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Inhibitory effect of *Zingiber cassumunar* Roxb. (*Phlai*) on nasal cytokine productions and eosinophilic recruitment in patients with allergic rhinitis

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Abstract

Background: *Zingiber cassumunar* Roxb. (*Phlai*) has been used for the treatment of allergies including allergic rhinitis (AR). Although the anti-histamine effects have been reported, assessment of nasal cytokine and eosinophil production had not been investigated.

Objective: This study aimed to examine the effect of *Phlai* on alterations in nasal pro-inflammatory cytokine levels and eosinophil counts in nasal mucosa.

Methods: This was a randomized, double-blinded, three-way crossover study. Nasal concentrations of cytokines, namely interleukin (IL)-4, IL-5, IL-13 and interferon-gamma (IFN-γ), nasal smear eosinophilia as well as total nasal symptom score (TNSS) were evaluated before and after a 4weeks treatment with 200 mg *Phlai* capsules or placebo in 30 AR patients.

Results: We observed significant ($p < 0.05$) reduction in IL-5, IL-13 as well as the number of eosinophils in subjects given *Phlai*. The degree of improvement of TNSS after *Phlai* treatment was initially manifested in week 2 with the greatest effect in week 4. In contrast, there were no significant differences in all nasal cytokines, eosinophil counts or TNSS between before and after receiving placebo.

Conclusions: These findings provided the first evidence for the anti-allergic effect of *Phlai* which possibly involved inhibition of nasal pro-inflammatory cytokines production and eosinophilic recruitment. *Phlai* thus represents a promising herbal medicine for alleviating inflammation and AR symptoms.

Key words: *Zingiber cassumunar*, *Phlai*, allergic rhinitis, eosinophils, Th2 cytokine

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Abbreviations:

ALT	alanine aminotransferase
AR	allergic rhinitis
AST	aspartate aminotransferase
B	blood sample
Der F	*Dermatophagoides farinae*
Der P	*Dermatophagoides pteronyssinus*
IFN-γ	interferon-gamma
INCS	intranasal corticosteroid
IL	interleukin
mg	milligram
mm	millimeter
n	number of subjects
N	nasal fluid and mucosa sample
nm	nanometer
OAH	oral H1-antihistamine
OD	once daily
pg/mL	picograms per milliliter
SEM	standard error of mean
Th1	T helper 1
Th2	T helper 2
TNSS	total nasal symptom score
U	urine sample

Introduction

Allergic rhinitis (AR) is one of the most common phenotype of chronic upper respiratory tract disease that affects quality of life in population worldwide.[1,2] The prevalence of AR accounts for at least 50% of upper respiratory tract diseases in both adult and children.[2] The four main symptoms of AR are nasal congestion, itching, sneezing and rhinorrhea.[1] AR is sustained by inflammatory response in the nasal mucosa which is characterized by activation of mast cells, eosinophils and T-lymphocytes.[3] It refers to the predominant cytokine production by Th2 over IFN-γ expression by Th1. Previous investigators have shown that elevated levels of Th2 cytokines and defect in IFN-γ production contribute to allergic inflammation in AR patients.[4,5] Emerging evidence clearly showed the symptom severity of AR being associated with the elevated levels of nasal Th2 cytokine and nasal eosinophil counts.[4-6]

Pharmaceutical treatment of AR has been conventionally divided into two groups which are oral H1-antihistamine (OAH) and intranasal corticosteroid (INCS) regardless of symptom severity or persistence.[7] It is evidently accepted that curative effect of medicine for relieving AR symptoms is primarily the result of the reduction in pro-inflammatory cytokine levels and eosinophils in nasal fluid specimens.[8,9]

Currently, traditional herbal medicine has played a role in the treatment of various ailments including AR. This is partly because it is more affordable and accessible for local communities and aligns with patient's ideology concerning the adverse effects of chemical (synthetic) medicines.[10] Likewise, natural remedies in AR may provide an alternative treatment.

Zingiber cassumunar Roxb., locally known in Thai as *Phlai*, has long been used in traditional medicine for treatment of allergic and allergic-related diseases.[11,12] Compound D, ((E)-4-(3′,4′-dimethoxyphenyl)-but-3en-1-ol) is proposed to be the main bioactive component in the hexane extract of *Phlai*.[11,12] Pharmacokinetic study of compound D in rats showed that less than 1% of unchanged compound D was excreted in the urine and feces suggesting a high stability of this bioactive compound in blood. Therefore, compound D is commonly used as a standardized reference of *Phlai* dosage.[13]

There have been a number of reports illustrating anti-allergic activity and anti-inflammatory effects of *Phlai* extract in both *in vivo* and *in vitro* models.[14-17] *Phlai* is considered to have antihistamine action similar to that of OAH.[17] Furthermore, *Phlai* in a 200 mg capsule exerted an inhibitory effect on histamine-induced skin reactivity and also improved the total nasal symptoms score (TNSS) in AR patients.[17]

To date, no clinical reports are available on the ameliorative effect of *Phlai* on nasal pro-inflammatory cytokine production and eosinophil number in AR. Therefore, this study aimed to examine the effect of *Phlai* on nasal cytokine levels and nasal epithelial eosinophil numbers in Thai AR patients.

Methods

Patient selection

Thirty patients aged between 18-35 years were recruited. All subjects underwent history and physical examination to confirm AR of mild to moderate level of severity. All experiments were performed in accordance with the guidelines for the Human Research Ethic Committee of Thammasat University, Faculty of Medicine, Thammasat University (Institutional Review Board: approval No. 096/2018). The trial was registered with the Thai Clinical Trials Registry (registered number TCTR20190501004). Each subject was well informed of the protocol and possible undesirable effects and signed informed consent was obtained prior to participation in the study. Only subjects who fulfilled the following criteria were recruited (i) a positive percutaneous skin prick test with a wheal ≥ 3 mm diameter in response to one of the following inhalant allergens; aeroallergens house dust mites (Der F, *Dermatophagoides farinae*; Der P, *Dermatophagoides pteronyssinus*), cockroaches, cat epithelium, dog epithelium and bermuda grass allergen extracts (AllerVACtest, Greater Pharma). Glycerine saline (50% weight per volume) and histamine phosphate (0.1% weight per volume) served as negative and positive controls, respectively (ii) TNSS score of 2-8 (from a scale of 0-12) in 3 consecutive days of initial screening visit. Exclusion criteria were: (i) patients with negative skin prick test or TNSS score > 8, (ii) with asthma-related comorbidities, (iii) on immunotherapy treatment, (iv) with underlying chronic diseases, for example, chronic pulmonary disease, coronary disease, chronic kidney disease and chronic liver disease, (v) with a history of nasal cavity surgery or abnormalities interfering with nasal airflow (eg. nasal polyp, nasal septum deviation) and onset of rhinosinusitis, (vi) pregnant (positive urine pregnancy test) or lactating women and (vii) patients who received antidepressants, sedative, anxiolytic, opioids or neuroleptic drug.

Prior to screening visit and the start of the experimental trials, patients enrolled into this study were ask not to take the following prohibited medications, OAH for 1 week, INCS for 1 month and corticosteroid oral tablet for 1 month. The patients were allowed to use normal saline solution during study. Previous reports suggested that the saline irrigation has no effect on nasal cytokine levels.

Withdrawal criteria for individual participants are as follow: (i) subject's withdrawal of consent, (ii) illness or pregnancy during the experiment, (iii) onset or diagnosis of neoplasm, severe systemic inflammation or any other systemic diseases that affect AR, (iv) occurrence of a serious adverse event leading to TNSS score > 8 and (v) medication compliance < 80%

Study design

This prospective, block 4 randomized, double-blinded, placebo-controlled, three-way crossover study was conducted at the Center of Excellence for Allergy, Asthma and Pulmonary Diseases, Thammasat University Hospital. The effects of two interventions comprising of placebo and *Phlai* capsules were compared. The trial consisted of two sessions separated by two weeks interval for washout period. Each session was performed for 4 weeks (28 days). Only one test medication was administered to each subject in one session and two sessions were required to complete the experiment (**Figure 1**). Placebo and *Phlai* capsule were kept at room temperature (25°C) and were determined based on random assignment of subjects to a balanced design. The enrolled patients were randomly allocated to two treatment groups in a double-blind manner. Neither all the experimenters nor AR patients know who is receiving a particular treatment. The medication was assigned to the patient by a medical staff who did not involve in the experiment.

Clinical trial protocol

The enrollment took place from June 2019 to May 2020. Eligible patients were randomized to receive placebo or 200 mg *Phlai* capsule daily in the first session. The second session was performed with another treatment separated by a 2 weeks wash out period. Based on previous result,[17] two-weeks of wash out period are sufficient time to nullify the effect of the first intervention. Each session consisted of 4 weeks with two visits; before and after treatment. Therefore, the complete set of experiments consisted of 2 sessions with 4 visits (**Figure 1**). Blood (B), urine (U) and nasal fluid/nasal mucosa sample (N) were collected before and after treatment. All samples were frozen immediately after collection and subsequently analyzed.

Blood was analyzed for complete blood count (CBC), liver function test, blood urea nitrogen (BUN) and creatinine level for the kidney function. Urinalysis, nasal secretion and nasal cytology were analyzed for pregnancy, cytokine levels and eosinophil number, respectively.

Of note, a patient was not allowed to take OAH, INCS, oral corticosteroid, leukotriene receptor antagonist, immunosuppressive agents or any medications or products with anti-inflammatory/anti-allergic activity throughout the experiment.

Medication

Phlai and placebo capsules provided by the Thai Government Pharmaceutical Organization were identical by their appearance. Each *Phlai* capsule (100 mg of standardized *Phlai* extract) contained 4 mg of compound D and the following inactive ingredients: dibasic calcium phosphate, microcrystalline cellulose, corn starch, sodium starch glycolate and polyvinyl pyrrolidone K30. Each volunteer received either a single dose of 2 *Phlai* capsules (200 mg standardized *Phlai* extract, which was equivalent to 8 mg of compound D) or 2 capsules of placebo. The medication was taken once daily. Notably, *Phlai* can take immediately after meal without the effect of drug-food interaction. The drug and placebo were encoded with capsules, the smell and taste were not absolutely difference.

AR severity and symptom evaluation by ARIA and TNSS

Prior to enrollment, all the patients were classified AR severity according to ARIA (Allergic Rhinitis and its Impact on Asthma) guideline to be intermittent, mild persistent and moderate persistent. However, TNSS was used for tracking the improvement of patient's symptom. TNSS including

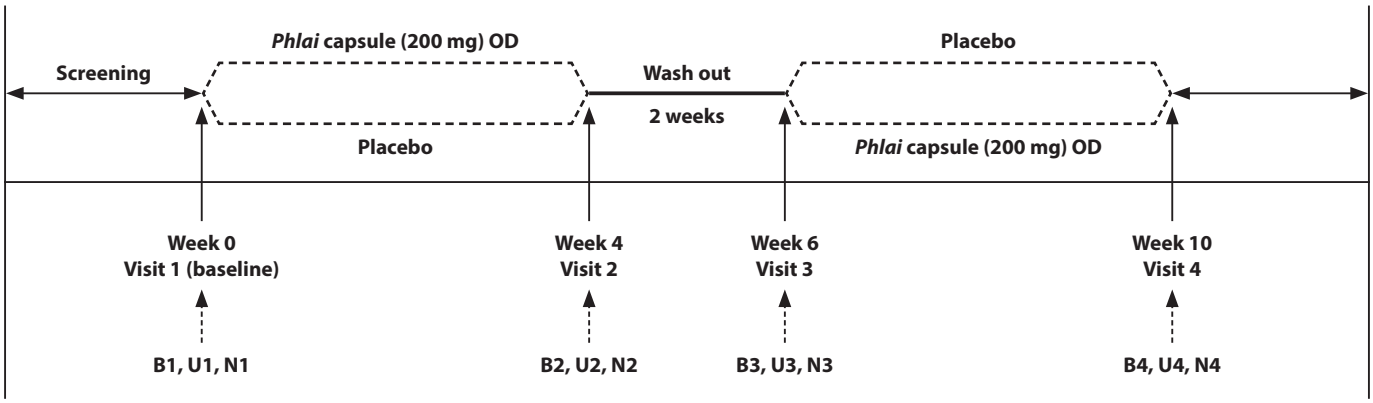

Figure 1. Clinical trial protocol.
Prospective, randomized, double-blinded placebo-controlled, crossover study; OD: once daily; B, U, N: blood, urine and nasal fluid/nasal mucosa sample, respectively.

nasal obstruction, sneezing, rhinorrhea and itchy nose was assessed every day throughout the study. Each symptom was evaluated on the following scale: 0 = absent, 1 = mild (symptom was present but was not annoying or troublesome), 2 = moderate (symptom was frequently troublesome but did not interfere with either normal daily activity or sleep) and 3 = severe (symptom was sufficiently troublesome to have interfered with normal daily activity or sleep). The total score range is from 0 to 12. The patients recorded daily TNSS by using diary provided by the researcher.

Nasal lavage fluid

The collection of nasal lavage was performed according to standard methods described previously.[18] Briefly, the patient was maintained with a ± 30 degree head extension. A 2 × 0.6 cm absorbable nasal sponge (Merocel®) was inserted into the floor of nasal cavity between nasal septum and inferior turbinate in each nostril. A volume of approximately 5.5 mL of physiologic saline solution was aerosolized into each nostril for 10 minutes, while alternately occluding the other. The nasal washing was then collected in a specimen cup. The supernatant was immediately separated by centrifugation at 2,000 × g, 4°C for 10 minutes. The sample was collected and maintained at -20°C until subsequent analysis.

Nasal cytology

Nasal mucosa specimen was obtained by scraping the middle portion of the inferior turbinate with Rhinoprobe™ curettes (Arlington Scientific Inc, Arlington, TX), as described in previous reports.[19] The retrieved tissues were placed on a glass slide and then fixed by air drying and stained by Wright-Giemsa staining method.[20] Quantification of eosinophil number was counted under light microscopy with 1000 magnification. At least 10-well spread, high power epithelium fields were examined by highly-trained technicians. Results were expressed as the number of cells per mm.

Cytokines measurement in nasal lavage fluid

Levels of IL-4, IL-5, IL-13 and IFN-γ in nasal fluid were analyzed using commercial human ELISA kit (abcam® product, Waltham, MA, USA) according to the manufacturer's instructions. ELISA were performed on absorbance microplate reader 340-900 nm (Metertech M965, Taipei, Taiwan). The detection limits of IL-4, IL-5, IL-13 and IFN-γ assays in pg/mL were 0.31, 5.00, 0.15 and 0.69, respectively.

Statistical analysis

Numerical and graphical representations of the results were presented as means ± standard error of mean (SEM). Data were tested for homogeneity of variance using Shapiro-Wilk test, before subjected to further analyses. The differences in cytokine levels and nasal eosinophils before and after treatment in each treatment were performed by paired Student's t-test. A two-way repeated analysis of variance (two-way repeated ANOVA) with Dunnett's multiple comparison tests were used to compare the significant changes over time in TNSS after both treatments. The level of significance for all statistical tests was set at p-value < 0.05 (two-tailed). All tests were analyzed by GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Patient demographics

Thirty allergic rhinitis patients were initially enrolled in this study, 9 males and 21 females. Of the 30 patients, five patients were excluded from the experiment because of an intentional withdrawal from the study (one male), concomitant use of other medications (2 females) and poor medical compliance (2 females). Therefore, 25 patients completed the experiment. The baseline of patient characteristics from excluded 5 patients including age, TNSS, symptom severity, skin prick test and cytokine levels are absolutely similar to those in included 25 AR patients (data not shown).

Table 1. Baseline patient characteristics.

Variables	Total (n = 30)
Age (years ± SEM)	25.96 ± 1.41
TNSS (mean ± SEM)	4.58 ± 0.25
ARIA class, symptom severity (n, %)	
Mild persistent	13 (43.3)
Moderate persistent	17 (56.7)
Skin prick test (n, %)	
Dermatophagoides pteronyssinus (Der P)	27 (90)
Dermatophagoides farinae (Der F)	25 (83.3)
Cockroach	22 (73.3)
Dog	4 (13.3)
Cat	9 (30)
Bermuda	9 (30)

Value in parenthesis represents percentage of total number of patients (n = 30). ARIA: Allergic Rhinitis and its Impact on Asthma; SEM: standard error of mean; TNSS: total nasal symptom score.

Baseline patient characteristics are listed in **Table 1**. The mean age of the patients was 25.96 ± 1.41 years. The percentage of female (70%) was higher than male (30%). There were no significant differences in the mean age between female (26.66 ± 1.92 years) and male (24.33 ± 1.44 years). On the first day of the experiment, TNSS were ~ 4.58 ± 0.25. Participants presented with only mild (43%) to moderate (57%) persistent symptom of severity. The highest to the lowest responses by skin prick test after allergen challenge were Der P (90%), Der F (83%), cockroach (73%), Bermuda or cat (30%) and dog (13%), respectively.

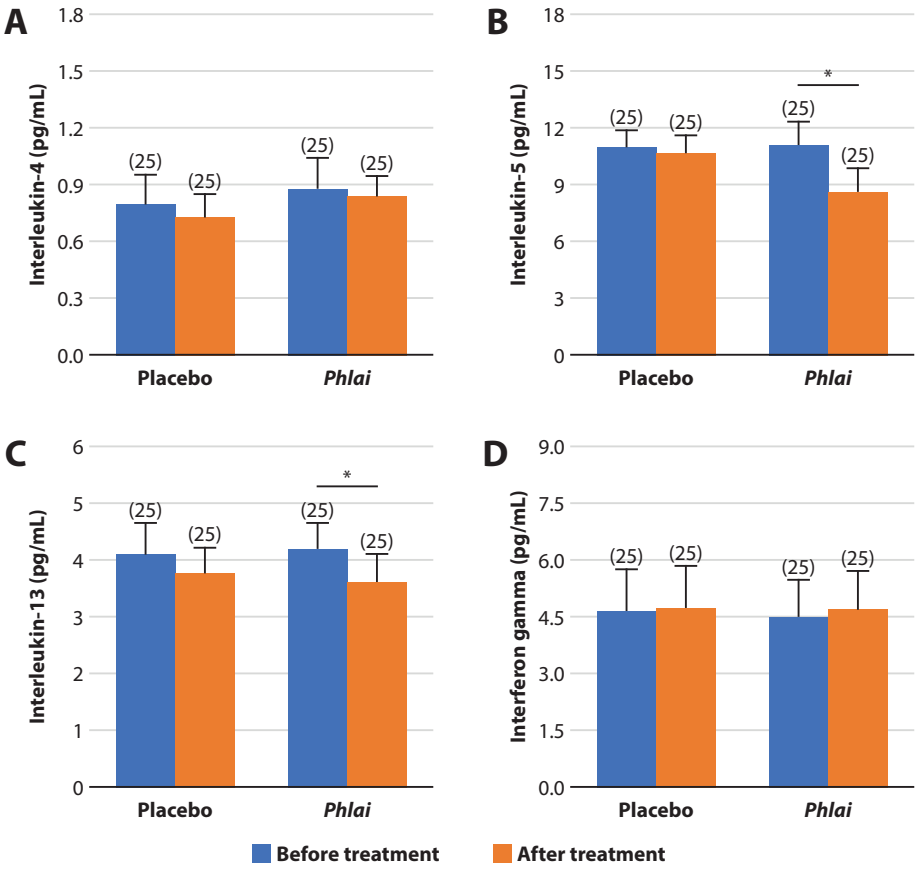

Figure 2. Levels of pro-inflammatory cytokines in nasal fluid after 4 week-treatment with placebo or *Phlai*.
IL-5 and IL-13 concentrations were significantly decreased after *Phlai* treatment. Values are mean ± SEM. Number in parentheses are number of patients. *$p < 0.05$ vs. pre-treatment.

Nasal cytokine levels

Levels of IL-4, IL-5, IL-13 and IFN-γ in nasal lavage fluid samples before and after treatment in each group were presented in **Figure 2** and **Table 2**. The pretreatment baseline levels of four cytokines in the nasal fluid of placebo and *Phlai* groups were similar. As seen in **Table 2**, treatment of placebo had no effect on the concentrations of cytokines in nasal fluids. *Phlai* treatment significantly ($p < 0.05$) reduced the nasal levels of IL-5 from 11.06 ± 1.25 to 8.58 ± 1.26 pg/mL and IL-13 from 4.17 ± 0.47 to 3.59 ± 0.50 pg/mL.

Total nasal eosinophil count

The numbers of eosinophils in the nasal mucosal scraping are summarized in **Table 2**. The before treatment number of the eosinophils in both placebo and *Phlai* groups were not significantly different; 2.08 ± 1.22 and 3.25 ± 0.94 cells/mm^2, respectively. There were no significant differences in the cell numbers between before and after treatment in the placebo group. On the other hand, treatment with *Phlai* significantly ($p < 0.05$) reduced the eosinophil count from 3.25 ± 0.94 to 0.95 ± 0.51 cells/mm^2 (**Table 2**).

Table 2. Nasal lavage fluid cytokine, eosinophil count in nasal mucosal scraping, blood and urine profiles in AR patients before and after 4 week-treatment with placebo or *Phlai*.

	Placebo		*Phlai*	
	Before	After	Before	After
Nasal cytokine and eosinophil				
IL4 (pg/mL)	0.78 ± 0.16	0.71 ± 0.13	0.86 ± 0.16	0.82 ± 0.11
IL5 (pg/mL)	10.93 ± 0.90	10.61 ± 0.96	11.06 ± 1.25	**8.58 ± 1.26***
IL13 (pg/mL)	4.07 ± 0.57	3.75 ± 0.46	4.17 ± 0.47	**3.59 ± 0.50** *
IFN-γ (pg/mL)	4.61 ± 1.11	4.70 ± 1.12	4.45 ± 1.00	4.64 ± 1.04

Table 2. (Continued)

	Placebo		*Phlai*	
	Before	After	Before	After
Eosinophil count (cells/mm²)	2.08 ± 1.22	1.85 ± 0.72	3.25 ± 0.94	**0.95 ± 0.51***
Complete blood count				
White blood cell (× 10³/ul)	5.28 ± 0.22	5.95 ± 0.48	5.14 ± 0.64	6.40 ± 0.91
Hemoglobin (gm %)	13.8 ± 0.36	13.40 ± 3.70	13.68 ± 0.55	13.60 ± 0.65
Platelet count (× 10³/ul)	238.00 ± 18.69	245.00 ± 16.8	233.40 ± 27.58	234.00 ± 24.86
Renal function test				
Blood urea nitrogen (mg/dL)	9.82 ± 1.07	12.01 ± 1.67	9.56 ± 0.75	11.61 ± 0.98
Creatinine (mg/dL)	0.77 ± 0.10	0.84 ± 0.11	0.73 ± 0.04	0.70 ± 0.04
Liver function test				
Albumin (g/dL)	4.42 ± 0.13	4.22 ± 0.16	4.10 ± 0.16	4.24 ± 0.10
Total protein (g/dL)	7.60 ± 0.13	7.26 ± 0.21	7.72 ± 0.22	7.88 ± 0.22
AST (< 50 U/L)	23.00 ± 2.23	21.20 ± 1.59	18.00 ± 2.16	23.00 ± 4.27
ALT (< 50 U/L)	28.40 ± 3.58	25.00 ± 2.75	25.20 ± 4.09	19.20 ± 4.96
Urinalysis				
Protein	Negative	Negative	Negative	Negative
Glucose	Negative	Negative	Negative	Negative

Data are expressed as means ± S.E.M. (n = 25). AST: aspartate aminotransferase; ALT: alanine aminotransferase; g/dL: gram per deciliter; mg/dL: milligram per deciliter; pg/mL: picogram per milliliter; U/L; units per liter. *$p < 0.05$ vs. pre-treatment.

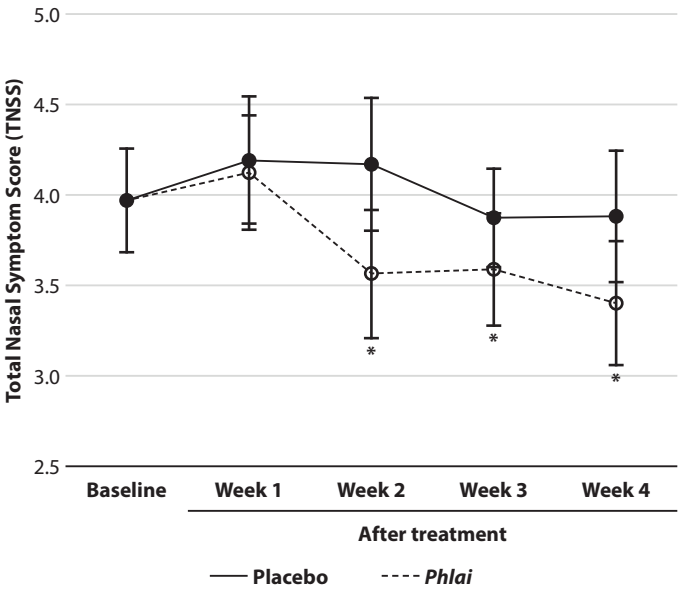

Figure 3. TNSS scoring over time during 4 weeks after treatment of placebo and *Phlai* capsule.
Values are mean ± SEM (n = 25). *$p < 0.05$ significantly different from baseline and week 1 in the same treatment.

Total nasal symptoms scoring

Severity of AR symptom was assessed by determining TNSS before and after receiving daily treatment every day and data was presented as weekly averages. Increases and decreases in the TNSS indicated worsening and relieving symptoms, respectively. **Figure 3** illustrates TNSS as functions of time after 4 weeks of treatment. Compared to the baseline (3.96 ± 0.28), treatment with placebo caused virtually no change in TNSS throughout the experiment and ranged from 4.19 ± 0.35 to 3.88 ± 0.26. In contrast, *Phlai* treatment significantly ($p < 0.05$) reduced TNSS at weeks 2 (3.56 ± 0.35), 3 (3.58 ± 0.30) and 4 (3.40 ± 0.34) when compared to baseline and week 1 post-treatment (4.12 ± 0.31). The lowest TNSS occurred at week 4.

Safe use of Phlai capsule

The adverse effect of *Phlai* in this study was evaluated by kidney and liver function. As shown in **Table 2**, markers of renal function including BUN and creatinine (Cr) level were normal before (BUN = 9.56 ± 0.75 mg/dL and Cr = 0.73 ± 0.04 mg/dL) and after *Phlai* treatment (BUN = 11.61 ± 0.98 mg/dL and Cr = 0.70 ± 0.04 mg/dL). Protein and glucose found a negative in urine evaluated by urinalysis both pre- and post-treatment of *Phlai*.

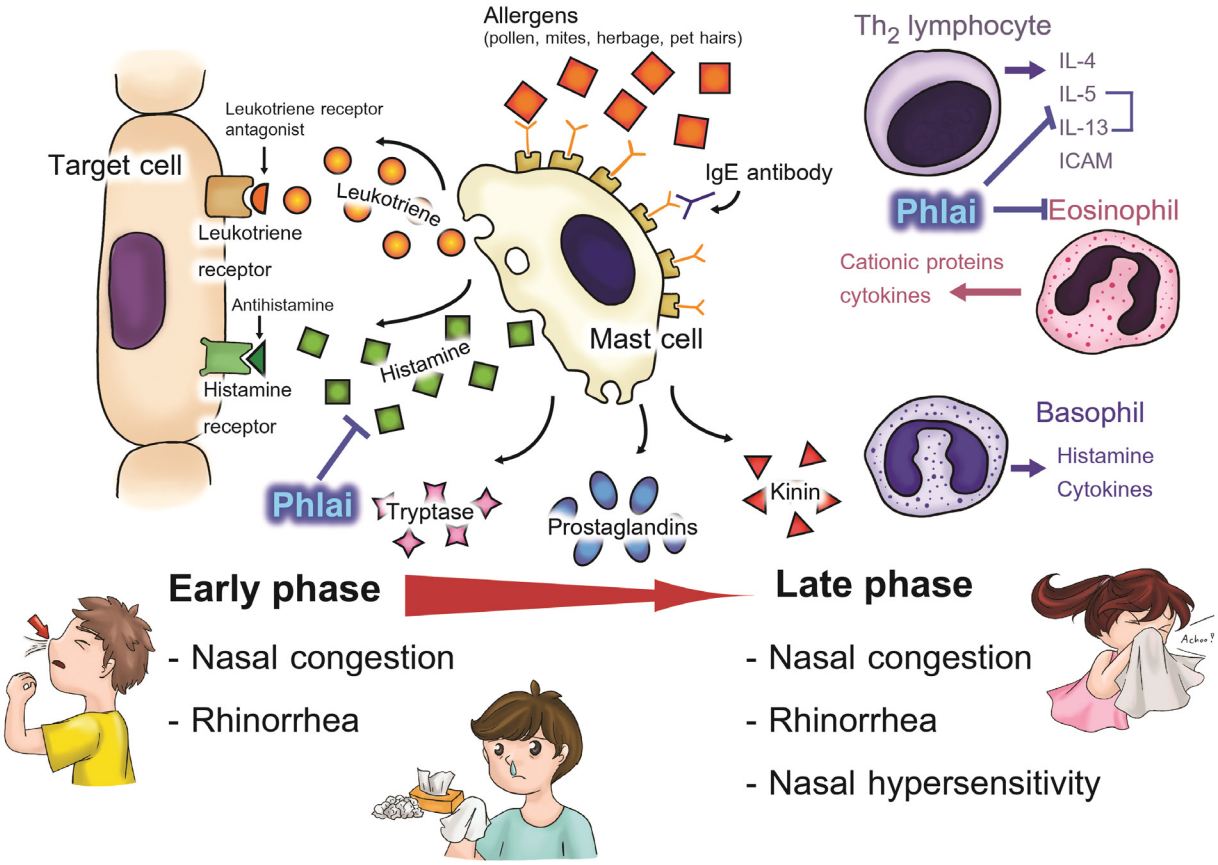

Figure 4. Proposed biological effects of *Phlai* in AR.
Phlai effectively reduce both reactivity to histamine and Th2 cytokine production, in particular IL-5 and IL-13 and the number of eosinophils. The beneficial effect of *Phlai* is scrutinized both early and late-phase of AR.

Liver function test including albumin (4.24 ± 0.10 g/dL), total protein (7.88 ± 0.22 g/dL), alanine aminotransferase (ALT) enzyme (19.20 ± 4.96 U/L), and aspartate aminotransferase (AST) enzyme (23.00 ± 4.27 U/L) were all normal value after 4 week of taking oral *Phlai* capsules (**Table 2**). As for CBC results, both receiving of placebo and *Phlai* showed a normal range of total number of white blood cells, hemoglobin concentration and platelet cell count (**Table 2**).

Discussion

To the best of our knowledge, we herein provided for the first time evidence showing that *Phlai* exerted inhibitory effect on nasal cytokine production and eosinophil numbers in AR patients possibly by reducing IL-5 and IL-13 levels, with no effect on IL-4 or IFN-γ. We showed that *Phlai* was able to improve TNSS beginning in week 2 and exerting the most potent effect in week 4.

Diagnosis and symptom severity of AR can be evaluated by nasal symptoms and positive results of allergic tests by skin prick test.[21,22] Our results corresponded to previous reports showing house dust mite and the cockroach are the first two important aeroallergen sensitizers among the Thai population.[23]

Since clinical manifestations alone may not precisely reflect the degree of allergic inflammation, the levels of cytokine and inflammatory cells in nasal fluid and on nasal mucosa should be investigated. The present study showed that upon the treatment of 200 mg standardized *Phlai* extract for 4 weeks, IL-5, IL-13 and eosinophil numbers were remarkably decreased. Nevertheless, *Phlai* had no effect on Th1 cytokine, IFN-γ.

Intrleukin-5 plays a crucial role in eosinophil differentiation in the bone marrow, recruitment and migration at active sites of allergic inflammation as well as prolongation of eosinophilia could be supported by this concept.

IL-13 in AR is proposed to contribute to development of a late nasal response without effect on the early phase or nasal eosinophilic inflammation.[25] IL-13 shares a number property with IL-4 in parts of receptor subunits, IgE switching and VCAM-1 upregulation.[26,27] The present study showed a significant decreasing in nasal level of IL-13 but not IL-4 after *Phlai* treatment. This was in agreement with the previous evidence that indicated inhibition of the allergen-induced late nasal response after corticosteroid treatment was associated with a marked decrease in IL-13 mRNA-positive and immunoreactive cells compared to IL-4 from nasal biopsy specimens in AR subject.[27]

Furthermore, the increase in IL-13 mRNA was much more pronounced than the increase in IL-4 after allergen challenge.[27] *In vitro* study, it was clearly demonstrated that IL-13, but not IL-4, could promote the chemotaxis and prolongs the survival of eosinophil.[28] Although elevation of IL-4 level in AR was reported, the differences were not statistically significant when compared to control.[29]

On the contrary, one study found decreased levels of IL-4 in seasonal AR patients.[30] Thus, it is likely that the development of allergic inflammation is dependent on IL-13 rather than IL-4.

Another possible explanation for an absence of *Phlai*'s effect on IL-4 was probably due to the profile of the patients in the present study. Our patients had previously been treated with OAH or topical antihistamines and INCS, which might expound the missing elevations of baseline level of cytokine. However, selectivity in the repressing effect of *Phlai* and a potential hierarchy in cytokine function in the nasal mucosal microenvironment could not be ruled out.

In high Th2 cytokine-secreting individuals, the correlation between IL-5 and IL-13 levels in nasal fluid has been addressed. Our data confirmed the previous findings of Erin et al,[31] who found nasal steroid has the capacity to selectively abolish IL-5 and IL-13 responses following nasal allergen challenge in patients with seasonal AR. It was feasible that anti-allergic activity of *Phlai* had an underlying mechanism similar to that of corticosteroid. Interestingly, recent study has revealed only the level of IL5 and IL13, were decreased in AR patient during treatment with *Zataria multiflora*, which is another local herb found in Iran and other parts of the Middle East.[32]

Although allergic diseases have been linked to an enhanced Th2 immune response, a decreased Th1 immune response by IFN-γ is also important in the pathogenesis. In the present study, surprisingly we found no significant alteration in IFN-γ level in both placebo and *Phai* treated groups, contradicting an expected upregulation. Similar results were also reported in AR patient during treatment of the other herbal medicines.[32] Previous investigation provided the evidence for the balancing of IL-4 to IFN-γ level in subjects with AR.[33] Compared to non-atopic individuals, the ratio of IFN-γ to IL-4 was markedly decreased among grass pollen-sensitive individuals suggesting some interconnections between IFN-γ and IL-4.[33] Also, one-week treated-Jiawei Yupingfeng (Chinese medicine) mouse apparently altered the IL-4/IFN-γ ratios in allergic responses.[34] Thus, the failure to demonstrate the influence of *Phlai* on IFN-γ probably resulted from concomitant IL-4 effect. The definite explanation for this this investigation is unclear. The interaction of Th1- with Th2 function seems to be more complex.

In line with clinical features of AR, as expected, in this study showed the degree of improvement of TNSS is robustly correlated with the degree of decrease in nasal cytokines level and eosinophil numbers. Alleviative effect of *Phlai* was initially observed in week 2 towards the greatest effect in week 4. This suggests that one mechanism of anti-allergic activity of *Phlai* is attributable to attenuation of certain pro-inflammatory cytokines and eosinophil counts.

Accordingly, the present study indicates duration of beneficial *Phlai* effect is similar to corticosteroids. Yet, in the aspect of antihistamine effects of *Phai*, our group noticeably showed that 100 mg (equivalent to compound D 4 mg) and 200 mg (equivalent to compound D 8 mg) *Phlai* capsules could inhibit skin reactivity to histamine and mite skin prick tests in AR Thai patients.[17] Based on our findings, it appeared that *Phlai* could exert a potent anti-histamine and anti-inflammation activity indicating therapeutic potential for both early- and late-phase of AR (**Figure 4**).

Concerning the safe use of *Phlai*, our group recently reported that oral *Phlai* capsule once daily (200 mg/day) over 12 weeks did not any cause serious effect on CBC, kidney function, liver function, or fasting blood glucose.[35] The safety profiles of *Phlai* in this study confirmed our previous finding. There were no liver or kidney toxicity and no abnormal CBC, BUN and creatinine level. Nevertheless, longer use of *Phlai* for treatment of a larger group of patients needs to be further investigated.

Regarding research design and methodology, two weeks washout interval between treatments lessened any carryover effects, as seen in the same baseline levels of cytokine in each pre-treatment.

Our study had certain limitations. First, we did not examine the direct effect of bioactive constituents of *Phlai* as represented by compound D on nasal cytokine and eosinophil production. In contrast to the rat, compound D bioavailability in human, is rapidly metabolized or converted to another chemical forms, which cannot be measured in the blood, urine or feces (unpublished data). It is plausible that the effectiveness of *Phlai* in human relies on the synergistic interaction among active constituents. We used compound D as the only measurable standardized reference ingredient for *Phlai* capsule dosage. However, *in vitro* study would confirm or modify our conclusions from this study and add to the extensive knowledge on the effect of *Phlai* in AR. Second, there is considerable discrepancy in the literature regarding quantitative results for nasal biomarkers. Such variability is likely to be largely accounted for by different nasal fluid collection techniques and the different immunoassays used.

Highlight of our findings rests on the fact that we provided the first clinical evidence to support the use of traditional *Phlai* herb for anti-inflammation in AR patients.

Conclusion

The present study reveals a biological activity of *Phlai* as an anti-inflammatory herbal medication in AR patients. The underlying mechanism involves an inhibition of Th2 nasal cytokines, in particular IL-5 and IL-13 production and nasal eosinophilic recruitment. Further research and development of *Phlai* may lead to the much needed alternative medicine for the treatment of AR.

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Declaration of competing interest

No conflicts of interest, financial or otherwise, are declared by the authors.

Author's contributions

- N.A., P.T., O.P., P.K., P.S.R., and S.N. conceived and designed of research.
- N.A., P.T., and P.S.U. performed experiments.
- N.A., P.T., and N.P. analyzed data and prepared figures.
- N.A., P.T., and O.P. interpreted results of experiments.
- N.A. and P.T. drafted manuscript.
- N.A., P.T., N.K., and O.P. edited and revised manuscript.
- All authors reviewed and approved final version of manuscript.

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