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Tracheal relaxant effect of column chromatographic elutes of chloroform fraction of *Adhatoda schimperiana* leaves in guinea-pigs

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ABSTRACT
Adhatoda schimperiana has been used in Ethiopian traditional medicine as a remedy for bronchial asthma. In the present study, tracheal relaxant effect of column chromatographic elutes (CCEs) of the chloroform fraction of the leaves of the plant was investigated on guinea-pigs. The intermediate polar CCE of the chloroform fraction showed tracheal relaxant effect as observed by a right-ward shift of the dose-response curve. The maximum response to histamine in presence of the intermediate polar CCE was significantly lower than that of the chloroform fraction ($p<0.05$). These results suggest that bioactivity-guided fractionation could show improved tracheal relaxant activity, and the alkaloid-rich fraction of the crude extract might be responsible for the claimed anti-asthmatic effect of the plant.
KEY WORDS: *Adhatoda schimperiana*, Alkaloid, Bronchial sthma, Guinea-pig trachea

INTRODUCTION

Among several non-infectious respiratory disorders affecting human being, bronchial asthma is the most common chronic disease that can impede breathing (1). Bronchial asthma is an airway inflammatory disease characterized by bronchial hyper-responsiveness, intermittent and reversible airway obstruction that leads to recurrent episodes of cough, wheezing, shortness of breath and chest tightness. It is belived that up to 10% of adults and 20% of children are affected globally (2). The etiology of bronchial asthma appears to have genetic and environmental components (3).

The standard of care in managing bronchial asthma is avoidance of exposure to allergens and non-specific exacerbating factors such as cigarette smoke, cold air, vigorous exercise and sensitizing agents (4). Currently available drugs for mangement of bronchial asthma are of two general categories: drugs that inhibit smooth muscle contraction and anti-inflammatory agents. Despite availability of wide range of drugs, the relief offered is mainly symptomatic and not curative. Moreover, their effects are short lived and the side effects are also quite disturbing. Thus, there is a need to have more effective and safe pharmacological agents that could interfere with the pathogenesis of bronchial asthma. One of these potential sources of therapeutic agents could be traditionally used plants.

Adhatoda schimperiana (Family: Acanthaceae) is a fast growing plant abundant in the highlands of Ethiopia and some other countries of East Africa (5). The plant is an erect shrub up to 4m high and usually much branched from the base. The leaves are simple, opposite and ovate in outline. The decoction of the dried leaves of the plant mixed with local beer ('Tela') is taken as a remedy for bronchial asthma (6). The chloroform fraction of the crude hydro-alcoholic extract of the leaves of the plant was found to have better bronchodilatory and respiratory distress protective effect than the other solvent fractions, and it was also found to be safe when taken orally (7). The

aim of the current study was, therefore, to evaluate trachea relaxant effect of column chromatographic elutes of the chloroform fraction. This could help in finding novel anti-asthmatic compound(s).

MATERIALS AND METHODS

Collection of plant material

Adhatoda schimperiana leaves were collected in February 2006 in Addis Ababa, Ethiopia. The leaves were identified and voucher specimen (No AS-2035) was deposited in the herbarium of the Department of Drug Research, Ethiopian Health and Nutrition Research Institute, Addis Ababa, Ethiopia.

Experimental animals

Guinea pigs (400-600g) were obtained from Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa, Ethiopia. They were kept in an animal house of EHNRI under room temperature of about 24°C. Prior to the experiment, the animals were acclimatized to the test environment for an hour and randomly assigned to a control, test and standard groups. The experiment was conducted in accordance with the accepted international laboratory animals care, and guidelines and rules of the EHNRI for animal experiments.

Drugs and chemicals

Histamine dihydrogen phosphate (Sigma-Aldrich, Germany), diphenhydramine hydrochloride (Loba chem., India), silica gel (Fluka, Switherland), salts for physiological solution (Labort chem., India), bismuth subnitrate and potassium iodide (Mayer and Baker lab., England) were used in the experiment.

Extraction and solvent fractionation

Dried powdered leaves (2.5kg) of the plant were extracted with 5L of 80% (v/v) methanol by percolation. The extract was filtered and concentrated using rota vapor. Half of the crude hydro-alcoholic (HA) extract (325gm) was suspended in 2% HCl (500ml) stirred for an hour and filtered. Alkalinization of the filtrate with 10% NH_4OH (to pH 10) was followed by repeated extraction with chloroform. The yield of the chloroform fraction was 25.5g, 8% of the crude HA extract. Phytochemcial screening indicated the presence of alkaloids in the chloroform fraction.

Column chromatographic fractionation

Fifteen grams of the chloroform fraction was suspended in 100 ml chloroform and adsorbed in 10 gm silica gel (silica gel F_{254}, mesh size 60). The chloroform was removed under reduced pressure and a dry silica-adsorbed sample remained. Silica gel (95 g) slurry was made using chloroform and packed into a column pre-plugged with a small piece of cotton at the bottom and fixed in a clamp. Silica-adsorbed sample was transferred to the column. The column was then filled with eluting solvent and allowed to run at a rate of 40 drops per minute (8).

The column was initially eluted with chloroform to facilitate the elution of pigment materials. This was followed by increasing polarities of a mixture of solvents: chloroform/acetone (10:1), chloroform/ acetone/ diethylamine [(5:2:1), (3:2:1), (2:5:1), and (2:6:1)] respectively. Finally methanol was used to elute the rest components. The column was run continuously and the fractions were collected sequentially in labeled flasks. The solvent was removed under reduced pressure, and components of each chromatographic elute was analyzed by thin layer chromatography (8). Solvent and column chromatographic fractionation is shown in figure 1.

For thin-layer chromatography (TLC), sample from the column chromatographic elute was dissolved in chloroform and spotted onto TLC plates (Silica gel F_{254}) by means of a micro-pipette. The plates were placed and allowed to run in pre-saturated glass tank containing chloroform/acetone/diethylamine (5:2:1). This solvent was chosen for its better resolution of maximum number of constituents with clear separation. The plates were then air dried and examined under UV light of wavelength 256 *n*m and 360 *n*m. Fluorescent spots were en-circled with a pencil. The plates were subsequently sprayed with freshly prepared Dragendroff's reagent and heated for 10 min at 110°C to facilitate the development of coloured spots. The position of the spots on the TLC plate was noted by calculating the retention factor (R_f), the distance the components traveled divided by the distance the solvent traveled from the base (8).

Testing relaxant effect on isolated guinea-pig trachea

Guinea pigs were sacrified by a blow at the back neck against a table edge. The trachea was rapidly removed and kept in Kreb's solution (gm/l): NaCl (6.8), $MgSO_4 \cdot 7H_2O$ (0.25), $CaCl_2$ (0.28), KCl (0.35), $NaHCO_3$ (2.1), KH_2PO_4 (0.16) and glucose (2.0) (9). The trachea was then cut transversely between segments so as to give five rings. A cotton thread was tied to the cartilages to form a tracheal chain. The chain was suspended in a 25 ml thermo-regulated organ bath containing Kreb's solution, maintained at 37°C and supplied with air. One end of the tracheal chain was attached to a tissue holder at the base of the organ bath and the other end to a recording device.

The suspended tracheal chain was allowed to equilibrate for at least an hour. During equilibration,

the bath was supplied with fresh Kreb's solution every 15 minutes (9). Then cumulative concentration-response to histamine (10^{-6}-10^{-2}) M in absence and presence of 400µg/ml concentration of the column chromatographic fractions were recorded. The tissue was exposed to the fractions for 10 minutes before addition of histamine. The responses were recorded with Grass recorder model 7E polygraph with force-displacement transducer equipped with time and event marker. The chart speed was 5mm/minute.

Tracheal-relaxant effect of each fraction was observed from the reduction of contractile effect of histamine. In presence of each fraction, the percentage maximum response to histamine and concentration of histamine that produces half maximum response were compared to those of the control and diphenhydramine, the standard drug.

Statistical analysis

The results of the experiment were expressed as mean ± SEM. For group comparison, analysis of variance followed by Tukey's HSD multiple comparison test with SPSS version 10 was used. The difference among means considered statistically significant when p-value was less than 0.05.

RESULTS

Column chromatographic elutes obtained from the chloroform fraction

Twenty column chromatographic elutes (CCE) were obtained from chloroform fraction (table 1). Thin layer chromatography (TLC) comparison of these CCEs led to their combination into three larger fractions: CCE-A (477mg, 0.25%), CCE-B (489mg, 0.26%), and CCE-C (342mg, 0.18%) containing fractions F_{2-5}, F_{6-10} and F_{11-18} respectively. Fractions F_{19} and F_{20} were discarded since there were no spots observed. TLC of the chloroform fractionate followed by a spray with Dragendroff's reagent revealed the presence of at least three different alkaloid compounds (R_f 0.80, 0.67, and 0.27) which appeared as orange-red spots.

Effect of CCEs on the maximum response to histamine

The maximum response to histamine in presence of 400 µg/ml concentration of CCE-B was significantly ($p<0.05$) lower than that of the control, the crude hydroalcoholic (HA) extract and the chloroform fraction (table 2). In the same concentration, the maximum response to histamine in presence of CCE-A and CCE-C were not statistically significant as compared to the chloroform fraction. There was no statistically significant difference between the maximum response to histamine in the presence of CCE-B and that of diphenhydramine (0.01 µM). In the presence of CCE-B, with further increase in the concentration of histamine up to 1M, it was difficult to achieve 100 % maximum response.

Effect of CCEs on cumulative concentration-response curve of histamine

In presence of 400 µg/ml concentrations of the chloroform fraction and CCE-B, cumulative concentration response curve of histamine clearly shifted right ward with decreased maximum response (figure 2). With activity guided fractionation, the inhibitory effect against histamine-induced contraction was improved. The relaxant effect of CCE-B was better than that of the chloroform fraction.

The effective concentration of histamine producing 50% of the maximum response (EC_{50}) in presence of the chloroform fraction and CCE-B were statistically significant ($p<0.05$) as compared to that of the control (table 3). About 25 times higher concentration of histamine in presence of CCE-B, and about 10 times higher concentration of histamine in presence of the chloroform fraction was needed to achieve the same half maximal response as in the absence of these fractions.

DISCUSSION

The leaves of *Adhatoda schimperiana* has been used traditionally to relieve respiratory disorders such as bronchial asthma (6). In the present study, the chloroform fraction of the crude hydro-alcoholic extract of the leaves of the plant showed tracheal relaxant effect. This is in agreement with bronchodilatory effect of the chloroform fraction obtained by a previous study (7).

The result showed tracheal relaxant effect of the chloroform fraction was higher than that of the crude hydro-alcoholic extract. With further fractionation, the intermediate polar column chromatographic elute (CCE) of the chloroform fraction was found to have better tracheal relaxant effect than the chloroform fraction. The reason for increased activity of the CCE could be due to increased concentration of active components. Bioactivity-directed fractionation could therefore, help isolate and identify active bronchodilatory compounds.

In the presence of the chloroform fraction and the intermediate polar CCE, there was a rightward shift of the concentration-response curve of histamine with reduced maximum response. In addition, in the presence of the intermediate polar CCE, it was not possible to recover the maximum response to histamine in spite of increased histamine

concentration. These results hint the non-competitive or irreversible competitive antagonistic effect of the test substance on histamine H_1 receptors (10). The tracheal relaxant effect might also be mediated through activation or inhibition of ion channels or other receptors, for example, calcium channel blockage might have contributed to the effect observed (11).

A previous phytochemical study showed that the chloroform fraction of the crude hydro-alcoholic extract of *Adhatoda schimperiana* leaves contains alkaloids (7). Our study also revealed the presence of alkaloids in the CCE of the chloroform fraction. From a study on a closely related Indian plant *Adhatoda vasica*, trachea relaxant alkaloids vasicine and vasicinone were isolated (12). Thus, the alkaloid constituents of *Adhatoda schimperiana* may be novel or similar to those of *Adhatoda vasica*.

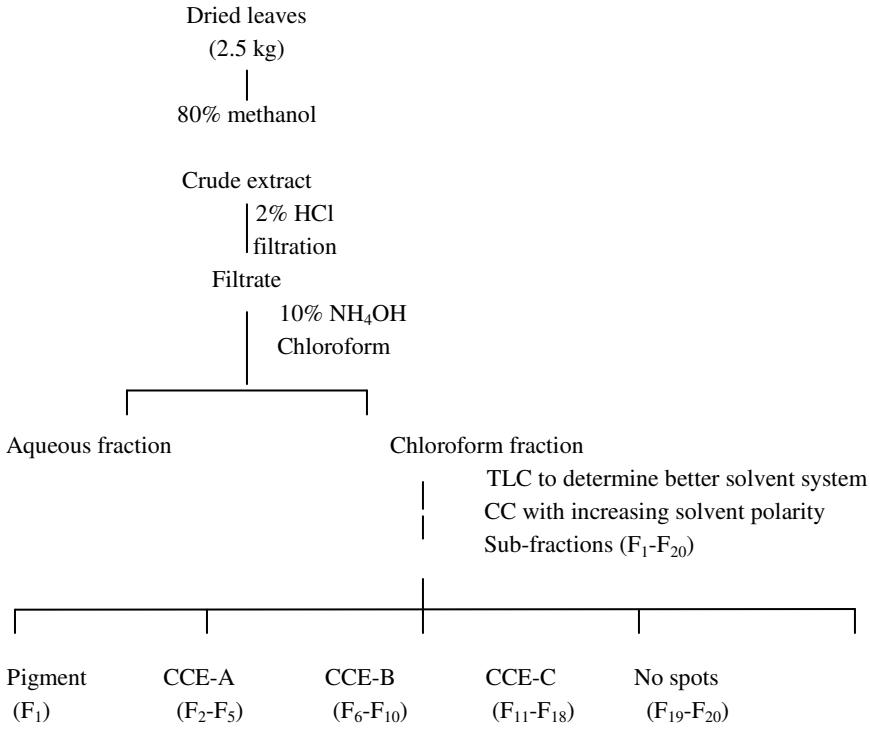

Figure 1: Solvent and column chromatographic fractionation of A. schimperiana

TLC- thin layer chromatography, CCE-column chromatographic elute,

Table 1: Column chromatographic elutes of the chloroform fraction of A. schimperiana

Mobile phase	Volume (ml)	fractions collected	pooled fractions	R_f
Chloroform	150	F_1	Discarded	
Chloroform/acetone (10:1)	290	F_2, F_3	CCE-A	0.80
Chloroform/acetone/diethylamine (5:2:1)	320	F_4, F_5		
Chloroform/acetone/diethylamine (5:2:1)	450	F_6, F_7, F_8	CCE-B	0.67
Chloroform/acetone/diethylamine (3:2:1)	360	F_9, F_{10}		
Chloroform/acetone/diethylamine (2:5:1)	520	F_{11}, F_{12}, F_{13}, F_{14}	CCE-C	0.27
Chloroform/methanol/diethylamine (2:6:1)	480	F_{15}, F_{16}, F_{17}, F_{18}		
Methanol	250	F_{19}, F_{20}	Discarded	

CCE- column chromatographic elute, R_f- retention factor

Table 2: Maximum response to histamine in presence of fractions of A. schimperiana

Vehicle/fraction	Maximum response (%)
Saline (control)	100.0 ± 0.0
Crude HA extract, 400µg/ml	87.7 ± 1.8 [a]
Chloroform fraction, 400µg/ml	77.2 ± 3.7 [a]
CCE-A, 400µg/ml	93.8 ± 1.7
CCE-B, 400µg/ml	64.6 ± 3.2 [a, b, c]
CCE-C, 400µg/ml	84.8 ± 2.4 [a]
Diphenhydramine (0.01µM)	58.6 ± 3.2 [a, b, c]

Values are expressed as means ± SEM, n=6, CCE- column chromatographic elute
a, b, c- p<0.05 compared to the control, crude HA extract and chloroform fraction, respectively

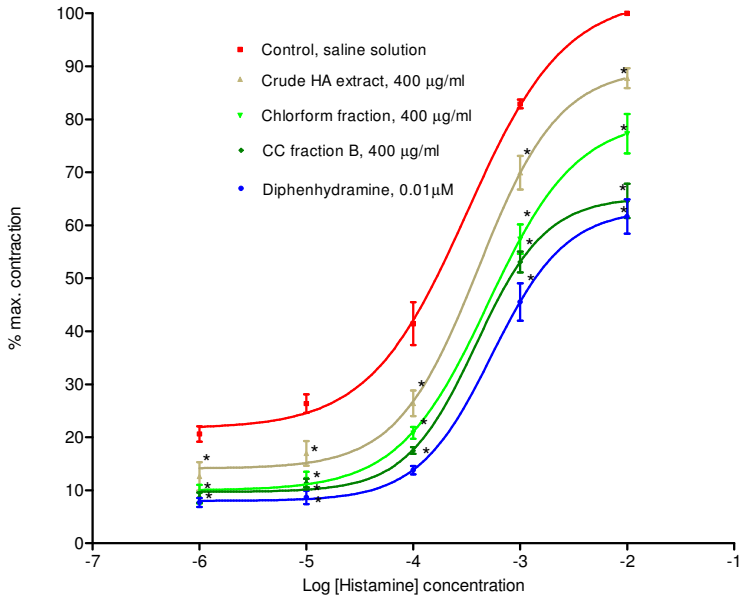

Figure 2: Concentration-response curve of histamine in presence of A. schimperiana fractions
*Data expressed as means ± SEM, n=6, * p<0.05 compared to the control*
HA- hydro-alcoholic, CC- column chromatography

Table 3: EC_{50} of histamine in presence of fractions of A. schimperiana

Solution	EC_{50} (10^{-4} M)
Control (Saline solution)	0.7 (0.6 – 0.8)
Crude HA extract, 400 µg/ml	2.0 (1.9 – 2.1)
Chloroform fraction, 400 µg/ml	6.7 (6.1 – 6.7)*
CCE-B, 400 µg/ml	17.3 (16.4 – 18.1)*
Diphenhydramine, 0.01 µM	46.6 (44.2 – 48.9)*

*Values are expressed as EC_{50} (95 % CI), n=6, * p<0.05 compared to the control*
HA- hydro-alcoholic, CCE- column chromatographic elute
EC_{50}- concentration of histamine producing half the maximum response

In conclusion, the intermediate polar CCE of the chloroform fraction of the crude hydro-alcoholic extract of the leaves of *Adhatoda schimperiana* was found to have trachea relaxant effect in the present study, and the alkaloid components are most likely responsible for the observed effects. Further studies, however, are needed to determine the exact mechanism(s) of action of the active components and to structurally elucidate them.

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