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Research Article

COMPARATIVE ANALYSIS OF LECTINS ISOLATED FROM SEED AND TESTA OF *Artocarpus heterophyllus* LAM.

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Abstract

Artocarpus heterophyllus or jackfruit is the national fruit of Bangladesh. The objective of this study is to characterize and compare the lectin proteins isolated from seeds and testa of *Artocarpus heterophyllus*. Thin layer chromatography was used to detect the presence of protein in seeds and testa.Hemagglutination assay was done to identify the isolated proteins as lectins. The lectin profiles of seeds and testa were analyzed using SDS-PAGE technique. Antioxidant effects were measured by DPPH scavenging assay. Anti-inflammatory assay was also investigated.Carbohydrate induced and bacterial induced hemagglutination inhibition was done. The seed lectin showed stronger hemagglutination activity and antioxidant activity compared to testa lectin. However, testa lectin showed potent anti-inflammatory activity at low concentration compared to seed lectin. Therefore, in the future these lectins may have the potential to play role as biotechnological tools.

Keywords: *Artocarpus heterophyllus* Lam., SDS PAGE, anti-oxidant, anti-inflammatory, haemagglutination assay.

Introduction

Many plant species contain carbohydrate-binding proteins, which are commonly referred to as either lectins or agglutinins.[1]Plant lectins can be defined as all plant proteins that possess at least one non-catalytic domain that binds reversibly to a specific mono- or oligosaccharide. Lectins from different plant species often differ with respect to their molecular structure and specificity.[2]Plant lectins are present in seeds but a lot of them are also found in different vegetative tissues such asstems, bulbs, tubers, barks, cladode, flowers, heartwood, leaves, rhizomes, roots, fruits.[3,4]Lectins have been widely used for preparative and analytical purposes in biochemistry, cell biology, immunology and related areas. They are used for structural characterization of glycocongugates of unknown structure, identification of lectin-reactive structures in biological materials (body fluids, cells, tissues), fractionation and purification of glycocongugates by affinity chromatography, comparative studies *etc*.[5]

Artocarpus heterophyllus Lam., belonging to the familyMoraceae (mulberry family) and popularly known asJackfruit (English), Kathal or Panas (Hindi), Kanthal (Bengali) & Kantakiphal (Sanskrit), is one of the important and commonly found treesin the home gardens of India and Bangladesh.[6] The synonyms of the species are: *A. philippinensis* Lam., *A. maxima* Blanco, *A. brasiliensis* Gomez,*Polyphema jaca* Lour., *Soccus arboreus major* Rumph.[7]Each jackfruit can contain between 100 and 500 seeds, and each seed is enclosed in a yellowish, juicy sheath with a strong flavor. The seed has thin and leathery testa. The testa is rather thick, tough, parchment-like, and crinkly when dry. The inner seed coat or tegmen is a thin, brownish membrane.[8]Preclinical studies have shown that jackfruit possesses antioxidant, anti-inflammatory, antibacterial,anticariogenic, antifungal, antineoplastic, hypoglycemic, wound healing effects and causes a transientdecrease in the sexual activity.

Phytochemicalstudies have shown that jackfruit contains useful compounds like the flavonoids, sterols and prenylflavoneswhich may have been responsible for the various pharmacological properties.[9]The methanolic extract of testa showed antioxidant, membrane stabilization, antimicrobial,and hemagglutination inhibition activities.[10] Seeds are known to contain a lectin, namely Jacalin, which has the unique ability of specifically recognizing immunoglobulin A from human samples. It has been used as a tool to investigate the glycosylation patterns of IgA in IgA1 nephropathy syndrome, to identify O-linked glycoproteins in histochemical studies and in HIV research given its selective mitogenicity for human $CD4^+$ T-cells.[11]ArtinM, a D-mannose-binding lectin from *Artocarpusheterophyllus*seeds, acts as a very potent suppressor of cell growth offering a novel potential strategy for anti-leukemia treatment.[12]

The present work deals with the isolation of the lectins from seeds and testa of *Artocarpus heterophyllus* and establishment of its antioxidant properties, anti-inflammatory activity and hemagglutination activity, including phytochemical evaluation. This would help in future isolation of their active constituent as they have found to contain various important constituents.

Materials and Methods

Plant Collection and Identification:

The seeds of the plant along with the testa were collected from the Reazuddin Bazar, Chittagong, Bangladesh during June, 2012 and identified by the taxonomist of the Bangladesh National Herbarium, Mirpur, Dhaka as *Artocarpus heterophyllus* Lam. A voucher specimen of the plant has been deposited (Accession No.: 38308) in the herbarium for further reference.

Isolation of the lectin:

The seeds along with testa were shade-dried and later dried testa was separated from the dried seeds. The testa and seeds were grinded separately. 5g of seed and testa powder was weighed and soaked in 50 ml 0.15 N NaCl solution separately for 3 days at 4°C.The mixture was then filtered and the filtrate was centrifuged at 10,000 rpm for 30 minutes. After centrifugation, the supernatant was collected in a beaker using a pipette. 20 g of Ammonium sulphate was added into each, dissolved, and kept at 4°C for 24 hours. The solution was then centrifuged at 10,000 rpm for 30 minutes at 4^oC. After centrifugation, the supernatant was discarded and the residue was collected. The residue was the isolated protein which was stored at -80°C to prevent protein denaturation.

Thin Layer Chromatography(TLC) Analysis:

The presence of protein in each extractwere analyzed by performing TLC method as previously described.[13] TLC was done under the solvent consisted of n-butanol, glacial acetic acid, and water (8:2:2). For charring, the plates were exposed to 2% ethanolic nin hydrin, dried and then heated to 100ºC for charring purpose. This helped in the fixation of spot and allowed it to be prominently visible.

Hemagglutination Inhibition Assay:

Agglutination of the red blood cells by the seed and testa protein was carried outusing human erythrocytes in a 96-well microtiter platewith slight modifications to previously described.[14] A serial twofolddilution of the protein solution was mixed with an equal volume (50 µl) of a 1% suspension of erythrocytes (A, AB, and O) in 0.9%NaCl at room temperature. The plate was leftundisturbed for 1h at room temperature in order to allow for agglutination of the erythrocytesto take place. Smooth button formation in bottom indicated negative activity, while a rough granular deposition at bottom showed positive activity. The intensity of hemagglutination was determined from the extent of deposition.

Carbohydrate Inhibition of Hemagglutination:

Hemagglutination inhibition assay with the seed and testa lectin wereperformed in 96-well microtiter plate with slight modifications to previously described.[15] Serial two-fold dilutions of sugarsamples were prepared in 0.9% NaCl. All the dilutions were mixed with an equalvolume (50 µl) of the seed and testa lectin solution of known hemagglutination units. The mixture wasallowed to stand for 1 hr. at room temperature and then mixed with 100µl of a 1% humanerythrocyte (O and A) suspension in 0.9%NaCl. The hemagglutination titers obtained were compared with a non-sugarcontaining blank. In this study, the sugars used were: galactoseand lactose. The concentration of the sugar in the final reactionmixture which completely inhibited hemagglutination units of the lectin sample were obtained.

Bacterial Inhibition of Hemagglutination:

This assay were performed in 96-well microtiter plate with slight modifications to previously described.[16]Fifty microlitres of the *Salmonella typhi* suspensionwereserially diluted into the successive wells with 0.9%NaCl. Then, 50 L of 1% human erythrocytesuspension was added to all the wells. Sedimentation of erythrocytes in presence of bacteria wasvisualized in the plate after 30 min. of incubation at 37^oC. Inbacterial inhibition of hemagglutination studies in

presence of lectin, the seriallydiluted lectin was previously incubated with bacteria at 37°C for 30 min. Later, 100 L of 1% erythrocyte suspensionwas added and the plate was incubated for 30 min at 37°C.The concentration of lectin at which bacteria inhibited hemagglutinating activity was obtained.

Sodium–Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Separation of the seed protein and testa protein was done by using 10% SDS-PAGE.[17]SDS-polyacrylamide gel electrophoresis was carried out in a vertical gel. Stacking gel contained: dH_2O, 30% acryl amide mix, 1M TRIS (pH 6.8) and 10% SDS, 0.1% TEMED and 10% APS. Running gel contained: dH_2O, 30% acrylamide mix, 1.5M TRIS (pH 8.8), 10% SDS, 0.04% TEMED and 10% APS. Samples were prepared of four parts of a dissolved extracted proteins and one part of Loading buffer, which contained: 0.25 M TRIS (pH 6.8), 30% glycerol, 10% SDS, 5% -mercaptoethanol, and 0.02% bromophenol blue.The electrophoresis was performed at 110V for 60 minutes in running buffer, which contained: 2l dH_2O, 144.13g glycine, 30.28g Tris and 10ml 10% SDS. The gel was stained using 25mg Commassie Blue R-250 in buffer that consisted: 10ml methanol, 2ml acetic acid, and 8ml dH_2O. The gel was destained by the same buffer. Obtained electrophoreograms were analyzed using ADOBE PHOTOSHOP program.

DPPH Radical Scavenging Activity:

The scavenging activity of the extracts was estimated by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) as a free radical scavenging assay and a previously described method was adapted.[18] 1000 µl of various concentration of the seed lectin (25, 250, 500 µg/ml, in dH_2O) and testa lectin (10, 20, 30 mg/ml, in dH_2O) extracts were added to 4 ml of 0.004% methanol solution of DPPH. Different concentrations of L-Ascorbic acid (2-10 mg/ml) were used as the standard antioxidant. After 30 min incubation period at room temperature, the absorbance was read against a blank at λ = 517 nm. Inhibition of free radical by DPPH in percent (%I) was calculated by the following formula:% inhibition = $(A_0 – A_1 / A_0) \times 100$ where, A_0 is the absorbance of the blank, and A_1 is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration.IC_{50}values denote the concentration of the sample required to scavenge 50% of DPPH radicals.

In-Vitro Anti-Inflammatory Test (Erythrocytes Membrane Stabilization Method):

This was done by collecting blood from the healthy volunteersand mixing with equal volume of sterilized Alsever solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% sodium chloride in water).[19] The blood was centrifuged at 3000rpm and packed cells were washed with isosaline (0.85%, pH 7.2) and a suspension was made with isosaline (10% v/v). The assay mixture contained 1ml of phosphate buffer (0.15M, pH 7.4), 2ml of hyposaline (0.36%), 0.5ml of HRBC suspension and 1ml of various concentrations of the extract. Diclofenac sodium was used as reference drug. In the control solution instead of hyposaline, 2ml of distilled water was added. The mixtures were incubated at 37°C for 30min and centrifuged at 3000rpm for 5 min. The absorbance of the supernatant solution was read at 560nm. The percentage hemolysis was calculated by assuming the hemolysis produced in the presence of distilled water as 100%. The percentage of HRBC membrane stabilization was calculated using the formula: Percentage membrane stabilization = 100 – [(O.D of drug treated sample/O.D of control) *100].

Results

Phytochemical Screening:

For the detection of the presence of protein, the solvent system consisted of butanol, glacial acetic acid, and water (8:2:2). The separation performed by the solvent is shown in Fig. 1. Charring with ninhydrin solution in high temperature the separated compounds transformed into brown color indicating the presence of amino acids in the samples.

Hemagglutination Inhibition Assay:

Various concentrations of seed protein (0-51 mg/ml) and testa protein (0-51 mg/ml) were taken to investigate hemagglutination inhibition activity on different types of human blood groups. Hemagglutination inhibitionactivities of the seeds and testa proteins were tested against A, AB, O types of human blood groups and the results are shown in Fig.2. The concentration required for inhibiting hemagglutination is shown in Tab.1. The hemagglutination inhibition concentration for testa proteins against A, AB and O blood types is same, 12.75mg/ml. The hemagglutination inhibition concentration for seed protein against A, AB and O blood types are different, 0.005mg/ml, 0.0009375mg/ml, and nil respectively.Seed protein did not show any hemagglutination inhibition against O blood type.

Carbohydrate Inhibition of Hemagglutination:

0.1M lactose and 0.01M galactose were taken to investigate hemagglutination inhibition activity on different types of human blood groups. The results of the hemagglutination inhibition activity tested against A and O types of human blood groups are shown in Figs. 3 and

4. The concentration required for inhibiting hemagglutination is shown in Tab. 2 and 3.The concentrations of lactose that inhibited hemagglutination of the testa lectin are 100mmol and 0.39mmol against O and A blood type respectively whereas lactose showed no inhibition of hemagglutination of the seed lectin in any blood type.The concentrations of galactose that inhibited hemagglutination of the testa lectin are 5mmol and 10mmol against O and A blood type respectively whereas galactose showed no inhibition of hemagglutination of the seed lectin in any blood type.

Bacterial Inhibition of Hemagglutination:

25 fold diluted *Salmonella typhi* stock solution in 0.9%NaCl was used. The hemagglutination inhibiting concentration of seed lectin and testa lectin for A^{+ve} erythrocytes in presence of *Salmonella typhi*are shown in Tab.4 and Fig. 5. Seed and testa lectin showedhemagglutination at every concentration.

Sodium–Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

SDS-PAGE electrophoretogram of testa showed a single band with a molecular weight of approximately 90 kDa, whereas SDS-PAGE electrophoretogram of seed showed three bands with approximate molecular weight (MW) of 80 kDa, 56 kDa and 38 kDa (Fig. 6). These results confirm that the seed lectin is a trimeric protein and testa is a single protein.

DPPH Radical Scavenging Activity:

From the analyses of Figure 7, we found the scavenging effect of seedlectin and testa lectin in a concentration dependent manner. An IC_{50} value is the concentration of the sample required to scavenge 50% of the DPPH free radicals present in the system. The 50% inhibitory concentration (IC_{50}) values of the seed lectin and testa lectin was 1.384mg/ml and 27.961mg/ml respectively. Whereas, the 50% inhibitory concentration (IC_{50}) values of ascorbic acid was 1.295mg/ml. Therefore, the seeds and testa of *Artocarpus heterophyllus* showed strong radical scavenging activities as that of ascorbic acid.

In-Vitro Anti-Inflammatory Test (Erythrocytes Membrane Stabilization Method):

The seed and testa lectin of *Artocarpus heterophyllus* were subjected to the in-vitro anti-inflammatory test using diclofenac as a standard as shown in Fig. 8. As the concentration of the extracts goes up, their membrane stabilizing capacity also goes up. The percentage membrane stabilization of RBCs is considered to correlate to the anti-inflammatory activity.

Discussion

Plant lectins are a class of predominantly multimeric, carbohydrate-binding proteinsof non-immune origin, possessing at least one catalytic domain.[20] Thin layer chromatography (TLC) was used to separate mixtures of compounds from the isolated precipitates of seed and testa. It provided a qualitative idea about the components that are present in the crude seeds and testa. The developed plates tested with Ninhydrin solution turned into brown color indicating the presence of amino acids in isolated seed and testa precipitates (Fig. 1). To identify these isolated proteins as lectins, hemagglutination inhibition assay was performed (Fig.2 and Tab.1).Lectins generally occur as multimers, and because of their multivalency they can form cross-links between cells. One such example may be the ability to agglutinate blood cells, where the lectins interact with sugar moieties on the surface of the blood cells and essentially interconnect them, enabling the formation of multi-cellular aggregates. This process is called hemagglutination. Due to this particular property of lectins, hemagglutination assay has become a very important lectin detection method.[21] The receptor binding affinity of the compounds present in the proteins on human erythrocytes can be understood from Fig.2 and Tab.1. The isolated proteins from seed and testa can be considered to be non-blood type specific since it agglutinates all human blood types although the minimum concentration at which hemagglutination takes place is different. Non-blood type specificity of the lectin may be due to the presence of multiple binding sites where it can recognize all the determinants for each blood type.[22]Hence, the proteins might be included in the category of lectin as they agglutinated the erythrocytes. The first indication that the ABO blood group system might be based on carbohydrate came from observation by Watkins and Morgan, who studied animal and plant lectins. They suggested that -L-fucose was a component of H(O)-specificity, galactose of B-specificity and -linked *N*-acetyl galactosamine of A-specificity. Theblood group AB contains both trisaccharides.[23,24] The evidence that lectins bind specific carbohydrate groups on surface membranes comes from the observation that when particular sugars are present in mixtures of blood cells with lectin, agglutination is inhibited. Fig.3 and 4, and Tab.2 and 3 indicates that the sugars (galactose and lactose) in the final reaction mixture completely inhibited hemagglutination units of the lectin samples at different concentrations.Inhibition occurred may be because the sugar-lectin interaction is stronger than the erythrocyte-lectin attraction and resulting in formation of a distinct button of the erythrocyte at the bottom of the well.Non-inhibition of agglutination by the sugar molecules to the erythrocyte-lectin interaction may be due to the following reasons: (a) there is tolerance for substitution at certain

Tab. 1: Concentration for hemagglutination inhibition assay

	Hemagglutination inhibition concentration (mg/ml)	
Blood Group	Testa lectin	Seed lectin
A+ve	12.75	0.005
AB+ve	12.75	0.0009375
O+ve	12.75	-

(-) – hemagglutination inhibition not observed.

Tab. 2: Concentration of lactose for inhibition of hemagglutination

	Hemagglutination inhibition concentration of lactose (mmol)	
Blood Group	Testa Lectin	Seed Lectin
O+ve	100	-
A+ve	0.39	-

(-) – hemagglutination inhibition not observed.

Tab. 3: Concentration of galactose for inhibition of hemagglutination

	Hemagglutination inhibition concentration of galactose (mmol)	
Blood Group	Testa Lectin	Seed Lectin
O+ve	5	-
A+ve	10	-

(-) – hemagglutination inhibition not observed.

Tab. 4: Concentration of lectins for bacteria -induced hemagglutination inhibition

	Hemagglutination inhibition concentration (mg/ml)	
Blood Group	Testa lectin	Seed lectin
A+ve	-	-

(-) – hemagglutination inhibition not observed.

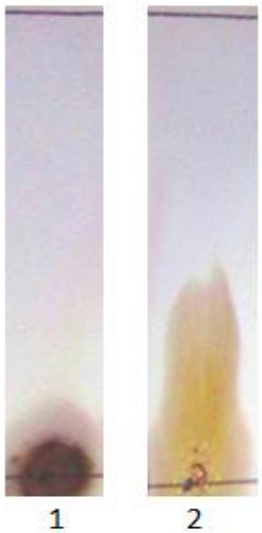
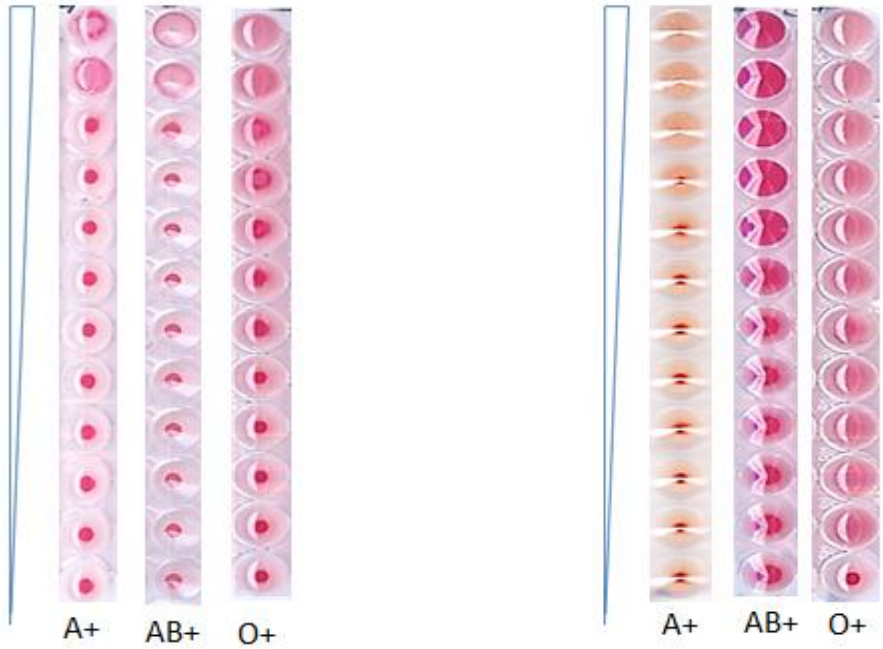


Figure 1: TLC chromatograms for protein detection in isolated precipitate from seed and testa of *Artocarpus heterophyllus*. Key: 1= Seed; 2= Test

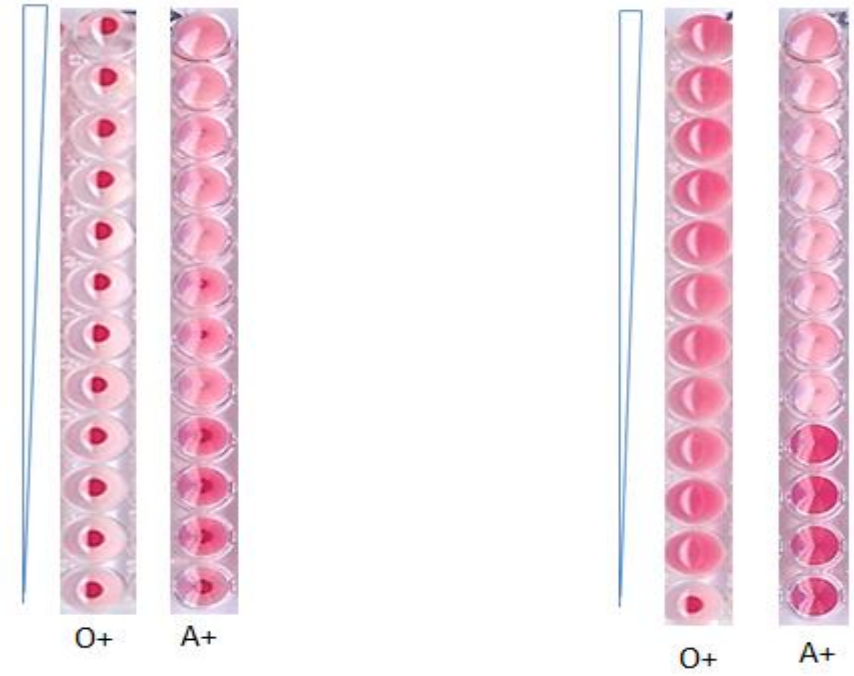
A. TESTA LECTIN (51mg/ml) B. SEED LECTIN(0.04mg/ml; 0.06mg/ml;



51mg/ml)

Figure 2: Hemagglutination inhibitory concentration determination of seed and testa proteins of jackfruit.

A. TESTA LECTIN (0.1M of lactose) B. SEED LECTIN (0.1M of



lactose)

Figure 3: Lactose- induced hemagglutination inhibition on seeds and testa lectins of jackfruit.

.TESTA LECTIN(0.01M of galactose) B.SEED LECTIN(0.01M of galactose)

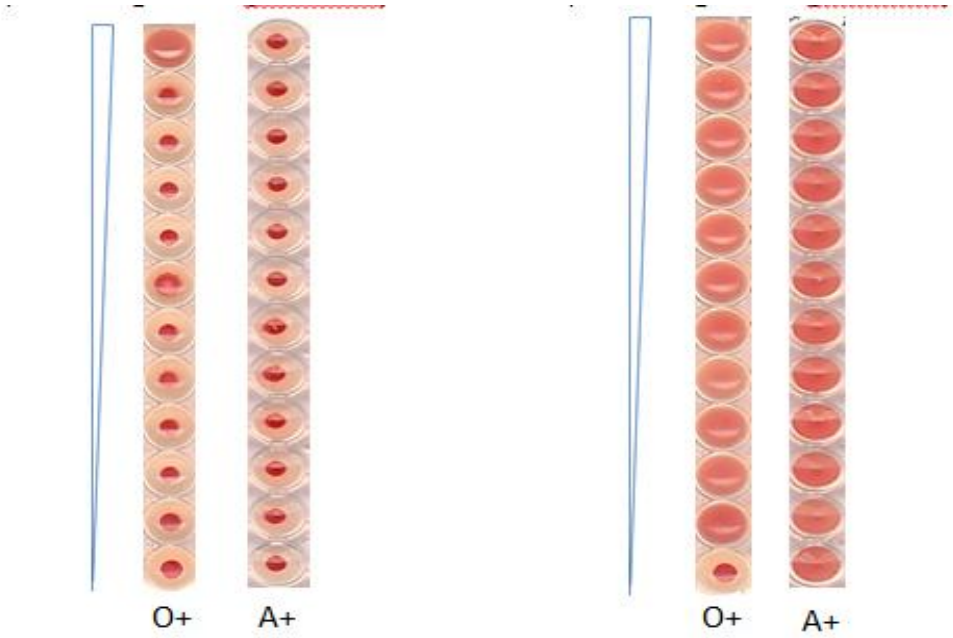


Figure 4:.Galactose-induced hemagglutination inhibition on seeds and testa lectin of jackfruit.

A. TESTA LECTIN (25mg/ml) B. SEED LECTIN (25mg/ml)

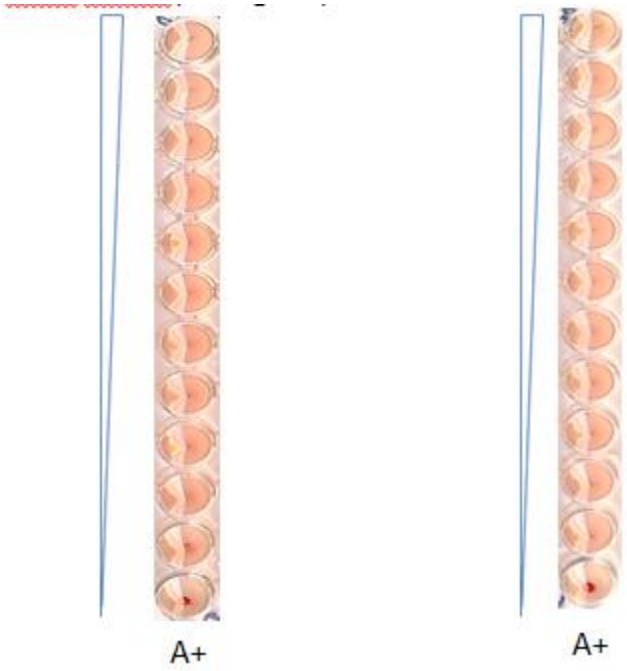


Figure 5: Bacteria (*Salmonella typhi*)- induced hemagglutination inhibition on seeds and testa lectin of jackfruit.

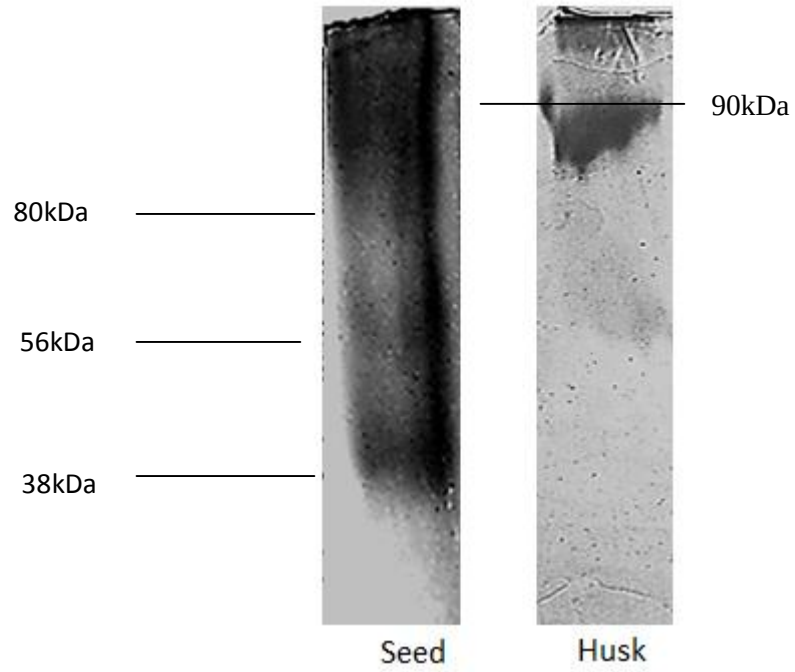


Figure 6: SDS-PAGE electrophoretograms of seeds and testa proteins of *Artocarpus heterophyllus*.

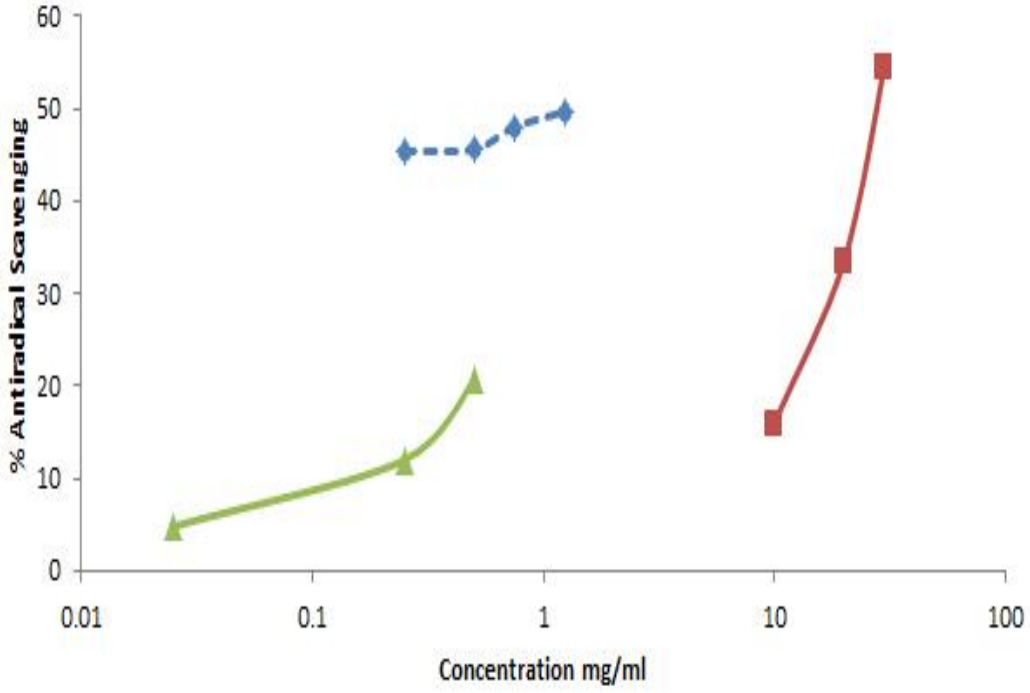


Figure 7: DPPH scavenging activity of the seed and test a proteins of *Artocarpus heterophyllus.* Key: Dotted rhombus line: Ascorbic acid; Solid rectangular line: Testa protein; Solid triangular line: seed protein.

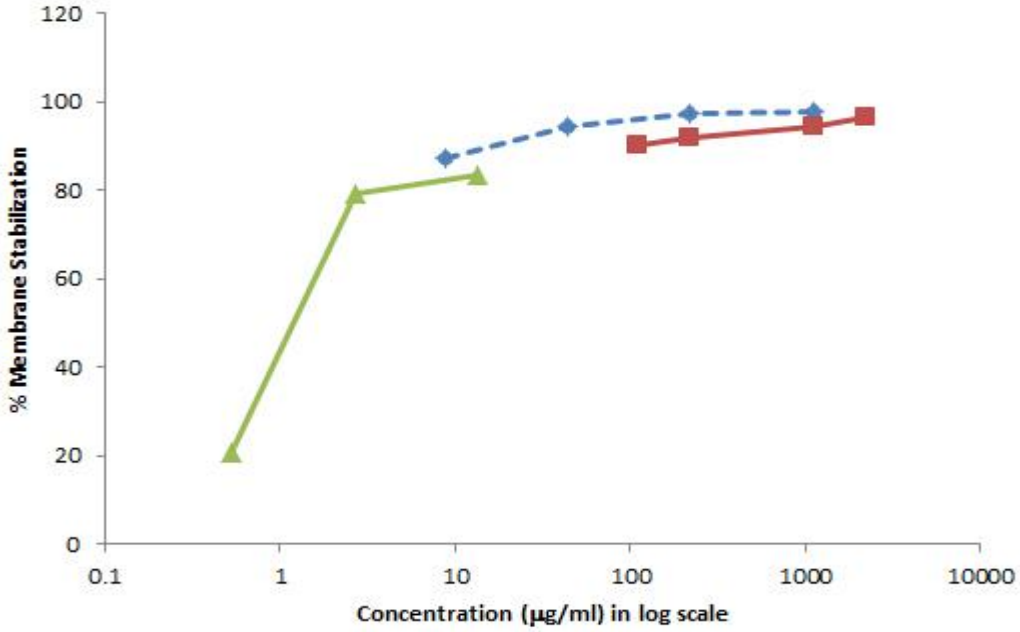


Figure 8: In-vitro anti-inflammatory activity of the seed and testa proteins of *Artocarpus heterophyllus.* Key: Dotted rhombus line: Diclofenac sodium; Solid rectangular line: Seed protein; Solid triangular line: Testa protein.

positions of the sugar moiety which determines their specificity; (b) lectin binding sites are non-specific for sugar anomers; (c) size and shape of sugar residues does not affect the erythrocyte-lectin binding; and (d) the carbohydrate containing the sugar or its derivatives cross react with the lectin.[25] Fig.5 and Tab.4 clearly exemplifies that seed and testa lectins serve protection against*Salmonella typhi* induced erythrocyte damage, may be due to masking of adhesion sites. Almost all microorganisms express surface-exposed carbohydrates which may be covalently bound, as in glycosylated teichoic acids to peptidoglycan, or non-covalently bound, as in capsular polysaccharides.[26] Every surface-exposed carbohydrate is a potential lectin-reactive site.Hemagglutination occurred might be because the erythrocyte-lectin attraction is stronger than the bacteria-lectin interaction and resulting in rough granular deposition of the erythrocyte at the well. A study has also reported that *Artocarpus heterophyllus* seed lectin extract showed killing effect against microorganisms insinuating that the lectin is bactericidal.[27]Hence, these lectins can be exploited as potential drug targets.

From SDS-PAGE analysis (Fig.6), we found that testa lectin is a single protein with molecular weight of approximately 90kDa and seed lectin is a trimeric protein with bands approximately at 38kDa, 56kDa, and 80kDa. In a previous study,[28]electrophoretic pattern of protein sample of *Artocarpusheterophyllus* seed extract showed the presence of a number of fractionated proteins ranging in molecular weights from 61.10kDa to 11.00kDa.The presence of a number of different lectins in a single plant may be explained either by the differences in post-translational protein modification, or by the presence of a set of nonallelic lectin-coding genes in the genome.[29]

To evaluate scavenging activity of antioxidants in the seed and testa lectin, DPPH assay was used (Fig.7). DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule, with an absorption band around 515-528 nm and thus, it is a useful reagent for evaluation of antioxidant activity of compounds.[30] It is considered to be a model for a lipophilic radical.A chain in lipophilic radicals is initiated by the lipid auto-oxidation.[31]Reaction between antioxidants and DPPH leads to decrease in absorbance of DPPH radicalwhich results in scavenging of the radical by hydrogendonation and causes discolouration from purple to yellow.[32]From Fig.7 we can conclude that seed and test a lectin showed strong DPPH scavenging activity with a lower IC_{50} value. Seed and testa lectins were also subjected to in vitro anti-inflammatory test for assessing the red blood cell membrane stabilizing activity (Fig.8). The inflammatory response involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown, and repair,which are aimed at host defense and usually activated in most

disease condition. The erythrocyte membrane is analogous to the lysosomal membraneand its stabilization implies that the extract may as well stabilize lysosomal membranes.Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release.[33] The non-steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane.Since HRBC membrane are similar to lysosomal membrane components, the prevention of hypotonicity induced HRBC membrane lysis is taken as a measure of anti-inflammatory activity of drugs.[34]In comparison with diclofenac sodium, testa and seed lectin of *Artocarpus heterophyllus* showed a considerable anti-inflammatory activity (Fig. 8) via stabilizing erythrocyte membrane implying that the extracts may as well stabilize lysosomal membranes. This can be implied that the extracts have anti-inflammatory properties because they have antioxidant molecules that prevent oxidative stress.

Therefore, from our present study on the proteins isolated from seeds and testa of *Artocarpus heterophyllus* can be concluded that the isolated proteins are lectins as they showed significant hemagglutination activity. The lectins have antioxidant property and anti-inflammatory effects also. They showed hemagglutination activity in presence of bacteria. Hence they have antimicrobial activity too. In the future, the protein isolates may have the potential to play role as biotechnological tools. Further studies may be conducted on the biological activities and mechanisms of action of these lectins, so that the production of lectins can be improved and new applications of these lectins can be found.

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