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PHYTAL PROTEINS OF JACKFRUIT (*Artocarpus heterophyllus* Lam.) AND THEIR IMMUNO-COMPATIBILITY ASSESSMENT WITH INDOOR FUNGAL ANTIGENS AND HUMAN ANTIBODY

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ABSTRACT

Several phytal extracts in Ayurveda are used in controlling certain allergies and bronchial disorders. In present study, the suspected anti-allergic activity of a tropical perennial tree, jackfruit (*Artocarpus heterophyllus* Lam), roots were evaluated against allergens that cause bronchial allergy/asthma. The chemical composition of phytal extracts indicated the presence of alkaloids, flavonoids, phytosterols, etc. with suspected anti-allergic capabilities. The binding capabilities between extracts, indoor fungal antigens and human antibodies (Ig E) based on different immunoassays indicated that jackfruit root protein of 75 kDa can be considered as possible and novel treatment option for common respiratory allergies.

Keywords: Allergens, jackfruit, immuno-binding, phytochemicals

INTRODUCTION

Jackfruit (*Artocarpus heterophyllus* Lam.) belongs to the family Moraceae and grows abundantly in Asian sub-continent, Southeast Asia and Africa (Abedin *et al*., 2012). The nutritive properties of jackfruit are known since ancient times but its medicinal properties have recently received the attention of researchers. Jackfruit seeds possess high contents of thiamine and ascorbic acid (Shrikant *et.al,* 2012), while fruit pulp contains niacin and a variety of saponins (Bello and Falade, 2008). The ascorbic acid, riboflavin, vitamins and carotenes present in perianth of fruit have anti-carcinogenic and antioxidants properties (Shrikant *et al.,* 2012; Valipour *et al.,* 2015). The plant possesses tetra-methoxy flavones, phenolic compounds (artocarpesin, norartocarpetin and oxyresveratrol) which have anti-inflammatory effect (Venkataraman, 1972). Other flavonoids like cycloartomunin cyclomorusin, cyclocommunin, cudraflavone, artomunoxanthone, artonin, artocarpanone, heteroflavanones, cycloheterohyllin, etc. present in jackfruit have shown anti-inflammatory and inhibitory activities on chemical mediators released from mast cells, neutrophils and macrophages (Jagtap and Bapat, 2010). Jackfruit has been exploited as cure for stomach ulcers, gastric problems, skin burns and skin-related infections, as well as to recover osteoporosis and aid in thyroid gland metabolism (Abedin *et al.,* 2012; Shrikant *et al,* 2012). The roots of jackfruit are suspected to exhibit anti-hypersensitive and anti-asthmatic properties and help in curing diarrhea and fever (Kanwar and

Verma, 2006; Ashok and Ramaswamy, 2014). Lectin protein jacalin from jackfruit made easy to study O-linked glycoproteins such as human IgA (Abedin *et al.*, 2012).

The human immune system is a complex system which protects an individual from micro-organisms, chemicals, viruses and fungi. An immune response is a function based on recognition and response activity. The response towards an antigen or allergen is brought about by complex chain of reactions by the various effector molecules of an immune system. An inappropriate immune response is termed as hypersensitivity or allergy. Potential allergens such as mold spores, dust mites, etc. are common in daily indoor and outdoor environments. Most molds found in home (e.g. *Aspergillus* and *Penicillium*) are more closely associated with allergic reactions in individuals with impaired immune functions. Although a variety of chemotherapeutic and allopathic agents are available to treat these allergies, they may cause side-effects (Chunduri, 2014). The development of plant-based medicines with active phytochemicals that can treat these allergies at preliminary stages is gaining popularity. The use of various plants or their parts as medicine is known to India for centuries. Plants are also the major source of drugs used in allopathy and continue to provide cure for mankind as herbal remedies (Halilu *et al*., 2012). Previous studies on root extracts suggested possible bronco-dilatory activity in albino rats (Bersabal *et al.*, 2012). The present study was aimed to assess jackfruit root extracts against common fungal allergens and elucidate their possible binding ability to excessive antibodies that may cause chronic allergies.

MATERIALS AND METHODS

Sample collection and preparation

Jackfruit root samples were collected from the Western suburbs of Mumbai (India). The roots were randomly selected, dried at 50˚C for 24 h and finely powdered.

Phytochemical analysis

For terpenoids and phytosterols analysis, the root extract was prepared by dissolving 5 g powdered root sample in 15 mL of 80% methanol and the material kept overnight as such at room temperature. The supernatant was used to analyze samples for terpenoids (ferric chloride, bromine water and sulphuric acid tests), phytosterols (Salkowski's and Liberman's tests), alkaloids (Mayer's, picric acid and Wagner's tests) and flavonoids (Shinoda's, NaOH and lead acetate tests) [Halilu *et al.* (2012), Manik *et al.* (2014)]. For carbohydrate analysis, 2.5 g dried root powder was dissolved in 10 mL methanol and the extract concentrated in a water bath to carry out for Molisch's and Fehling's tests.

FT-IR analysis

FT-IR of jackfruit root samples was performed by standard procedure (Starlin *et al.*, 2012; Ashok and Ramaswamy, 2014). The methanolic root extract prepared (1 g mL^{-1} methanol) and dried for 3-4 days. The dry extract was subjected to FT-IR analysis to screen various phytochemical functional groups as per Sawant (2011).

Total flavonoid estimation

Flavonoids are phytochemicals with immuno-enhancing and antioxidant activity. The total flavonoid content was estimated by $AlCl_3$ assay (Yadav *et al*., 2014). Roots (5 g) were crushed in 15 mL 20% methanol to prepare methanolic extracts of sample. To 0.5 mL methanolic extract, 2 mL distilled water, 0.15 mL 5% $NaNO_2$, 0.15 mL 10% $AlCl_3$ and 2 mL 1N NaOH were added and mixed well. The absorbance was noted at 510 nm using Equiptron spectrophotometer. Quercetin was used as standard.

Total phenol estimation

The total phenolic content of methanolic extracts of jackfruit root was estimated by Folin-Ciocalteau method (Rao, 2013; Yadav *et al.,* 2014). For this, 2.5 mL Folin-Ciocalteau reagent and 2 mL 7% Na_2CO_3 were added to 0.5 mL root extract, incubated at room temperature for 30 min and absorbance taken at 765 nm using Equiptron spectrophotometer. The total phenolic content was estimated in terms of gallic acid equivalents. The samples and standards were colorimetrically (Equiptron 650) read at 590 nm. The protein concentration of the root sample was estimated in μg mL^{-1}

Root protein extraction

For protein extraction, 5 g root powder was dissolved in 10 mL protein-extraction buffer [containing tris, EDTA and mercaptoethanol (pH 7.2)]. The mixture was boiled, filtered, concentrated and stored under refrigerated conditions. The protein concentration of filtered extract was estimated by Bradford's assay using BSA as standard (Valipour *et al,* 2015). The samples and standards were colorimetrically (Equiptron 650) assayed at 590 nm and protein concentration expressed in terms of μg protein mL^{-1} extract.

Gel based protein separation

The native polyacrylamide gel electrophoresis protocol was followed for purification and separation of proteins from root extract samples. The samples were mixed with gel loading in 1:10 ratio. The sample (30 μL) and protein marker (14-66 kDa) were loaded in the wells of 15% polyacrylamide gel. A steady current of 100 V was passed during electrophoresis process. The gel was stained using standard CBB staining technique (45 min) followed by de-staining (2-3 h). Protein bands were estimated on the basis of matching with protein marker bands. Protein was eluted by homogenizing the band in tris HCl (pH 6.8) as elution buffer (Rosenberg, 2004). These protein samples were used for further immunological assays.

Extraction of fungal allergens

For extraction of fungal allergens, fungal (*Aspergillus niger)* extracts were prepared from their spores using coca's buffer and from mycelium using phosphate buffer. The samples were homogenized and centrifuged; and supernatant stored under lyophilized conditions till further use. Standardized Ig E antiserum sample (359 kU L^{-1}) was used to check the antigenic binding capacity of phytal extracts.

Immunological assays of eluted proteins

Immuno-binding ability of separated proteins with fungal antigenic extracts and human antibodies (Ig E) was evaluated by various immunological assay methods (Mayer and Walker,1987). The immunoassays were standardized and replicated thrice as per standard methods (Collins *et al.*, 2004). These assays enable us to assess the binding efficiencies between the protein components and establish the plant protein extracts as suitable for future medicinal sources.

Double diffusion assay: Double diffusion assay was performed as per standard protocol of Collins *et al.* (2004). For this, 1.5% agar (prepared in borate buffer, pH 8.2) was poured on plates and 10 μL each of eluted protein, fungal antigens, Ig E antibodies, and buffer loaded in individual wells (Abedin *et al.,* 2012) and incubated at 37˚C for 3 days under moist conditions. The plates were washed by borax washing solution, stained with standard CBB stain and unstained (Rosenberg, 2004). Negative and positive controls were also run along with the test.

Counter immuno-electrophoresis: The binding ability between fungal antigen and protein sample, and Ig E titer and fungal antigen was assessed by using counter immuno-electrophoresis (Indumathi *et al.,* 2008; Csako, 2019). Agar gel (1%) slides were used to load the eluted protein samples. Electrophoresis was performed at a steady current of 100 V. After the run was complete, troughs were made on either side of the slide and filled with std Ig E and fungal antigen extract. The slides were incubated at 37˚C for 2 days under moist conditions and stained using CBB staining technique followed by de-staining.

Radial immuno-diffusion: The interaction between protein and Ig E was assayed by radial immuno-diffusion technique (Mayer and Walker, 1987). For this, wells were bored in 1% agarose gel containing 300 µL fungal antigenic extract. Then 10 µL each of eluted protein and high Ig E titer were loaded in separate wells and slides incubated under moist conditions at 37˚C for 3 days. Precipitation zones were assessed after CBB staining and destaining procedure. Negative control plates having extraction buffer alone were also maintained.

Rocket immuno-electrophoresis: The standard protocol of Csako (2019) was followed. For this, 1% agarose in TBE buffer (pH 8) and 350 µL fungal antigenic extract was used to coat glass slide. Wells were loaded with eluted protein sample and subjected to electrophoresis at a steady current of 100 V. Standard CBB staining technique was carried out for 45 min followed by for 2-3 h destaining.

RESULTS AND DISCUSSION

Phytochemical screening

In present study, an attempt was made to evaluate the phytochemical composition of jackfruit roots qualitatively and quantitatively. The qualitative phytochemical screening revealed the presence of tannins, alkaloids. flavonoids, phytosterol, etc. (Table 1). The positive results for bromine water (orange precipitate) and H_2SO_4 (yellow precipitate) tests confirmed the presence of tannins. Positive Mayer's test (insoluble precipitate) and Wagner's test (brown colour) confirmed the presence of alkaloids. Molisch's and Fehling's test were negative revealing the absence of carbohydrates. However, positive results for Shinoda's test (yellow precipitate), lead acetate test (white insoluble precipitate) and NaOH test indicated the presence of flavonoids. Phytosterol screening by Liberman's test showed brown ring formation indicating positive test and Salkowski's test showed a golden yellow precipitate indicating the presence of phytosterols. The qualitative phyto-chemical screening revealed the presence of tannins, alkaloids, flavonols and phytosterols.

Table 1: Qualitative phytochemical analysis of jackfruit roots

Phytochemical constituents	Tests	Results
Tannins	Bromine water test	+
	H_2SO_4 test	+
Alkaloids	Mayer's test	+
	Wagner's test	+
Carbohydrates	Molisch's test	-
	Fehling's test	-
Flavonoids	Shinoda's test	+
	NaOH test	+
	Lead acetate test	+
Phytosterols	Liberman's test	+
	Salkowski's test	+

FT-IR analysis

FT-IR analysis of root extract was read with standard FT-IR interpretation chart (Coates, 2000; Sahu and Saxena, 2013). FTIR analysis confirmed the presence of terpenoids, flavonoids, alkaloids, phenols and phytosterols as phytochemical constituents. FT-IR showed a sharp peak at 3029, 2384, 1819 and 1746 nm which corresponded to CH stretch, tertiary amines, acid halides and tertiary linked esters/alkyl carbonates. The peak at 1413 nm indicated the presence of phenols or tertiary alcohols, OH bond and the presence of phenolic groups was confirmed by a sharp and clear peak at 1203 nm. Peaks were also observed at 1302, 884 and 646 nm revealing the presence of sterol ring, peroxide stretch and thioesters, respectively. FTIR analysis confirmed the presence of terpenoids, flavonoids, alkaloids, phenols and phytosterols as phytochemical constituents of root extracts (Fig. 1).

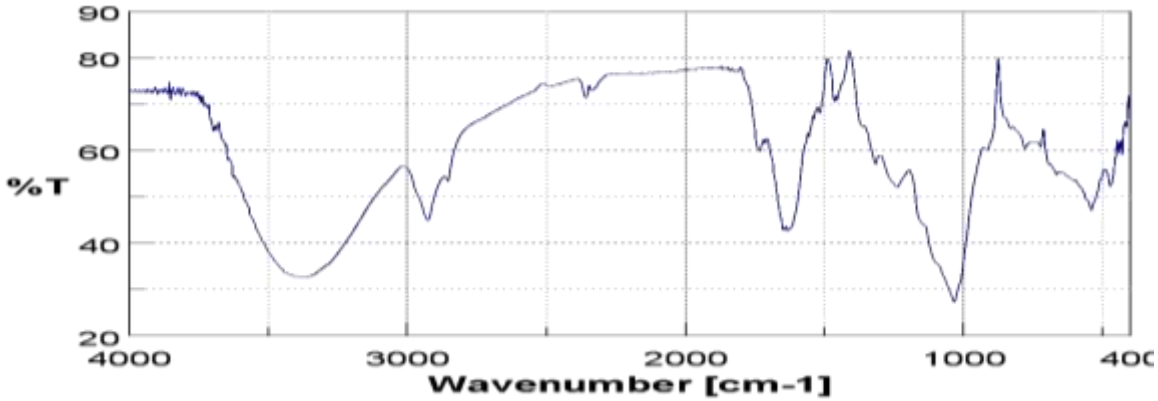


Fig. 1: FTIR based phytochemical analysis of root extracts of jackfruit

Quantitative analysis of phytochemicals

For determining the total flavonoid content, the methanolic extract of jackfruit root was assayed by $AlCl_3$ method using quercitin as a standard. The concentration of flavonoid was 1.028 mg g^{-1} sample. For total phenolic content, the methanolic extracts of jackfruit root was assayed by Follin-Ciocalteau method using gallic acid as a standard. Total phenolic content was 0.364 mg g^{-1} sample.

Immuno-assays

Immuno-diffusion methods are the techniques that play an important role in immunology. These powerful tools work on the principle of reactions between antigens and antibodies based on the principles of zone electrophoresis and immunodiffusion. These assays were conducted to know the binding capacities of plant proteins with that of antigens or antibodies and evaluate the best interactions.

Soluble proteins (antigen or antibody) diffuse through a matrix of gel towards each other and form an immune complex (Kanwar and Verma, 2006) in the immunodiffusion process. Immunobinding studies were earlier considered to determine the binding ability between viral particles and vaccine (Ullman *et al.*, 1989). In present study of DID, isolated protein band number 2 (45.7 kDa) from jackfruit root could form an immune complex with fungal antigen while other proteins were unable to do so.

Root proteins identification and purification

Native polyacrylamide gel electrophoresis of root extract indicated four different protein bands. The Rf values of the isolated protein band number 3 and 4 from the root sample matched with that of marker protein band number 4 and 5 indicating their molecular weights being 20 and 14 kDa, respectively. On contrary, the Rf values of isolated protein bands number 1 and 2 did not match with any of the protein marker bands. The molecular weights of these bands were calculated as per Rosenberg (2004). The molecular weight of isolated protein band number 1 and 2 from root extract was found to be 1000 and 45.70 kDa, respectively (Fig. 2). Thus, on gel-based protein isolation, 4 bands of 1000, 45.7, 20 and 14 kDa were obtained.

Immuno assays

The immunoassay of separated plant protein, human Ig E and fungal antigen was done by double immune-diffusion method. A distinct zone of precipitation measuring 1.1 cm was seen in positive control whereas negative control showed no zone of precipitation. A clear band of precipitate was seen in case of isolated root protein band number 2 when loaded along with fungal antigen and Ig E. No distinct precipitate formation was seen for other isolated protein bands (Fig. 3). In current study of DID, isolated protein band number 2 (45.70 kDa) from the jackfruit root could form an immune complex with the fungal antigen while the other proteins were unable to do so.

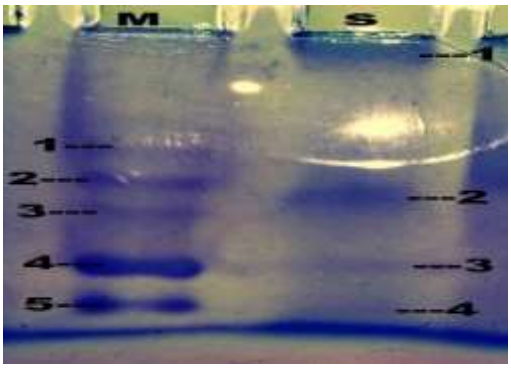
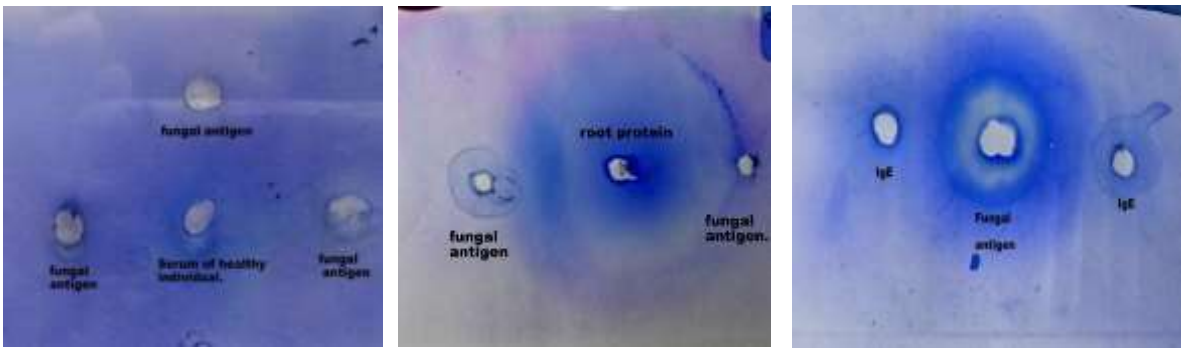


Fig. 2: Protein bands separated from the root extracts of jackfruit by PAGE

Immuno-diffusion methods are powerful tools working on the principle of reactions between antigens and antibodies based on the principles of zone electrophoresis and immunodiffusion. These assays were conducted to know the binding capacities of plant proteins with that of antigens or antibodies and evaluate the best interactions. Soluble proteins (antigen or antibody) diffuse through a matrix of gel towards each other and form an immune complex (Kanwar and Verma, 2006) in immunodiffusion process. Immuno-binding studies were earlier considered to study binding ability between the viral particles and the vaccine (Ullman *et al.,* 1989).



Negative control Sample control (protein band 2) Positive control

Fig. 3: Double immunodiffusion exhibited by the protein extract of jackfruit

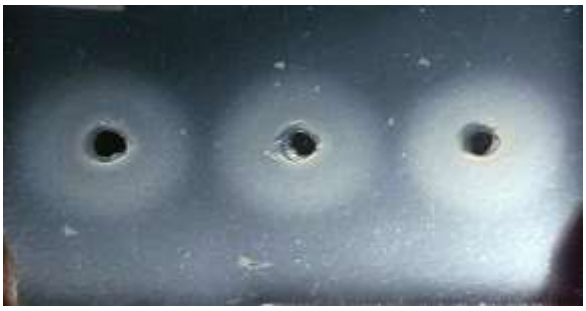


Fig. 4: Radial immunodiffusion results of root extraction; Distinct zone of precipitation for protein band number 2 with fungal antigen

Single radial immuno-diffusion

This test is performed to assess the quantitative level of an antigen. Positive control with fungal (*Aspergillus niger*) antigen in gel and human Ig E in well showed a clear circular zone of precipitate formation measuring 0.9 cm in dia. The negative control with fungal antigen in gel and extraction buffer in well showed no such zone of precipitation. Root protein sample was loaded in wells in the agar containing fungal antigen, a distinct zone of precipitation of 0.6 cm was seen for isolated protein band number 2 and faint zone of precipitation of 0.35 cm for isolated protein band number 3. Faint zone of precipitation of 0.4 cm was seen for isolated protein band number 2 and human Ig E (Fig. 4) Single radial immuno-diffusion indicated an equivalence of protein band number 2 with fungal antigen as well as human Ig E with plant protein 2 inferring the ability of protein to bind to human Ig E.

Counter immuno-electrophoresis (CIE)

CIE was carried out to assess the immune-binding ability of antigen with antibody. The sensitive immuno-diffusion technique such as counter immuno-electrophoresis is considered for disease diagnostic purpose works based on binding ability between antigen and antibody represented by formation of the precipitin line. The root extract protein 2 with fungal antigen showed faint blotches of precipitate formation at the edges of slide after CBB staining. The CIE assay indicated that white

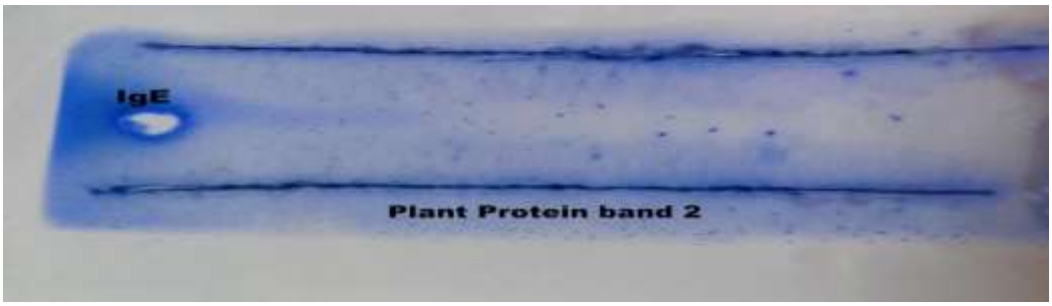


Fig. 5: Counter immuno-electrophoresis results of root extracts of jackfruit; Protein band 2 and Ig E

precipitates formed near the trough loaded with isolated protein band number 2. Similar results were noticed when fungal antigen reacted with Ig E and with isolated protein from band 2 with Ig E (Fig. 5).

Rocket immuno-electrophoresis

Rocket immuno-electrophoresis assay can determine the appropriate antigen-antibody concentration to initiate a serological reaction (Hornok and Szecsi, 1977). In present study the binding ability of fungal antigen (gel) with isolated proteins (well) was observed. Well No. 2 loaded with Ig E showed a rocket of highest peak of 5.2 cm. The remaining four wells (1, 3, 4 and 5) loaded with isolated protein band No. 2 showed peaks of 4. 6, 4.9, 4.9 and 4.8 cm, respectively, with average height/peak of 4.8 cm. No distinct rocket formation was seen for other isolated proteins (Fig. 6), which may indicate that there is no immuno-binding between isolated protein and fungal antigen. The binding ability of fungal antigen (gel) with isolated proteins (well) was observed during the study (Fig. 6).

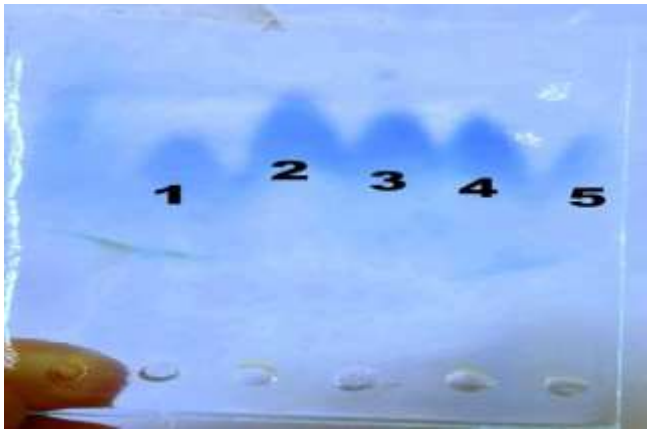


Fig. 6: Rocket immuno-electrophoresis results exhibited by jackfruit root protein; Well No. 1, 3, 4, 5 are protein Band 2; well No. 2 - Ig E.

Different immunoassays carried out between fungal antigen, antibodies and proteins isolated from jackfruit roots indicated that there exists a certain immuno-binding between the two. It was demonstrated at primary level by means of immune-complex formation. The isolated protein of molecular weight 45.70 kDa showed a distinct immuno-binding with fungal antigen and Ig E. The assay showed that the protein displayed the ability to bind to the allergic antigen and Ig E, and may play a vital role in hypersensitivity reactions caused by the indoor fungal allergen. The study further can lead to the production of simple herbal medicine against common fungal allergens.

The sensitive immuno-diffusion technique such as counter immuno-electrophoresis is considered for disease diagnostic works based on binding ability between antigen and antibody represented by formation of the precipitin line. The CIE assay indicated that white precipitates formed near the trough loaded with isolated protein band No. 2. Different immunoassays carried out between fungal antigen, antibodies and proteins isolated from jackfruit roots showed that there exists a certain immuno-binding between the two which can be demonstrated at a primary level by means of immuno-complex formation. The isolated protein of molecular weight 45.7 kDa showed a distinct immuno-binding with fungal antigen and Ig E. Immunoassay indicated that the protein displays an ability to bind to the allergic antigen and Ig E and can play a role in hypersensitivity reactions caused by the indoor fungal allergen. The study further can lead to the production of simple herbal medicine against common fungal allergens. Phytal extracts as trusted sources for allergic remedies (Agarwal and

Singh, 1999) and medicinal and nutritional value of jack fruit (Prem *et al.,* 2015) are well documented. Herbal extracts could be safest and choicest options against bronchial allergies such as asthma (Pravin *et al.,* 2014; Saxena and Saxena, 2014). Phenol and flavanol concentrations of the bark as well could be a good source of medication (Ozcan *et al.,* 2014) The previous assumption of jackfruit root with suspected anti-bronchial allergic properties (Bersabal *et al.,* 2012) has been proved true during the current study.

Conclusion: Phytal extracts jack fruit could be a cheap and safe option for remedy of bronchial allergies. The binding ability of 47 kDa jackfruit root protein with indoor fungal antigen and human Ig E indicated that it can exert a certain level of control over allergies. Characterization and molecular structure analysis of this isolated protein can be of great use due to its potential pharmaceutical properties in future.

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Conflict of interest: The authors declare that they have no competing conflict of interest.

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