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Reduction of Allergenicity of *Litchi chinensis* Flowers Pollen Protein Conjugated with Polysaccharide by Maillard Reaction

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Abstract

Background: Allergy to pollen from gymnosperms is well documented in the west. The objective was to define the allergologic protein from *Litchi chinensis* (Litchi) pollen and conjugate the protein with polysaccharides by Maillard reaction to reduce the allergic effect of that protein.
Methods: Total soluble proteins were extracted from the pollen of Litchi flower pollen and subjected to ammonium sulphate precipitation at 80% saturation. Pollen antigen from *Litchi chinensis* (Litchi) was prepared by gel cutting method and characterized by biochemical and designated by LFPP. The homogeneity of this protein was demonstrated by a single band on SDS-PAGE. The protein then conjugated with galactomannan through Maillard Reaction. The resulting purified pollen protein and conjugated protein were administered to the Swiss albino mice as amount of 5.8mg/kg body weight.
Results: The total protein was then separated on a 12% SDS-Polyacrylamide gel which revealed 5 bands between molecular weight range of 29kDa and 69kDa. Each band was recovered from the gel by electroelution and sent for skin tests. 28kDa proteins was the only allergenic protein while others were not shown reactivity in patients. Intraperitoneal injection of the purified protein (LFPP) caused a significant rise in the levels of neutrophils (38-81%) and eosinophils (3-14%) compared to control ($P<0.001$) whereas conjugated protein caused only a 2% increase of both neutrophils and eosinophils level. On the other hand treatment with LFPP-galactomannan conjugate causes no such change in physical appearance with eosinophils and neutrophils level.
Conclusion: The present study demonstrates that the protein extracted and purified from Litchi flowers pollen has been recognized as a new allergen from Bangladesh for the first time and the allergic effects can be reduced by conjugation with polysaccharides.

Keywords: *Litchi chinensis* (Litchi); sensitization; Swiss albino mice, Eosinophils & Neutrophils.

1. Introduction

In Bangladesh, the incidence of allergen pollen is comparatively higher than neighboring countries. An experiment conducted by M.K. Pasha, Chittagong University revealed that in Bangladesh the major allergenic pollen grains are released by plants are: *Lantana camara, Acacia species, Cassia fistula, Cocos nucifera, Eleusine indica, Datura metel, Amaranthus spinosus, Cynodon dactylon, Achyranthes aspera, Albizia lebbeck, Brassica campestris, Cannabis sativa, Carica papaya, Mangifera indica,* and *Xanthium* (Pasha *et al.*, 2009).

Pollen causes respiratory allergy to the sensitive individual when it comes in contact with the upper respiratory tract, the nosal or the oral cavity releasing the allergens, which are located in the exine and other cytoplasmic sites of the pollen grains on reaching the

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watery surface of the mucosa. Thus, pollen grains are the carrier of the allergens and not the allergen itself (Rudramuniyappa, 1996). The role of pollen as a causative agent of respiratory allergic disorders is very well established, as is evident from the recent increase of reports from across the world (Ortega *et al*., 1992; Singh *et al*., 1994; Amato *et al*., 1998; Arnon *et al*., 1998). Pollen extracts are widely used both for diagnosis and treatment of allergic diseases. One of the most successful treatments for allergic rhinitis is Immunotherapy, often termed hyposensitization, involving repeated subcutaneous injections of gradually increasing concentrations of the allergens. Another method used for testing human reactions is the immunological cross-reactivities measured by ELISA. However, a major drawback which eclipses the importance of this method is that each type's pollen has its own immunologic identity. Although immunotherapy or hyposensitization vaccine is the best method for bringing permanent relief to patients suffering from respiratory allergy due to pollen, particularly for atopic patients (Bapat & Bapat, 1994; Chanda, 1996), doctors generally prescribe antihistamine drugs and steroids, which render only short-term relief to such patients. For preparation of a specific hyposensitization vaccine, for clinical application, proper identification and characterization of pollen allergens is an essential prerequisite.

The infiltration of eosinophils into the airway walls and lumen has long been recognized as a defining feature of asthma, and absence of eosinophils has been shown to modulate key features of airway allergy in experimental models (Lee *et al*., 2004; Humbles *et al*., 2004). In contrast, the role of neutrophils in airway allergy has received increasing attention relatively recently. There is a robust correlation between airway neutrophils and human asthma: the severity of airway disease appears to correlate with number of neutrophils in the airway in both asthma (Little *et al*., 2002) and allergic bronchopulmonary aspergillosis, and neutrophils are over represented in patients with asthma exacerbations, severe or poorly controlled asthma, and in those who die of asthma (Wenzel *et al*., 1997). In the context of this strong association noted in human disease, the study found long-term elevations in lung neutrophils in a mouse model of chronic airway allergy to clinically relevant ages of a ubiquitous environmental mold. Neutrophils may represent a common effector mechanism of airway inflammation in both Th-1- and Th-2-acquired immunity. Increased eosinophils, basophils and neutrophils level due to plant pollen is supported by the study of seasonal changes of laboratory data in patients with Japanese cedar pollinosis (Fujiwara *et al*., 1997).

Bangladesh is blessed with the richest delicious seasonal fruits flora on the earth. Every locality has a distinct type of forage plants. Again, the concentration and composition of forage plants are also variable. From a study of pollen load, it is estimated that about 110 plants are common as forage plants in Bangladesh. The most common fruit trees being mango, coconut, Indian jujube, litchi, Goran, Golpata, *Hibiscus* species, Cucurbits, many leguminous, Cruciferous and Solanaceous crops. Most of these are either agricultural or horticultural plants. At the flowering time of these plants peoples around these groves suffers severs allergy diseases (according to the local doctors and hospital reports). Thus, sensitive patients generally get a recurrence of symptoms during those particular seasons and or places. We reported earlier, mango pollen is one of the important components of airborne allergenic pollen (Talukder *et al*., 2012).

Although, studies on the allergenic properties of airborne pollen from various species have been carried out by several workers (Shivpuri *et al*., 1979; Singh *et al*., 1987; Singh *et al*.. 1993; Mondal *et al*., 1997) information on allergy to aerial pollen from *Litchi chinensis* tree species has been completely lacking. The present study was undertaken to assess the allergenic role of the pollen of litchi and to identify, isolate and partially characterize the allergenic components of this pollen.

This study strongly correlated with our findings because in our case the level of eosinophils and neutrophils significantly increased with treatment of LFPP in the mice model. So, our protein modulates key features of pollen allergy. Finally, we conjugate the LFPP with galactomannan. The SDS-PAGE pattern of the LFPP-galactomannan conjugate prepared by the Maillard reaction showed broad bands, suggesting the attachment of galactomannan. The treatment of purified LFPP-galactomannan in mice did not cause any significant change of physical appearance and eosinophil and neutrophil level. This result is supported by the reduction of antigenicity of Cry j I, Major allergen of Japanese cedar pollen, by the attachment of polysaccharides (Masakatsu Usui *et al*., 2003). These results suggest that the allergenicity of LFPP is greatly reduced by the conjugation with galactomannan. Dry heating of LFPP in the absence of galactomannan was not effective in reducing the level of eosinophils and neutrophils. Therefore, the attachment of galactomannan is important in reducing the level of eosinophils and neutrophils

1. Materials and Methods

1.1 Source material

The pollen was collected from Nawabganj, Rajshahi Town and Rajshahi University ranges of the Rajshahi region, Bangladesh during the pollination period in March to April. These pollen grains were then processed for > 95% purity by sieving through different grades of sieves (100, 200 and 300 mesh/cm^2). All the samples were analyzed under the microscope which revealed pollen purity varying from 85% to 90%. To

remove lipids and irritants of low molecular mass, the pollen sample was defatted with diethyl ether by repeated changes, until the ether becomes colorless. The defatted pollen powder was then completely dried and stored at 40°C in airtight containers until further use.

2.2 Protein extraction

The defatted pollen was then used for protein extraction. Proteins were extracted in 0.2M Tris- HCl buffer, pH 7.4 by continuous stirring at 40°C for 24 hours. The extract was clarified by centrifugation at 15,000 x g for 20 min. at 4°C. The supernatant was collected and was subjected to fractional precipitation by solid ammonium sulphate. It was made up to 80% saturation by slow addition of the salt at 40°C. After centrifugation, the precipitate was re-suspended in 0.1M Tris HCl buffer, pH 7.4 and desalted by dialyzing against distilled water for 48 hr at 40°C by frequent changes of the distilled water using dialysis sacs (MW cut off 9 kDa). Finally, the supernatant was passed through a Millipore filter membrane (0.45µm), lyophilized in small aliquots, and stored at -200°C until further use.

2.3 Estimation of protein

The protein concentration in the extract, as well as in the various eluted fractions, was estimated by the modified method of Lowry (Lowry *et al*., 1951). A calibrated solution of bovine serum albumin was used as a standard.

2.4 Gel electrophoresis

The protein sample was heated with an equal amount of sample buffer [0.06M Tris HCl (pH 6.8), 1% SDS, 10% sucrose, 0.5% β-mercaptoethanol, 0.01% bromophenol blue] at 100°C for 3 min. 10µl of the sample containing 85µg of protein was loaded in the well of a 12% T mini-gel (8 x 7cm gel) Mini-Protean II slab gel apparatus (Bio-Rad, Hercules, CA, USA) and the gel was run using Laemmli buffer system [1971]) (0.05M Tris, 0.192 M Glycine, 0.1% SDS, pH 8.4) at room temperature for 2 hours 30 min., at 70 V. The molecular mass of the fractions was calculated by calibrating with standard marker protein, lysozyme (14,000 kDa), trypsin inhibitor (20,000 kDa), carbonic anhydrase (29,000 kDa), ovalbumin (45,000 kDa), albumin (BSA, 67,000 kDa) (Pharmacia, Uppsala, Sweden). After electrophoresis, the gel stained with 0.1% Coomassie Brilliant Blue R-250 and destined with methanol: acetic acid: water (4:1:5) mixture.

2.5 Isoelectric focusing (IEF)

IEF was performed, as described by Grafin (1990), with precasted, broad-range (3.5 to 9.5) Ampholine PAG plates (Pharmacia, Uppsala, Sweden). Pollen extract was electro-focused on gel along with pI marker (Pharmacia, Uppsala, Sweden) to calibrate the pI of proteins in *Litchi* pollen extracts. IEF was run for 15 min. at 100 V, followed by another 15 min. at 200 V, and finally at 450 V for 2 hours. The gel was stained with Coomassie Brilliant Blue R-250, as in SDS-PAGE.

2.6 Recovery of protein from the gel

Protein was eluted from the gel, following the method of Wilson & Goulding (1986). After electrophoresis, one side of the gel (covering 2 lanes) was cut vertically and stained in order to ascertain the banding positions. Only those portions corresponding to the protein bands to be recovered were cut out with a sterile scalpel from the other half of the gel which was not stained. The gel pieces were then transferred into pre-treated dialysis bags filled with electroelution buffer (containing 0.05M Tris and 0.192M Glycine, pH 8.4) so that each gel piece was surrounded by a small amount of buffer with no air bubbles.

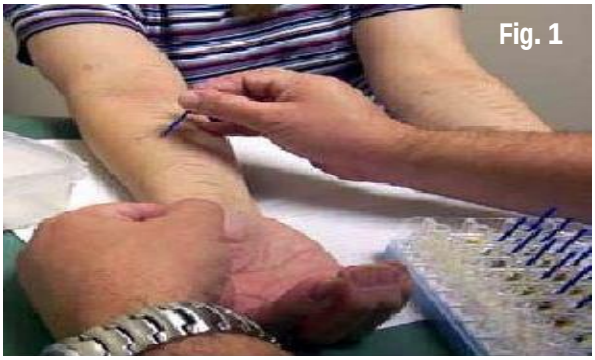
These dialysis bags were immersed in electrode buffer in an electrophoresis tank of a horizontal gel apparatus. Electric current (120V) was passed through the bags for nearly 3 hours to elude the proteins out of the gel. At the end, the current was reversed for 30 seconds in order to release the protein from the wall of the sac. The buffer containing the eluted proteins was then transferred into a cotton plugged Eppendorf tube and centrifuged for 2 min. to remove contaminating gel particles. The entire process was repeated 5 times to get sufficient quantities of each fraction for loading in the gel and for further skin tests. The protein in each fraction was quantified and each fraction was again electrophoresed to check its homogeneity.

2.7 Skin prick test

The skin tests were performed on patients suffering from nasobronchial allergy as well as healthy volunteers at the Rajshahi Medical College and Rajshahi University students. Each patient was tested by placing 10µl of each allergen; at least 5cm apart on the volar surface of his/ her forearm and each site was then pricked with a disposable hypodermic needle. Negative and positive controls were also performed. The negative control was the buffer saline in which the allergen was resuspended and the positive control was histamine acid phosphate injection diluted with buffered saline to 1:10,000 i.e. 1µg of histamine acid phosphate. The patients were prohibited from using antihistamine, steroid and ephedrine for 48 hrs before the skin prick tests. The skin reactions were read after 15 to 20 min. from the commencement of the test. The test was quantified on the basis of the wheel diameter and graded 1^+ to 4^+. The skin tests were conducted at the Rajshahi University Medical center and Rajshahi Medical College Hospital, Bangladesh. The patients were selected on the basis of their suffering from respiratory allergic disorders.

2.8 Skin Tests (Practical)

A diluted extract of each kind of pollen is applied to a scratch or puncture made on the patient's arm or back or injected under the patient's skin. With a positive reaction, a small, raised, reddened area with a surrounding flush (called a wheal and flare) will appear at the test site. The size of the wheal can provide the physician with an important reaction diagnostic clue. Skin testing remains the most sensitive and least costly diagnostic tool.



2. Result and Discussion

An attempt was made to extract and purify *Litchi chinensis* flower pollen protein (LFPP). The protein content of *Litchi* flower collected from different region showed considerable variation. The concentration of protein varied between 7.6 to 9.2mg ml^{-1} with the highest concentration observed in the sample collected from Nawabganj sadar (Table 1). The lowest concentration of protein in the litchi pollen extract is in conformity with the general observation of low protein content in the pollen of gymnosperms (Lacovacci, P. *et al*., 1998; Cimignoli, E. *et al*., 1992; Pettyjohn, M.E. *et al*., 1997).

Table 1. Protein content of pollen extracts of *Litchi* flower pollen from different places.

Place of Collection	Protein (mg ml^{-1})
Nawabganj Sadar	9.2
Thanor Sadar	8.4
Rajshahi University	7.6
Rajshahi Town	8.6

3.1 Protein Purification

The protein, extracted from the Lychee (*Litchi chinensis*) flower pollen was partially purified by ammonium sulphate precipitation and then followed by gel filtration and Ion exchange chromatography respectively. High purified protein for the allergenic skin test was not found after Ion exchange chromatography (Figure not shown).

The SDS-PAGE protein profile of the extracted pollen protein after Ion exchange chromatography shows in Fig. 2. There are five protein bands (three deep and two faint) found on SDS-PAGE between 26 kDa to 67 kDa. Multiple protein fractions of 10-100 kDa in other gymnosperm species have been reported from Europe (Lacovacci *et al*., 1998; Cimignoli *et al*., 1992; Pettyjohn *et al*., 1997). We decided to separate proteins by gel cutting methods. Only one protein band (28 kDa) had shown allergenic in character, which was found after gel cutting and which was confirmed by skin test (Table 3). By IEF, we were able to detect 9 protein bands mostly in the acidic range (3.5 - 9.5), except for two fractions observed in the basic region. Our new allergen protein band pI was 6.5 (Fig. 3). The molecular weight of the purified Litchi flowers allergen pollen protein (LFPP) as determined by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE) to be 28,000 also (Fig. 2). Whereas, Salvatore Feo *et al*., purified allergen protein from *Parietaria judaica* pollen having molecular weight 10,400 by HPLC on SDS-PAGE (Salvatore *et al*., 1984).

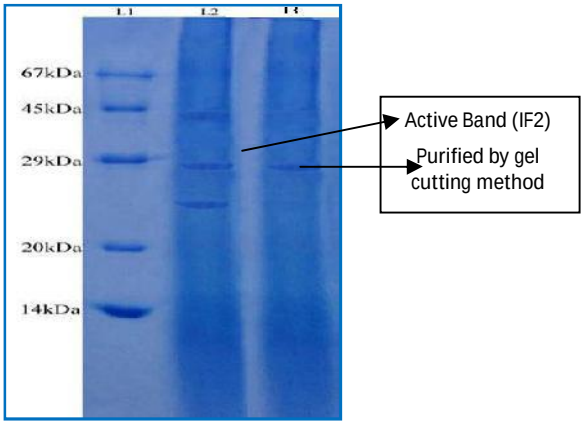


Fig. 2. Photographic representation of sodium dodecyl sulfate polyacrylamide slab gel electrophoretic pattern of the protein profile after Ion exchange chromatography and purified protein by gel cutting method. L-1: Marker protein solution containing lysozyme (14,000), trypsin inhibitor (20,000), carbonic anhydrase (29,000), ovalbumin (45,000), albumin (BSA, 67,000). L-2: Fraction from DEAE-cellulose column. L-3: Purified fraction eluted from gel cutting method.

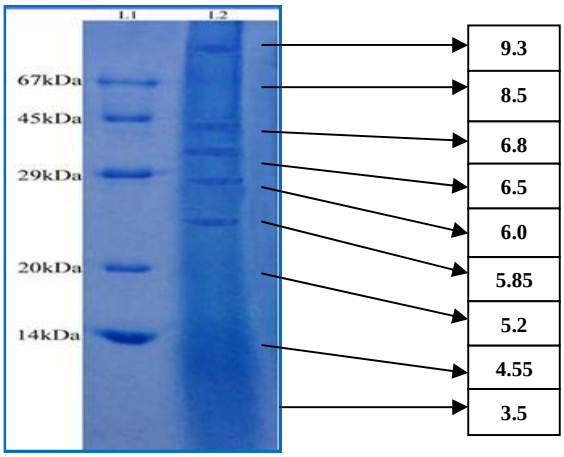


Fig. 3. IEF pattern of proteins of Liches flower pollen extract (1.2) pI range 3.5 to 9.5) PAG plates. (1,1) pI markers.

Variation in skin sensitivity was also observed in the different area samples (Table 2). Skin prick tests with the extracts of pollen sampled at different locations were performed in 30 patients already suffering from respiratory allergic disorders, with the ages ranging between 12-65 years. The highest degree of skin reactivity was observed in the case of the Nawabganj sadar sample with 28 (93%) showing positive reactions, of whom 4 (13.3%) showed 1+ reaction, 6 (20%) showed 2+ reaction, while 3+ reaction was obtained in 18 patients (60%). The lowest degree of skin reactivity was observed in case of the Rajshahi University campus sample with 21 patients (70%) reacting positively and only 3 (10%) showing 3+ reaction. The pollen of *Litchi* flower proves to be one of the important aeroallergens of Rajshahi Regions capable of inducing respiratory allergic diseases in Bangladesh. We reported earlier (Talukder *et al*., 2012) a new allergen from mango flower pollen, the M.W. 27 kDa. The differences in the protein profiles, as well as the protein content in the *Litchi* flower pollen samples collected from the various regions of Rajshahi, could be due to variable climatic conditions prevailing in these areas and soil conditions, as has been earlier suggested by Singh *et al*., 1993. The highest rate of skin sensitivity in the sample collected from Nawabganj sadar may be due to the higher protein content as well as the additional protein bands, resulting in sensitivity to some patients due to these protein fractions may another factor for increasing the allergenicity of pollen in air pollution. This is effected by changing the kind and proportions of exinic mineral elements (Amato *et al*., 1998; Newmark *et al*., 1967) or by affecting pollen morphology or protein profile (Arnon *et al*., 1998; Shivpuri *et al*., 1979). Thus, the atmosphere of Rajshahi (Nawabjanj sadar), being the most polluted due to brick, rice mills, industrialization and dust particle in the air from the dry Padma river by the wind, may be responsible for the increased rate of allergenicity of the pollen grains.

IEF: Altogether, 10 bands were detected by IEF, of which 6 were in the acidic region (3.5 - 6.55) and only two bands at pI 8.5 and 9.3 were observed in the basic region (Fig. 4). Protein bands at pI 4.55, 5.2, 5.85, 6.0, 6.5, 6.8, 8.5 were prominent and intensely stained, while bands at pI 5.8, 6.0, 6.5 and 6.8 were distinct but fine. However, other fractions were weak and poorly stained.

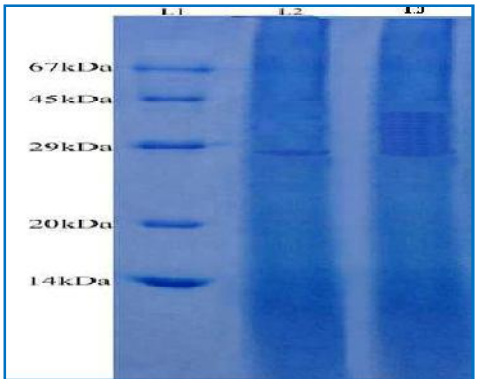


Fig. 4. SDS-PAGE pattern of LFPP and LFPP-galactomannan conjugate.

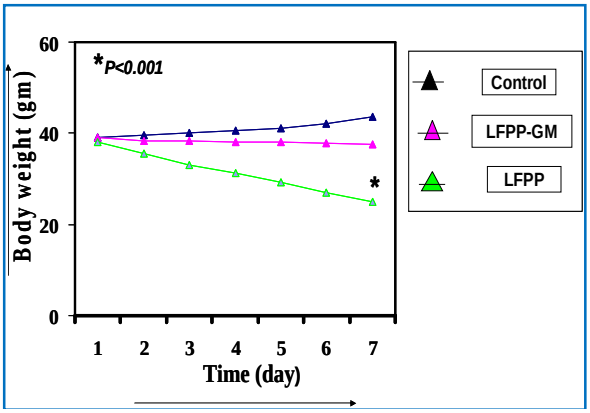


Fig. 5. Effects of LFPP and LFPP-galactomannan conjugate on body weight in mice model: Body weight of control and treated mice were also measured. Body weight was found to be decreased by 34% and 3% for LFPP and LFPP-galactomannan conjugated protein respectively compared to control.

Table 2. Results of skin tests with the antigen of *Litchi* flower pollen from different places.

Place of collection	Total no. of Test	Negative	1+	2+	3+
Nawabganj Sadar	30	2 (6.7%)	4(13.3%)	6(20%)	18(60%)
Thanor Sadar	30	12 (40%)	9(30%)	6(20%)	3(10%)
Rajshahi University	30	11(36.7%)	9(30%)	7(23.3%)	3(10%)
Rajshahi Town	30	9(30%)	11(36.7%)	5(16.7%)	5(16.6%)

Table 3. Result of skin tests against total extract and individual antigenic fraction of *Litchi* Pollen sensitive patients.

No. of patient	Age (yrs)	Sex	Total Protein	IF_1	IF_2	IF_3
5	9-12	F	2+	-ve	3+	1+
8	21-27	F	1+	1+	3+	-ve
5	50-60	F	3+	1+	3+	-ve
6	31-45	M	2+	-ve	3+	-ve
12	55-60	M	3+	1+	2+	1+
14	20-26	M	2+	-ve	3+	-ve

3.2 Purification of LFPP-galactomannan conjugates prepared by Maillard Reaction

The LFPP-galactomannan conjugate was purified by cation exchange chromatography to remove the free LFPP and galactomannan. The SDS-PAGE pattern of purified LFPP-galactomannan conjugate shows high molecular mass broad bands. The broadband is attributed to the widely distributed molecular size and the binding number of galactomannan. The attachment of galactomannan to LFPP increased with dry heating time and reached a maximum after two weeks. These results indicated that LFPP was covalently attached with galactomannan through the Maillard reaction during the dry heating. Similar results were reported for an ovalbumin-dextran conjugate, (Babiker *et al*., 1998) and a soybean allergen p34-galactomannan conjugate (Salvotor *et al*., 1984). Cross-reactivity of carbohydrate moieties with specific IgE has also been reported by Afferni *et al*., (1999).

3.3 Effects of LFPP and LFPP-galactomannan conjugate on physical appearance

Application of LFPP by regular doses to the experimental mice causes watery and red eyes, sneezing, nasal mucus, and itchy throat and hair, ears, nose and skin rash. The mice also become week. But in case of normal and LFPP-galactomannan treatment, there is a little significant change in physical appearance.

3.4 Effects of LFPP and LFPP-galactomannan conjugate on Eosinophils and Neutrophils level

The LFPP and LFPP-galactomannan conjugate applied on the experimental mice for seven days. Intraperitoneal injection of the purified protein (LFPP) caused a significant rise in the levels of neutrophils (38-81%) and eosinophils (3-14%) compared to control ($P<0.001$) whereas conjugated protein caused only a 2% increase of both neutrophils and eosinophils level.

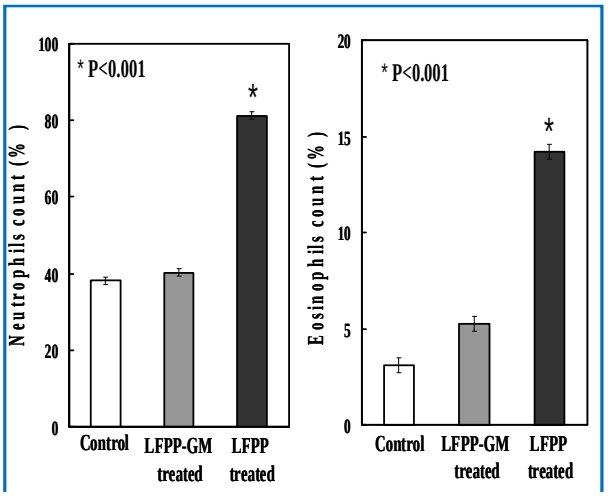


Fig. 6. Total count (%) of neutrophils and eosinophils.

We also examined lymphocytes and monocytes level. In case of lymphocytes, the level decreased significantly after LFPP treatment and almost similar in case of LFPP-galactomannan treatment. But the level of monocytes not significantly changed in both cases. Although IgE is an important parameter in allergic responses, the result of this experiment not included here. Furthermore, LFPP-galactomannan conjugate is constructed by a naturally occurring reaction without any synthetic chemicals. Therefore, LFPP-galactomannan conjugate may be a safe material and this protein polysaccharide conjugation is a promising approach to reduce the allergenicity of allergens.

Fig. 7. Physical appearance of experimental and control mice.

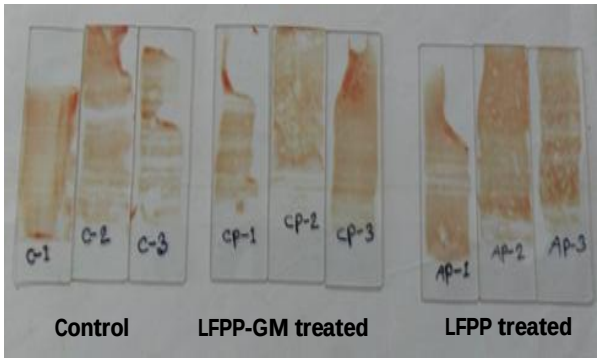


Fig. 8. Slide of the experimental and control mice's blood.

4. Conclusion

Skin tests are the major diagnostic tool for allergy and reveal the pollen of *Litchi chinensis* to be a potent allergenic offender. Among the 5 proteins, bands obtain after Ion exchange chromatography only one protein which showed a positive response in all the 30 patients can thus be said to be the only allergenic band. With the advent of immunotherapy, the need for standardization of allergenic extracts is well known. Pollen extract content complex mixtures of proteins, complex carbohydrates, lipids, enzymes, lectins etc. (Mondal & Mandal, 1997, Mondal *et al*., 1997, a, b, 1998; Parui & Mandal, 1998, Parui *et al*., 1998, 1999) and the crude

antigenic extracts usually contain several other components other than proteins to which the patients may show some allergic reaction. The method followed in this investigation enabled us to isolate particular protein bands from a mixture of total proteins and to test these purified fractions to identify the allergenic determinants. A considerable degree of allergen crosses reactivity with carbohydrate moieties is suspected in the *Litchi* pollen allergen. The LFPP and LFPP-galactomannan conjugate applied on the experimental mice for seven days. Intraperitoneal injection of the purified protein (LFPP) caused a significant rise in the levels of neutrophils (38-81%) and eosinophils (3-14%) compared to control ($P<0.001$) whereas conjugated protein caused only a 2% increase of both neutrophils and eosinophils level. Therefore, this protein polysaccharide conjugation is a useful technique for reduction of allergenicity. With present day knowledge of epitope mapping and molecular cloning, the present study will contribute to the design of immunotherapeutic vaccines and the production of unlimited quantities of defined allergens.

Acknowledgment

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