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Original Research

Effect of the Hydroalcoholic Extract from the Leafy Stems of *Waltheria indica* L. (Malvaceae) on Acetylcholine and Barium Chloride-Induced Contractions on Isolated Rat Tracheal Tissue

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Abstract

A previous study has reported the interesting relaxant effect of the hydroalcoholic extract from *Waltheria indica* L. (Malvaceae) leafy stems, a plant with several therapeutic uses. The present study aimed to investigate the preventive effect of this plant using an *ex vivo* model of the rat trachea. Two agonists, acetylcholine (10^{-6} - 1.5×10^{-5} M) and barium (10^{-5}- 10^{-1} M) were used to induce contractions. The preventive effect was assessed on rat tracheal rings pretreated with hydroalcoholic extract (1.92 mg/mL), glibenclamide, atropine, and papaverine, all at 10 µM. Acetylcholine and barium provoke contractions in a concentration-dependent manner, with a maximum contractile response of 3.953 ± 0.692 g and 2.999 ± 0.326 g, respectively. The EC_{50} values were 3.711 ± 0.823 µM and 9.502 ± 12.354 mM, respectively, for acetylcholine- and barium-induced contraction. Glibenclamide caused a rightward shift of the acetylcholine-response curve, followed by a reduction of the maximum contraction (from 3.953 ± 0.692 g to 3. 116 ± 0.244 g). The hydroalcoholic extract, atropine (ATROP), papaverine (PAP), and their combinations induced a complete suppression of the contractile response to acetylcholine ($p < 0.0001$) and barium ($p < 0.0001$). The hydroalcoholic extract exhibited a potent relaxant effect comparable to that of atropine and papaverine. It can be concluded that the hydroalcoholic extract of *W. indica* can potently prevent acetylcholine-and barium chloride-induced contractions. The possible mechanisms by which the extract exerts its relaxant effect may involve the blockade of muscarinic receptors, the inhibition of phosphodiesterase activity, and/or the calcium channel.

Keywords: *Waltheria indica*; Rat trachea; Acetylcholine; Barium chloride; Glibenclamide; Papaverine

Introduction

Worldwide, chronic respiratory diseases account for the majority of common non-communicable diseases. A recent study of the Global Burden of Diseases, Injuries, and Risk Factors estimated the number of people suffering from chronic respiratory diseases at about 545 million people in 2017 [1]. Among chronic respiratory diseases, chronic obstructive pulmonary disease (COPD) and asthma are the most common causes of death worldwide [1,2]. In Burkina Faso, a landlocked nation situated in West Africa, lower respiratory tract diseases, including pneumonia, asthma, and bronchitis, are the leading causes of death, accounting for 14.3% of deaths [3]. Although the

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management of diseases through modern medications exists, the availability and cost associated with several side effects of these treatments have led numerous people in lower-income countries to develop novel therapeutic approaches. Worldwide, many communities have used medicinal plants effectively against significant respiratory disorders [4-8].

Waltheria indica L. (syn. *Waltheria americana*) (Malvaceae) is a short-lived, perennial plant used worldwide against many ailments, including cancer, respiratory, inflammatory, and infectious diseases. The plant has been the subject of research papers and two reviews demonstrating the considerable benefit of using *W. indica* to treat several disorders [9,10]. Previous studies have proved the relaxant effect of hydroalcoholic and aqueous extracts of *W. indica* on Wistar rat tracheal smooth muscle [11,12]. It has been hypothesized that the aqueous decoction of the leafy stems of *W. indica* relaxant effect involves ATP potassium channels. In contrast, other mechanisms are responsible for the relaxant activity of the hydroalcoholic extract [11]. Indeed, despite the blockade of ATP potassium channels, the relaxant effect of the hydroalcoholic extract was enhanced, suggesting other mechanisms of action. The hydroalcoholic extract contains alkaloids, tannins, coumarins, steroids, flavonoids, saponins, and triterpenoids. However, only saponins, flavonoids, and alkaloids were found in the aqueous decoction. Various studies suggested that most of these phytochemicals may alleviate some respiratory diseases and protect the lung from function loss [7,13-16]. *W. indica* is still under investigation in our laboratory. The present manuscript aimed to verify if the hydroalcoholic extract can prevent the acetylcholine (Ach)-and-barium chloride ($BaCl_2$)-induced contractions on rat tracheal tissue. Moreover, the manuscript sought to examine the underlying mechanisms capable of explaining the relaxant effect of the hydroalcoholic extract from the leafy stems of *W. indica*.

Materials and Methods

Drugs and Chemicals

ACh, $BaCl_2$, papaverine, rutin, gallic acid, and aluminum trichloride were purchased from Sigma-Aldrich (St. Louis, MO). Folin-Ciocalteu's phenol reagent was obtained from Carlo Erba (France) and Trolox from Fluke (France). ACh and $BaCl_2$ were used as chlorides. Atropine and potassium chloride (KCl) were obtained from Labosi (France).

Ethanol and methanol were procured from Prolabo (France), and water was distilled in our lab. Sodium carbonate was supplied from Merck (Germany).

A modified Krebs-Henseleit physiological solution prepared in 1L of distilled water was used. The modified solution had the following composition (in mM): NaCl 118; $NaHCO_3$ 24.1; KCl 4.7; KH_2PO_4 1.2; $MgSO_4$ 2.5; $CaCl_2$ 3.33; Glucose 3.33 and the pH was adjusted to 7.4.

Plant material collection and identification

The leafy stems of *W. indica* were harvested in Gomboussougou, located in the Centre-South region of Burkina Faso. The sample was taxonomically identified by a specialist from the Plant Biology and Ecology Laboratory, University of Joseph KI-ZERBO, Burkina Faso. A voucher specimen bearing the number ID 16876 was deposited in the Herbarium of the Laboratory of Plant Biology and Ecology, University of Joseph KI-ZERBO.

Plant material extraction

The leafy stems of *W. indica* were shade-dried at room temperature and got powdered using a grinder. The hydroalcoholic extract (HE) was prepared by maceration. Briefly, 150 g of plant part powder is placed in a water and ethanol mixture (1.5 L) in the proportion of 20: 80 (v/v) at room temperature and under mechanical stirring for 48 h. Then, the extract was filtered, concentrated using a rotary evaporator, and lyophilized. The lyophilisate was stored in a freezer for further biological studies.

Determination of Flavonoid content

Flavonoid content was measured using an aluminum chloride ($AlCl_3$) colorimetric assay [17]. Briefly, 100 µL of methanolic extract (1 mg/mL) was mixed with 100 µL of 2% $AlCl_3$ methanolic solution. The reaction mixture was allowed to incubate at room temperature for 40 min. Then, the absorbance was read at 415 nm with a Bio-Rad model 680 microplate reader against a blank (100 µL of methanol +100 µL of 2% $AlCl_3$). Rutin was used as a standard for the calibration curve and total flavonoid content (TFC) was expressed as mg rutin equivalent per g dry weight (mg RuE/g) of three independent experiments.

Determination of total polyphenol content

The total polyphenol content was quantified spectrophotometrically using the Folin-Ciocalteu reagent [18]. In the presence of phenolics, the Folin-Ciocalteu reagent (FCR) is reduced resulting in the production of molybdenum-tungsten blue whose intensity increases linearly with the concentration of phenolics. The blue color is read in a spectrophotometer at 760 nm [19]. The reaction mixture was prepared with 1 mL of the extract (1 mg/mL) and 1 mL of FCR (2N). After 5 min at room temperature, 3 mL of 20% (w/v) sodium carbonate was added. The mixture was left to incubate for 40 min at room temperature and the absorbance was read at 760 nm using a Shimadzu UV-Vis Spectrophotometer against a blank sample (extract was replaced

by distilled water). Gallic acid was used as standard for the calivration curve and total polyphenol content (TPC) was expressed as mg gallic acid equivalent per g dry weight (mg GAE/g).

Animals

The experimental animals were Wistar strains rats (300–400 g) of either sex obtained from the animal facility of the Institute. The experimental protocols respected the principles and regulations about laboratory animal care and ethical use set in the eighth edition of the Guide for the Care and Use of Laboratory Animals (Guidelines set by the European Union on the protection of animals (CEC Council 86/609). Ethical approval for the research protocol was obtained from the Ethical Committee on the use of animals for research of the University Joseph KI-ZERBO, Ouagadougou, Burkina Faso with agreement code NO CE-UJKZ/2022-06. The rats were maintained under standard laboratory conditions (temperature of 22 ± 3°C, 12/12 h light/dark cycle, and relative humidity of 50 – 70%) with ad *libitum* access to food and water. However, food was drawn back before the experiment, but the animals still had free access to water.

Preparation of tracheal rings and tension measurement

The rats were fasted for 24 h before starting the experiment and anesthetized with ketamine (150 mg/kg of body weight). The tracheal preparations were isolated according to a modified method [20]. Briefly, the chest of the animals was opened, and the trachea was removed and placed quickly in a dish containing the modified Krebs-Henseleit physiological solution. After removing the connective and adipose tissues, the isolated tracheas were cut into five rings of about 5 mm in length. Each ring was mounted between two stainless steel hooks in a 20 mL organ bath chambers containing a modified Krebs-Henseleit physiological solution, continuously aerated and maintained at the temperature of 37°C. An experimental apparatus that included isometric transducers linked to a computer for data acquisition was used to record the isometric force and the changes in isometric force. The tracheal rings were left to equilibrate under an isometric tension of 1 g for one hour, during which the bathing solution was refreshed every 15 min.

KCl at 80 mM was added to the bath during the plateau phase to verify the contractile reactivity of tracheal chains. After that, the tracheal chains were washed several times with fresh Krebs-Henseleit physiological solution, left to recover, and returned to their initial state.

Experimental protocols

The mechanisms by which the preventive effect of the extract was exerted were evaluated using two different contracture compounds, namely ACh and $BaCl_2$. Two experimental conditions were adopted as follows:

(i) On non-incubated trachea rings, and On (ii) incubated trachea rings 15 min prior to the cumulative addition of contracture agents. The incubating substances were the extract, atropine, papaverine, and glibenclamide (GLI)

The antagonist effect of the HE, atropine and papaverine against the contractions elicited by ACh or $BaCl_2$ was determined by cumulative addition of ACh (10^{-6} - 1.5×10^{-5} M) or $BaCl_2$ (10^{-5} – 10^{-1} M) in the organ bath. The concentration-response curves were constructed in the absence or the presence of tested substances. Briefly, the tracheal rings were incubated with the extract at a submaximal concentration of 1.9 mg/mL, corresponding to approximately the $EC_{60\text{-}70}$ concentration obtained from a preliminary study in our laboratory (data not published). Atropine and papaverine were used at respective final concentrations of 10 µM. After 15 min of incubation, ACh (1- 15 µM) or $BaCl_2$ (10^{-5} – 10^{-1} M) were cumulatively added to the organ bath, and isometric contractions were recorded. ACh or $BaCl_2$ concentration was added each 2 min and 10 min, respectively.

To verify the hypothesis that the ATP-sensitive potassium channel is involved in preventing Ach- induced contractions, GLI (final concentration of 10 µM in the organ bath) was incubated for 10 min with the trachea tissue. Then, cumulative ACh concentrations (1- 15 µM) were added.

These different protocols are summarized in table 1.

Data analysis

The results of the quantification of flavonoids and total polyphenols were presented as means ± SD of three (3) experiments. The different pharmacological parameters data were expressed as means ± SD of at least six (6) experiments. The EC_{50} values corresponding to the concentration required to induce 50% of the maximal effect and the dose-response curves for the extract and the tested antagonists were obtained with GraphPad Prism® Software (version 6.07).

Groups were compared by a two-way ANOVA followed by Tukey's test for multiple comparisons between each experimental protocol. Differences were considered significant at a p value less than 0.05.

Results

The total flavonoid content and total polyphenol content

TFC was determined from the equation of the rutin calibration curve, $Y=2.2597x+0.0159$ ($R^2=0.9943$) and TPC from the gallic acid calibration curve, $Y=10.459x+0.0335$ ($R^2=0.9993$). The TFC and TPC

estimation gave respectively 262.638 ± 1.282 mg RuE/g and 443.959 ± 0.125 mg GAE/g.

Effect of the HE, GLI, and ATROP on ACh-induced contractions

ACh (10^{-6} - 1.5× 10^{-5} M) induced a concentration-dependent contraction in tracheal preparations as presented in figure 1.

The maximum contractile response to ACh was equal to 3.953 ± 0.692 g with an EC_{50} value of 3.711 ± 0.823 µM. GLI produced a rightward shift of the ACh dose-response curve, followed by a reduction of the maximum contraction from 3.953 ± 0.692 g to 3. 116 ± 0.244 g. An EC_{50} value of 5.622 ± 0.916 µM was determined. For agonist doses < 4 µM, p values were > 0.05, indicating no statistical difference between ACh and GLI. However, this difference was statistically significant ($p < 0.05$ at agonist dose of 4 µM) and highly significant ($p < 0.0001$) at agonist doses > 4 µM.

The pretreatment of the trachea preparations with ATROP and HE significantly ($p < 0.0001$) almost completely abolished the effect of ACh. Similarly, this result was obtained in the presence of the extract and glibenclamide (HE + GLI).

Comparisons between treatments, i.e., ATROP vs. HE, ATROP vs. HE + GLI, and HE vs. HE + GLI, gave no statistical differences ($p > 0.05$). Otherwise, there was a significant statistical difference ($p < 0.0001$) between the treatment GLI vs. HE + GLI.

Table 2 presents some pharmacological parameters for the different experimental protocols.

Based on their potency to relax the acetylcholine-induced contractions (obtained from table 2), the following classification can be drawn: HE (99%) > ATROP (97%) > HE+GLI (96%) > GLI (21%).

Table 1. Summary of the protocols used to evaluate the hydroalcoholic extract mechanisms of action

Experiment No	Contracture agent	Experiment condition	Incubating substance
1	Acetylcholine (10^{-6} - 1.5× 10^{-5} M)	*Non-incubating trachea rings*	----------
		Incubating trachea rings	1.9 mg/mL HE 10 µM ATROP 10 µM GLI
2	Barium chloride (10^{-5} – 10^{-1} M)	*Non-incubating trachea rings*	----------
		Incubating trachea rings	1.9 mg/mL HE 10 µM PAP

For each experimental condition, n = 6-10. HE: hydroalcoholic extract; ATROP: atropine: GLI: glibenclamide and PAP: papaverine

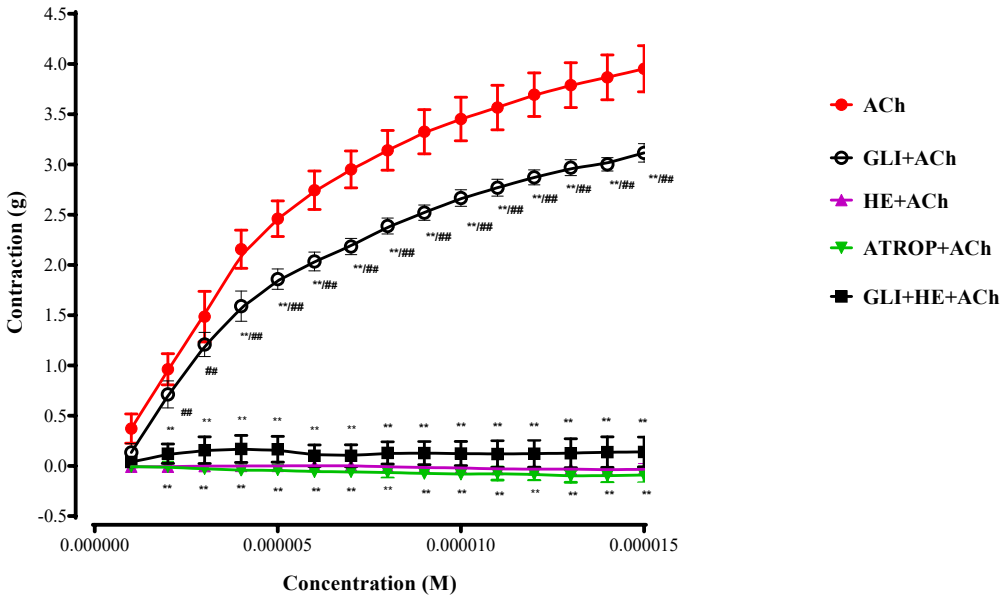

Figure 1. Cumulative concentration-response curves for acetylcholine in the absence (●ACh) and presence of glibenclamide (ɵ GLI), hydroalcoholic extract (▲HE), atropine (▼ATROP), and the combination of glibenclamide+hydroalcoholic extract (■ HE+GLI)

Data are expressed as mean ± SD (n = 6-10 individual experiments). *P < 0.05, **P < 0.0001 compared to ACh alone. ##P < 0.0001 compared to the combination HE+GLI

Table 2. Pharmacological parameters of Ach for the prevention of Rat tracheal ring's contracting

Experimental groups	Parameters		
	EC_{50} (µM)	Emax (g)	% of Contraction
ACh alone	3.711 ± 0.823[a]	3.953± 0.692[a]	100[a,*]
ACh + GLI	5.622 ± 0.916[b]	3.116±0.244[b]	78.838 ± 6.171[b]
ACh + HE	< 1[c]	-0.034 ± 0.180[c]	-0.865 ± 4.545[c]
ACh + ATROP	< 1[c]	-0.091 ± 0.193[c]	-2.290 ± 4.871[c]
ACh + HE + GLI	< 1[c]	0.139 ± 0.396[c]	3.520 ± 10.011[c]

Data are presented as mean ±SD (of at least six (6) independent experiments). Values within columns not followed by the same superscript letters are significantly different at $P < 0.0001$ (a-c, b-c) or $P < 0.05$ (a-b). ACh = acetylcholine; GLI = glibenclamide; ATROP = atropine; HE = hydroalcoholic extract; *ACh alone response was considered as 100% contracted and zero (0%) relaxed before the use of antagonists

Effect of the HE and papaverine on $BaCl_2$-induced contractions

$BaCl_2$ (10^{-5} - 10^{-1} M) concentration-dependently induced contractions in tracheal preparations as shown in figure 2.

Papaverine and HE almost totally inhibited the contractile response of barium chloride response curves. No significant statistical differences were found at lower concentrations of $BaCl_2$ (10^{-5}-10^{-4} M). However, from concentration 10^{-3} to 10^{-1} M, statistical differences were observed between the groups at $p < 0.05$ (concentration = 10^{-3} M: $BaCl_2$ vs. HE; $BaCl_2$ vs. PAP; $BaCl_2$ vs. HE+PAP), and at $p < 0.0001$ (when the concentration = 10^{-2}-10^{-1} M: $BaCl_2$ vs. HE; $BaCl_2$ vs. PAP; $BaCl_2$ vs. HE+PAP). Likewise, a statistical difference ($p < 0.05$) was found between HE and papaverine from 10^{-2} to 10^{-1} M.

The EC_{50} values of barium chloride shifted from 9.502 ± 12.354 mM to a value lower than 0.01 mM (the

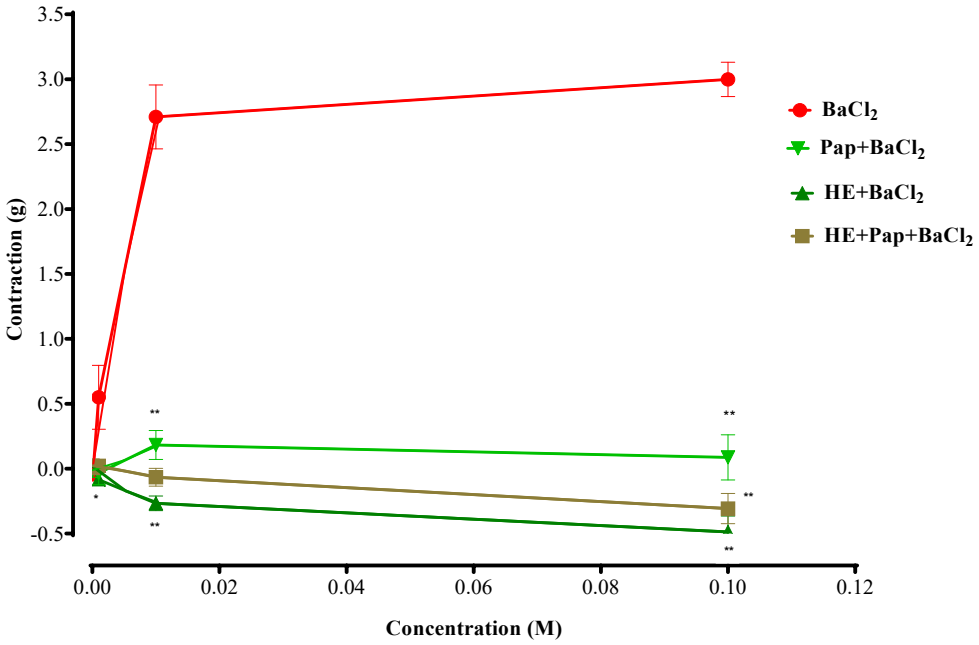

Figure 2. Cumulative concentration-response curves for barium chloride ($BaCl_2$) in the presence and absence of the hydroalcoholic extract (HE), papaverine (PAP), and the combination papaverine+hydroalcoholic extract (PAP+HE). (●$BaCl_2$; ▼Papaverine; ▲ HE; ■ Papaverine + HE)
Data are expressed as mean ± SD (n = 6-10 independent experiments). *P < 0.05 and **P < 0.0001 compared to $BaCl_2$ alone.

Table 3. Pharmacological parameters of $bacl_2$ for the prevention of rat tracheal ring's contracting

Experimental groups	Parameters		
	EC_{50} (mM)	E_{max} (g)	% of contraction
$BaCl_2$ alone	9.502 ± 12.354^{NS}	2.999 ± 0.326^{NS}	$100 \pm 0.000^{NS,*}$
$BaCl_2$ + HE	$<0.01^{NS}$	-0.487 ± 0.273^{NS}	-16.245 ± 9.103^{NS}
$BaCl_2$ + PAP	$< 0.01^{NS}$	0.087 ± 0.602^{NS}	2.895 ± 20.058^{NS}
$BaCl_2$ + HE + PAP	$< 0.01^{NS}$	-0.307 ± 0.371^{NS}	-10.223 ± 12.371^{NS}

Data are presented as mean ±sd (of at least six (6) independent experiments). No statistical difference ($P > 0.05$) was observed between groups within each column. $BaCl_2$ = barium chloride; PAP = papaverine; HE = hydroalcoholic extract; NS = Not significant (as performed by a two-way ANOVA followed by Tukey's multiple comparison test). * = $BaCl_2$ alone response was considered as 100% contracted and zero (0%) relaxed before using antagonists.

smallest $BaCl_2$ concentration used) (Table 3). There were no significant statistical differences ($p > 0.05$) in $BaCl_2$ EC_{50} values and maximal contractile response (E_{max}) between the different experimental treatments (Table 3).

Discussion

Previous studies with different parts of *W. indica* have revealed the interesting effect of this plant on relaxing ACh-and KCl-induced contractions on rat trachea tissue [11,12]. It was demonstrated that the hydroalcoholic extract of the leafy stems was more potent than the aqueous decoction. Therefore, the present research work was planned to explore the possible mechanisms involved in the relaxant effect of this extract. In this respect, the rat trachea preparations were pretreated with various substances, including hydroalcoholic extract, ATROP, GLI, and papaverine.

Ach induces contractions on the rat trachea rings in a dose-dependent manner. The mechanism by which ACh causes contraction is the stimulation of muscarinic M3 receptors present in the airway smooth muscle [21,22]. The stimulation of M3 receptors is accompanied by the increase of intracellular concentration of calcium ($[Ca^{2+}]_i$ through the generation of inositol 1,4,5-trisphosphate (IP_3) [23,24]. IP_3 acts on its receptors and induces calcium release from the sarcoplasmic reticulum [24]. It is well known that ATROP is one of the oldest treatments used to treat asthma because it prevents the binding of ACh on M3 receptors [21,22]. The results demonstrate that ATROP and the HE almost entirely inhibit the ACh-induced contractions. Therefore, this result suggests that the blockade of muscarinic M3 receptors is involved in the relaxant effect of HE.

ATP-sensitive potassium (K_{ATP}) channels are present in various muscle cell tissues, including smooth, cardiac, and skeletal cells [25]. It has been demonstrated that the inhibition of potassium activity leads to the depolarization of membrane potential followed by other biochemical events, including (i) activation of L-type Ca^{2+} channels, (ii) Ca^{2+} release, and (iii) vasoconstriction [25]. GLI exerts different effects on ACh-induced contraction. At low ACh concentrations (i.e. [ACh] <4 µM), GLI had practically no effect on the binding of ACH on muscarinic M3 receptors. However, GLI induces a rightward shift in the concentration-response curve to ACh at high ACh concentration. The maximal contractile response was 21.16% lower than in the absence of GLI. This result is different from some reports that GLI provokes membrane depolarization and contraction [23,25]. In our experimental conditions, it can be hypothesized that at high ACh concentrations, GLI can induce relaxation of the trachea rings probably by preventing the binding of ACh on its receptors or by other underlying mechanisms (eg. inhibition of the release of calcium).

Interestingly, complete relaxation was obtained when GLI and HE were combined. This result supposes that gGLI may potentiate the effect of HE, revealing a synergistic effect, or HE prevents the binding of GLI on the K_{ATP} channel. The extract contains compounds that may act as competitive inhibitors of GLI.

In the second experiment, we examine if the relaxant effect of HE involves the inhibition of phosphodiesterase. To this intent, the trachea rings were incubated with papaverine and contracted with BaCl2. HE and papaverine act synergistically to inhibit the contractile effect of $BaCl_2$.

In various smooth muscle tissues, barium ions induce (*i*) membrane depolarization and (*ii*) opening of the voltage-dependent calcium channels, which in turn result in a calcium influx and contraction [26,27]. Papaverine has been known to relax smooth muscle tissue through the inhibition of phosphodiesterase [28,29]. The results showed that HE induces a significant relaxant effect on the tracheal tissue, similar to that caused by papaverine.

HE contains various phytochemicals such as alkaloids, flavonoids, saponins, tannins, steroids, and triterpenoids [11]. These compounds act probably synergistically to prevent the ACh- and $BaCl_2$-induced

contractions. The estimation of total phenolics and flavonoids demonstrated the richness of HE with natural polyphenols. Regarding the large beneficial effects of polyphenols in human diseases [30,31], the results suggest that these compounds may be responsible for the relaxant effect of the extract as they can modulate or interfere with various cell signaling pathways.

Conclusion

W. indica (Malvaceae) has been claimed to treat respiratory diseases, including asthma. The present study sought to determine the underlying mechanisms capable of explaining the relaxant effect of the hydroalcoholic extract from this plant. The results showed a potent trachea-relaxant effect that may involve the blockade of muscarinic M3 receptors or the inhibition of phosphodiesterase activity but not the ATP-sensitive potassium channels (K_{ATP}). Moreover, it can be assumed that the relaxant effect of this extract may relate to the blocking of the calcium channel. After all, detailed studies are needed to identify more precisely the pharmacological mechanisms of action responsible for the relaxant effect of the hydroalcoholic extract.

Abbreviations

ACh: Acetylcholine; ATROP: atropine; $BaCl_2$: barium chloride; Ca^{2+}: calcium; GLI: glibenclamide; HE: hydroalcoholic extract; IP_3: inositol 1,4,5-trisphosphate; K_{ATP}: ATP-sensitive potassium channel; PAP: papaverine

Conflict of Interests

The authors declared no conflict of interest.

Acknowledgments

None.

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