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Determination of Optimum Excipients for *Platanus orientalis* Pollen Extract by Accelerating Chemical Stability Test and Their Synergistic Effect

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Abstract

Background: The quality of extracts used in the skin prick test directly influences the interpretation of the test. Accordingly, the outcomes and effectiveness of immunotherapy for the management of IgE-mediated allergies depend on the quality of the extracts used. Excipients, which are pharmacologically inert ingredients, are intentionally added to the active ingredients. The aim of this study was to address optimum excipients for stability *Platanus (P.) orientalis* extract.

Methods: In this study the excipients examined were l-lysine (20 mM), l-cysteine (20 mM), albumin (0.5%), sorbitol (2%), sucrose (750 mM), trehalose (20 mM), D-mannitol (2% w/v), urea (100 mM) and Tween-20 (0.1%). Their effects on *P. orientalis* extract stability were analyzed using an inhibition enzyme linked immune assay at 37 °C.

Results: A mixture of lysine (20 mM), trehalose (20 mM), and D-mannitol (2% w/v) conferred the greatest stability on the *P. orientalis* extract.

Conclusions: The *P. orientalis* extract stability was increased by a mixture of lysine (20 mM), trehalose (20 mM), and D-mannitol.

Keywords: Lysine, Mannitol, *Platanus orientalis*, Skin prick test, Trehalose.

Introduction

A literature review of the last two decades of medical media shows a growth in the prevalence of allergic diseases (1). The spectrum of allergic symptoms and signs varies from simple skin redness to fatal anaphylactic shock. The skin prick test (SPT) is the major diagnostic test used to identify IgE-mediated allergy (2, 3). A properly-performed SPT can identify the cause of allergic symptoms (4). The quality of extracts used in the SPT influences the results. Also, the outcomes and effectiveness of immunotherapy to manage IgE-mediated allergies depend on the quality of the extracts used. Hence, the formulation and stability of prepared extracts for SPTs have drawn much attention in the current decade (5).

The extract solutions of allergic materials usually contain a water-soluble compound of proteins, carbohydrates, and sometimes, biologically active molecules (6). The stabilities of these components with dissimilar properties and molecular weights may vary; thus the expressed changes may be complex (7). The biologically-active molecules can

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be susceptible to enzymatic degradation and compounds of allergenic materials that contains proteases may undergo self-degradation (8). In addition, storage conditions, including storage temperature, humidity, exposure to ice during manufacturing and shipping, and exposure to sunlight may affect extract stability (9, 10). These environmental factors can damage the extract components. To overcome these problems, manufacturers use excipients. Excipients, which can be synthetic or natural, are pharmacologically inert ingredients intentionally added to the active ingredient (11-13). Nearly all drugs used in medical and veterinary fields contain at least one excipient (14, 15).

Accelerated stability tests are widely employed to analyze the stability and quality of pharmaceutical products. Ensuring the safety, quality, and efficacy of prepared extracts is a requisite for product standardization (16).

The pollen grains of *Platanus (P.) orientalis* are a common aeroallergen found in many countries worldwide, including Asia (17, 18). *Planatus orientalis,* or sycamores, are widely grown along streets as shade trees. The *P. orientalis* pollen grain extract is used extensively in SPTs and many patients are sensitive to it (17-20).

Because of the importance of the stability of this allergen extract and because of the limited research on this topic, the present study was performed to identify desirable excipients for *P. orientalis* extract. This study employed a low-cost, sensitive, and accurate inhibition enzyme-linked immunoassay (ELISA) to evaluate *P. orientalis* extract with an accelerated stability test.

Materials and methods

Collection of the pollen grains

First, a *P. orientalis* branch was sent to the Academy of Herbal Sciences herbarium at Ferdowsi University to identify the genus. The *P. orientalis* pollen grains were collected from trees in March by hand. After drying the pollen clusters, the pollen grains were separated by progressively passing them through sieves of mesh numbers 50, 150, 270, and 500. The integrity of greater than 95% was determined using an optical microscope (Fig. 1).

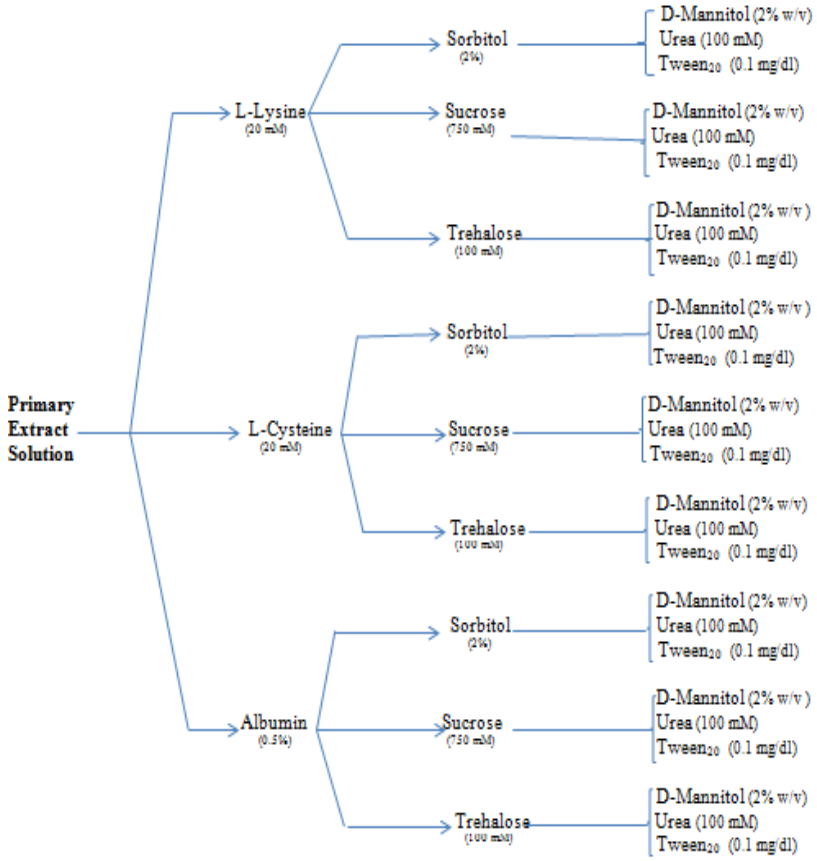


Fig. 1. Schematic of excipient mixtures for evaluation of their effects on *Planatus orientalis* extract stability

Extraction procedure

A 0.5 g aliquot of pollen grains was dissolved in 10 cc of phosphate-buffered saline (PBS) (NaCl, 8 g, Na2HPO4, 1.44 g, KH2PO4, 0.2 g, KCl, 0.2 g, up to 1 L with distilled water and pH=7.4) (1:20 w/v). The solution was mixed by vortexing and then shaken with 500 RPM at 4 °C overnight. Then the solution was then centrifuged at 1646 RCF for 10 min at 4 °C. The supernatant was collected and re-centrifuged at 5678 RCF for 13 min. At the next step, the extracts were dialyzed at 4 °C overnight against phosphate buffer (KH_2PO_4, 2.44 g, K_2HPO_4, 0.018 g). The pH of the extract was then adjusted to 7.4 and the solution was filtered through a 0.22 µm pore syringe filter (BiofilTM, Shanghai, China).

Excipient selection

The literature was reviewed using the PubMed search engine to idientify common excipients used in medicines (5, 8, 11, 13-15, 21-28). Accordingly, l-lysine (20 mM), l-cysteine (20 mM), albumin (0.5%), sorbitol (2%), sucrose (750 mM), trehalose (20 mM), D-mannitol (2% w/v), urea (100 mM) and Tween 20 (0.1%) were selected. Twenty-seven various excipient mixtures were prepared and analyzed with P. orientalis extract in triplicate (Fig. 2). All the excipient mixtures plus extracts were incubated at 57 °C for 24 h. At the end of incubation those that had precipitated were removed. The mixture stabilities were evaluated by inhibition ELISAs method. Three excipients that conferred the greatest stability on the extract were further studied (Table 1).

Table 1. Excipients selected for stability tests with *Planatus orientalis* extract

Excipients				
Samples	**Lysine 20 mM**	**Trehalose 100 mM**	**D-Mannitol 2% w/v**	**Urea 100 mM**
Sample 1	+	+	+	-
Sample 2	+	+	-	+
Sample 3	+	+	+	+

Accelerated stability test

First, 15 ml of *P. orientalis* extract was prepared as described. Three ml from this extract were aliquotted into 5 ml polyethylene tubes with the following excipient mixtures at the temperatures listed.

Sample 1. Three ml of extract + l-lysine (20 mM) + trehalose (20 mM) + D-mannitol (2% w/v) at 37 °C.

Sample 2. Three ml of extract + l-lysine (20 mM) + trehalose (20 mM) + urea (100 mM) at 37 °C.

Sample 3. Three ml of extract + l-lysine (20 mM) + trehalose (20 mM) + D-mannitol (2% w/v) + urea (100 mM) at 37 °C.

Sample 4. Three ml of extract with no excipient at 37 °C.

Sample 5. Three ml of extract with no excipient at 4 °C.

Storage conditions and duration

The pollen grains extracts containing excipients were evaluated by the normal stability test. Before the test each mixture was incubated at room temperature (24 ± 2 ºC) for 24 h to complete the emulsification process (29).

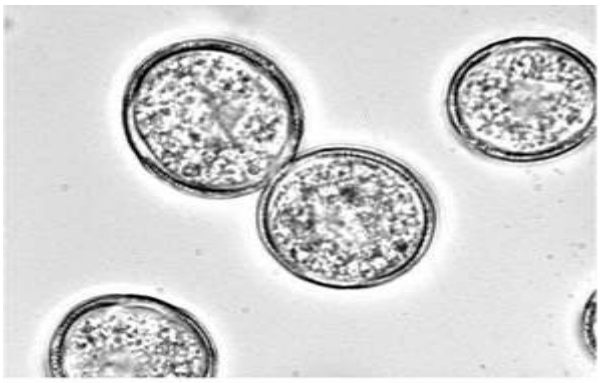


Fig. 2. Light microscopy image of *Platanus orientalis* grains (X100).

Storage conditions included low and high temperatures (4 ± 1 °C and 37 ± 0.5 °C, respectively) without exposure to sunlight. The chemical stabilites of the samples were analyzed by inhibition ELISAs.

The percentage inhibition was calculated by following formula:

% Inhibition = 100 – (mean of observances of test samples / mean of observances of control samples) X 100. Furthermore, each extract was examined weekly for aspect, color, and odor.

Patient selection by skin prick test (SPT)

The proposed research protocol was approved by the Vice Chancellor of Research at Mashhad University of Medical Sciences (approval number IR.MUMS.REC.1394.45). Of patients with allergic presentations referred to the allergy clinic in Ghaem hospital, Mashhad, 34 with positive SPTs to *P. orientalis* were entered into the study. After explaning the study to the patients, consent forms were given to them to read and sign. Before performing the SPT patients were asked whether they had taken any medications that might interfere with the SPT results. First, the patient's arm was cleaned with cotton moistened with 75% ethanol. Next a drop of pollen extract was placed on the patient's forearm. Then the patient's arm was scratched through the drip with a sterile needle. After 1-2 minutes the remaining extract was wiped away (30). Normal saline (9 gr/l) was used as a negative control and histamine hydrochloride (10 mg/ml) was used as a positive control.

Total IgE assay

Five ml of blood were collected from the patients with positive SPTs in accordance with the principles of the Declaration of Helsinki of 1965 (as revised in Brazil 2013). After clotting, the samples were centrifuged at 1,500 x g for 15 minutes to seperate the serum. The total IgE assay was performed using an Euroimmun ELISA Kit (Lübeck, Germany) according to the manufacturer's instructions. The samples with IgE concentrations greater than 100 IU/ml were used in this study. Ultimately, 14 samples from eight male and six female patients were used for making pooled serum. The prepared pooled serum from these 14 patients were aliquoted and stored at -20 °C.

Inhibition ELISA procedure

Here, inhibition ELISA measures proteins of *P. orientalis* extract that containing various excipients. Forasmuch as, the stability that has been induced by various excipients are different in the *P. orientalis* extract, the results of inhibition ELISA vary in each extract with special excipient. Inhibition mixture that is prepared before the main procedure provides sufficient time for intact proteins to be inhibited by antibodies against them in the pooled serum. In the extract with denatured proteins, antibodies do not adhere to the denatured proteins and remain in the solution. In the next step, when the solution is added to the wells of ELISA, there are free antibodies who can attach to coated antigens on the bottom of the wells.

Wells of micro-titer plates (Nunc-Immuno Modules, Roskilde, Denmark) were coated with 100 µl of 1: 2,000-diluted *P. orientalis* extract and incubated at 4 °C overnight. After washing, 200 µl of bovine serum albumin (BSA) (Sigma ® Cat. number: A7030) (2% w: v) was added and the plates were incubated at room temperature for 1 hr. To prepare the inhibition mixtures, 100 µl of *P. orientalis* extract mixed with the homogenized excipients described above were incubated with 100 µl of 1:5-diluted pooled serum in micro-tubes for 1.5 h at room temperature with shaking at 1646 RCF. The wells were washed and 100 µl of each inhibition mixture was added to the test wells in duplicate. The plates were incubated for 1 h at room temperature with continuous shaking at 200 RPM. Two wells received 100 µl of 1:10 diluted pooled plasma was added to two wells as controls. 100 µl of goat anti-IgE (KPL, Gaithersburg, USA) diluted 1: 2,000 (v:v) was added to each well and the plates were incubated for 1 h at room temperature with continuous shaking. After washing, 100 µl of 1: 10,000-diluted streptavidin-horseradish peroxidase (BD Biosciences, San Jose, CA, Cat number: 554066) was added to the wells and the plates were incubated on a shaker at room temperature for 1 h. After washing, 100 µl of substrate solution (prepared as described by Frey A. et al.) was added to each well and the plates were incubated for 15 min in the dark at room temperature (31). The reaction was stopped by adding 20 µl of 0.3M H_2SO_4. Absorbances were measured on an ELISA plate reader (Statfax 2600, Palm City, FL) at 450 nm with the filter at 630 nm. The results were expressed as a percentage of inhibition derived from the mean absorbance values for duplicate samples by the formula described in Materials and Methods.

Results

The total protein concentration in the *P. orientalis* extract was 2 mg/ml by bicinchoninic acid assay method.

The pH of the *P. orientalis* extract after dialysis was 7, but changed in some samples after adding the excipients (Table 2). No changes were observed in the color or odors of four of the five extract plus excipients mixtures; however, sample 4 precipitated after the first week and its odor changed.

Table 2. Physical properties of *Platanus orientalis* extract evaluated after the resting period (t_0) and at the end of the study (week 11).

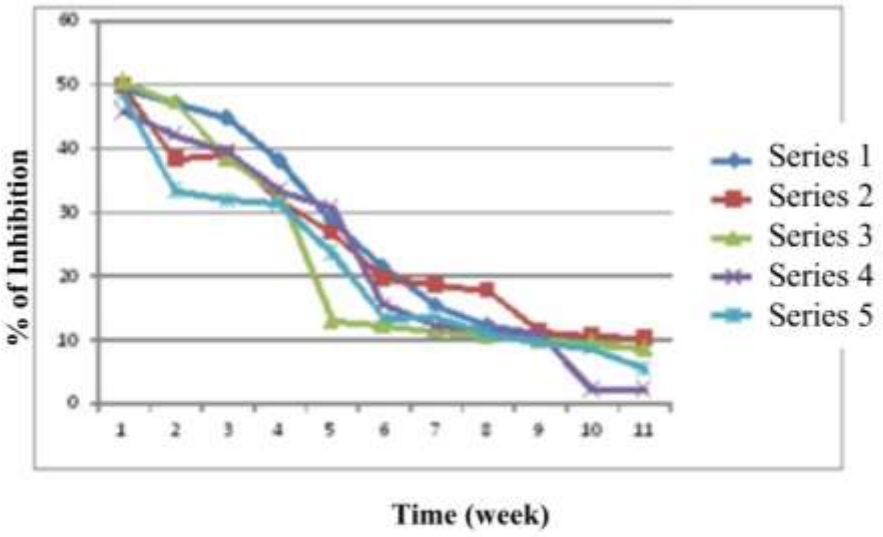
Parameters	t_0					Final stage				
	S1	S2	S3	S4	S5	S1	S2	S3	S4	S5
PH	8	8	8.5	7	7	7.5	8	8	7	8
Aspect	H	H	H	H	H	H	H	H	P	H
Color	S	S	S	S	S	S	S	S	S	S
Odor	C	C	C	C	C	C	C	C	I	C

S; sample, H; homogeneous, P; precipitated, t_0= time of resting, S; semi-yellow, I; increased, C; characterized.

Based on the total IgE concentrations, 14 serum samples were selected. The minimum and maximum IgE concentrations were 118 and 629 IU/ml, with a mean of 430 IU/ml.

The percentage inhibition was measured each week during the study. Overall, samples were measured 13 times (Fig. 3). The inhibition percentages were 46-66% during the first week and decreased to below 10% in the last week of the study (Week 11). The extract stability decreased in all the samples during the study and the decline was most severe in samples 4 and 5, which contained no excipients. At week 6 the stability of sample 1 was about 25% greater than that of sample 4 at 37 °C. The both samples that had a mixture of excipients, including urea had lower stability power. This means urea is not suitable excipient for *P. orientalis* extract.

On the whole, the samples with a mixture of excipients (except urea) had better stability results compared to *P. orientalis* extracts without any excipient.



Series 1: L-lysin+ Trehalose, Series 2: L-lysisn+Trehalose+Urea
Series 3: L-lysin+Trehalose+D-Manitol+Urea, Series 4: Extract without any excipient at 37 ◦C,
Series 5: Extract without any excipient at 4 ◦C.

Fig. 3. Percentage inhibition of excipient mixtures for *Planatus orientalis* extract evaluated by inhibition ELISA

Discussion

The stability of the various extracts, which may be stored for extended periods and used in SPTs, is a significant issue. It can directly affect the test result and subsequent patient treatment. Some surveys reported that pollen extracts are stable at 4 °C for up to15 months when prepared in PBS and 50% glycerol (7, 24), while an aqueous form of the extract without glycerol was less stable (32).

Establishing safe and effective excipients for pharmaceuticals is a responsibility of academia and pharmaceutical companies (33).

In the present study, the synergic effect of nine excipients in the *P. orientali*s extract was studied. *Platanus orientali*s was selected for the study because its pollen grains are among the most allergenic pollens in many regions.

Moreover, the inhibition ELISA was developed to measure the amount of active allergenic proteins in the *P. orientalis* extract. The extract plus excipients were incubated at 37 °C during the study to accelerate sample degradation to identify the best excipients for increasing stability of P. orientalis extract.

Our results showed that an excipient mixture containing l-lysine, trehalose, and mannitol conferred greater stability on the extract than the other combinations or controls and should be considered when preparing extracts for SPTs. The exact mechanisms for modulation of protein stability by excipients have not been elucidated (34). Liu Y and et al. reported that trehalose applies protein stabilization by inhibiting protein aggregation(25), l-lysine act as a perturbing factor for enzyme function (34), and D-mannitol prevents Maillard reactions (35).

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