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Studies on *Cassia* Pollen Grains of India

Key Words

Cassia
Pollen grains
Aerobiology
Allergenicity
Skin test

Abstract

The aerial distribution of the pollen grains *Cassia* species was found to be predominant in Kerala and Tamil Nadu, the adjacent states of South India. The present study deals with the allergenicity and biochemical aspects of four different species of *Cassia* which were found to be dominant in the atmosphere of India. The pollen grains of *Cassia occidentalis* were found to be the most and *Cassia roxburghii* the least reactive of the four different species studied. The individual analysis of the reactive patients also showed the possibilities of cross-reactivity. The protein, carbohydrate and amino acids of these members were analysed. The results obtained are useful as a step towards standardisation of allergen extracts of *Cassia* species and are also worthy of further investigations both in relation to cross-reactivity and characterisation of allergens.

Introduction

Aerobiological studies conducted over the last few decades have shown the relation of *Cassia* pollen with the allergic manifestations of human beings. Nasobronchial diseases, which progress faster in nature and intensity of reactivity, are mainly due to the inhalation of pollen and spore allergens. The nature and property of the allergenic particulates differ between species due to physiological and environmental factors. A pronounced progress was found in the clinical and immunological aspects of allergy research; however, not much attention has been paid to biochemical studies which are the basis for allergen characterisation. Aerobiological studies in India have shown the major incidence of different pollen grains which include members of the genus *Cassia* (Leguminosae). The

genus *Cassia* originating from India is widespread in tropical countries and is recognized for several uses. Aerobiological studies conducted in different zones of India showed that the pollen grains of *Cassia* spp. predominated [1–6]. In the air of Tamil Nadu and the adjacent state Kerala the pollen grains of *Cassia* spp. were also recorded to be predominant [4, 7–11]. In India, the clinical evaluation studies using the extracts of *Cassia* pollen grains were done in Andhra Pradesh [12], Delhi [13, 14], Bangalore [15, 16] and Kerala [17]. The results of the studies of allergy evaluation done using the common airborne pollen grains in the patients clearly suggested the importance of an aerobiological survey as a prerequisite for allergy evaluation studies [18]. The studies have taken note of the fact that the pollen grains of different species of *Cassia* could be allergenic to the people in almost all parts of

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1018–2438/94/1033–0280
\$5.00/0

India. Thus it was planned to have a preliminary approach to the clinicobiochemical aspects of the four dominant species of *Cassia* in the patients with respiratory allergy complaints.

Materials and Methods

Antigenic Extraction

The antigenic extraction of four species of *Cassia* was done following Sheldon et al. [19]. The major steps in the preparation of the extracts included purification, drying and defatting the pollen grains. The extraction was carried out for 24–72 h using phosphate-buffered saline of pH 8.0. These extracts were clarified by passing through millipore filters of pore size 0.45 µm attached to Sartorius filter adapters (Sartorius, FRG). For getting rid of the irritation and colouring materials which could stain the skin of the patient, dialysis using dialysing tubes (27 × 32 inches) was done for 24 h. Later the toxicity of these extracts was studied and its aerobic and anaerobic sterility using soya-bean casein digest medium (Hi-Media Lab, India) and Brewer thioglycolate medium (Difco Lab, Mich., USA) was examined after filtering the extracts through millipore filters of 0.22 µm. These extracts were lyophilised and later reconstituted as 1:500 for intradermal tests.

Clinical Studies

A total of 140 patients 10–60 years of age with a history of respiratory allergy represented the study group. Patients were selected among those attending the chest clinics in Tamil Nadu (India) with respiratory complaints of over 3–4 years' duration. The major group were females (n = 79). Of the total sample studied, 64.28% (n = 90) had a clinical history of rhinitis with asthma, 21.42% (n = 30) of rhinitis and 14.28% (n = 20) of asthma alone. Patients with an associated illness such as food allergy, drug allergy and skin allergy were excluded from the study. Intradermal skin testing was performed on the volar aspect of the forearm by injecting 0.02 ml of the antigen (1:500) using a sterile 1.0 ml tuberculin syringe. The needle was advanced until its entire bevel was in the skin penetrating entirely but not going deeper than the superficial layers, since the subcutaneous injection can lead to a false-negative test. Similarly a negative control with phosphate-buffered saline and a positive control with histamine phosphate (100 µg/ml) were used. The skin reactions were read after 15–20 min according to Chai et al. [20].

Biochemical Studies

The protein carbohydrates and amino acids of the four species of *Cassia* were analysed chemically.

Protein Content. The protein content of the extract was determined with three different methods, i.e. Lowry et al. [21] and modified Lowry's method [22] with bovine serum albumin as standards and the Micro-Kjeldhal method (% N × 6.25; using Tecator, Kjeltac Auto 1030 Nitrogen Analyser).

Carbohydrate Content. The phenol-sulphuric acid method [23] was used to determine the carbohydrate content of the crude pollen extract. A volume of 0.1 ml of aqueous solution of phenol (80%) was added to a 2 ml aliquot of the extract containing 10–20 µg protein and 5 ml of concentrated H₂SO₄ were added to the mixture stirring constantly. A reference solution was made by substituting water for the solution of allergen extract. This was allowed to stand for 30 min at

room temperature and the absorbance of the solution at 490 nm was measured in a Spectronic 20. A standard curve was established with mannose.

Amino Acid Content. The quantitative and qualitative analyses of amino acids were carried out with two-dimensional paper chromatography using Watman No. 1 paper. The extracts were hydrolysed with 6 N HCl in the presence of thioglycolic acid and phenol at 110–112°C for 24 h. The acid was removed in vacuo. The solvent used was n-butanol-acetic acid-water (4:1:5 v/v) and phenol-water (8:2 v/v). After developing, the paper was dried and sprayed with 0.4% ninhydrin, warmed at 75°C for 5 min and Rf values were noted and compared with those of a standard sample run at the same time. Colour reactions such as vanillin reagent [24] for glycine and tryptophan and sulphanilic acid reagent [25] for histidine were also used for identification. The spots were cut and eluted in a mixture of 75% ethanol and 1% copper sulphate (80:20 v/v) and colour density was measured at 540 nm in a Spectronic 20.

Results

Skin Testing

The data of the clinical study are presented in table 1. A total of 69 significant positivities (12.32%) among the total sites tested with all the *Cassia* spp. were studied. Those patients reacting to all the species of *Cassia* had a clinical history of rhinitis with asthma. The antigens of *Cassia occidentalis* were most reactive (n = 21; 15.0%) followed by *Cassia siamea* (n = 19; 13.6%), *Cassia auriculata* (n = 17; 12.1%) and *Cassia roxburghii* (n = 12; 8.6%) in the patients tested here. Of the 140 patients tested with all these four different species of the *Cassia* antigen, 10 showed significantly positive intradermal skin reactions to two species and 7 to three species, while 4 patients reacted significantly to all the four antigens. Thus, information both on the possibility of cross-reactivity and the intensity of skin reactions was obtained (fig. 1).

Protein-Carbohydrate Content

Data on the estimations of protein and carbohydrates are presented in table 2. Protein estimation of the aqueous extracts of the pollen of the four species of *Cassia* with Lowry's method gave values ranging from 7.14 to 9.37% w/w with the highest protein content in *C. occidentalis* and the lowest in *C. auriculata*. However, for the modified Lowry method, the range of protein estimated was 2.52–4.83% w/w. With this method, *C. roxburghii* showed the highest amount of protein as compared to others. With the Micro-Kjeldhal method, the estimated value of protein ranged from 4.85 to 7.13% w/w, with the lowest protein content in *C. siamea* and the highest in *C. auriculata*. For all the samples, protein estimation with Lowry's method showed higher values as compared to the other

Table 1. Clinical studies with *Cassia* pollen grains

	Positive reactions						Controls		
	total patients	2+	3+	4+	total significant reactions	%	normals	1+	%
<i>C. auriculata</i>	140	11	6	–	17	12.1	30	0	0.0
<i>C. occidentalis</i>	140	15	5	1	21	15.0	30	1	3.3
<i>C. roxburghii</i>	140	8	4	–	12	8.6	30	0	0.0
<i>C. siamea</i>	140	16	3	–	19	13.6	30	1	3.3

C. occidentalis had the greatest number of significant reactions followed by *C. siamea*. No significant reactions were observed in the control groups studies. 1+ = 3–5 mm > control; 2+ = 6–8 mm > control; 3+ = 9–11 mm > control; 4+ = 12 mm and above > control.

two methods. For each sample, the sensitivity and accuracy varied with the different methods used. The total carbohydrate content ranged from 7.81 to 12.47% w/w with the highest content in *C. roxburghii* and the lowest in *C. occidentalis*. The carbohydrate content also demonstrated a great variation between the four species. Except for one species, all the others showed a protein/carbohydrate ratio of less than one.

Amino Acid Analysis

The data on the assay of amino acids of the pollen extracts of the four species of *Cassia* are given in table 3. All the result are expressed in percent w/w.

The aqueous extract of *C. auriculata* contained a detectable level of ten amino acids of which arginine, aspartic acid, glutamic acid, leucine, proline and glycine were found to be predominant. The amino acid concentration varied from 2.5 to 20.7% w/w. The extracts of *C. occidentalis* showed the presence of thirteen amino acids ranging from 0.5 to 13.8% w/w. Of these, arginine, aspartic acid, glutamic acid, leucine and lysine showed higher concentrations. The *C. roxburghii* extracts indicated the presence of ten amino acids of which alanine, arginine, glutamic acid, lysine, and serine had higher concentrations in a range of 2.8–26.0% w/w. The amino acid concentrations of *C. siamea* ranged from 1.5 to 18.3% w/w and arginine, aspartic acid, glutamic acid and leucine showed higher values. But, amino acids such as lysine, tryptophan, cysteine and valine in *C. auriculata*; serine in *C. occidentalis*; glycine, leucine, methionine and valine in *C. roxburghii* and serine and cysteine in *C. siamea* were not observed. The absence of these amino acids in the different extracts studied and the presence in others may be due to some unforeseen factors as the same methodology for estimation was adopted in the analysis of all the extracts.

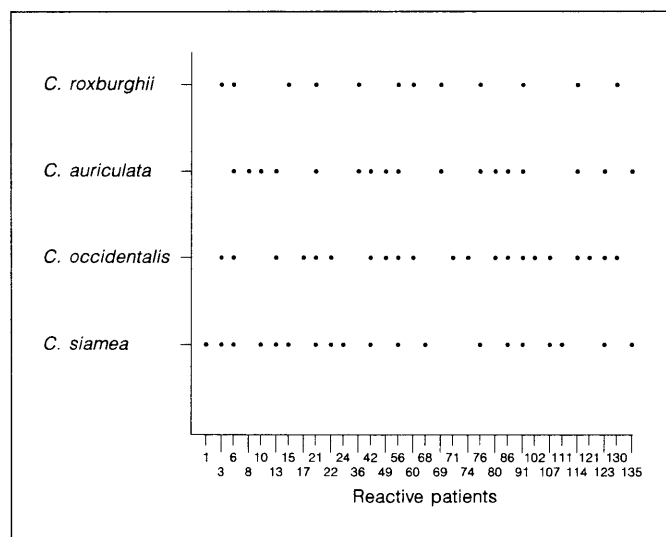


Fig. 1. Ten patients were significantly reactive to two species of the *Cassia* antigen, 7 patients to three species while 4 patients were significantly reactive to all the four species tested.

Discussion

It is well established that the airborne pollen grains are involved in human allergic diseases, such as allergic rhinitis and bronchial asthma. In India, the distribution of the pollen grains belonging to *Cassia* spp. in the air is of national relevance [4]. Similarly the aerobiological studies carried out in Thiruchirappally in the Tamil Nadu state [4, 10] and its adjacent state Kerala noted the predominant aerial distribution of the pollen grains of the *Cassia* spp. The intradermal skin test studies using these extracts elicited a higher percentage of markedly positive reactions of the patients. Similarly also in the patients of Kerala [17],

Table 2. Chemical composition of the aqueous extracts of the pollen of *Cassia* spp.

Plant name	Total protein, % w/w			Total carbohydrate % w/w	Protein/carbohydrate ratio
	Lowry's method	modified Lowry's method	Micro-Kjeldhal method		
<i>C. auriculata</i>	7.14	3.47	6.37	8.53	0.84
<i>C. occidentalis</i>	9.37	2.52	7.13	7.81	1.20
<i>C. roxburghii</i>	8.22	4.83	5.72	12.47	0.66
<i>C. siamea</i>	8.97	3.71	4.85	10.34	0.87

The protein content measured with Lowry's method was higher. The value of total carbohydrate was highest in *C. roxburghii* and smallest in *C. occidentalis*. Except for *C. occidentalis*, the protein/carbohydrate ratio was less than one.

the results of the clinical studies were almost on a par with our present findings. Different researchers used different levels of positivity percentage varying from 4 to 6% or above to consider an antigen as allergen. In the present study, all the markedly positive skin reactions (2+ to 4+) elicited by the *Cassia* antigens were taken into consideration for the recognition of their allergenic potency. A reliable diagnosis was reached as in many cases with a proper interpretation of skin test results taking into account the patients' own observations of the allergens eliciting symptoms [26, 27]. The absence of the markedly positive reactions in healthy volunteers in this study agrees with other findings [18, 28]. This could also indicate the absence of nonspecific irritants and colouring materials, which could be present in pollen preparations. Besides, for the reliability of intradermal skin testing and for its correlation with bronchial provocation testing, a recent study done by one of us showed that the skin testing positivity increased from 2+ to 4+, the BPT positivity also increased from 72 to 100% [18]. Thus, the intradermal skin testing procedure was found to be suitable and was recommended for the identification and diagnosis of the inhalant allergens in the patients of India [13, 18]. The percentage of markedly positive skin reactions elicited by *Cassia* pollen antigens ranged from 8.6 to 15.0% in the patients. Shivpuri [13] stressed the importance of 3+ and 4+ skin reactions alone while assessing the significance of an antigen through an intradermal skin test. In the present study, all the four antigens showed 3+ reactions and the *C. occidentalis* antigen even showed 4+ reactions in some patients. Considering the individual reactions, *C. occidentalis* was followed by *C. siamea* and *C. auriculata* (table 1). Individual analysis of the reactive patients also clarifies the possibility of the cross-reactivity between these different *Cassia* members (fig. 1). Of the total significant reac-

Table 3. Amino acid composition of the aqueous extracts of the pollen of *Cassia* spp. (% w/w)

Amino acid	<i>C. auriculata</i>	<i>C. occidentalis</i>	<i>C. roxburghii</i>	<i>C. siamea</i>
Alaine	7.50	1.50	8.30	2.10
Arginine	18.40	8.50	14.10	10.50
Aspartic acid	9.60	11.50	2.80	7.50
Glutamic acid	20.70	13.80	26.00	18.30
Glycine	8.30	3.50	—	18.30
Histidine	2.50	2.70	4.90	3.70
Leucine	9.10	12.40	—	11.50
Lysine	—	9.70	17.60	4.50
Proline	8.50	1.50	4.80	5.80
Serine	3.40	—	8.10	—
Tryptophan	—	2.30	4.20	2.70
Cysteine	—	1.60	2.70	—
Methionine	5.50	0.50	—	1.50
Valine	—	2.50	—	2.90

tants, a large group of patients is reactive to two or more of these antigens. This is highly suggestive of the higher intensity of reactivity, possibility of the presence of cross-allergenicity and minor allergens of these *Cassia* members to the reactive groups.

All the early studies suggested that the pollen grains have highly complex mixtures of allergenic proteins and carbohydrates, together with nonallergenic components [29]. Therefore, a preliminary study on the biochemical and clinical nature of the aqueous extracts of the pollen grains of *C. auriculata*, *C. occidentalis*, *C. roxburghii* and *C. siamea* was undertaken. This could help to understand its composition and the diagnostic value of the whole pollen extract, and is also a valid approach to extract standardisation.

The protein analysis play a greater role in the evaluation of allergen extracts. Many researchers have indicated that proteins are the active substances in the pollen grains [30]. Different methods are used for estimating protein in pollen extracts; in the present study three different methods commonly employed in the standardisation of pollen allergens were used and compared. With all the techniques the samples were analysed in triplicate at the same concentrations. Large variations were observed in the protein content estimated using different methods. In all the samples, the highest estimations were obtained with Lowry's method and the lowest with the modified Lowry's method. Noticeable differences were observed between Lowry's method and the Micro-Kjeldhal method. It is known that many substances including phenolic compounds could interfere with the Lowry method and it is probable that the high amount of phenols and phenolic pigments in the extract can be the cause for a higher protein value estimated with Lowry's method. Since the Kjeldhal procedure directly estimates only the nitrogen but not the protein, the protein value may be underestimated. The pollen extract of *C. occidentalis* showed the highest protein content which also showed significant skin reactions. This shows that by and large the Lowry method is more suitable for protein analysis with proper reference blanks and standard.

The carbohydrate content showed wide variations between the species and is in reasonable agreement with the values obtained earlier. Maheswary [31] showed that pollen grains contained carbohydrate from 3 to 15%, while in *Cassia* it ranged from 7.81 to 12.47%. However, studies have proved that the carbohydrate content is not a very common allergen. Moreover the smallest amount of carbohydrates present was observed with the most reactive *C. occidentalis* while the highest content was observed

with the least reactive *C. roxburghii*. This may be useful as a step towards standardisation of extracts, which supports the data in table 1 and figure 1.

The present study revealed the presence of several amino acids with a variation in concentration between species. The glutamic acid was found to have the highest concentration in all the samples. Alanine, arginine, aspartic acid, glutamic acid, histidine and proline were found to be present in all the samples in different quantities. Ritcher and Sehon [32] showed that the allergenicity of ragweed pollen was caused by peptides. Analysis of pollen extracts revealed a variation in the quantity and type of amino acids present. However, some of the amino acids such as glycine, serine, lysine and valine may not have been found in the extracts because of some unforeseen factors. The highest number of amino acids (i.e. 13) was present in *C. occidentalis*, which was the most significantly reactive species. This was followed by *C. siamea*, *C. auriculata* and *C. roxburghii* in number of amino acids and intensity of reactivity. An almost similar observation was made with regard to the number of amino acids present in the protein fractions. This appears to indicate that there may be a correlation between the number and quantity of amino acids and their allergenic effect. These findings agreed with those of Bathurst [33] and Shivpuri [34]. Thus, the analysis of the composition of proteins, amino acids and carbohydrates in the extracts may be a valid preliminary step towards extract standardisation in terms of protein and carbohydrate contents and also towards determining the cross-reactivity of these allergens.

Acknowledgment

The senior author wishes to thank the Council of Scientific and Industrial Research, New Delhi for financial assistance.

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