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Anti-asthmatic activity of alkaloid compounds from *Pericarpium Citri Reticulatae* (*Citrus reticulata* 'Chachi')†

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Pericarpium Citri Reticulatae (PCR, *Citrus reticulata* 'Chachi', Guangchenpi in Chinese) is one of the most famous Chinese citrus herbal medicines. The *in vivo* anti-asthmatic activity of 'Chachi' PCR was investigated using a histamine-induced experimental asthma model in Guinea pigs. Two alkaloid-type compounds, synephrine and stachydrine, were analyzed and identified in the 'Chachi' PCR alkaloid fraction. The alkaloid fraction and synephrine protected Guinea pigs against histamine-induced experimental asthma in a dose-dependent manner. The respective application of high, middle, and low doses of the 'Chachi' PCR alkaloid fraction significantly increased specific airway resistance by 284%, 328%, and 355%, and decreased dynamic compliance by 57%, 67%, and 75%. A similar change was observed for synephrine. The expression of eosinophils in bronchoalveolar lavage fluid (BALF) and serum IgE, IL-4, and IL-5 levels in histamine-induced experimental asthmatic Guinea pigs were significantly downregulated by the 'Chachi' PCR alkaloid fraction and synephrine compared to the control group, whereas stachydrine did not impart a statistically significant effect on the expression of tested inflammatory cells (leucocytes, eosinophils, neutrophils, and lymphocytes), immunoglobulin (IgE), or cytokines (IL-4 and IL-5). Pathological changes in lung tissues in each treatment group included the infiltration of inflammatory cells around the bronchia.

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1. Introduction

Bronchial asthma affects almost 10% of the general population.^{1,2} Asthma is a complex disease characterized by airway inflammation, bronchial hyper-responsiveness, airflow limitation, mucous hypersecretion, and inflammatory cell infiltration, ultimately resulting in lung dysfunction.³ It is defined as an acute symptom that can be readily reversed by bronchodilators or exacerbations that can be prevented or slowly reversed by anti-inflammatory drugs caused by chronic inflammation.¹ Nevertheless, currently there is no curative treatment for bronchial asthma. Pathobiologically, asthma promotes the release of proinflammatory mediators.^{4,5} Treatment schemes for asthma mainly focus on reducing airway inflammation that is

driven by T helper (Th) 2 lymphocytes and various cytokines, including interleukin (IL)-4, IL-13, and IL-5.^{6–8}

Epidemiological studies indicate that asthma is the most common chronic human disease. Many beneficial natural foods used in traditional Chinese medicine (TCM), such as citrus fruits, are associated with reduced risks of developing chronic diseases.⁹ The beneficial effects are partly due to their unique phytochemical content. *Pericarpium Citri Reticulatae* (PCR, *Citrus reticulata*, Chenpi in Chinese), the dried and matured tangerine pericarp, is one of the most famous Chinese citrus herbal medicines and has officially been listed in the Chinese Pharmacopoeia. PCR has been traditionally used as a health-beneficial food in southern China. It has been widely used as a folk remedy for the treatment of indigestion and inflammatory respiratory diseases such as bronchitis and asthma.^{10–12} To date, approximately 140 chemical components have been isolated and identified in PCR. Polymethoxy flavones (PMFs) such as hesperidin, nobiletin, 3,5,6,7,8,3',4'-heptamethoxyflavone, tangeretin and 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone, are the major constituents of PCR. Alkaloids, including synephrine, stachydrine and N-methyl tyramine have also been extracted from PCR. Small quantities of microelements, vitamins, pectin, polysaccharides, and volatile oils extracted from PCR have also been

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reported.¹³ Among the main Chenpi cultivars, the dried ripe pericarp of *Citrus reticulata* 'Chachi' (Guang Chenpi in Chinese) grown in the Xinhui District of Guangdong Province, China, is regarded as the source of genuine PCR based on its excellent quality.¹⁴

Pharmacological studies have shown that extracts of PCR and their ingredients may also be utilized in the treatment of other diseases, such as those affecting the cardiovascular and respiratory systems. The present study has revealed that the PCR alkaloid fraction has significant spasmolytic effects on acetylcholine chloride (ACh)-induced contractions in isolated Guinea pig trachea.¹⁵ It has also been reported that the major flavonoid hesperetin together with naringenin of PCR reduces airway inflammation and remodeling in a house dust mite-induced asthma murine model by significantly decreasing sub-epithelial fibrosis, smooth muscle hypertrophy in airways, and lung atelectasis.¹⁶ Moreover, the application of PCR extracts could decrease serum level total cholesterol (TC) and triglyceride (TG) levels in an acute hyperlipidemia mice model.¹⁷ Pretreatment of flavonoids from PCR significantly reduced the activities of serum aspartate aminotransferase and alkaline phosphatase in hypercholesterolemic rats, thus attenuating liver injury.¹⁸ However, due to the complex ingredients in PCR, the effects and related basis of the citrus-mediated reduction of asthmatic symptoms have not been systematically confirmed.

Therefore, the present study established a histamine-induced chronic asthma Guinea pig model. The objectives of the present investigation were to evaluate the protective effects of induced asthma and to elucidate the mechanism underlying the protective effects of 'Chachi' PCR *in vivo* using a histamine-induced experimental asthma model in Guinea pigs. The effects were evaluated by dynamic compliance (C_{dyn}) and specific airway resistance (sRaw), inflammatory cell infiltration, and release of cytokines and immunoglobulins. The findings of the present study provide evidence to promote the use of 'Chachi' PCR as a composition of a traditional multi-herb formulation for the treatment of asthma.

2. Materials and methods

2.1 Plant material

Approximately 50 kg of fresh *C. reticulata* 'Chachi' fruit were collected from the Xinhui District, Guangdong Province, China, in 2016. The citrus peels were removed and dried to a constant weight in a heat pump dryer (GHRH-20, Guangdong Agri-machinery Research Institute, China) at 45 °C. The *C. reticulata* 'Chachi' fruit was carefully examined and identified by Professor Jiwu Zeng, South China fruit germplasm resource evaluation scientist of Fruit Tree Research Institute, Guangdong Academy of Agricultural Sciences. A voucher specimen (No. 20161215) has been deposited at the laboratory of Sericultural & Agri-Food Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, China.

2.2 Chemicals

Salbutamol, bovine serum albumin (BSA), and carboxymethyl-cellulose-Na (CMC-Na) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Thiopental sodium was purchased from Samsung Pharmaceutical Co. (Seoul, Korea). All other chemicals used in this study were reagent grade and were purchased from Sigma Chemical Co. (St Louis, MO, USA). The composition of the Hank's balanced salt solution (HBSS)/BSA buffer (pH 7.4) was as follows: 132.0 mM NaCl, 6.0 mM KCl, 20.0 nM HEPES, 1.2 mM KH₂PO₄, 1.1 mM CaCl₂·H₂O, 1.0 mM MgSO₄, 5.5 mM dextrose, 10.0 mM EDTA and 1.0% BSA. Standards of synephrine and stachydrine (purity > 99%) were purchased from the National Institute for Food and Drug Control (NIFDC, Beijing, China). CH₃CN was HPLC grade. Deionized water was prepared using a Millipore water purification system. All other analytical grade reagents were purchased from the Guangzhou chemical reagent factory.

2.3 Sample preparation, HPLC and UHPLC/QTOF-MS analysis

The tested 'Chachi' fruit peel was extracted with methanol under reflux conditions, and the solvent was evaporated *in vacuo* to afford the 'Chachi' PCR extract. The methanol phase of the extract was dissolved in 1% HCl, and designated as the non-alkaloid fraction, and the solution was subsequently basified using ammonia water to pH 9–10. The basic solution partitioned with EtOAc, affording the alkaloid fraction. Synephrine was analyzed on an Agilent 1200 HPLC system (Waldbronn, Germany) equipped with an Agilent 1200 series VWD detector and autosampler, using a 250 mm × 4.6 mm, 5 μm Waters XBridge RP18 column (Milford Massachusetts, USA). The conditions used were: column temperature 30 °C and injection volume 20 μL. The mobile phase was CH₃CN-0.2% glacial acetic acid containing 0.1% SDS solution (32:68). Detection was set at a wavelength of 275 nm. Stachydrine was analyzed by using an ELSD detector and a Thermo Fisher Shodex Asahipak NH₂P-50 column (Waltham, MA, USA) using CH₃CN-0.2% glacial acetic solution (5:95) as the mobile phase. The mobile phase was pumped at a constant flow rate of 1.0 mL min⁻¹.

The alkaloid fraction was analyzed on an Agilent 1290 Infinity UHPLC system (Santa Clara, CA, USA) equipped with a binary solvent delivery system and a standard auto-sampler. The conditions used were as follows: column temperature, 30 °C; injection volume, 2.0 μL; mobile phase, 0.1% aqueous solution formic acid (solution A) and acetonitrile (solution B). The mobile phase was pumped at a constant flow rate of 1.0 mL min⁻¹ and programmed as follows: 0–12 min, solution B 10%–70%; 12–18 min, solution B 70%–95%.

Mass spectrometry was performed on an Agilent 6540 ultra-high definition (UHD) QTOF mass spectrometer (Santa Clara, CA, USA), equipped with a jet stream electrospray ionization (ESI) source. The mass range recorded was *m/z* 100–1600. The parameters were set as follows: positive ion mode, capillary 4000 V; nebulizer, 30 psi; drying gas, 8 L min⁻¹; gas temperature, 200 °C; nozzle voltage 300 V; skimmer, 65 V, OCT RF V

600 V; fragmentor, 150 V. The collision energy (CE) was set at 10 V.

2.4 Animals

Female Dunkin–Hartley Guinea pigs (weight: 250–350 g) were purchased from the Laboratory Animal Center of Southern Medical University. Animals were housed in an air-conditioned room (temperature: 24 ± 2 °C, humidity: $50 \pm 5\%$) with a 12 h light/dark cycle. The Guinea pigs were fed a standard laboratory diet and provided with tap water *ad libitum* in accordance with the national standards outlined in the “Laboratory Animal Requirements of Environment and Housing Facilities” (GB-14925-2010). All experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and animal handling followed the dictates of the National Animal Welfare Law of China. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of South China University of Technology and experiments were approved by the Animal Ethics Committee of South China University of Technology.

2.5 Animal treatment

All the Guinea pigs were randomized into 12 groups of 6 each. (1) normal control, (2) asthma-model control, (3) positive control (0.5 mg kg⁻¹, salbutamol), (4–6) preventive administration with the alkaloid fraction at doses of 20, 40, and 80 mg kg⁻¹, respectively, (7–9) preventive administration with synephrine at doses of 20, 40, and 80 mg kg⁻¹, respectively, (10–12) preventive administration with stachydrine at doses of 20, 40, and 80 mg kg⁻¹, respectively. All treatments lasted for four weeks, and a schematic diagram of the treatment schedule is shown in Fig. 1.

2.6 The anti-asthmatic test *in vivo*

To screen for sensitivity, the Guinea pigs were placed in a glass chamber (3 L) and sprayed with a mixture of 0.1% histamine and 2% acetylcholine chloride (1:1, v/v) under an average pressure of 450 ± 50 mmHg for 15 s.¹⁹ All groups were administered according to the treatment administered and given a single dose daily for 4 weeks, and the last dose was given 24 h before the measurement of the preconvulsive time. The

pulmonary function was recorded using the Anires 2005 system (Beijing Biolab, Beijing, China). The system automatically calculates and displays pulmonary parameters, including dynamic compliance (C_{dyn}) and specific airway resistance (sRaw), based on the changes detected. And the following formula was used:

$$sRaw = \Delta P_R / \Delta V$$

$$C_{dyn} = V_T / \Delta P_c$$

ΔP_R —the change in intrathoracic pressure, cm H₂O; ΔV —change in respiratory velocity, L s⁻¹; V_T —change in tidal volume, cm H₂O; ΔP_c —the change in intrathoracic pressure, cm H₂O.

$$\text{Increasing rate of sRaw} = (sRaw_1 - sRaw_0) / sRaw_0$$

$$\text{Decreasing rate of C}_{dyn} = (C_{dyn_1} - C_{dyn_0}) / C_{dyn_0}$$

sRaw₁ represents the maximum value of sRaw after histamine stimulation; sRaw₀ represents the average value of sRaw before histamine stimulation; C_{dyn}₁ represents the minimum value of C_{dyn}₁ after histamine stimulation; C_{dyn}₀ represents the average value of C_{dyn}₀ before histamine stimulation.

2.7 Bronchoalveolar lavage and cytological examination

Lung function parameters were measured 24 h after the allergen challenge, and animals were then anesthetized with thiopental sodium (30 mg kg⁻¹, i.p.). A tracheotomy was performed, after which the trachea was cannulated and the lungs were lavaged 4× with 5 mL aliquots of Ca²⁺, Mg²⁺-free HBSS containing EDTA (10 mM), and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) (10 mM), pre-warmed at 37 °C. The resulting bronchoalveolar lavage fluid (BALF) was centrifuged (200g for 10 min at 4 °C). The pellet was resuspended in 5 mL of HBSS containing 1% bovine serum albumin. A total leukocyte count of the BALF was performed on the resuspended cells with a hemocytometer (Superior, Germany), while differential leukocyte counts were performed with cytocentrifuged preparations (Cytospin II; Shandon Southern Instruments, Pittsburgh, PA, USA) stained with Wright's staining agent. More than 300 cells were counted using the standard morphological criteria for the classification of cells into basophils, eosinophils, neutrophils, lymphocytes, or mononuclear cells.

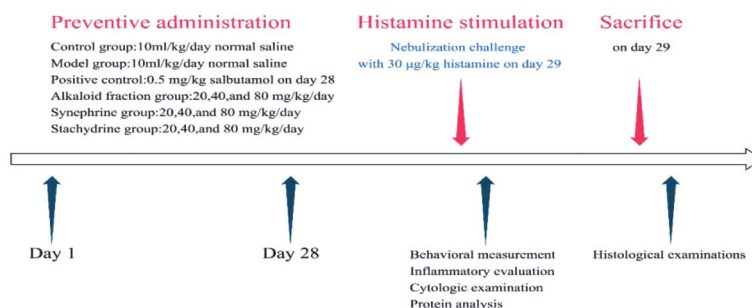


Fig. 1 Preventive administration and histamine stimulation of Guinea pigs with different groups.

2.8 IgE, IL-4, and IL-5 levels in serum

Serum IgE, IL-4, and IL-5 levels were determined using commercially available kits for IgE, IL-4, and IL-5 (USCN Life Science, Inc., Houston, TX, USA). All procedures were performed according to the guidelines provided by the manufacturers and methods in the literature.²⁰ Absorbance readings were obtained with an automatic micromatic plate reader (ELX800, Biotek Instruments, Winooski, VT, USA) at a wavelength of 450 nm, using 630 nm as a reference.

2.9 Histological observations

Histological examinations of the lungs were undertaken at the same time points as bronchoalveolar lavage (BAL) in animals from each group. Lung tissues were fixed by inflating the lungs with phosphate-buffered formalin saline *via* a tracheal cannula to a pressure of 15 cm H₂O and then removing the lungs, which were stored in phosphate-buffered formalin saline at 4 °C for histological examination. A length of trachea and two lung slices from similar sites were examined from each animal. Tissues were dehydrated in a graded series of aqueous ethanol solutions and embedded in paraffin. Sections were cut at 3 µm thickness and were stained with Mayer's H&E prior to mounting in Canada balsam. The sacrificed Guinea pigs were euthanized by cervical dislocation after full anaesthetization by inhaling diethyl ether.

2.10 Data analysis

All values are presented as the mean ± S.E. The results of airway function measurements were expressed as the present change from the baseline. The significances of the differences between two values were calculated by ANOVA followed by the Bonferroni method. *P* values <0.05 were considered statistically significant. All statistical analyses were performed with a sigma plot.

3. Results and discussion

3.1 Ingredients of the 'Chachi' PCR extract analyzed by HPLC

Fig. 2 shows the results of HPLC analysis of synephrine and stachydrine in the 'Chachi' PCR alkaloid extract. The above two alkaloid compounds could be detected in the chromatogram at lower concentrations.

To further identify the effective ingredients of the alkaloid fraction, functional UHPLC/QTOF-MS was performed. The mass spectra and structure of the responsible compounds are shown in Fig. 2E and F.

Previous studies have reported that PMFs are mainly distributed in the 'Chachi' PCR.¹³ HPLC analysis indicated that the main ingredients of the 'Chachi' PCR alkaloid fraction were large polar substances, and the contents of alkaloids such as synephrine and stachydrine were low (Fig. 2A–D), which agree with the findings of a previous report.¹⁵

3.2 Anti-asthmatic effects

After the administration of nebulized histamine in Guinea pigs, changes in airway resistance and dynamic lung compli-

ance were detected, which increased by $432 \pm 85\%$ and decreased by $92 \pm 3\%$ after stimulation, respectively (Fig. 3A and B). These findings were significantly different from those of the control group before histamine stimulation, thereby indicating modelling. Table 1 shows that the PCR alkaloid fraction and synephrine significantly increased the sRaw and decreased Cydn levels in a dose-dependent manner ($P < 0.01$). The application of high, middle, and low doses of the PCR alkaloid fraction significantly increased sRaw by $284 \pm 21\%$, $328 \pm 29\%$ and $355 \pm 33\%$, respectively, and decreased Cydn by $57 \pm 2\%$, $67 \pm 4\%$ and $75 \pm 4\%$, respectively, compared to that before histamine stimulation ($P < 0.05$). The application of high, middle, and low doses of synephrine significantly increased sRaw by $287 \pm 36\%$, $346 \pm 39\%$ and $386 \pm 16\%$, respectively, and decreased Cydn by $63 \pm 2\%$, $71 \pm 2\%$ and $78 \pm 2\%$, respectively, compared to that before histamine stimulation ($P < 0.05$). The application of high, middle and low doses of stachydrine increased sRaw by $441 \pm 28\%$, $435 \pm 24\%$ and $420 \pm 26\%$, respectively, and decreased Cydn by $84 \pm 3\%$, $85 \pm 4\%$ and $82 \pm 5\%$, respectively, compared to that before histamine stimulation. Salbutamol application at a dose of 0.5 mg kg^{-1} significantly increased sRaw by $177 \pm 17\%$ and decreased Cydn by $45 \pm 3\%$ ($P < 0.01$) (ESI Tables 1 and 2†).

Fig. 3 shows the effects of high, middle, and low doses of the alkaloid fraction of PCR on Guinea pigs compared with the control group, wherein slowed down the increase in sRaw (Fig. 3A) and enhanced the decrease in Cydn significantly ($P < 0.05$) (Fig. 3B). This trend represented a gradient change. The slower rate of increase in sRaw was observed with increasing dosage, and sRaw increased by 284% at a high dose. The rate of decrease in Cydn was gradually reduced at a high dose, and Cydn decreased by 57%. An anti-asthmatic effect was observed when the alkaloid fraction of PCR was administered, and the anti-asthmatic effect was significantly weaker than that of the positive control salbutamol.

Guinea pigs were given either high, middle, and low doses of synephrine and compared to the control group, the rate of increase in sRaw had significantly decreased, whereas the rate of decrease in Cydn was significantly enhanced. Similar to the PCR alkaloid fraction, this trend presented a gradient change. The rate of increase in sRaw decreased with a higher dosage. sRaw increased by 287% with a high dose. The rate of decrease in Cydn was reduced with a higher dosage, and Cydn was decreased by 63% with a high dose. These results showed that synephrine has an anti-asthmatic effect. The anti-asthmatic effect was weaker than that of the PCR alkaloid fraction and the positive control salbutamol. Otherwise, after administering high, middle, and low doses of stachydrine to the Guinea pigs, no significant differences in sRaw and Cydn from those of the control group were observed, including a dose-dependent pattern. Thus, stachydrine did not exhibit anti-asthmatic activity.

Synephrine is a pharmacologically active substance that has been used as a vasoconstrictor and bronchiectatic agent.²¹ It is a phenolic amine that promotes sympathomimetic activity through signaling by β_1 , β_2 , β_3 , and α -adrenoceptors.²²

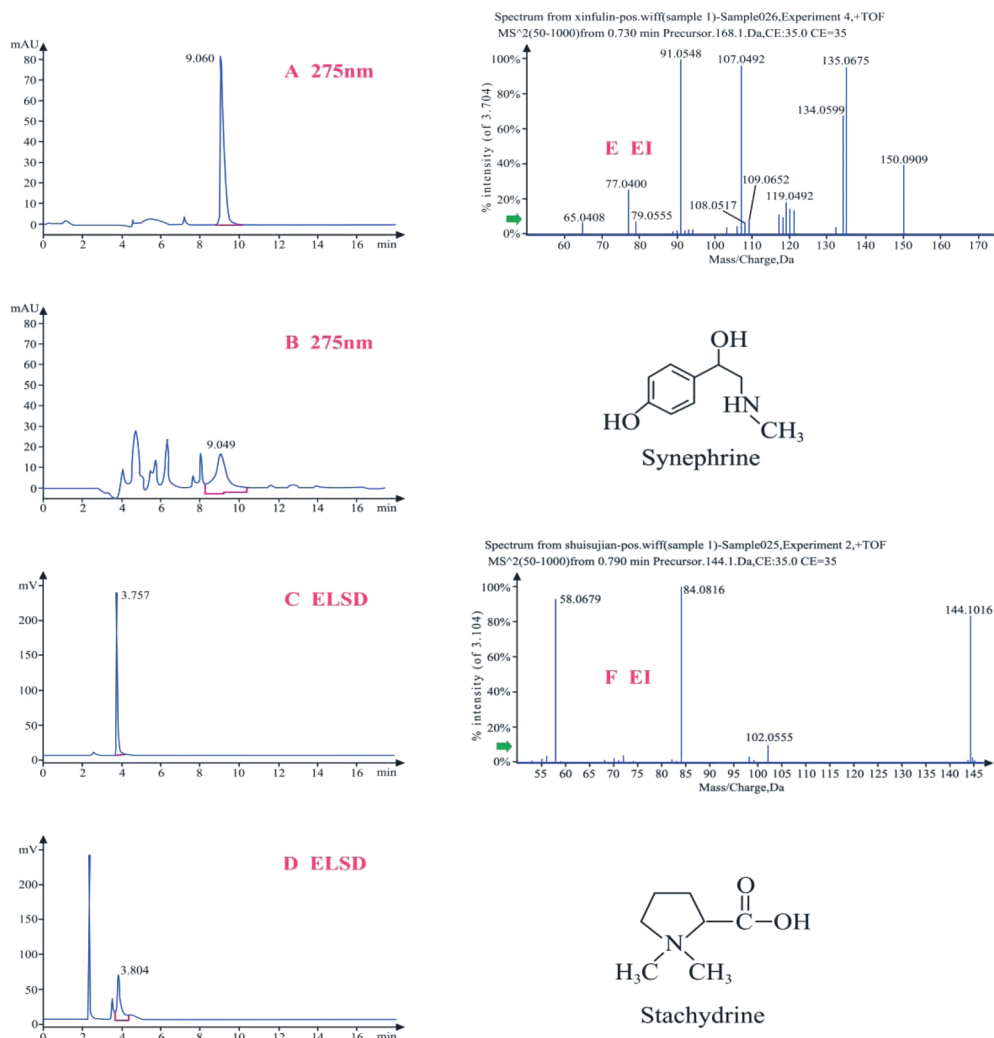


Fig. 2 Representative HPLC chromatograms (A: synephrine standard; B: alkaloid fraction at 275 nm with a VWD detector; C: stachydrine standard; D: alkaloid fraction with an ELSD detector), chemical structure and positive MS spectra of synephrine (E) and stachydrine (F).

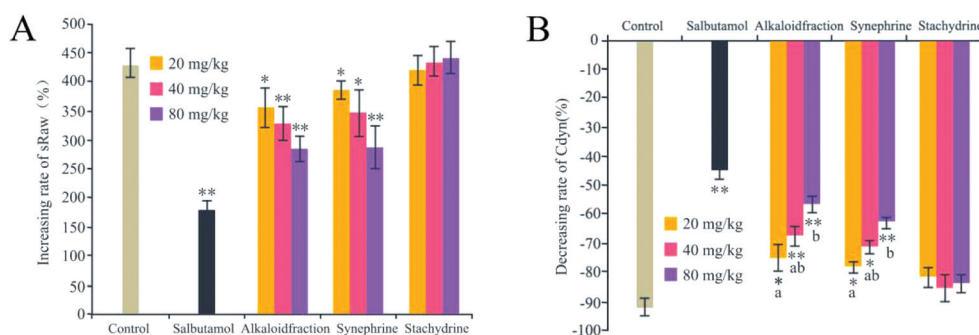


Fig. 3 Effects of different treatments on the increasing rate of specific airway resistance (A) and the decreasing rate of dynamic compliance (B) of asthmatic Guinea pigs. ** Indicates significant differences ($P < 0.01$) compared to the control group; * indicates significant differences ($P < 0.05$) compared to the control group; different lowercase letters indicate statistical differences ($P < 0.05$) among different doses in the same treatment by Tukey's test.

Stachydrine is a glycine betaine analogue. It is probably one of the secondary metabolites from various plants, particularly alfalfa (*Medicago sativa*)²³ and Capparis species.²⁴ Bioactivity

studies have demonstrated that synephrine induces relaxation in histamine-induced experimental Guinea pig tracheal strips in a dose-dependent manner. Although stachydrine had no

Table 1 Changes in specific airway resistance (sRaw) and dynamic compliance (C_{dyn}) before and after histamine stimulation (HS) in asthmatic Guinea pigs (mean ± SD, *n* = 6)

Treatments	Dose (mg kg ⁻¹ d ⁻¹)	sRaw ($\Delta P_R/\Delta V$)		C _{dyn} (L cm ⁻¹ H ₂ O)	
		Before HS	After HS	Before HS	After HS
Control	0	7.5 ± 0.28	40 ± 6.2**	0.21 ± 0.008	0.02 ± 0.006**
Salbutamol	0.5	6 ± 1.1	17 ± 1.4**	0.18 ± 0.004	0.10 ± 0.02**
Alkaloid fraction	20	6.8 ± 0.61	31 ± 3.1**a	0.27 ± 0.015a	0.07 ± 0.036**
	40	6.1 ± 0.15	26 ± 2.2**ab	0.24 ± 0.029b	0.08 ± 0.009**
	80	6 ± 1.6	22 ± 1.4**b	0.21 ± 0.029c	0.09 ± 0.025**
Synephrine	20	7.1 ± 0.98	34 ± 1.9**	0.26 ± 0.024a	0.06 ± 0.016**
	40	6.6 ± 0.43	30 ± 2.7**	0.25 ± 0.062a	0.07 ± 0.021**
	80	7.0 ± 0.61	27 ± 2.3**	0.19 ± 0.024b	0.07 ± 0.021**
Stachydrine	20	7.3 ± 0.37	38 ± 1.5**	0.24 ± 0.064	0.04 ± 0.01**
	40	7.5 ± 0.45	40 ± 2.6**	0.23 ± 0.018	0.03 ± 0.01**
	80	7.4 ± 0.85	40 ± 2.1**	0.22 ± 0.026	0.04 ± 0.006**

** Indicates statistical differences ($P < 0.01$) before and after histamine stimulation. Different lowercase letters indicate statistical differences ($P < 0.05$) among various doses in the same treatment by Tukey's test.

effect when administered alone, it enhanced the relaxation effect of synephrine, which was significantly higher than that obtained with a 10-fold higher concentration of synephrine alone. Further investigation indicated that the alkaloid fraction was responsible for activating the β 2AR signaling pathway.²⁵ Therefore, the total alkaloid fraction may be an active ingredient of 'Chachi' PCR that is responsible for the observed anti-asthmatic efficacy.

3.3 Inhibition of the recruitment of leukocytes in BALF

Table 2 shows that the recruitment of basophils, eosinophils, neutrophils, and lymphocytes significantly increased in the model group, which demonstrated typical inflammatory cell infiltration into the airway. The PCR alkaloid fraction and synephrine treatment obviously inhibited the recruitment of eosinophils in BALF compared with the model group ($P < 0.05$). A significant difference ($P < 0.01$) was observed in the high-dose treatment of the PCR alkaloid fraction. High and middle doses of synephrine inhibited the recruitment of lym-

phocytes, and significant differences compared to the model group were observed ($P < 0.05$). The inhibitory effect of stachydrine on white blood cells was not statistically significant compared to the model group.

3.4 Inhibition of the expression of IgE, IL-4, and IL-5 in blood serum

Table 3 shows that the IgE, IL-4, and IL-5 levels in the model group significantly increased. The high and middle doses of the PCR alkaloid fraction and the high dose of synephrine induced a significant decrease in serum IgE, IL-4, and IL-5 levels ($P < 0.01$). The application of a low dose of the PCR alkaloid fraction; middle and low doses of synephrine; and high, middle, and low doses of stachydrine treatment did not significantly reduce IgE, IL-4, or IL-5 levels compared to the model group.

3.5 Improvement of histopathological changes

Fig. 4 shows that the lungs in the control group showed no abnormal lesions of the bronchial tube wall, clear alveolar

Table 2 Effects of different treatments on the inflammatory cells in the BALF of asthmatic Guinea pigs (mean ± SD, *n* = 6)

Treatments	Dose (mg kg ⁻¹ d ⁻¹)	Inflammatory cells (10 ⁸ L ⁻¹)			
		Basophils	Eosinophils	Neutrophils	Lymphocytes
Control	0	1.02 ± 0.07	0.15 ± 0.07	1.18 ± 0.02	0.11 ± 0.04
Model	0	5.2 ± 0.21 [#]	2.77 ± 0.09 [#]	1.12 ± 0.04	0.22 ± 0.06 [#]
Salbutamol	0.5	1.25 ± 0.06	0.19 ± 0.08	1.06 ± 0.07	2.08 ± 0.04
Alkaloid fraction	20	4.6 ± 0.13	2.25 ± 0.04a	1.16 ± 0.06a	2.13 ± 0.05
	40	4.21 ± 0.09	1.8 ± 0.10*ab	1.06 ± 0.09b	1.94 ± 0.08
	80	4.5 ± 0.11	1.37 ± 0.06**b	1.05 ± 0.05b	1.82 ± 0.06
Synephrine	20	4.56 ± 0.03	2.48 ± 0.06a	0.87 ± 0.03b	1.97 ± 0.05a
	40	4.87 ± 0.08	2.27 ± 0.05b	1.15 ± 0.05a	1.51 ± 0.05*b
	80	4.75 ± 0.09	1.91 ± 0.03*c	1.17 ± 0.03a	1.15 ± 0.02*c
Stachydrine	20	4.84 ± 0.04	3.0 ± 0.14	1.23 ± 0.02	2.57 ± 0.01
	40	5.3 ± 0.67	2.8 ± 0.10	1.18 ± 0.04	2.25 ± 0.04
	80	4.8 ± 0.13	2.57 ± 0.08	1.17 ± 0.03	2.3 ± 0.11

[#] Indicates statistical differences ($P < 0.01$) between the control and model groups; ** Indicates statistical differences ($P < 0.01$) among the model and different treatments; * Indicates statistical differences ($P < 0.05$) among the model and different treatments; different lowercase letters indicate statistical differences ($P < 0.05$) among different doses in the same treatment by Tukey's test.

Table 3 Effects of different treatments on IgE, IL-4, and IL-5 in the serum of asthmatic Guinea pigs (mean $\pm$ SD, $n = 6$)

Treatments	Dose (mg kg ⁻¹ day ⁻¹)	IgE (IU mL ⁻¹)	IL-4 (pg mL ⁻¹)	IL-5 (pg mL ⁻¹)
Control	0	77 $\pm$ 3.0	14.9 $\pm$ 0.42	19.4 $\pm$ 0.15
Model	0	198 $\pm$ 18 [#]	39.4 $\pm$ 0.11 [#]	36.5 $\pm$ 0.19 [#]
Salbutamol	0.5	99 $\pm$ 4.1	20.4 $\pm$ 0.13	24.6 $\pm$ 0.16
Alkaloid fraction	20	182 $\pm$ 14a	36.2 $\pm$ 0.12a	37.7 $\pm$ 0.20a
	40	122 $\pm$ 13*b	22.4 $\pm$ 0.11*b	30.5 $\pm$ 0.26*b
	80	90 $\pm$ 7.4*c	17.6 $\pm$ 0.16*c	25.2 $\pm$ 0.22*c
Synephrine	20	202 $\pm$ 16a	40.1 $\pm$ 0.27a	34.4 $\pm$ 0.18a
	40	184 $\pm$ 8.1a	34.9 $\pm$ 0.21ab	35.5 $\pm$ 0.30a
	80	131 $\pm$ 12*b	31.2 $\pm$ 0.19*b	29.7 $\pm$ 0.19*b
Stachydrine	20	191 $\pm$ 11	38.1 $\pm$ 0.14	41.6 $\pm$ 0.16
	40	207 $\pm$ 14	37.7 $\pm$ 0.14	40.3 $\pm$ 0.10
	80	206 $\pm$ 12	40.7 $\pm$ 0.12	37.1 $\pm$ 0.15

[#] Indicates statistical differences ($P < 0.01$) between the control and model groups; * indicates statistical differences ($P < 0.05$) among the model and various treatments; different lowercase letters indicate statistical differences ($P < 0.05$) among various doses in the same treatment by Tukey's test.

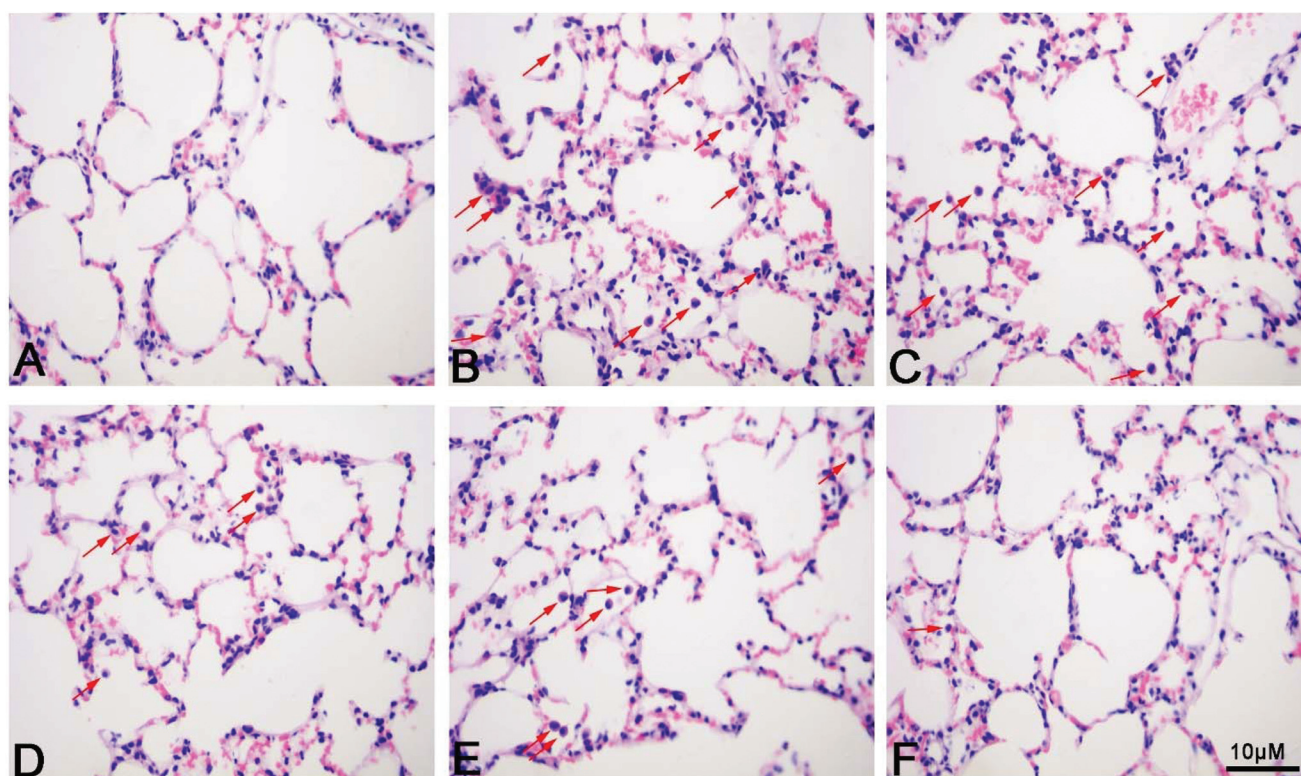


Fig. 4 Photomicrograph of a section of the lung stained with H&E. (A) Non-histamine stimulation control group; (B) histamine-induced model group; (C) asthmatic Guinea pigs treated with the alkaloid fraction, 80 mg kg⁻¹ day⁻¹; (D) asthmatic Guinea pigs treated with synephrine, 80 mg kg⁻¹ day⁻¹; (E) asthmatic Guinea pigs treated with stachydrine, 80 mg kg⁻¹ day⁻¹; (F) asthmatic Guinea pigs treated with salbutamol, 0.5 mg kg⁻¹ day⁻¹. The red arrows indicate inflammatory cell infiltration.

duct, or alveolar structure and had a normal structural architecture with no inflammatory cell infiltration around the terminal bronchioles. The positive control showed that the partial alveolar interval exhibited mild incrustation and local pulmonary hemorrhage. In contrast, the model group showed inflammatory cell infiltration. The groups that received a high dose of the PCR alkaloid fraction or synephrine treatment showed partial inflammatory cell infiltration.

Respiratory disease comprises a series of symptoms, such as airway constriction and coughing. Effective asthma treatment is generally associated with reductions in the severity of various symptoms.²⁶ In particular, it will be an alternative path to search for effective medicines in the rich traditional medicine in the world.²⁷ Ge *et al.* have documented that the water extracts of three *Elaeagnus* leaves and *Anchomanes difformis* (Blume) Engl. Leaf significantly prolonged the preconvulsive

time ($P < 0.01$) in Guinea pigs and lengthened the latent period of cough.^{28,29} However, there are few published data establishing the mechanisms and effective constituents of the traditional Chinese medicine responsible for the anti-asthmatic activity. Citrus species are acclaimed to be used as effective anti-asthmatic remedies thousands of years ago in the view of the “Huangdi Nei Jing” (The Yellow Emperor’s Classic of Medicine). In the present research, we successfully used a histamine-induced asthmatic system to assess the effect of ‘Chachi’ PCR. We found that the ‘Chachi’ PCR alkaloid fraction and synephrine at high doses significantly inhibited not only sRaw and dynamic lung compliance, but also significantly inhibited the recruitment of eosinophils and lymphocytes. Furthermore, serum IgE, IL-4, and IL-5 levels decreased. IL-4, IL-5, and IgE may play an important role in the pathogenesis of asthma, and they may be useful in the diagnosis and severity evaluation of asthma. It means that synephrine dissolved in the alkaloid fraction may be the main active component of ‘Chachi’ PCR against histamine induced pulmonary inflammation. These results agree with the findings of previous studies that demonstrated that alkaloids are the main components of ‘Chachi’ PCR.¹⁵

Many phytochemicals possess anti-asthmatic activity and support bronchial function.³⁰ A previous study reported that *in vivo* evaluation with the alkaloid fraction from *Alstonia scholaris* resulted in a strong anti-asthmatic effect when it was administered to Guinea pigs that experienced bronchoconstriction induced by a mixed spray of histamine and acetylcholine chloride.²⁵ Another study reported that a PCR alkaloid extract significantly reduced ACh-induced contractions in isolated Guinea pig tracheal rings by activating β_2 adrenoceptors. It also protected against histamine-induced experimental asthma in Guinea pigs by prolonging the latent period of asthma (Shi *et al.*, 2009).¹⁵

When compared with the positive control, it showed that the partial alveolar interval exhibited mild incassation and local pulmonary hemorrhage. In contrast, the model group showed inflammatory cell infiltration. The groups that received a high dose of the PCR alkaloid fraction demonstrated improvement in pathological changes and inflammatory cell infiltration. The groups that received a high dose of synephrine treatment showed partial alveolar interval incassation and focal interstitial inflammatory cell infiltration. The groups that received a high dose of stachydrine treatment exhibited focal interstitial inflammatory cell infiltration.

After comparing the effects of synephrine, stachydrine and the alkaloid fraction, we found that the effects of administering the alkaloid fraction were more effective than administering synephrine alone. The reason may be due to the synergistic effects of synephrine and other effective ingredients. Previous reports found that the combination of synephrine and stachydrine increased the anti-asthmatic effects to a greater extent than that at a higher concentration of synephrine alone.¹⁵ Therefore, we deduced that synergistic interactions exist among the alkaloid mixtures in ‘Chachi’ PCR.

4. Conclusions

PCR imparts protective effects against asthmatic attacks in a histamine-induced Guinea pig asthma model, and these effects are specifically mediated by the PCR alkaloid fraction and synephrine by measuring sRaw and Cdyn. The PCR alkaloid fraction appears to be more significantly active than synephrine in relieving asthma *in vivo*. Stachydrine did not exhibit any anti-asthmatic effect *in vivo*. These effects serve as evidence that ‘Chachi’ PCR has anti-asthma activity. Additionally, ‘Chachi’ PCR has good immunoglobulin (IgE)- and inflammatory cytokine-regulatory activity. The proposed mechanism of action for the observed activities requires further investigation.

Conflicts of interest

There are no conflicts to declare.

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