Antitussive activity of *Adhatoda schimperiana* (Hochst.) Nees on ammonium hydroxide-induced cough model in mice

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Abstract - Cough is a natural reflex mechanism that removes foreign materials and secretions from the airways. It is the most common symptom of airway inflammatory diseases. Clinically, cough suppressant drugs are used to relief cough. However, the most frequently used antitussive drugs in clinical practice produce adverse effects like drowsiness, constipation, hypotension and respiratory depression that limit their use. Hence, currently there is unmet need for the development of safe and effective antitussive therapeutic options in the treatment of persistent cough as alternative to existing medications. Medicinal plants, claimed to have antitussive activity from traditional medicine, could be potential sources of alternative therapy.

***Adhatoda schimperiana* (Hochst.) Nees (Family: Acanthaceae) has been used in Ethiopian traditional medicine as a remedy for cough and bronchial asthma. In the present study, antitussive activity of 80% methanol extract of *Adhatoda schimperiana* leaves at various doses (100, 200 and 400 mg/kg/day, orally for 3 days) were investigated by the classic ammonium hydroxide-induced cough model in mice. The antitussive activity was assessed by the latent period of cough and the percent inhibition of cough frequency compared to that of the negative control (Tween 80) and a standard drug (codeine phosphate, 10 mg/kg).**

The crude 80% methanol extract of *Adhatoda schimperiana* leaves showed a statistically significant dose-dependent increase in the latent period of cough ($P < 0.05$), and reduction in cough frequency ($P < 0.05$) compared to that of the negative control group. The maximum antitussive activity of the extract was observed at the dose of 400 mg/kg/day which achieved 57.5% cough frequency suppression. However, the antitussive activity was lower in comparison to that of codeine phosphate, the strongest narcotic antitussive agent which achieved 74.0% cough frequency suppression. The findings of this study suggest that *Adhatoda schimperiana* leaves might be helpful for reducing cough, supporting the claimed traditional use of the plant for the relief of symptoms of respiratory illnesses. Further investigation with activity-guided fractionation is required to elucidate the principal components responsible for the antitussive activity, and to determine the mechanism of action of the active components of the plant.

Key words: *Adhatoda schimperiana* leaves, Ammonium hydroxide, Antitussive, Cough, Mice

Introduction

Cough is a sudden and forceful expiration of air from the lungs caused by an involuntary contraction of the muscles controlling the process of breath [1]. It is a physiological reflex process to remove foreign materials and secretions from the airways [2]. Cough is the most common symptom of various airway inflammatory diseases such as bronchial asthma, bronchitis, chronic obstructive pulmonary disease (COPD) and lung cancer [3]. It could also be symptom of extra-pulmonary conditions such as gastro-esophageal reflux disease (GERD) or heart disorders [4]. In addition, cough could be due to side-effects of certain medications (e.g. Angiotensin-converting enzyme inhibitors) [5], or without any associated cause, often referred to as idiopathic cough [6]. Cough is an important public health concern that can significantly impair quality of life [3], [7]. Cough is associated with reduced comfort, sleep disturbance, nausea, chest pain and fatigue, and patients with chronic cough often experience social discomfort, urinary incontinence, and mood disturbance [8]. There is a significant economic cost for the individual with chronic cough, and the society when it leads to absence from work and lost productivity [4].

Cough reflex is evoked when sensory receptors in the airways are excited (i.e., activation of afferent nerves terminating in the larynx, trachea, and bronchi) by mechanical and/or chemical stimuli [9]. The common mechanical stimuli for these sensory receptors are accumulated mucus and agents that alter mucociliary factors (eg, mucus volume, production, consistency, or ciliary activity) [10]. Airway hyper-responsiveness as a result of inflammation and/or infection in the respiratory system [11] can lead to activation of sensory nerves at the level of the airway lumen following the release of inflammatory mediators, increased mucus secretion or damage to the airway epithelium [5].

The stimuli that initiate the cough reflex stimulate sensory nerve fibers that are broadly divided into three main groups: A-delta fibers respond to mechanical stimuli, C-fibers activated by both mechanical and chemical stimuli, and slowly adapting stretch receptors (SARs) sensitive to mechanical stimuli, particularly stimuli that evoke bronchospasm [5]. Other afferent nerves are also likely to modulate the cough reflex, e.g. Rapidly adapting receptors, are sensitive to mechanical stimuli [4]. Afferent signals are transmitted through the sensory fibers to the cough center in the nucleus tractus solitarius (nTS) in the dorsal medulla within the brain, the brainstem cough-generation system transmits excitatory information to spinal motor neurons innervating respiratory muscles, i.e., from the cough center, the impulses travel through the efferent pathways through the vagal, phrenic, and spinal motor nerves to the expiratory musculature (diaphragm, intercostal, and abdominal muscles) which results in the production of cough [9], [4]. Under inflammatory or disease conditions, many pathological changes can occur around and within sensory nerve fibers, leading to increased excitability or sensitize the cough reflex, leading to heightened response [5].

Cough, apart from being traditionally classified as either productive, i.e. producing mucus usually with expectoration or non-productive (dry) cough, can also be classified based on the duration into acute, sub-acute or chronic [12]. Treatment of cough mainly consists of treating the underlying cause [12]. Hydration of respiratory tract by steam inhalation is helpful in reducing majority of cough symptoms [11]. However, persistent cough is the common reason for seeking medical attention [4]. Antitussives or more often, referred to as cough suppressants are used for symptomatic relief of dry cough by reducing the frequency and intensity of cough by acting centrally and/or peripherally [12]. The most frequently used drugs in clinical practice are the centrally acting agents such as codeine and dextromethorphan that are used mainly to suppress dry, painful, annoying and debilitating cough without influencing the underlying condition [13]. These agents depress the cough center that is located in the medulla, thereby raising cough threshold [13]. Peripherally acting antitussives may act on either the afferent or efferent side of the cough reflex [12]. These agents reduce afferent fiber nerve inputs (inhibit the excitability of airway sensory receptors by resetting the cough reflex sensitivity), or anesthetize the stretch receptors located in the airways, lungs and pleura by dampening their activity and thereby reducing the cough reflex [14].

However, the effectiveness of the antitussive drugs in the Western medicine has been challenged recently, and in general remains unsatisfactory, with questionable clinical benefit despite being the widely used drugs in the world [4, 15]. In addition, adverse effects such as constipation, drowsiness, respiratory depression, decreased secretion in the bronchioles and inhibition of ciliary activity, increased sputum viscosity, decreased expectoration, hypotension, addictive liability, and hallucination (in large doses) limited their therapeutic benefit [14]. Hence, currently there is a serious unmet need for the development of safe, effective antitussive therapeutic options in the treatment of persistent cough as alternative to existing medications [15], [16]. Herbal medicines and active ingredients of natural products have got growing attention as potential therapeutic agents to prevent and treat cough due to their efficacy and low risk of adverse effects [17], [14]. Thus, herbal therapy may be effectively used for the treatment of mild to moderate cases of cough with fewer side effects than the conventional drugs [18].

Adhatoda schimperiana (Family: Acanthaceae), also called *Justicia schimperiana*, is a fast growing perennial herb abundant in the highlands of Ethiopia (local name 'sensel') and some other countries of East Africa [19]. Traditionally, *Adhatoda schimperiana* is used for many diseases locally. The decoction of the dried leaves of the plant mixed with local drink ('Tela') is taken as a remedy for cough and bronchial asthma [20], [21]. It is also used for stomach burning [22], scabies [23], tooth ache [24], wound healing [25], [26] and diarrhea management [22].

So far, many beneficial biological activities were obtained thorough pharmacological screening of the hydro-alcoholic extract of the dried leaves of *Adhatoda schimperiana*. The plant has trachea relaxant and respiratory distress protective effects [27], [28], hepato-protective activity [29], anti-malarial effect [30], [31], [32], anti-diarrheal activity [33], anti-diabetic role [34], anti-inflammatory effect [28], and antibacterial activity against different pathogenic bacterial strains [35], [36]. Phytochemical constituents of this plant have also been studied [28, 37]. The aim of the current study was to evaluate antitussive activity of the hydro-alcoholic extract of *Adhatoda schimperiana* leaves in ammonium hydroxide-induced cough model in mice. This could help in finding novel antitussive herbal product.

Materials and Methods

Plant material collection and Extraction

Adhatoda schimperiana leaves were collected from Semin-mazegaja area 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 Public Health Institute the then called Ethiopian Health and Nutrition Research Institute, Addis Ababa, Ethiopia. Dried powdered leaves of the plant (2.5 kg) were macerated in 80% (v/v) methanol for two days with intermittent agitation. It was then filtered, and the residue was re-extracted with fresh solvent. The combined filtrates were concentrated under vacuum in a rotary evaporator and further dried in a water bath. The yield of the crude extract was 650 g, which is 26% of the dried leaves. The extract was then kept in a refrigerator until antitussive activity is evaluated. For oral administration of the extract, the crude methanol extract of the leaves of the plant was suspended in 3% Tween 80 solution to enhance its solubility.

Animals

In order to investigate antitussive activity of multiple doses of 80% methanol extract of *Adhatoda schimperiana* leaves (MEASL), 6-8 weeks old healthy Swiss albino mice of either sex weighing 20-30 g were used. The mice were subjected to natural lighting conditions (12 hrs light/dark cycles) and kept in the animal house in the Department of Pharmacology, College of Health Sciences - Addis Ababa University. The mice were moved into the experimental room for acclimatization one week before the experiment. Six mice were put in standard transparent plastic cages bedded with a wood chips and equipped with continuous-flow nipple watering devices. The cages were clearly labeled with experimental details and the mice were fed on pellets and water *ad libitum*. The wooden chip dressings in the cages were changed every day.

Pharmacological evaluation

Antitussive activity of 80% methanol extract of *Adhatoda schimperiana*

Antitussive activity of the 80% methanol extract of *Adhatoda schimperiana* leaves (MEASL) was investigated on a standard mouse cough model-induced by ammonium hydroxide liquor [38], [39], [40], with slight modifications. Briefly, each mouse was placed in a desicator with 0.3 mL 25% ammonium hydroxide liquor soaked in a piece of cotton ball for 2 minutes. The mice were then taken out from the desicator and placed in an observation chamber for counting of bouts of cough (cough frequencies) produced within 5 minutes. Cough was detected as a contraction of thoracic and abdominal muscles followed by the mouth opening with a coughing sound and jerking of the front body of the mouse [41]. Mice with coughing 5-20 times in 5 minutes were chosen as eligible animals for the experiments [42], [43].

After 24 hours of recovery, the eligible mice were randomly divided into five groups (6 mice per group) and treated orally for three consecutive days (from 9-10 A.M daily) as follows: mice of control groups were treated with 1mL 3% Tween 80 solution/day, while three dose levels of MEASL (100, 200 and 400 mg/kg/day) were administrated to mice of the test groups. The positive control group was treated with codeine phosphate (10 mg/kg/day). Half an hour after oral administration of the last treatment dose, each mouse was placed in the desicator with 0.3 mL 25% ammonium hydroxide soaked in a piece of cotton ball for 2 minutes. Then, the mice were taken out, the latent period and the cough frequencies produced within 5 minutes were counted. The animals were monitored continuously by the trained technician blinded to the treatments given and number of coughs and latency time to initial cough response were noted. The antitussive activity was assessed as the percentage of inhibition of the number of coughs in terms of that in the control group using the following equation [44]: % Inhibition = $[(C_0 - C_t)/C_0] \times 100\%$, where C_0 and C_t are number of coughs in the control and the treatment groups, respectively.

Statistical Analysis

The results of the study were reported as mean and standard error of the mean. For the comparison of changes in latent period of cough and suppression of cough frequency after expose to ammonium hydroxide-induced cough, analysis of variance (ANOVA) with Tukey's HSD post hoc test using SPSS (version 20.0) were used. Differences between means of treated groups and the control were regarded as statistically significant when *P*-value was less than 0.05 ($P < 0.05$).

Results

The results of the 80% methanol extract of *Adhatoda schimperiana* leaves (MEASL) on ammonium hydroxide-induced cough in the mice test are shown in Table 1. Codeine phosphate (10 mg/kg), the positive control drug, significantly delayed the latent (incubation) period of cough by 155.2%. MEASL increased the latent period of cough in a dose-dependent manner. Pre-treatment with MEASL at doses of 100, 200 and 400 mg/kg/day delayed the latent period of cough by 3.8%, 35.3%, and 92.9%, respectively. The MEASL at moderate and high doses caused a statistically significant delay of the latent period of cough compared to the control group ($P < 0.05$). However, the low dose (100 mg/kg) of MEASL did not show significant delay of the latent period of cough compared with the control.

With regard to the frequency of cough, codeine phosphate (10 mg/kg) significantly suppressed the cough response by 74.0% (Table 1). The MEASL suppressed the cough response in a dose-dependent manner. Pre-treatment with MEASL at doses of 100, 200 and 400 mg/kg caused 9.6%, 24.7%, and 57.5% inhibition of the cough frequency, respectively. The MEASL at moderate and high doses caused a statistically significant reduction in cough frequency compared to the control group ($P < 0.05$). However, the low dose (100 mg/kg) of the MEASL did not show significant antitussive activity compared with the control. The increased latent period of cough and the cough suppressive effect as a result of the MEASL, at the experimentally used treatment doses, however, were less than that of codeine phosphate.

Table 1. Effects of 80% methanol extract of *Adhatoda schimperiana* leaves (MEASL) on ammonium hydroxide-induced cough in mice

Group	Dose (mg/kg)	Latent period of cough		Frequency of cough	
		Time (Seconds)	Increase (%)	Number	Inhibition (%)
Control	Tween 80	75.2 $\pm$ 3.4	-	12.2 ± 0.8	-
MEASL	100	78.0 $\pm$ 4.7	3.8	11.0 ± 0.6	9.6
	200	101.7 $\pm$ 3.0*	35.3	9.2 ± 0.7*	24.7
	400	145.0 $\pm$ 10.0**	92.9	5.2 ± 0.4**	57.5
Codeine phosphate	10	191.8 $\pm$ 3.2.0**	155.2	3.2 ± 0.6**	74.0

Values expressed as Mean $\pm$ Standard error of the mean (n = 6)
$^{*}P < 0.05$ and $^{**}P < 0.01$ for comparison of treated groups with the control group

Discussion

Since long time, human beings have been relying on plants as therapeutic resource to restore and maintain health, and plants are well known as important source of many biologically active compounds [45]. In fact 25% of all medical prescriptions are based on substances derived from plants or plant derived synthetic analogues [46]. There has been a growing interest in plants as a significant source for the discovery of new drugs [47]. Most of the drugs from plants which have become important in modern medicine had a folklore origin in traditional medicine [45]. Research on plants with medicinal properties and identification of the chemical components responsible for their activities have supported the traditional uses of ancient healing knowledge and have proven the continuing healing potential of many plant medicines even in today's hi-tech community [48]. The correct identity of the crude herbal material and standardized extracts, the validation of the popular uses and their safety, contribute to the development and rational use of phytomedicine [49].

It has been estimated by World Health Organization (WHO) that approximately 80% of the world's population, mainly residing in developing countries, still depends on the complementary and alternative systems of medicine, while about half of the population in industrialized countries use herbal medicines [50]. Ethiopia is among those developing countries where traditional medical practice is significantly utilized [51]. While it is apparent that there exist different medicinal plants in Ethiopia claimed to have cough suppressive effect, there are limited studies conducted to evaluate their activity. The aim of the present study was, therefore, to investigate the antitussive activity of the 80% methanol extract of *Adhatoda schimperiana* leaves (MEASL) at various dose levels (100, 200 and 400 mg/kg, orally) by the classic ammonium hydroxide-induced cough model in mice for validation of traditional use of the plant. The extract showed significant inhibition of cough like the standard drug (codeine), in a dose-dependent manner.

Antitussive animal models could be established by mechanical, electrical or chemical stimuli [40], [52]. Of these methods, chemical stimulation is more similar to the physiological event than is obtained with other tussigenic stimuli, and is also the experimental model used most often [53]. In this model, the cough incubation period and the frequency of cough are used as assessment indices [53]. Ammonium hydroxide-induced cough model, which we used in the current study, is a commonly used chemical stimuli model with relatively easy procedure for evaluating the antitussive effects of bioactive components in new drug development from traditional medical practice [3], [40]. This model is a valid method used previously by several investigators [49], [38], [39], [40]. Therefore, in the current study, the antitussive activities of MEASL were demonstrated in the *in vivo* experiment by prolonging the incubation time, and reducing coughing frequency in ammonium hydroxide-induced cough model. In this experiment, a strict selection procedure was used for the mice enrolled in the study to ensure the exclusion of insensitive or oversensitive animals, i.e., animals having low or high cough threshold were not entertained for further studies [15]. The model also required careful observation to monitor the mice as the cough reflex is observed as a contraction of abdominal muscles [44], [54], [41].

In our study, the MEASL at moderate-to-high doses significantly prolonged cough incubation time, and reduced coughing frequency in mice in dose-dependent manner. The cough incubation period exhibited the potential of the drug on delaying cough. The longer cough incubation period showed stronger effect of the extract on relieving cough, and the less cough frequency exhibited its stronger antitussive effect. To our knowledge, the current study is the first to report the antitussive effects of *Adhatoda schimperiana* (Hochst.) Nees leaves using animal models. This study indicates that the antitussive pharmacological properties of the extract of *Adhatoda schimperiana* might be helpful for reducing cough, and support the popular traditional use of this plant in cough related to respiratory illnesses in the Ethiopian traditional medical practice.

Phytochemical screenings of the crude MEASL done before revealed that it is rich in alkaloids, glycosides, steroids, terpenoids, and saponins [28] [37]. One or more of these secondary metabolites of *Adhatoda schimperiana* leaves may be responsible for the antitussive bioactivity of the plant. From a study on a closely related Indian plant *Adhatoda vasica*, the crude methanol extract of the plant showed antitussive activity [55], [56].The vast variety of pharmacological uses of *Adhatoda vasica* including trachea relaxant and antitussive effects are believed to be the result of its rich concentration of alkaloids isolated, mainly vasicine and vasicinone [57]. Hence, the alkaloid constituents of *Adhatoda schimperiana* may be novel or similar to those of *Adhatoda vasica*. On the other hand, the secondary metabolites responsible for the trachea relaxant effect [27], respiratory distress protective effect [20], anti-inflammatory [28], spasmolytic [33], and antibacterial effects [35] of *Adhatoda schimperiana* leaves identified in earlier studies may contribute for the antitussive activity of MEASL. Further investigation with activity-guided fractionation of the crude MEASL is required for elucidating the principal ingredient(s) responsible for the antitussive activity.

A previously study conducted to evaluate acute toxicity effect of the crude MEASL in mice showed that no acute or delayed toxic symptoms were observed after an oral administration of up to 5000 mg/kg as a single dose [20], and the currently estimated effective antitussive dose is very much lower than the median lethal dose on experimental mice. This may support the safe use of the plant as an antitussive herb in Ethiopian traditional medical practice. It may also shows that the plant may be a good candidate for future work-up as a standardized antitussive herbal product, and to find lead compounds. Further investigation with activity-guided fractionation of the crude extract is required to elucidate the principal components responsible for its antitussive activity, to determine the mechanism of action and to structurally elucidate the active components.

Conclusion

In the present study, the methanol extract of *Adhatoda schimperiana* leaves has shown promising dose-dependent antitussive activity against ammonium hydroxide-induced mouse model. The results of this study support the traditional use of the plant for cough related to respiratory illnesses in Ethiopia. Further investigation is required for designing a standardized herbal preparation for local use.

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