness in Senegal. In the respiratory system, antitussive activity of alkaloids of *G. senegalensis* has been demonstrated (Diatta et al., 2007; Faye et al., 1980), although other pharmacological activities of this plant were reported by numerous works, including trypanocidal (Aderbauer et al., 2008), antimalarial (Ancolio et al., 2002; Benoit et al., 1996), anti-diarrhoeal and ulcer-protective effects (Aniagu et al., 2005) and a lack of toxicity (Diouf et al., 2000). Similarly, *G. barbadense* extract has real potential therapeutic dominated by its antitumorigenic effects (Amara et al., 2008), hypotensive (Hasrat et al., 2004) but with many cytotoxic effects (Thomas et al., 1991; Stipanovic et al., 2009; Amara et al., 2008). Among the many studies reported on *G. barbadense*, none have re-ported beneficial effects on the respiratory system.
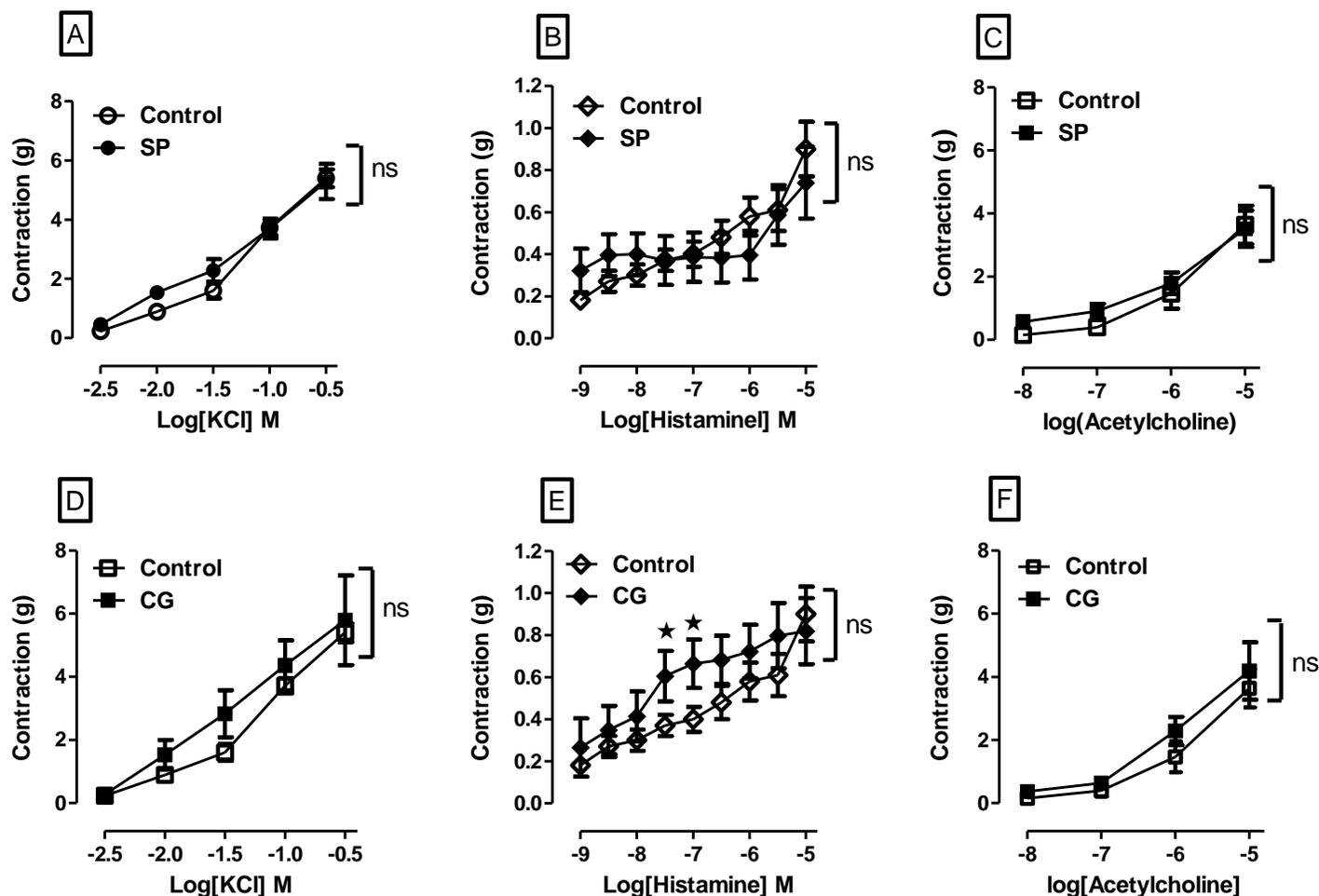
Figure 1. Effects of *Salvadora persica*, SP (top panel) and *Cymbopogon giganteus*, CG (bottom panel), administered at a dose of 0.1 mg/ml for 30 min on the KCl (A and D), histamine (B and E) and acetylcholine (C and F) dose-response curves of rat isolated tracheal smooth muscle. Results are expressed as mean contraction tension in g ± SEM for at least six experiments obtained from different rats. ns: Not significant and *p < 0.05.

system.

Moreover, among the plants studied, *H. acida* is the only effective plant that inhibits the three agonists studied. If no phytochemical study or toxicity of this plant have been developed during this study, the presence of carbohydrates, tannins, flavonoids, saponins, alkaloids, cardiacglycosides, resins, terpenes and steroids in its roots were reported (Ibrahim et al., 2006; Ward et al., 2002; Sofidiya et al., 2009). In addition, this plant has proved positive effects in sickle cell disease (Ibrahim et al., 2006; Mpiana et al., 2007) and studies have reported an antiparasitic activity (Hoet et al., 2004; Vonthron-Senecheau et al., 2003) or cytotoxic (Sowemimo et al., 2007). However, none of these studies reported pharma-cological effects on the respiratory system, which is the originality of our work. It is important to note that in our experimental protocol, no toxicity was observed with the extract of *H. acida*. Indeed, contractile responses to various agonists, identical to those obtained with controls

tracheal rings, were obtained after impregnation of the validate their preventive role in respiratory disorders trachea followed by several washings (data not shown). This suggests an absence of disturbance of the tracheal contractile apparatus by treatment with the extracts of this plant.

Conclusion

This preliminary work opens interesting perspectives on the impact of medicinal plants on bronchial tone, especially on the control of agonists responsible for airway hyper-responsiveness. This should lead to a better understanding of mechanisms underlying the inhibitory effect of tracheal tone by the extracts of these plants. On the long run, this should allow us (using animal models of respiratory diseases) to scientifically
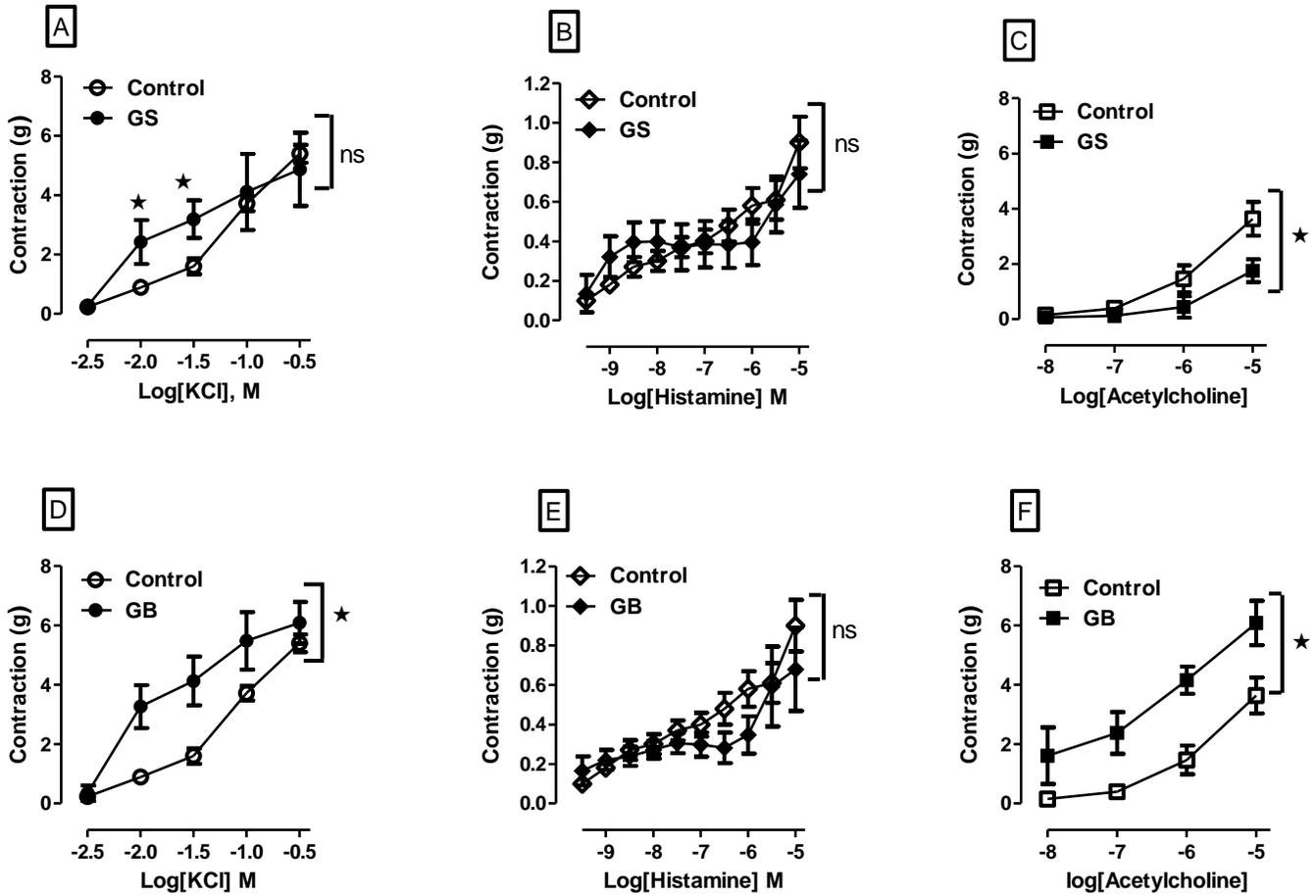
Figure 2. Effects of *Guiera senegalensis*, GS (top panel) and *Gossypium barbadense*, GB (bottom panel), administered at a dose of 0.1 mg/ml for 30 min on the KCl (A and D), histamine (B and E) and acetylcholine (C and F) dose-response curves of rat isolated tracheal smooth muscle. Results are expressed as mean contraction tension in g ± SEM for at least six experiments obtained from different rats. ns: Not significant and *p < 0.05.
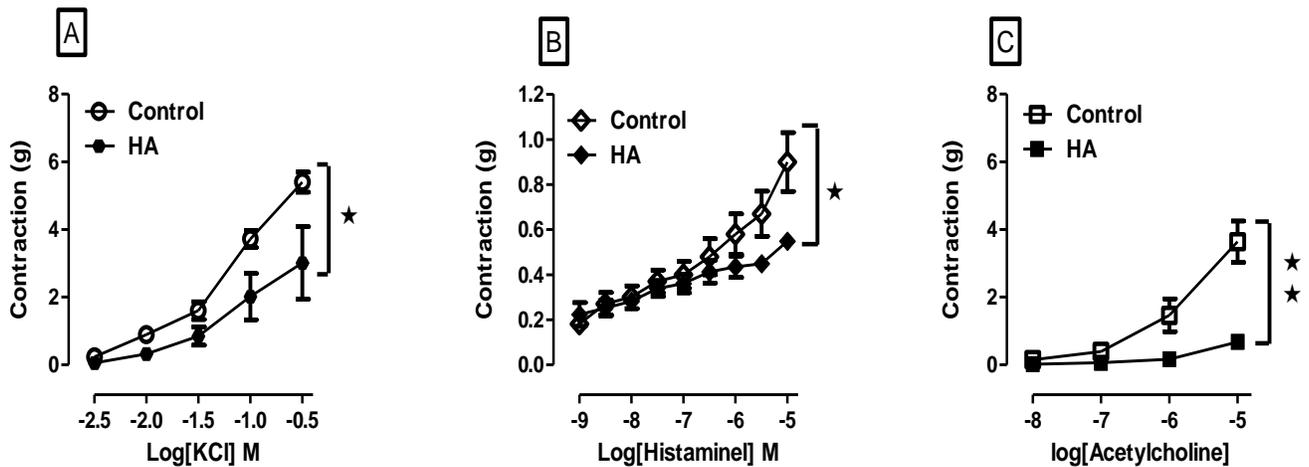

Figure 3. Effects of *Hymenocardia acida*, HA, administered at a dose of 0.1 mg/ml for 30 min on the KCl (A), histamine (B) and acetylcholine (C) dose-response curves of rat isolated tracheal smooth muscle. Results are expressed as mean contraction tension in g ± SEM for at least six experiments obtained from different rats. ns: Not significant and *p < 0.05.
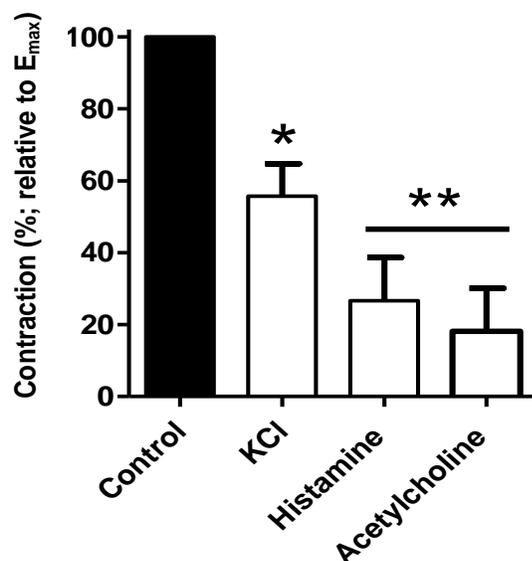
Figure 4. Percent inhibition of contractile responses induced by different agonists after rat trachea impregnation by *Hymenocardia acida* crude extract. Results are expressed as mean ± SEM for at least six experiments obtained from different rats. ns: Not significant and *$p < 0.05$.

validate their preventive role in respiratory disorders particularly asthma, but it also will allow us to eliminate inefficient or potentially dangerous plants.

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