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PROTEIN PROFILES OF PHYTOAGGLUTININS FROM INDIGENOUS SPECIES OF EUPHORBIACEAE, LEGUMINOSAE AND MORACEAE FROM PAKISTAN

HASEENA GULZAR[1,2*], JAWERIA GUL[2] AND GUL HASSAN[3]

[1]*Institute of Biotechnology and Genetic Engineering, University of Agriculture, Peshawar, Khyber Pakhtunkhwa, Pakistan*
[2]*Department of Biotechnology, Shaheed Benazir Bhutto University, Sheringal, Dir Upper, Khyber Pakhtunkhwa Pakistan*
[3] *Department of Agriculture, Shaheed Benazir Bhutto University, Sheringal, Dir Upper, Khyber Pakhtunkhwa Pakistan*
**Corresponding author email: haseenagulzar@gmail.com*

Abstract

The present study was aimed at the identification of plant lectins from indigenous plants belonging to Leguminosae (*Albizia lebbeck, Cassia fistula, Pongamia glabra, Acacia catechu*), Moraceae (*Morus nigra, M. alba, Ficus indica, Ficus* sp.*, Broussonetia papyrifera*) and Euphorbiaceae (*Euphorbia helioscopia, Jatropha hastata, Putranjiva roxburgii*). Soluble proteins were selectively isolated in 0.15 M NaCl and the presence of agglutinin activities against erythrocytes of blood groups A, B, AB and O was monitored in different organs of the plants. The protein content of the extract was determined by Pyrogallol Red method. An SDS gel electrophoresis protocol was developed to separate proteins in crude extracts as well as fractions obtained by fast protein liquid chromatography of some selected plants. The gel electrophoresis was carried out using a Bio-Rad Mini gel assembly. Several distinct protein profiles were observed both for the crude protein extracts and samples fractionated by fast protein liquid chromatography (FPLC). These proteins corresponded with strong agglutinin activities particularly in *Albizia lebbeck* (black siris), *Cassia fistula* (Amaltas), *Pongamia glabra* (Sukh chaen), *Aacacia catechu* (Katha) *Morus nigra* (Siah Toot), *M. alba* (Bedana) and *Putranjiva roxburghii* (Patanjan).

Key words: Agglutinin activities, Indigenous plant species, Lectins, Protein profiles, FPLC and SDS-PAGE.

Introduction

The presence of proteins in plant seeds that are capable of binding to and agglutinating cells was identified during the last century; the proteins were designated hemagglutinins for their ability to agglutinate erythrocytes. Lectins are known as carbohydrate binding glycoproteins that recognize only certain type of free sugars or specific sugar residues of polysaccharides, glycoproteins, and glycolipids, a trait that has been used extensively over the past 30 years as a laboratory tool for studying glycans (Barondes, 1981; Oda *et al*., 2000). More importantly perhaps, plant lectins have been known for sometime to interact with human cells inducing an array of physiological, biochemical, and immunological responses including changes in membrane permeability (Sheue-Fang *et al*., 2001), lymphocyte transformation (Kabir *et al.*, 2011) and apoptosis (De Miejia *et al*., 2003). Studies over the past few years have demonstrated the lectin's ability to inhibit inflammatory responses and bacterial proliferation in infected tissue (Alencar *et al*., 2005), stop HIV I infection of lymphocytes (Pashov *et al*., 2005) and act as anticarcinogenic phytochemicals (Barandos, 1981). This rapidly increasing interest in the potential therapeutic applications of plants, (Fatima, *et al*., 2014) has created a potential demand for new sources of active constituents of plants like lectins and it appears that plants known for health benefit are strong candidates for the presence of potent lectin activities.

Lectins are widely distributed in nature, particularly in the plant kingdom where they can be found in seeds, leaves, bark, bulbs, rhizomes, roots, cotyledons and tubers (Oliveira *et al*., 2002; Konozy *et al*., 2002 and 2003). Seeds of leguminous species often contain large amounts of lectins that are similar to those present in other tissues of the same plant, including the latex. Recently, highly potent abrin like lectin proteins have been shown to be present in black mulberry seeds (Yeasmin *et al*., 2001). Pakistan has three different types of mulberry fruit species widely distributed throughout the country including the northern regions of Khyber Pakhtunkhwa. There is a need to examine all three fruit mulberry species, *Morus alba*, *M. nigra*, and *M. serrata*, for the distribution of lectins in their various tissues. Other than Moraceae, Leguminosae and Euphorbiaceae also contain a wide range of potent lectins. Lectins are particularly abundant in the seeds of legumes. They constitute up to 10% of the soluble protein in the seed extracts (Talbot & Etzler, 1978). The legume lectins, despite the high sequential and structural similarity of their subunits, show a remarkable range of sugar specificities (Hamelryck *et al*., 1998 & 1999).

The aim of the present study was to develop specific electrophoreic profiles at different phases of chromatographic fractionation of plant lectins isolated from some indigenous members of Leguminosae, Moraceae, and Euphorbiaceae.

Material and Methods

Plant material: Following indigenous plant species were collected from different regions in Khyber Pakhtunkhwa including botanical gardens, natural habitats, from Pakistan Forest Institute Peshawar (PFI) medicinal garden, from roadside etc. Some were also cultured in water cultures to harvest different organs of the plants easily.

A. Family Leguminosae: *Acacia catechu, Albizia lebbeck, Cassia fistula, Pongamia glabra*

B. Family Moraceae: *Ficus indica, Ficus sp., Morus alba, Morus nigra, Broussoetia papyrifera*

C. Family Euphorbiaceae: *Euphorbia helioscopia, Jatropha hastata, Putranjia roxburghii*

Water cultures and harvesting: Seedlings of *Euphorbia heloiscoia* and stem sprouts of *Morus nigra* were raised in 3 L containers with modified Arnon and Hoagland nutrient solution under green house conditions. Plants were harvested for protein and lectin analysis. Roots, shoots, leaves, and reproductive organs were separated and weighed fresh.

Protein extraction: A 10-20 g specimen of reproductive organs, leaves, shoots, and roots was blended with 0.15 M NaCl to isolate lectins and other soluble proteins, and the slurry was strained through 2 layers of cheese cloth. Crude extracts were clarified by centrifugation at 10,000 g (10,000 times Earth's gravitational force) for 30 min at 15ºC using a JA-10 rotor on a Beckman Coulter Avanti J-26 XP refrigerated floor model centrifuge machine. The clarified supernatant solutions were collected as protein extracts, and stored at -70ºC prior to further processing.

Protein determination: Total protein content of the extract was determined by Micro Pyrogallol Red procedure. An aliquot of 10-50 µl was mixed with 1 ml reagent containing 50 mM succinate buffer, 7 mM sodium dodecyl sulfate, 4 mM sodium molybdate and 60 mM pyrogallol. After 10 min incubation at room temperature, the absorbance was read at 600 nm on a Bio-Rad Smart Spec UV-Visible spectrophotometer. Bovine serum albumin was used as a standard.

Hemagglutination of human erythrocytes: Human erythrocytes of blood groups A, B, AB, and O obtained from a local hospital were diluted 50 times with 0.15 M NaCl and a drop of 100 µl placed on a microscope slide. A 100 µl of lectin extract was added carefully to the centre of the blood drop, and after a 10 min incubation at room temperature, the degree of agglutination was recorded as either very strong (++++), strong (++ to +++), weak (+) or absent (0).

Sodium Dodecyl Sulfate gel electrophoresis (SDS-PAGE): Proteins were separated by SDS-PAGE using a Bio-Rad Mini Gel assembly. Resolving gel and stacking gel were prepared according to the standard procedures. The final running buffer composition was 196 mM glycine + 0.1% SDS + 50mM Tris-HCl pH 8.3 used for both top and bottom tanks. Samples were prepared with and without mercaptoethanol as the reducing agent. For samples with mercaptoethonol, a 100µl aliquot was mixed 2:1 (v:v) with SDS-PAGE disruption mixture containing125mM Tris-HCl pH 6.8 / 10% 2-mercaptoethanol / 10% SDS / 10% glycerol, and 0.05% w/v of the dye bromophenol blue. Mercaptoethanol was omitted from the formulation for meraptoethanol free samples. A 0.2% Coomassie Brilliant Blue (CBB) solution was prepared in 45:45:10 % methanol: water: acetic acid and was used as staining solution while distaining was achieved by agitating gel with 25%:65%:10% methanol water acetic acid mixture. The gel was illuminated with white light, and documented using a gel documentation system.

Results

Protein content of plants: A list of indigenous plant species as shown in Table 1 and their various organs obtained either directly from the field, or from the greenhouse grown material were examined for the protein content, blood agglutination activity and protein profiling.

In the legume family the protein contents of the leaves were highest in all species except for *Acacia catechu* and *Pongamia glabra* where the protein content of the seeds was the highest. In general, stem extracts were relatively low in their protein content and concentration. The protein contents of Moraceae extracts (Table 1) revealed that the protein content and concentration of fruit and leaves were high in these species.

In contrast to the members of Leguminosae and Moraceae studied, several species of Euphorbiaceae showed highest protein content and concentration in the stem fractions and their leaves were relatively low.

Erythrocytes agglutination: Erythrocytes agglutination by Leguminosae extracts is also presented in Table 1. In *Albizia lebbeck,* all three organs examined viz. leaves, stems and seeds, showed strong agglutinating activities in the crude extracts. Of the two specimens of *Cassia fistula* studied, the one collected from Pakistan Forest Institute showed somewhat stronger agglutination activities than the one collected from a roadside. Agglutination activities were similarly high in other two legume species; *Pongamia glabra* and *Acacia ctechu.*

Erythrocytes agglutination by some Moraceae extracts are also shown in Table 1. *Morus alba*, and *M. nigra* fruit and *M. nigra* leaves showed strong agglutinating activities in their crude extracts. The stem extract of *M. nigra* also contained a significant agglutination activity. The two fig species (*Ficus indica* and *Ficus* sp.) contained high agglutination activity in their fruit extracts. The leaves of *F. indica* also contained a high agglutination activity. The agglutination activities in the male and female plants of paper mulberry were detectable in the crude extract.

Erythrocytes agglutination activities in Euphorbiaceae extracts are shown also in Table 1. Strong agglutination activities were observed in the crude extracts obtained from *Euphorbia helioscopia, Jatropha hastata,* and *Putranjiva roxburghii.* In all the three families studied, all the extracts exhibited similar agglutination activities against blood groups A, B, AB and O.

Table 1. List of indigenous plant species, their organs selected for SDS Gel Electrophoresis analysis, their protein content and hemagglutination activity

No	Moraceae	Organs and protein Content (mg/ml extract)	Blood Agglutination test			
			Blood Groups			
			A	B	AB	O
1	*Morus Niger*	Fruit(0.372)	++++	++++	++++	++++
2	*Morus alba*	Fruit(0.101)	++++	++++	++++	++++
3	*Ficus indica*	Leaves(0.530)	+++	++++	++++	++++
		Stem(0.256)	+++	+++	++++	++++
4	*Ficus species*	Leaves(0.240)	+++	++++	++++	+++
		Fruit(0.991)	++++	++++	++++	++++
5	*Broussonetia papyrifera* (male)	Leaves(0.511)	++++	++++	++++	++++
6	*Broussonetia papyrifera* (female)	Leaves(0.402)	+++	+++	++++	++++
	Leguminosac					
7	*Albizia lebbeck*	Leaves(1.807)	++++	++++	++++	++++
		Stem(0.369)	++++	++++	++++	++++
		Seeds (0.401)	++++	++++	++++	++++
8	*Acacia catechu*	Seeds (0.947)	++++	++++	++++	++++
9	*Cassia fistula1* (PFI)	Leaves(2.770)	++++	++++	++++	++++
		Seeds (0.369)	++++	++++	++++	++++
10	*Pongamia glabra*	Leaves(1.176)	++	++	++	++
		Stem(0.583)	++	++	++	++
		Seeds (2.390)	+++	++++	+++	++++
11	*Cassia fistula 2* (roadside)	Leaves(1.257)	+++	++++	+++	++++
		Fruit (0.647)	+++	+++	+++	+++
	Euphorbiaceae					
12	*Putranjiva roxburghii*	Fruit (1.129)	++++	++++	++++	++++
13	*Jatropha hastate*	Fruit (0.021)	+++	+++	+++	+++
14	*Euphorbia helioscopia*	Stem(0.215)	++++	++++	++++	++++

Very Strong (++++), Strong (++ to +++), week (+)

†PFI= Pakistan Forest Institute

Electrophoresis profiles of protein fractions: Figures 1 (from 1a to 1p) shows the SDS gel electrophoresis profiles of clarified crude extracts of plants including *Albizia lebbeck*, *Acacia catechu*, *Cassia fistula*, and *Pongamia glabra* (legumes), *Putranjia roxburghii*, *Jatropha hastata*, *Euphorbia heliscopia* (Euphorbiaceae) and *Ficus* sp., *Broussonetia papyrifera* (Moraceae). In *Albizia lebbeck* leaves the detection of protein was apparently enhanced in the absence of mercaptoethanol as shown in Fig. 1 (a). The protein detection in the seed extract of *Acacia catechu* (Fig. 1g), the leaf extract of *Cassia fistula* 1 and 2 (Figs. 1 c & d) and the stem extract of *Pongamia glabra* (Fig. 1 h), on the other hand, was apparently not influenced by the exposure of samples to mercaptoethanol.

The SDS gel electrophoresis profiles of protein extracts obtained from leaves, seeds and stem of *Albizia lebbeck* are shown in Figs. 1a, b & m. In all three extracts the detection of protein was enhanced by the absence of mercaptoethanol but the separate bands look clear only with mercaptoethanol. Proteins were most abundant and numerous in seeds and very few proteins were detected in the stem extract (Figs. 1: e, g, i, n, h, m, p). Similarly, Proteins in leaf extract were more abundant than the stem extract (Fig. 1: a, c, d, o).

Figure 1c shows the SDS gel electrophoresis profiles of *Cassia fistula* 2 leaves and Fig. 1e shows the profile of *Cassia fistula*1 seeds. Proteins in this legume specimen collected from a road side (2) were most abundant in leaves, whereas not many proteins were detected in its seeds, pod and stem. The results obtained for *Cassia fistula*1 pods, fruit stem and leaves collected from PFI site were similar to those obtained from the road side samples. The SDS gel electrophoresis profiles of seeds of the *Cassia fistula*1 seeds are shown in Fig. 1e. The detection of proteins on gel was better without the presence of mercaptoethanol in the sample buffer.

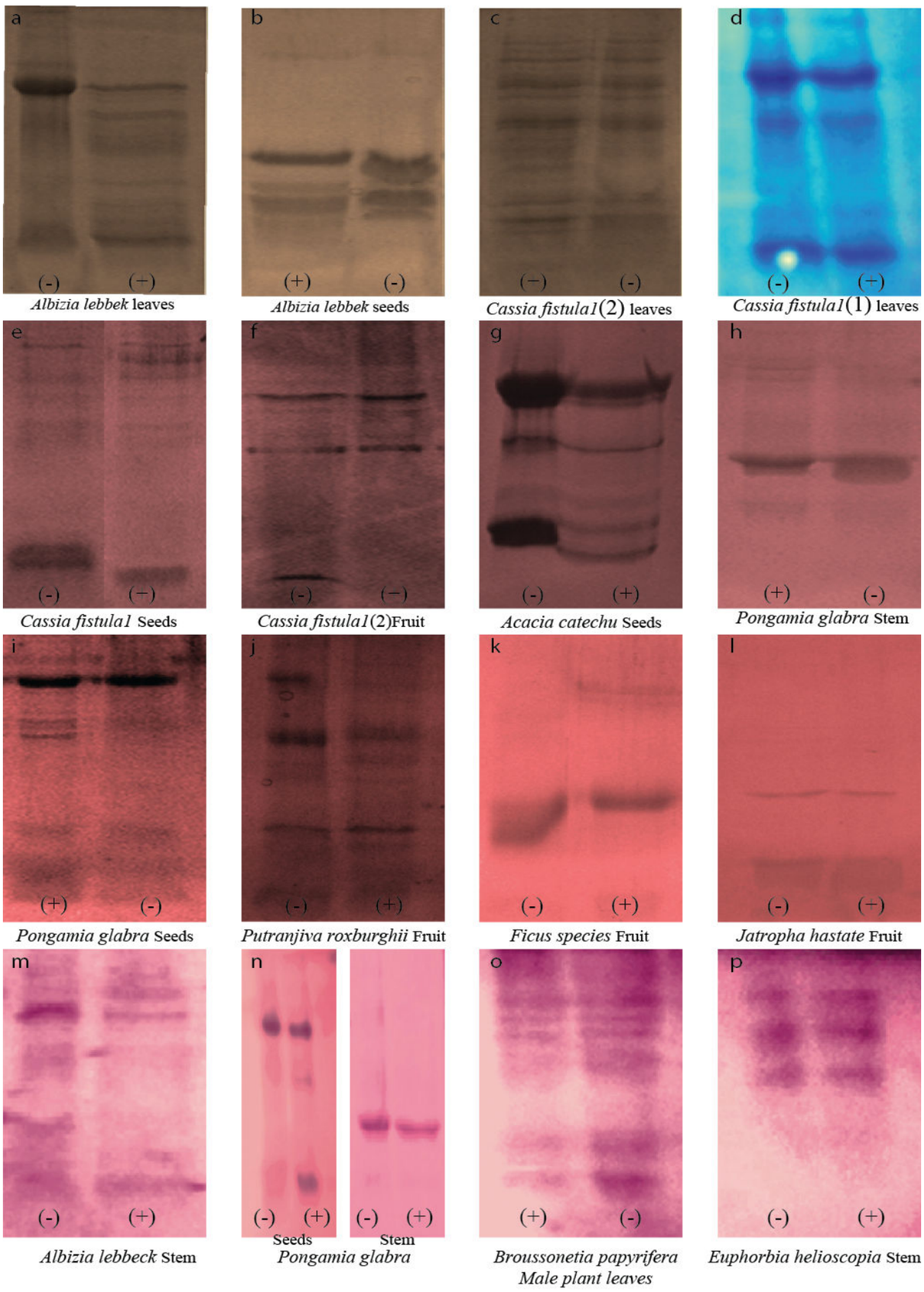

Fig. 1. SDS Gel Electrophoretic profiles of clarified crude extracts of plants (+) and (–) indicate the presence and absence of mercaptoethanol in the sample buffer.

Figures 1(h & i) shows the SDS gel electrophoresis profiles of clarified crude extracts of *Pongamia glabra* stem and seeds. The protein distribution in these two organs was distinct with a high molecular weight protein being the dominant one in seeds and a low molecular weight protein being the dominant one in stems. The FPLC profile of *Pongamia glabra* seeds was not detectable.

Figure 1(k & o) shows the SDS gel electrophoresis profiles of Moraceae species i.e. *Ficus* fruit and *Broussonetia papyrifera* male plant leaves. The protein profile of seeds shows that low molecular weight proteins are dominant. The leaves of the paper mulberry (*B. papyrifera*) plant shows the presence of a number of proteins in these extracts.

Among Euphorbiaceae, the fruit extract of *Putranjia roxburghii* had the most abundant protein distribution (Fig. 1 j). Other organs of this species showed much lower protein distribution. Similar results with most proteins being found in the fruit organ of another Euphorbiaceae species *Jatropha hastata* (Fig. 1 l). The SDS gel electrophoresis of FPLC fractions of *Euphorbia helioscopia* stem was also not visible.

Figure 2 (a, b & c) shows the SDS gel electrophoresis profiles of protein fractions of *A. lebbeck* leaves, seeds and *Acacia catechu* seeds obtained by anion exchange separation by fast protein liquid chromatography (FPLC). Proteins in the *A. lebbek* leaves extract showed major peaks in fractions no. 12 in the absence of mercaptoethanol in the sample buffer (Fig. 2 a). The elution of proteins from *A. lebbeck* seeds, on the other hand, was found in fractions 11, 12 and 13 (Fig. 2 b). The protein bands look dense in the presence of mercaptoethanol while sharp in its presence. *Acacia catechu* seeds illustrated major peaks in fractions 11 and 12 as shown in Fig. 2c. It also showed that the detection of proteins with or without mercaptoethanol is similar.

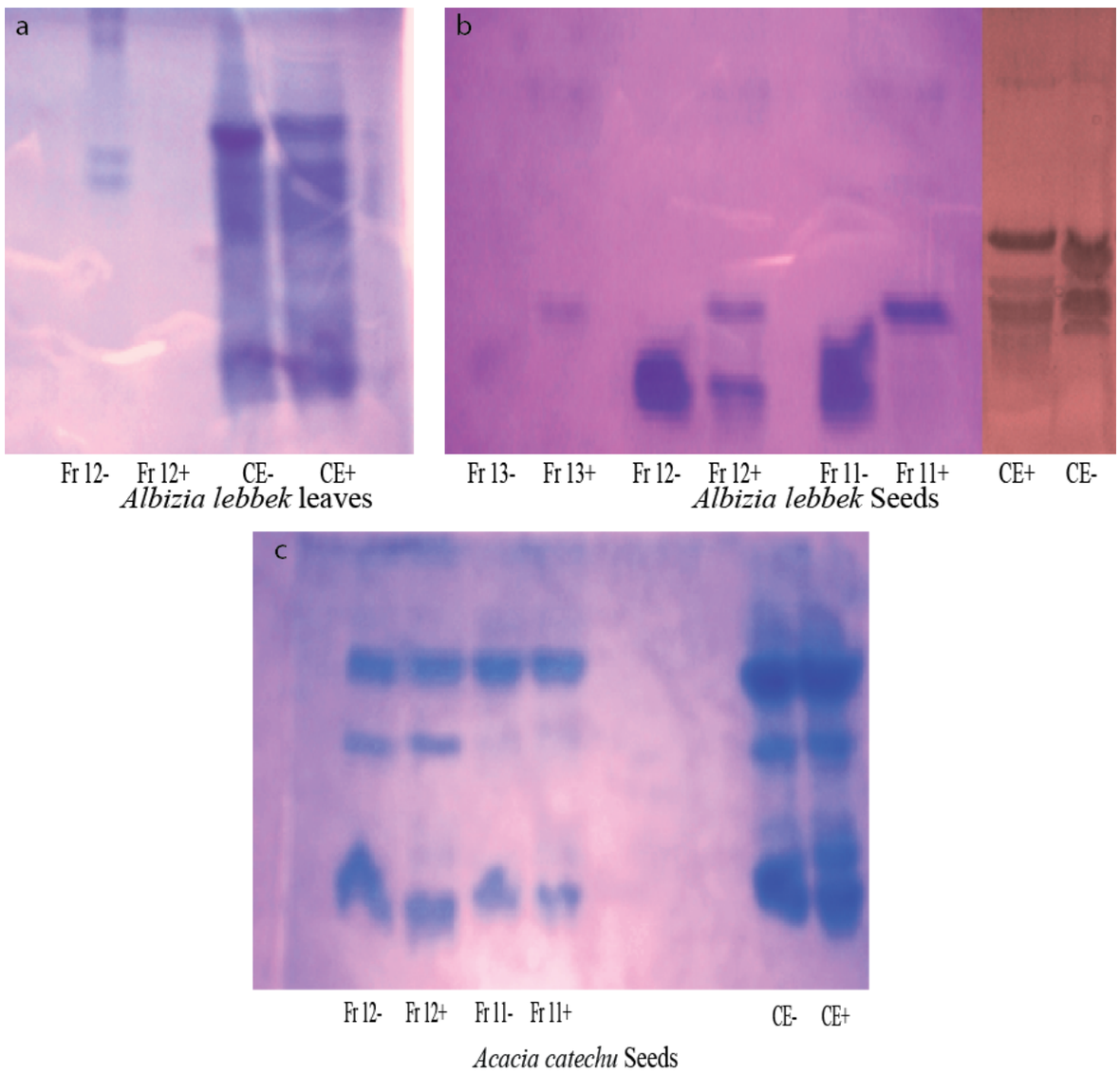

Fig. 2. SDS Gel Electrophoretic profiles of FPLC fractions (Fr.) of plants, CE indicates profiles of clarified crude extracts, (+) and (-) indicate the presence and absence of mercaptoethanol in the sample buffer.

Discussion

The existence of a rich and diverse medicinal flora and a wealth of traditional knowledge of their usage in various parts of the country have allowed us to look at the distribution of potent lectin proteins in our indigenous plants. The present study has thus benefited from the ethnobotanical tradition of the region in these regards (Ali & Qaiser, 2009; Ali *et al*., 2011; Hazrat *et al*., 2011). The selection of plant materials has taken a note of the local distribution of taxonomic groups (Shinwari *et al*., 2006 & 2010) that have been reported in the literature to contain strong hemagglutinating activities, a trait that is used to detect the presence of lectins in plant extracts (Louis *et al*., 1990; Zahoor *et al*., 2009; Kabir *et al*., 2011; Sultana *et al*., 2014; Saha *et al*., 2014). The present study has developed standardized methods for an efficient isolation of proteins from herbaceous and woody plants, and ensured the detection and preliminary potency assessment of these phytoagglutinin activities.

The present study has isolated and partially purified hemagglutinating lectins from various organs of Leguminosae, Moraceae and Euphorbiacea species. Seeds and plant parts were harvested either directly from wild sources or procured from botanical gardens, and local nurseries. A number of species were grown either in soil or soil less water cultures in the greenhouse. Other taxa of ethnobotanical interest were raised from seeds obtained from ethnobotanical sources in the region.

A number of electrophoretic profiles have been identified in the current studies. The protocols have been developed for both crude extracts and partially purified protein fractions obtained in our laboratory during fast protein liquid chromatography (FPLC) procedures. Our studies indicate that a somewhat selective extraction of soluble proteins in 0.15 M NaCl is a suitable starting material for plant lectin profiles (Bezerra *et al*., 2013). In the literature buffered saline solution is also used for this purpose (Yeasmin *et al*., 2001; Saha *et al*., 2014; Damme *et al*., 1999). The electrophoretic profiles of crude extracts appear less crowded than what one would expect from a more complete extraction in strongly buffered media not comparable to the study of genetic diversity based on protein profiling (Ali *et al*., 2007). The monitoring of phytoagglutinin activities in the crude extract is also standardized in the present study. The electrophoresis gel with 12% acrylamide appears to be ideal for separating proteins in 0.15 M NaCl extracts.

Despite the enormous interest in plant lectins, little is known about their functions in the plants. Many leguminous lectins are found in seeds, but the same lectin and homologs are also found in other parts of the plant, such as the bark, stem, and leaves. Lectins probably have many different and important functions in plants. There is a high level of research interest in the possibility that leguminous lectins may be involved in rhizobial attachment and root nodulation (Hirsch, 1999). For example, transgenic expression of the soybean agglutinin gene in *Lotus corniculatus*, which is normally nodulated by *Rhizobia loti*, changes the nodulation pattern of the transgenic plant to *Bradyrhizobium japonicum*, which normally nodulates soybeans (Rhijn *et al*., 1998). Some of the nodulation factors released by the bacteria are glycol conjugates, and it is intriguing to consider that some of the members of the plant lectin group may be involved in this important plant signaling pathway (Geijtenbeek & Gringhuis, 2009). Plant lectins are toxic to many plant pathogens, and this may be a major role within seeds and other peripheral tissues of the plants. For example, the snowdrop lectin *Galanthus nivalis* agglutinin is toxic toward the sap-sucking insect called the rice brown plant hopper. Transgenic expression of the gene encoding *G. nivalis* agglutinin in rice plants decreases survival and fecundity of insects attacking the transgenic plants (Powell *et al*., 2011). Although we usually think of plant lectins as only having an ability to bind carbohydrate, evidence exists that these proteins may have additional activities. For example, some lectins, such as *Dolichus biflorus*, bind adenine residues with high affinity and specificity in regions of the protein outside the common carbohydrate-binding site (Hamelryck *et al*., 1999).

Plant lectins have made an enormous contribution to the development of interest in animal cell glycobiology. They have provided important clues to the vast repertoire of carbohydrate structures in animal cells and the possibilities that animal cells might also contain carbohydrate-binding proteins. Undoubtedly, plant lectins will continue to have an important role as reagents in their own right. The increased understanding of the carbohydrate-binding specificity of lectins and their three-dimensional structures allows the possibility in the future of engineering lectins to recognize specific glycoconjugates. In addition, the increased attention of biologists worldwide to the phenomenal part carbohydrates play in cellular interactions and regulation may finally contribute to a better understanding of the natural functions of the interesting plant proteins. Genetically altering plants to allow expression of different lectins may afford protection to plants from infectious diseases and may also enhance the nodulation and nitrogen-fixing ability of the plants (Peumans & Van Da, 1995; Hamelryck *et al*., 1999; Powell *et al*., 2011; Saha *et al*., 2014). As the “superbugs” or “superstrains” microbes are imposing the need for new drugs and identifying new sources of naturally occurring antimicrobial peptides (Bagley, 2014), our indigenous medicinal flora including *F. indica* offers enormous potential to be used as an active antibacterial component in modern drug formulations (Toor *et al*., 2015; Malik, *et al*., 2015; Hussain, *et al*., 2015).

Conclusions and Recommendations

The above study was a preliminary screening of SDS Gel Electrophoretic profiles of plant extracts. The aim here was to standardize the polyacrylamide concentration, reducing conditions and buffering regimes for detecting proteins of various molecular sizes present both in the crude extracts as well as in FPLC fractions. These exercises

were aimed at the visualization of the spread of polypeptides in the protein extracts of various plant organs for which our agglutination stets were positive for the presence of hemagglutinins. These procedures need to be standardized further for making the gel electrophoresis more specific lectin proteins. Obviously, a reliable and sensitive method for separating plant lectins on SDS gels is critical for screening biologically active, clinically significant, and commercially important plant lectins from the rich and diverse flora of Pakistan. Only after a verifiable isolation of purified hemagglutinins, one would be able to evaluate their efficacy and potency and suggest their biochemical and biomedical applications. The key at this stage is to refine the electrophoretic procedures and collate this part of the work with FPLC profiles.

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