

In Vitro and In Vivo Evaluation of the Anti-Asthmatic Activities of Fractions from *Pheretima*

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Abstract:

Pheretima (family Megascolecidae) has been documented as a potent agent for the treatment of cough and breathing difficulty in traditional Chinese medicine for nearly 2000 years. The water extract of *Pheretima* was separated into three fractions of the ethanolic precipitate, the alkaline fraction and the acidic fraction. Among the three fractions, the acidic fraction showed the most potent spasmolytic effects on histamine-induced contractions in isolated guinea pig tracheal rings, and the most inhibitory activities on increase of short circuit current induced by carbachol in isolated rat tracheal epitheliums with the IC₅₀ values of 0.15 and 0.08 mg/ml, respectively. Further in vivo studies also displayed that the acidic fraction could protect experimental asthma model induced by the combination of histamine and acetylcholine chloride in guinea pigs to prolong the latent periods of asthma ($P < 0.05$) and significantly decrease the cough frequency caused by ammonia water in mice ($P < 0.001$).

Article:

INTRODUCTION

Asthma is a chronic inflammatory disease of the respiratory tract that is characterized by increased airway hyper-responsiveness and mucus production that leads to episodes of wheezing, coughing and shortness of breath. Asthma is common among the individuals (up to 10% in adults and 35% in children), and as a result, large quantities of asthma medications are needed to stop the asthma prevalence increase (Annesi-Maesano, 2005). Because of little adverse effect compared to those of steroids or other synthetic drugs to prevent and treat such repetitious chronic disease, there are increasing demands for the use of traditional natural drugs in the therapy of asthma. In traditional Chinese medicine (TCM), *Pheretima* (family Megascolecidae) has been documented as an anti-asthmatic remedy in the earliest material medica "Shennong Bencao Jing" (Donghan dynasty, about 1900 years ago). It has been acclaimed that *Pheretima* is a potent agent for the treatment of cough and breathing difficulty owing to various exogenous or interior heat reasons in the folk medicine. Four different kinds of earthworms, *Pheretima aspergillum* (E. Perrier), *Pheretima guillelmi* (Michaelsen), *Pheretima vulgaris* Chen and

Pheretima pectinifera Michaelsen are collected in the Pharmacopoeia of People's Republic of China (2005, edition I) for anti-asthmatic remedies and all named as *Pheretima*. Among the four kinds, the first one is conventionally called as Guang-*Pheretima* (mainly collected from Guangdong province, China) while the rest of ones are called as Hu *Pheretima* (collected from Shanghai area).

Dried powder and hot water extract of *Pheretima* are the two main pharmaceutical forms to manage asthma in TCM (Li et al., 1997), however, the active components and the pharmacological mechanisms have not been thoroughly investigated so far due to its complexity of the ingredients. The present study was undertaken to separate *Pheretima* into three parts of extracts, and to screen the active parts of *Pheretima* with spasmolytic effects on the carbachol and histamine contracted guinea-pig tracheal smooth muscles in vitro and with mucus secretion inhibitory effects on carbachol-evoked isolated rat tracheal epitheliums in vitro. Additionally, the protective effect of the acidic fraction on broncho-constriction and the antitussive effect were evaluated in vivo.

MATERIALS AND METHODS

Collection and preparation of material

The materials were purchased from Huayu Pharmaceutical Company in Shanghai. The authenticated samples were identified as *Pheretima vulgaris* Chen by Dr. Zhong Liu, Associate Professor in Pharmacognosy at School of Pharmacy, Shanghai Jiao Tong University (SJTU) and the voucher specimen (SJTU 05-12-01) were deposited in the Herbarium of School of Pharmacy, SJTU. The dried earthworms were previously eviscerated with the viscera and organic components washed away by the supplier, and reduced to coarse powder in our laboratory for experimental use. Other chemicals reagents were obtained from Sigma Chemical Co. The test samples were dissolved in distilled water, and distilled water served as negative control. All animal experiments were conducted in accordance with the internationally accepted principles for laboratory animal use and care in the European Community guidelines (EEC Directive of 1986; 86/609/EEC) and all efforts were made to minimize animal suffering.

Extraction and separation of three fractions from Pheretima

Each of the extractive fractions of *Pheretima* was obtained according to the procedure described in Fig. 1. The yields were expressed as the weight of prepared extracts in the total weight of starting crude material, specifically, 6.13% ethanolic precipitate, 7.25% alkaline fraction and 13.23% acidic fraction respectively.

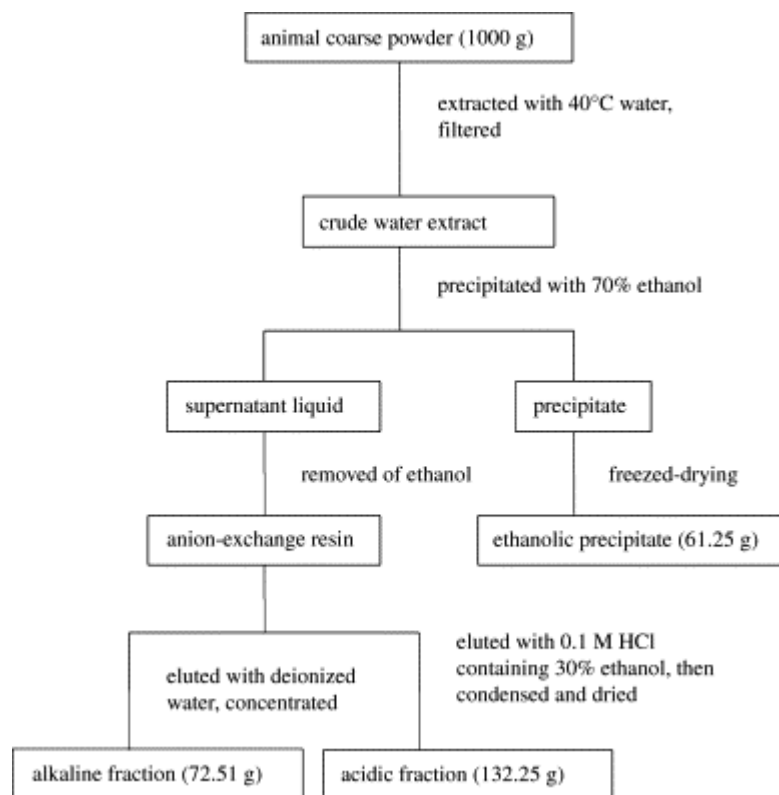


Figure 1: Procedures for isolation of ethanolic precipitate fraction, acidic fraction and alkaline fraction from dried *Pheretima* powder.

Analysis of three fractions from Pheretima

Analysis of the acidic fraction with pH 4 of *Pheretima* was performed using combined gas chromatography and mass spectrometry (GC–MS) method with ethyl chloroformate (ECF) derivatization agents (Qiu et al., 2007) which revealed that the extract consists of amino acids and fatty acids. Chemical analyses indicated that alkaline fraction with pH 11 contains mainly guanidines, and the ethanolic precipitate fraction contains mainly proteins.

Protocols

Spasmolytic tests of three fractions in vitro. Guinea pigs of male sex (200–250 g) starved overnight, but free access to water, were used. The animals were sacrificed by intraperitoneal injection of sodium pentobarbital, and the section of trachea between the larynx and sternum was removed and dissected into 6–8 small rings. The small rings were then suspended in a 8 ml organ bath containing K–H (Krebs–Henseleit) solution of the following composition (mM): NaCl 118.4, KCl 4.7, KH₂PO₄ 1.2, NaHCO₃ 25.0, CaCl₂ 2.5, MgSO₄ 1.2, glucose 11.1, pH 7.4 ± 0.05. The K–H solution was maintained at 37 °C and gassed with 95% O₂ and 5% CO₂. Tissue was suspended under isotonic tension of 1.5 g and allowed to equilibrate for at least 1h before experiment during which the solution was replaced for once a time. Maximum contraction was elicited by carbachol (10 µM) or histamine (10 µM) in each tracheal ring at the beginning of the experiment. The responses of the extracts (up to 2.03 mg/ml) on elicited contraction were recorded after exposing tissue to the dose-accumulating solution for 5 min. The spasmolytic effects were examined by producing cumulative log concentration–response curve. A decrease in

tone was considered as relaxant effect (Fig. 2). The relaxant responses were expressed as percentages of inhibition on maximal contraction produced by the agonists. Responses were recorded on ML740 (Power Lab data acquisition systems) with Chart V 3.4.6 recorder and analysis software (Shibata et al., 1998).

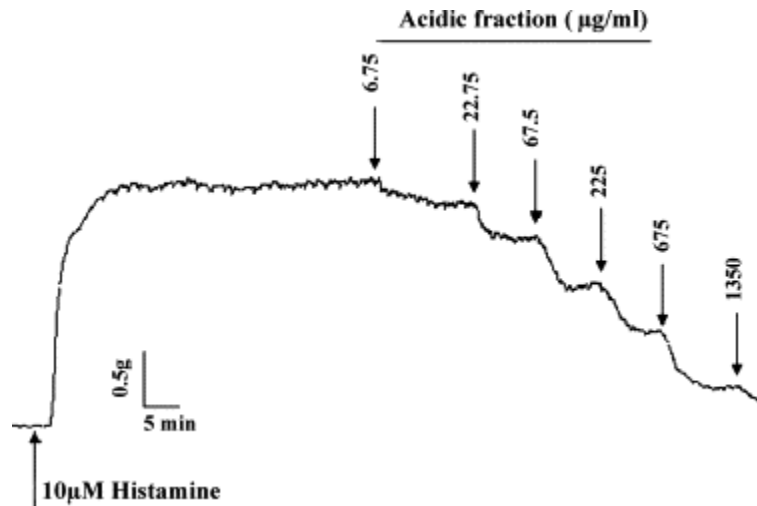


Figure 2: Representative of tracings of tension in isolated guinea pig tracheal rings evoked by 10 μM histamine resulting in a significant increase in tension. After the increased tension was sustained at relatively stable levels, the acidic fraction solution with increasing concentrations was added, leading to a decrease tone in dose-dependent manner. The down arrows showed cumulative concentration of the extract in the organ bath.

Transepithelial ion transport tests of three fractions in vitro. Male Sprague Dawley rats (200–250 g) were sacrificed by intraperitoneal injection of sodium pentobarbital. The distal two-thirds of the tracheas were separated, longitudinally opened on the ventral side and mounted in modified Ussing chambers. The lucite chambers exposed 0.1 cm^2 tracheal surfaces to separate reservoirs (5 ml) containing gassed (95% O_2 , 5% CO_2) K–H solution (in mM: NaCl 115, K_2HPO_4 2.2, KH_2PO_4 0.4, NaHCO_3 25, CaCl_2 1.2, MgCl_2 1.2, glucose 5.1, pH 7.4 ± 0.05) at 37 $^\circ\text{C}$. Constant potential pulses (mV) were passed through the samples using a pulse generator. The transepithelial short circuit current (I_{sc}) and potential were measured using CEZ-9100 amplifiers (Physiologic Instruments, San Diego, CA) and a voltage current clamp (VCC MC-2, Physiologic Instruments, San Diego, CA) connected to the mucosal and submucosal solutions by calomel half cells and 3 M KCl–4% agar bridges, with tips closely opposed to the tissue sample (Roche et al., 2000). The transepithelial resistance (R_{te}) was calculated according to Ohm law ($R_{te} = \Delta V_{te}/I$). After the establishment of a steady state, as indicated by maintenance of stable basal I_{sc} readings, 10 μM carbachol was added to evoke a sharp increase in I_{sc} . When the increases in I_{sc} were sustained at relatively stable levels for at least 10 min, one of the test extracts (ethanolic precipitate, alkaline or acidic fraction) with increasing concentration was added to the mucosal reservoir at 10 min intervals (final concentration up to 2.00 mg/ml) (Fig. 3). Tested extracts remained fully soluble in the bathing solution for the duration of the experiment. Tissues with abnormal baseline values of I_{sc} or conductance were considered as damaged and excluded. Curves of cumulative concentration–response relationships were generated by measuring the peak to baseline increases in I_{sc} induced after each concentration of test extract was added cumulatively to the serosal bath.

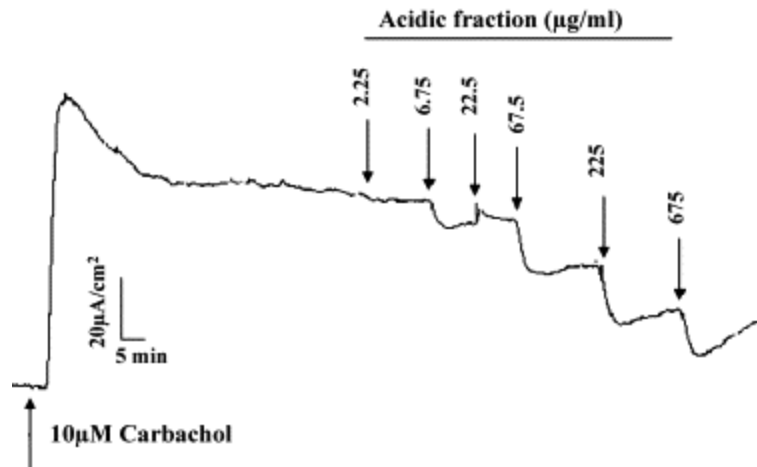


Figure 3: Representative of tracings of I_{sc} through isolated rat tracheal epithelium challenged with 10 μM carbachol resulting in a significant increase in I_{sc} . After the increases in I_{sc} were sustained at relatively stable levels, the acidic fraction solution with increasing concentrations was added to the mucosal reservoir, leading to a decrease tone in dose-dependent manner. The down arrows showed cumulative concentration of the extract in the mucosal reservoir.

The bronchoprotective tests of acidic fraction in vivo. To screen the sensitivity of guinea pigs, guinea pigs of both sex (150–200 g) were placed in a plexiglass chamber and sprayed with 0.1% histamine and 2% acetylcholine chloride mixed with the same volume under the average pressure of 450 ± 50 mmHg for 15 s. The time to onset of respiratory distress (preconvulsive time) during challenge with these agents was measured, the guinea pigs with preconvulsive time of more than 120 s were considered to be insensitive and discarded. The eligible guinea pigs were randomly allotted to different groups with 10 per each. The negative control of animals received distilled water orally, and the positive control animals received aminophylline by gastric perfusion, the other two groups were treated with acidic fractions of low and high doses respectively. All groups were treated with a single dose daily for 3 days prior to the challenge, the last dose given 1 h before the challenge. The methods of challenge were the same as those of screening the sensitive guinea pigs. The delitescence of convulsion for each guinea pig and tumble numbers for each group during challenge within a 6 min exposure period were recorded. Protection from convulsion was expressed relative to control (Okpo and Adeyemi, 2002).

Antitussive tests of the acidic fraction in vivo. ICR mice of both sex weighing about 20 g were divided into four groups, 10 mice per group. The negative control of animals received distilled water orally, and the positive control received pentoxyverine, the remaining groups received low and high doses of acidic fraction by gastric perfusion respectively. The in vivo antitussive activity was investigated on a classical mouse cough model induced by ammonia liquor (Xu et al., 1991). Briefly, mice were placed in a special glass chamber and exposed for 5 s to a 28% NH_4OH aerosol which was produced through a nebulizer by compressed air at a pressure of about 400 mmHg. The cough frequency produced during a 3-min exposure period was counted. The cough frequency for the test groups (C_1) was compared with those of control group (C_c), the antitussive effect was expressed as below: % inhibition = $(C_c - C_1)/C_c \times 100$.

Statistical analysis

Data from experiments was analyzed and expressed as mean \pm S.E.M., paired *t*-tests and the software of origin 7.0 were employed.

RESULTS

Spasmolytic effect of three fractions in vitro

On carbachol-contracted isolated guinea pig tracheal rings, the maximal bronchodilatory effects of all three fractions were statistically significant as compared to those of control (Table 1), indicating that all three extracts had bronchodilatory effects on carbachol-induced contraction dose-dependently (Fig. 4). The order of bronchodilatory effect was acidic fraction > alkaline fraction > ethanolic precipitate (Table 1).

Table 1: Comparison of maximal relaxations of the extracts on isolated guinea pig tracheal rings contractions induced by two different agonists (*n* = 6)

Group	Carbachol		Histamine	
	Dose (mg/ml)	Relaxation (%)	Dose (mg/ml)	Relaxation (%)
Control	–	8.29 \pm 0.34	–	12.08 \pm 3.13
Acidic fraction	1.35	35.53 \pm 4.07**	1.35	78.28 \pm 3.75**
Alkaline fraction	1.05	27.47 \pm 3.01**	0.53	26.47 \pm 8.87 $\Delta\Delta$
Ethanolic precipitate	1.35	24.58 \pm 7.75*	0.45	41.74 \pm 12.01* Δ

Values are presented as mean \pm S.E.M. **P* < 0.05 and ***P* < 0.01 statistical significance vs. control; Δ *P* < 0.05 and $\Delta\Delta$ *P* < 0.01 statistical significance vs. the acidic fraction.

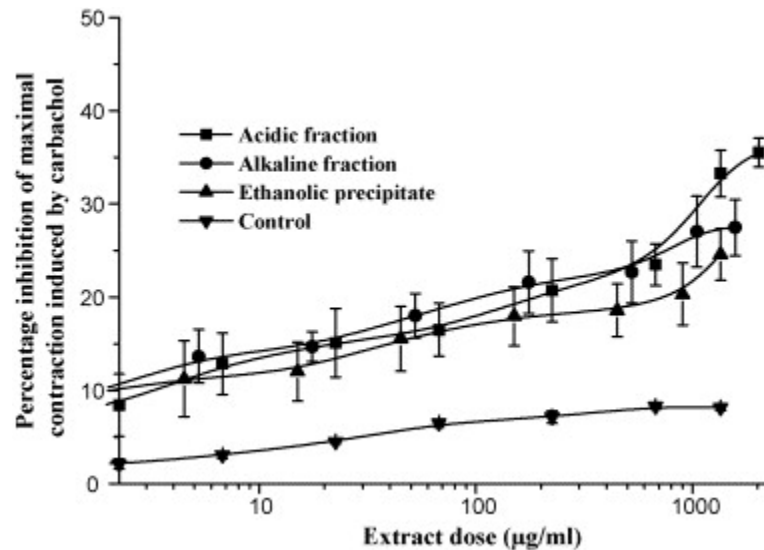


Figure 4: Effects of cumulative addition of extracts on carbachol-induced contraction in guinea pig tracheal rings.

On histamine-contracted isolated guinea pig tracheal rings, the maximal bronchodilatory effects of the acidic fraction and ethanolic precipitate were statistically significant as compared to those of control (Table 1), suggesting that the two fractions had relaxant effects on histamine-contracted tracheal smooth muscles. Additionally, the inhibitory effect of the acidic fraction on histamine-induced contraction with IC_{50} (the concentration causing 50% inhibitory effect) being 0.15 mg/ml was much more potent than that of the ethanolic precipitate fraction (Table 1) without producing 50% inhibitory response (Fig. 5). On the other hand, the effect of the alkaline

fraction was not statistically significant as compared to that of negative control (Table 1), suggesting that the alkaline fraction might have little or no bronchodilatory effect on histamine-evoked contraction.

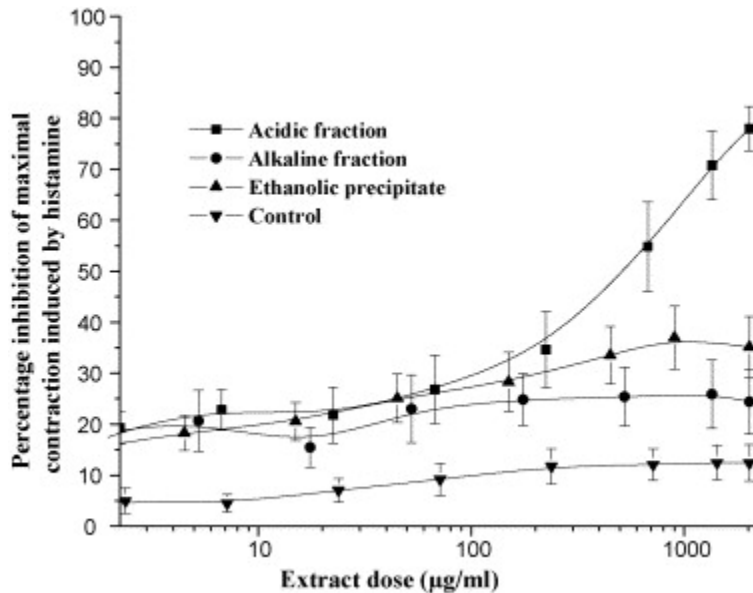


Figure 5: Effects of cumulative addition of extracts on histamine-induced contraction in guinea pig tracheal rings.

The above results suggested that the acidic fraction was the most effective fraction with spasmodic activity on isolated guinea pig tracheal rings in dose-dependent manner, and the maximal relaxant effect of 78.00% on histamine-induced contraction is greater than that (35.53%) on carbachol-induced contraction.

Inhibitory effects on carbachol-induced I_{sc} in vitro

Baseline values for I_{sc} , V_t and R_t in intact rat tracheas ($n = 7$) were $34.4 \pm 6.0 \mu A/cm^2$, $-5.3 \pm 0.6 mV$, $106.8 \pm 12.0 \Omega cm^2$ respectively. The $10 \mu M$ carbachol evoked a rapid I_{sc} increase response within 2 min. The maximal reduction in carbachol-induced I_{sc} by the acidic fraction were significantly different from that of control (Table 2, Fig. 6), indicating that this fraction had inhibitory effect on I_{sc} increase evoked by carbachol, in a dose-dependent manner (Fig. 3) with IC_{50} 0.08 mg/ml. The maximal reduction in carbachol-induced I_{sc} by the alkaline and ethanolic precipitate fraction were not statistically significant compared with control (Table 2, Fig. 6), suggesting that the alkaline fraction and ethanolic precipitate had little or no effect on ion transport evoked by carbachol.

Table 2: Comparison of maximal inhibition of extracts with corresponding doses on carbachol-induced I_{sc} increases through isolated rat tracheal epitheliums ($n = 6$)

Group	Dose (mg/ml)	Inhibition (%)
Control	–	20.72 ± 6.33
Acidic fraction	0.68	$83.76 \pm 6.52^{**}$
Alkaline fraction	0.53	$34.60 \pm 9.54^{\Delta\Delta\Delta}$
Ethanolic precipitate	0.45	$20.73 \pm 4.34^{\Delta\Delta}$

Values are presented as mean \pm S.E.M. $**P < 0.01$ statistical significance vs. control; $\Delta\Delta P < 0.01$ and $\Delta\Delta\Delta P < 0.001$ statistical significance vs. the acidic fraction.

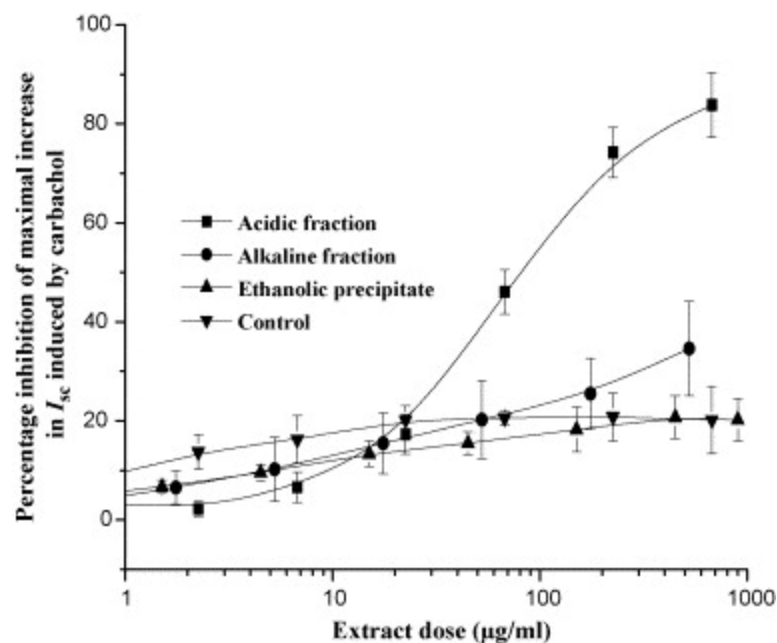


Figure 6: Effects of cumulative addition of extracts on carbachol-induced increase in I_{sc} through isolated rat tracheal epitheliums.

Spasmolytic effects of the acidic fraction in vivo

The effects of the acidic fraction on sensitive guinea pigs exposed to mixture spray of 0.1% histamine and 2% acetylcholine chloride were shown in Table 3. A high dose (504 mg/kg) of this fraction produced good prolongation effects of preconvulsive time ($P < 0.001$), not significantly different from that of the positive control, aminophylline. On the other hand, in this dose, this fraction decreased the tumble numbers of guinea pigs compared to vehicle, which also showed a bronchoprotective effects on mixture of histamine and acetylcholine chloride induced bronchoconstriction. However, at the low dose (252 mg/kg), this fraction did not exhibit significant bronchoprotective effect.

Table 3: Effect of acidic fraction on guinea pigs bronchoconstriction induced by mixture spray of histamine and acetylcholine chloride ($n = 10$)

Group	Dose (mg/kg)	Tumble numbers	Preconvulsive time (min)
Negative control	–	10	45.57 ± 3.48
Aminophylline	171	6	78.00 ± 3.89***
Acidic fraction	252	9	54.17 ± 4.67 ^{△△}
	504	5	79.86 ± 11.71*

Values are presented as mean ± S.E.M. * $P < 0.05$ and *** $P < 0.001$ statistical significance vs. negative control; ^{△△} $P < 0.01$ statistical significance vs. positive control.

Antitussive effects of the acidic fraction in vivo

The cough frequency obtained in the presence of low dose (252 mg/kg) and high dose (504 mg/kg) of acidic fraction of *Pheretima* extract compared to those obtained in the presence of vehicle and positive control were shown in Table 4, both groups showed significant antitussive activities ($P < 0.01$ and $P < 0.001$) which were comparable with that of positive control, pentoxyverine.

Table 4: Effect of acidic fraction on cough mice induced by ammonia ($n = 10$)

Group	Dose (mg/kg)	Cough frequency within 3 min	Inhibition (%)
Negative control	–	66.40 ± 3.08	–
Pentoxyverine	33.33	45.78 ± 3.45**	31.05
Acidic fraction	252	45.60 ± 3.91**	31.33
	504	45.17 ± 3.30***	31.97

Values are presented as mean ± S.E.M. ** $P < 0.01$ and *** $P < 0.001$ statistical significance vs. negative control.

DISCUSSION

In the present study, the water extract from *Pheretima* was separated into three fractions of the ethanolic precipitate, the alkaline fraction and the acidic fraction over anion resin chromatography. Through the pharmacological evaluation of relaxant effects on contraction induced by histamine and carbachol on isolated guinea pig tracheal rings as well as the inhibitory effects on carbachol-induced I_{sc} increase in isolated rat tracheal epitheliums, the acidic fraction appeared to be the most active part in asthma-relieving in vitro. Results from in vivo evaluation with the acidic fraction on the bronchoconstriction induced by mixed spray of histamine and acetylcholine chloride in guinea pigs, strongly supported the in vitro observation of the bronchodilatory effect of this fraction. Additionally, this acidic fraction also showed significant antitussive effects on cough model induced by ammonia water in mice in vivo. All these results persuaded us to draw a conclusion that the acidic fraction played an important role in asthma-relieving among the three water extractive fractions from *Pheretima*.

The measurement of I_{sc} has widely been used to estimate the ion transportation through epithelium, since the ion transport forms local osmotic gradients across epithelia which regulate transepithelial water movement (Davis and Nadel, 1980), consequently regulate airway surface liquid (ASL) volume (mucus) (Tarran et al., 2001). Furthermore, the ionic composition of the tracheal fluid can be manipulated by cholinergic stimulation (Vanthanouvong et al., 2005). Therefore the inhibitory activity of the acidic fraction of *Pheretima* on the increase of I_{sc} induced by carbachol in rat tracheal epithelium suggested that this fraction might be effective in inhibiting excess mucus (sputum) secretion to relieve airway obstruction owing to mucus hyper-responsiveness.

GC–MS analyse indicated that the acidic fraction consists of a number of amino acids and fatty acids, which may be beneficial for the altered regulatory network and certain ion transportation pathways. However, due to the nature of multiple chemical components involved in the drug intervention as well as the multi-factorial condition of asthma, it is difficult to ascertain the mechanism of action with regard to the inhibition of spasmogen activity, regulation of specific ion channel in tracheal epithelium and antitussive effect involved in asthma.

CONCLUSION

In summary, the anti-asthmatic fraction from the water extract of *Pheretima* was successfully obtained through a series of pharmacological evaluation in vitro and in vivo on the basis of traditional use of the *Pheretima* extraction as anti-asthmatic agent. The acidic fraction of the *Pheretima* inhibited increase of carbachol-induced I_{sc} in vitro, showed significant bronchodilatory effect both in vitro and in vivo, and exhibited significant antitussive effects in vivo. The bioactive components as well as the mechanisms of action responsible for the observed activities have not been established, and thus warrant further investigation.

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