RESEARCH PAPER

Design and Development of Phytoantigen Containing Nasal Vaccine for Defense Against Seasonal Allergy

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Abstract
Seasonal allergy to *Parthenium hysterophorus* pollen is a considerable respiratory burden in India, yet drug therapy is only symptomatic and allergen immunotherapy needs prolonged supervised treatment. A needle-free nasal formulation of a plant-derived antigen and evaluated it preliminarily in rats. A freeze-dried ethanolic extract was characterised by micromeritic tests, phytochemical screening, FTIR spectroscopy and Folin–Lowry protein estimation. Acute toxicity was assessed at 400, 600 and 800 mg/kg and the LD_{50} estimated by probit analysis. A nasal drop containing aluminium hydroxide as adjuvant, with glycerin, HPMC and CMC as viscosity modifiers, was optimised with a 15-run, three-factor response-surface design (target viscosity below 300 cP). Rats received the pure extract (G1) or the optimised formulation (G2) intranasally, and antigen-specific IgG was measured by indirect ELISA on days 0, 7 and 21. The extract was a poorly flowing powder containing alkaloids, sesquiterpene lactones, flavonoids, saponins and tannins, with about 90 µg/mL protein in a 10% solution. FTIR showed no loss of characteristic bands in an extract–excipient mixture. The LD_{50} was approximately 600 mg/kg. A quadratic model described viscosity well (adjusted R^2 0.985); only runs with 1% CMC stayed below 300 cP, and Runs 10 (92 cP) and 1 (290 cP) were selected. Corrected OD_{450} rose above baseline by day 7 (1.82-fold for G1, 1.41-fold for G2) and stayed elevated on day 21 (1.47- and 1.38-fold). The formulation is feasible to prepare and the antibody signal is encouraging but preliminary; allergen-specific and protective effects, and stability, remain to be shown.

Keywords: *Parthenium hysterophorus*; phytoantigen; nasal vaccine; seasonal allergy; response-surface design; indirect ELISA

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1. Introduction

Allergic diseases are a large and growing public-health problem, with prevalence rising among both children and adults [1]. Warming and air pollution are thought to worsen allergic disease by lengthening pollen seasons and intensifying exposure [2], and ambient pollen levels have been linked to asthma-related emergency visits over a 26-year period in Atlanta [3]. Seasonal allergic rhinitis and allergic asthma carry substantial health-care and productivity costs [4], socioeconomic circumstances shape who is affected [5], and the burden is considerable in India [6,7].

Parthenium hysterophorus L. (Asteraceae), known in India as congress grass, is an invasive weed that has spread across wastelands, roadsides and croplands, where it causes a distinctive airborne contact dermatitis [8]. Its pollen is also an allergen source. A proteomic study identified 258 proteins in feverfew pollen, 47 of them from known allergen families, and recombinant Par h 1 was recognised by sera from 60% of Austrian and 40% of Indian patients tested [9]. Because exposure to this weed is widespread and hard to avoid, it is a reasonable antigen on which to base a region-specific allergy product.

Antihistamines and other drugs relieve symptoms but do not change the course of disease, whereas allergen immunotherapy can induce lasting tolerance but needs prolonged, supervised treatment [10]. Mucosal delivery is therefore of interest as a simpler route. The nasal cavity offers a large, well-vascularised mucosal surface, needle-free self-administration and access to local immune tissue. An intranasal live attenuated influenza vaccine has been licensed in the United States since 2003 [11], and several intranasal COVID-19 vaccines have since been developed [12]. A phytoantigen also avoids the need for cultured pathogens or animal-derived media.

In the literature we reviewed, we did not find a nasal formulation of a plant-derived antigen intended to prevent seasonal allergy. This study therefore aimed to (i) characterise a *P. hysterophorus* extract as a candidate phytoantigen, (ii) estimate its acute toxicity margin in rats, (iii) optimise the viscosity of a nasal drop formulation with a statistical design, and (iv) make a first assessment of antibody responses after intranasal administration.

2. Materials and Methods

2.1 Materials

A freeze-dried ethanolic extract of *P. hysterophorus* was purchased from AMSAR Pvt. Ltd., Indore, India [plant part used; batch or voucher number]. Aluminium hydroxide (adjuvant), glycerin, hydroxypropyl methylcellulose (HPMC) and carboxymethylcellulose (CMC) were used as formulation components [grade and supplier]. Bovine serum albumin (BSA), Folin–Ciocalteu reagent and horseradish peroxidase (HRP)-labelled goat anti-rat IgG were used for protein estimation and ELISA.

2.2 Pre-formulation studies

Bulk and tapped densities were measured, Carr's index and Hausner's ratio were calculated from them, and the angle of repose was determined. Phytochemical screening used standard qualitative tests: Wagner's and Hager's tests (alkaloids), Molisch's test (carbohydrates), Baljet's test (sesquiterpene lactones), the sulfuric acid test (flavonoids), the foam test (saponins) and the ferric chloride test (tannins).

2.3 FTIR spectroscopy and compatibility

Infrared spectra of the extract and of a physical mixture of the extract with the formulation excipients [composition and ratio] were recorded on a Shimadzu spectrometer over 4000–400 cm^{-1}. Compatibility was judged from the persistence, shift or loss of the characteristic bands.

2.4 Protein estimation

Protein was estimated by the Folin–Lowry method [13] using BSA standards of 60–180 µg/mL (0.2–0.6 mL of standard made up to 1 mL with distilled water). Each tube received 5 mL of alkaline copper reagent (Folin I) followed by 0.5 mL of Folin–Ciocalteu reagent (Lowry II), and absorbance was read at 680 nm in a digital colorimeter. A 10% solution of the extract was assayed in the same way, and its concentration was read from the linear regression of the standards.

2.5 Acute toxicity

Acute toxicity was assessed in [species/strain, sex and body weight] rats, two animals per group, at 400, 600 and 800 mg/kg [route of administration; observation period]. All procedures were [approved by the Institutional Animal Ethics Committee; approval number]. Mortality was recorded and the LD_{50} was estimated by the logarithmic-probit method [14]. Mortality of 0% and 100% was corrected as 100(0.25/n) and 100(n − 0.25)/n, respectively, with n = 2.

2.6 Formulation development and optimisation

Six feasibility batches (F-I to F-VI) with different levels of glycerin, CMC and HPMC were first prepared to identify workable ranges. The nasal drop contained the extract, aluminium hydroxide as adjuvant [concentrations], and glycerin (A, 10–50%), HPMC (B, 0.1–1.5%) and CMC (C, 1–5%) as viscosity modifiers. A 15-run, three-factor, three-level response-surface design with three centre points was built in Design-Expert version 13 (Stat-Ease). The response was viscosity, measured with a rotational viscometer (spindle no. 2, 100 rpm), with a target below 500 cP. Linear, two-factor interaction, quadratic and cubic models were compared using sequential p-values and adjusted and predicted R^2.

2.7 Immunisation and antibody estimation

Rats [strain, sex, age; number per group] received either the pure extract at 60 mg/kg, one-tenth of the estimated LD_{50} (G1), or the optimised formulation, Run 10 (G2), by the intranasal route [extract dose in G2, volume per nostril, number and timing of doses]. Blood was collected on days 0, 7 and 21. Serum IgG was measured by indirect ELISA on plates coated with [coating antigen and concentration], using sera at [dilution] and HRP-labelled goat anti-rat IgG, with absorbance read at 450 nm. Each plate carried a blank (OD 0.108), a negative control (day 0 serum) and a positive control. Optical densities were corrected by subtracting the blank, and the fold increase was the corrected OD divided by the corrected day 0 OD. Accelerated stability testing (40 ± 2 °C/75 ± 5% RH) of the selected batches was planned; those data are not part of this report.

3. Results and Discussion

3.1 Physicochemical characteristics of the extract

The extract was a dark green, fine powder with an earthy, musky odour (Table 1). From the measured bulk (0.62 g/cm³) and tapped (0.86 g/cm³) densities, Carr's index was 28% and Hausner's ratio 1.39, and the angle of repose was 38°. Together these indicate a cohesive, poorly flowing powder, as is common for freeze-dried plant extracts. Because the extract is dispersed in a liquid vehicle, this is mainly a handling issue (weighing, transfer, dispersion) rather than a limit on the final dosage form.

Table 1. Micromeritic properties of the *P. hysterophorus* extract.

Parameter	Observation
Description	Dark green fine powder; earthy, musky odour
Bulk density	0.62 g/cm³
Tapped density	0.86 g/cm³
Carr's index	28% (calculated from the densities)
Hausner's ratio	1.39 (calculated from the densities)
Angle of repose	38°

Phytochemical screening (Table 2) was positive for alkaloids, carbohydrates, sesquiterpene lactones, flavonoids, saponins and tannins. Sesquiterpene lactones are expected, since parthenin and related lactones are characteristic constituents of the plant and its main contact sensitisers [8]. The extract is chemically complex, so the antigenic material cannot be assumed to be a single protein.

Table 2. Phytochemical screening of the extract.

Test	Constituent	Observation	Inference
Wagner's	Alkaloids	Purple precipitate	Alkaloids present
Hager's	Alkaloids	Yellow precipitate	Alkaloids present
Molisch	Carbohydrates	Purple ring	Carbohydrates present
Baljet	Sesquiterpene lactones	Orange to red colour	Sesquiterpene lactones present
Sulfuric acid	Flavonoids	Yellow-orange colour	Flavonoids present
Foam	Saponins	Foam layer	Saponins present

Test	Constituent	Observation	Inference
Ferric chloride	Tannins	Blue colour	Tannins present

3.2 FTIR spectroscopy and extract–excipient compatibility

The spectrum of the extract (Fig. 1) showed a broad O–H stretching band centred at 3280 cm^{-1}, aliphatic C–H stretching at 2893 cm^{-1}, a band at 1614 cm^{-1} assigned to C=C of an unsaturated carbonyl system, O–H bending at 1402 cm^{-1} and a strong C–O stretching band at 1010 cm^{-1} (Table 3). All of these bands were present in the extract–excipient mixture (Fig. 2), with no new bands and no loss of the main peaks, which suggests no chemical interaction. Three bands moved by 23–44 cm^{-1} (C–H, C=C and C–O). These shifts most likely reflect overlap with the many hydroxyl and ether bands of glycerin, HPMC and CMC, and weak hydrogen bonding, rather than a change in the extract.

Two cautions apply. The extract is a mixture, so the assignments indicate functional-group classes only. Also, no distinct band is resolved above 1700 cm^{-1}, so the spectrum should not be taken as confirming parthenin itself. Identity would need a marker-based method such as HPLC or HPTLC against a parthenin standard.

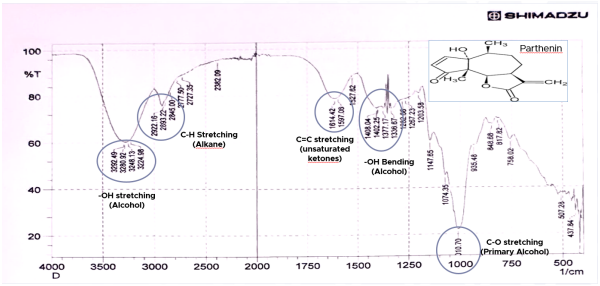

Fig. 1. FTIR spectrum of the *P. hysterophorus* extract with the main band assignments (inset: structure of parthenin).

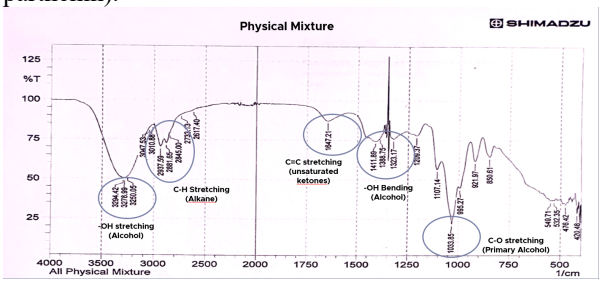

Fig. 2. FTIR spectrum of the physical mixture of the extract with the formulation excipients.

Table 3. FTIR band assignments for the extract and the physical mixture (cm^{-1}).

Band	Class	Standard range	Extract	Mixture	Shift
O–H stretching	Alcohol	3200–3550	3280	3278	−2
C–H stretching	Alkane	2840–3000	2893	2937	+44
C=C stretching	α,β-Unsaturated ketone	1610–1620	1614	1647	+33
O–H bending	Alcohol	1330–1420	1402	1411	+9
C–O stretching	Primary alcohol	1000–1085	1010	1033	+23

3.3 Protein content

Absorbance rose with BSA concentration between 60 and 180 µg/mL (Table 4, Fig. 3; A = 0.0062C − 0.300, R^2 = 0.981). The sample reading of 0.26 corresponded to about 90 µg/mL protein in the 10% solution. This value should be read with care. The Folin reagent also reacts with phenolic compounds, and the extract contains tannins and flavonoids (Table 2), so true protein is probably lower. The standards also deviate slightly from a straight line (residuals up to about 0.05 absorbance units). An SDS-PAGE profile, ideally with immunoblotting against sera from sensitised patients, would show which proteins are present.

Table 4. Folin–Lowry estimation of protein (absorbance at 680 nm).

Tube	Standard (mL)	Distilled water (mL)	Concentration (µg/mL)	A_{680}
1	0.2	0.8	60	0.11
2	0.3	0.7	90	0.23
3	0.4	0.6	120	0.39
4	0.5	0.5	150	0.67
5	0.6	0.4	180	0.82
Sample	–	–	≈ 90 (read from curve)	0.26

Tube	Standard (mL)	Distilled water (mL)	Concentration (µg/mL)	A_{680}

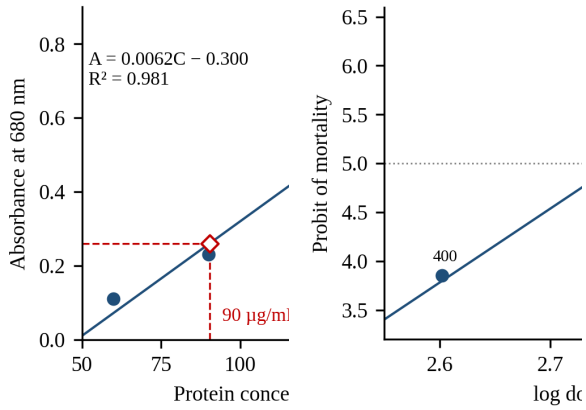

Fig. 3. Folin–Lowry standard curve; the diamond is the sample reading.

Fig. 4. Logarithmic-probit plot of acute toxicity (n = 2 per dose); the dashed line marks 600 mg/kg.

3.4 Acute toxicity

No animal died at 400 mg/kg, one of two died at 600 mg/kg and both died at 800 mg/kg (Table 5). After correction for 0% and 100% mortality, the probit plot (Fig. 4) gave an LD_{50} of about 600 mg/kg, the dose that produced 50% mortality. A least-squares line through the three points gives a similar value (577 mg/kg). With only two animals per dose the estimate is imprecise and no confidence interval can be calculated, so it should be treated as a rough indication of the toxic range. The immunisation dose of 60 mg/kg is one-tenth of this estimate, which leaves a margin for a first immunogenicity study. Repeated-dose toxicity and local nasal tolerance were not studied.

Table 5. Probit calculation for acute toxicity.

Group	Dose (mg/kg)	Log dose	Deaths/n	% dead	Corrected %	Probit
1	400	2.60	0/2	0	12.5	3.85
2	600	2.78	1/2	50	50	5.00
3	800	2.90	2/2	100	87.5	6.15

Corrected %: 100(0.25/n) = 12.5 for 0% and 100(n − 0.25)/n = 87.5 for 100% mortality, n = 2.

3.5 Formulation optimisation

Viscosity ranged from 92 to 1950 cP across the 15 runs (Tables 6 and 7, Fig. 5). All three modifiers raised viscosity and CMC had the strongest effect. At 30% glycerin and 0.1% HPMC, raising CMC from 1% to 5% increased viscosity from 290 to 1600 cP. At 0.8% HPMC and 1% CMC, raising glycerin from 10% to 50% increased it from 92 to 438 cP. HPMC mattered most at low CMC (290 to 980 cP between 0.1% and 1.5% HPMC at 30% glycerin and 1% CMC) and little at 5% CMC (1600 to 1800 cP), which points to non-additive behaviour. The three centre-point runs all gave 1100 cP.

Table 6. Factors and levels of the experimental design.

Factor	Name	Low	Centre	High
A	Glycerin (%)	10	30	50
B	HPMC (%)	0.1	0.8	1.5
C	CMC (%)	1	3	5

Table 7. Experimental runs and measured viscosity (spindle no. 2, 100 rpm).

Run	Glycerin (%)	HPMC (%)	CMC (%)	Viscosity (cP)	Run	Glycerin (%)	HPMC (%)	CMC (%)	Viscosity (cP)
1	30	0.1	1	290	9	10	0.1	3	650
2	10	1.5	3	875	10	10	0.8	1	92
3	30	0.8	3	1100	11	50	0.1	3	1400
4	50	0.8	1	438	12	50	1.5	3	1750
5	30	0.8	3	1100	13	10	0.8	5	900
6	50	0.8	5	1950	14	30	1.5	1	980
7	30	0.1	5	1600	15	30	1.5	5	1800
8	30	0.8	3	1100					

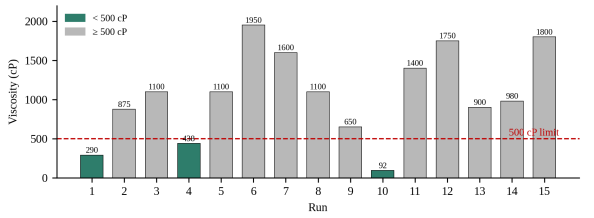

Fig. 5. Viscosity of the 15 experimental runs; the dashed line is the 500 cP target.

Only Runs 1, 4 and 10 stayed below the 500 cP target, and all three contained 1% CMC. Model selection (Table 8) favoured a quadratic model (sequential p = 0.0032; adjusted R^2 0.985; predicted R^2 0.914). The small gap between adjusted and predicted R^2 suggests the model is not over-fitted, and the cubic model was aliased. Because the three centre points gave identical viscosity, pure error was zero and lack of fit could not be tested. Runs 1 and 10 were taken forward. Run 4 (438 cP) met the limit but was set aside because it sat close to it, leaving little tolerance for batch variation. Run 10 (10% glycerin, 0.8% HPMC, 1% CMC; 92 cP) was used in the animal study. Only viscosity was optimised here. Droplet or spray behaviour, pH, osmolality, mucoadhesion and content uniformity are not reported and matter for nasal delivery.

Table 8. Fit summary for the viscosity response.

Model	Sequential p-value	Adjusted R^2	Predicted R^2	Remark
Linear	< 0.0001	0.8565	0.7593	
2FI	0.2560	0.8778	0.6311	
Quadratic	0.0032	0.9850	0.9142	Suggested
Cubic	–	1.0000	–	Aliased

3.6 Antibody response

Corrected OD_{450} values are given in Table 9 and Fig. 6. Against a day 0 baseline of 1.567, the pure extract (G1) gave 2.861 on day 7 (1.82-fold), falling to 2.306 on day 21 (1.47-fold). The optimised formulation (G2) gave 2.209 on day 7 (1.41-fold) and 2.170 on day 21 (1.38-fold). Both groups were therefore above baseline at both time points. G1 was higher on day 7 and comparable to the positive control (2.814; 1.79-fold), but fell by about 19% by day 21, whereas G2 fell by only about 2%. One possible explanation is that the viscous, adjuvanted vehicle releases antigen more slowly, but this study cannot test that.

Table 9. Indirect ELISA results (OD at 450 nm), raw and corrected values.

Sample	Rep 1	Rep 2	Mean OD	Corrected OD	Fold vs. day 0
Blank	0.108	0.108	0.108	–	–
Day 0 negative control	1.675	1.675	1.675	1.567	1.00
Day 7, G1	2.969	2.969	2.969	2.861	1.82
Day 7, G2	2.317	2.317	2.317	2.209	1.41
Day 21, G1	2.414	2.414	2.414	2.306	1.47
Day 21, G2	2.278	2.278	2.278	2.170	1.38
Positive control	2.922	2.922	2.922	2.814	1.79

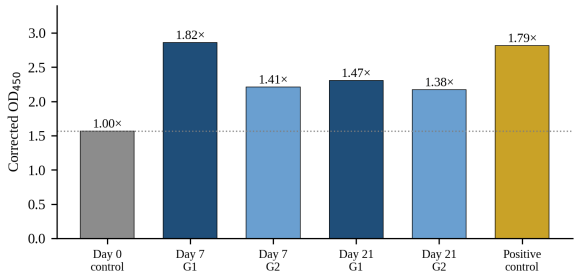

Fig. 6. Corrected OD_{450} by group and day; fold increase over day 0 is shown above each bar.

These results should be read as preliminary, for several reasons. First, no statistical test was possible, because between-animal replicates are not reported. Second, the day 0 control already read 1.675 against a blank of 0.108, which points to substantial background reactivity, and no group reached a two-fold rise. Third, several readings approached 3.0, where plate readers lose linearity, so a higher serum dilution would be needed to avoid compressing differences. Fourth, the assay measures IgG binding to the coating antigen, not allergen-specific IgE, IgG subclasses, blocking activity or protection from challenge, so the data support immunogenicity rather than defence against allergy. Finally, the nature of the coating antigen and the practicality of dosing 60 mg/kg within nasal volume limits for a rat need to be justified in the full report.

4. Summary and Conclusion

A freeze-dried ethanolic extract of *P. hysterophorus* was characterised as a poorly flowing powder rich in sesquiterpene lactones and other phytochemicals, with about 90 µg/mL protein in a 10% solution and no sign of incompatibility with the formulation excipients by FTIR. Its LD_{50} in rats was approximately 600 mg/kg. A response-surface design showed that CMC chiefly governs the viscosity of an aluminium hydroxide-adjuvanted nasal drop, and only formulations with 1% CMC met the 500 cP target; Runs 1 and 10 were selected. After intranasal dosing in rats, OD_{450} rose above baseline by day 7 and stayed elevated on day 21, with a more sustained signal for the formulation than for the pure extract.

The work shows that a Parthenium-based nasal drop can be prepared with controlled viscosity and gives an encouraging, but preliminary, antibody signal. Characterisation of the antigenic proteins, a better-controlled ELISA with replicates and statistics, allergen-specific readouts, challenge studies, nasal tolerance and

the pending stability data are needed before efficacy against seasonal allergy can be claimed.

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