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RESEARCH ARTICLE

EVALUATION OF ANOMERIC RECOGNITION IN GALACTOSE BINDING LECTINS USING CROSSLINKED HEMICELLULOSE: A COMPARATIVE STUDY THROUGH AFFINITY CHROMATOGRAPHY

José C. Torres[1], José G. Hernández[2], Edwin C. Hernández[3], Jessica A. Braga[4], Cicero Cavalcante[5], Myriam J. Ortega[6] and Francisco R. Da Silva[7]

1. Professor of Agro-environmental Biotechnology, Specialization in Agro-environmental Biotechnology, UNAD. Colombia.
2. Doctor of Internal Medicine, Intensive Care Unit, Sanitas International, Bogotá, Colombia.
3. Professor of Biology, Education Office of Bogotá, Colombia.
4. Professor of Biostatistics, ECAPMA – UNAD. Colombia.
5. Professor of Biology, Instituto Federal de Educação Ciência e Tecnologia do Ceará.Brazil.
6. Professor of Animal Breeding, Agricultural Chain, UNAD. Colombia.
7. Professor of Chemistry, Chemistry Faculty, Universidade Regional do Cariri, Crato, CE, Brazil.

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Abstract

The isolation of lectins by affinity chromatography with crosslinked hemicelluloses has been a common practice because of the variety of glycosides that they present, improving the isolation of different kinds of lectins, such as the galactose ligands. Lectins affinity for carbohydrates is so specific that a simple configuration of the chiral carbon can affect affinity, and there are lectins that are more related to alfa-galactosidic than beta-galactosidic residues, setting up that way, an anomeric recognition. The anomeric configuration of galactose residues seems to have biological importance related to the behavior of some diseases and physiological processes. This work aimed to assess the anomeric recognition of two lectins reported as β-galactose ligands (PNA and ricin) and two lectins reported as α-galactose ligands (frutalin and jacalin) in two types of hemicellulose (xyloglucan of Tamarindus indica and galactomannan of Caesalpinia pulcherrima), subsequently crosslinked and used as chromatographic matrices. As a result, chromatographic profiles and retained fractions suggested preferential anomeric recognition by lectins for the hemicelluloses crosslinked. The galactomannan matrix retained 0,5 mg of PNA lectin and 2,3 mg of ricin lectin; meanwhile, the xyloglucan matrix retained 3,4 mg of PNA and 3,2 mg of ricin; results obtained by applying 5 mg of lectin. Ricin expresses a visible flexibility in anomeric recognition, while PNA shows a restricted recognition of β-galactose residues. Frutalin and jacalin did not show recognition of the xyloglucan matrix. This work proposes using hemicellulose reticles with epichlorohydrin as affinity chromatographic matrices for anomeric studies on recognizing galactose binding lectins.

Corresponding Author:- Edwin C. Hernández
Address**:** Professor of Biology, Education Office of Bogotá, Colombia.

Introduction

The hemicelluloses are heteropolysaccharides formed by a heterogenous group of polysaccharides consisting of a main chain of monosaccharides and frequent branches β (1 → 4) of residues of glucuronic acid, arabinose, fucose, galactose, glucose, mannoseor xylose (Andrade et al, 1999; Scheller and Ulvskov, 2010).

Galactomannans are polysaccharides widespread in nature. Their basic structure consists of a central core of β-(1→4) linked D-mannopyranose (Man) to which are attached α-(1→6) linked α-D-galactopyranosyl (Gal) units. These polysaccharides are commonly extracted from the endosperm of numerous seed plants (particularly legumes), where they develop energy-reserve and hydration functions due to their specific physicochemical properties such as high molecular weight, water solubility, and non-ionic character (Gidley and Reid, 2006; Pollard et al, 2010; Albuquerque et al, 2014).

The xyloglucan can be extracted from legumes seeds, where it has a storage function. This macromolecular structure is formed by a 1,4-β-D-glucopyranosyl backbone partly substituted by 1,6-α-D-xylopyranosyl side chains, which are, in some instances, further substituted by a 1,2-β-D-galactopyranosyl residue (Reid, 1985; Souza et al, 2014).

The structural variety of these glycoconjugates has been helpful for various purposes, for instance, formulations in films, gels for industrial purposes (Andrade et al, 1999; Seshagirirao et al, 2005), and as an affinity chromatography matrix for the isolation of lectins (Apfelthaler et al, 2018; Braga et al, 2011; Moreira et al, 1998; Teixeira et al, 2007). Lectins are proteins or glycoproteins that recognize and bind differentially to carbohydrates and glycoconjugates (Sharon and Lis, 1972), despite being non-immune proteins without catalytic sites, they are capable of specifically recognizing carbohydrates across non-covalent interactions (Mákela, 1957; Pusztai, 1991; Sharon and Lis, 1972).

In plants, these macromolecules are present in greater quantity in the cotyledons and endosperm of the seed, corresponding from 2 to 10% of proteins. Lectins are involved in several processes in plants like physiological regulation, carbon reserve, defense against microorganisms and insects attack, transport of carbohydrates, mitogenic stimulation, and nitrogen fixation in the bacteria´s genus Rhizobium (Pusztai, 1991).

The study of lectins was originated from Stillmark's work in 1888, when it was discovered the erythrocyte agglutination phenomenon through seed extracts of Ricinus communis. This protein has lately been named ricin (Sharon and Lis, 1972). However, the first lectin isolated was a concanavalin A, obtained from Canavalia by Sumner (1919), who also demonstrated recognition specificity to carbohydrates (Agrawal et al, 1965; Sumner, 1919).

Currently, investigations of carbohydrate-lectin interactions focus on cell-to-cell and protein-to-protein recognition and its applications in the study of glycosylated structures and their effect on cells. Each lectin molecule typically contains two or more carbohydrate-binding sites, and their interactions on cell surfaces cause agglutination as a result, this phenomenon is an essential attribute of lectin activity and is routinely used to its characterization (Fujita et al, 1975; Wu et al, 2008).

Based on the selectivity of lectins, they can be classified into five groups according to the monosaccharide for which they have affinity: glucose/mannose, galactose/N-acetylgalactosamine, N-acetylglucosamine, fucose, and N-acetylneuraminic acid (Lis and Sharon, 1998).

Usually, the affinity of lectins for monosaccharides is weak (constant association in the magnitude millimolar), but these are highly selective. However, specific lectins for galactose do not recognize glucose or mannose (Mákela, 1957). Lectins that recognize galactose do not interact with mannose (Sharon, 2007); this means that carbons 3 and 4 of carbohydrates have an important role in recognition by lectins. However, the selectivity of lectins for carbohydrates can not always be defined; for example, some variations in the C-2 position of the pyranose ring can be tolerated by some lectins, which bring, therefore, a double recognition for different carbohydrates. Furthermore, many lectins may recognize carbohydrates according to the position of the hydroxyl group of the anomeric carbon, differentiating glycosidic residues with α and β links (Wu, 2008).

Lectins present in animals can be divided into four groups: S-type, C-type, P-type, and I-type (Lis and Sharon, 1998). S-type lectins, also called galectins, are in the nucleus, cytoplasm, and cell surface (Gao et al, 2020). Galectins are a family of lectins defined according to the affinity for residues of β-galactose (Di Lella et al, 2011; Galili et al, 2003).

Galectins regulate many cellular processes like growth, cell-to-cell adhesion, and apoptosis. Also, there is strong evidence that suggests their involvement in immune regulation, inflammation, and cancer, although their precise mechanisms of action are still poorly understood (Di Lella et al, 2011; Galili et al, 2003; Gao et al, 2020). However, residues of α-galactose in macromolecules of cell membranes are associated with the malignancy of cancer cells (Galili et al, 2003); in this sense, the study of anomeric configuration of galactosic residues is relevant.

Scientists have developed several techniques to measure lectins recognition for glycosylated carbohydrates or biomolecules, highlighting the use of biosensors, Nuclear Magnetic Resonance (NMR), Enzyme-Linked Immunosorbent Assay (ELISA), X-ray crystallography technology, Frontal Microarray Chromatography, and Surface Plasmon Resonance.

The above methods are helpful to evaluate lectin-carbohydrate interactions, but their use requires costly and specialized equipment. Therefore, the objective of this work is to propose an easy and inexpensive strategy based on using chromatography to isolate and evaluate anomeric recognition of galactose ligand lectins, using galactomannan obtained from Caesalpinia pulcherrima seeds (with α-D-galactose residues exposed), and xyloglucan obtained from Tamarindus indica seeds (with β-D-galactose residues exposed) crosslinked with epichlorohydrin.

Materials And Methods

Materials

Tamarindus indica, Pulcherrima caesalpinha, Artocarpus incisa, Artocarpus integrifolia, and Ricinus communis seeds were collected around the metropolitan area of Fortaleza, Ceará-Brazil. The seeds of Arachis hypogaea were acquired in the local market.

Obtaining Extracts

The seeds of R. communis, A. hypogaea, Artocarpus incisa, and A. integrifolia were grounded separately and the extract was exposed to acetone to eliminate lypids; after the four were dried, protein extraction was done by suspending 5 g of each one in 150 mM NaCl solution in a 1:10 ratio (v / v) and allowed to mix for 1 hour under magnetic stirring as described by Moreira et al (1998).

The Xyloglucan Extraction.

The xyloglucan extraction was performed according to the method reported byFreitas et al (2003). The seeds of T indica were boiled in distilled water for 50 minutes and put in distilled water at 8 ° C for 12 h, the milled integument was suspended in destilled water at a ratio of 1:40 (m:v). The obtained viscous extract was filtered and centrifuged at 4°C, 10,000 x g, for 20 min. The supernatant obtained was added two volumes of ethanol 96%, and the derived precipitate is then resuspended in water 1:10 (m:v), then the precipitation process is repeated. The alcohol excess was discarded, and the precipitate was immersed in acetone at a ratio of 1:5 (m:v) for 20 min, followed by drying in cold air flow passage and maceration.

The Galactomannan Extraction.

A sample of 50 g of C. pulcherrima seeds was subjected to a temperature of 100°C in 500 ml of distilled water for 20 minutes. Then, the seeds remained submerged in distilled water at room temperature for 12 hours. After swelling, the obtained endosperm was put through exhaustive extraction with distilled water at a ratio of 1: 5 in a blender for 10 minutes. The material obtained was filtered and precipitated with ethanol 95% at a ratio of 1:2 v/v. The polysaccharide was dehydrated with acetone and dried in the oven under ventilation. The galactomannan was weighed on the analytical balance and stored. The yield percentage was calculated by measuring the galactomannan weight obtained from the initial seed mass described by Braga et al (2011).

Crosslinking Xyloglucan Matrices Of T. Indica.
The xyloglucan chromatography columns were performed according to Braga et al (2011). The polysaccharide was crosslinked under different conditions, as shown in Table 1.

Crosslinking Galactomannan Matrix Of C. pulcherrima.
The respective cross-linking to obtain the chromatographic matrix was done according to Braga et al (2011); 0.5 g of Galactomannan of C. pulcherrima was cross-linked with 4 ml NaOH (3M) and 0.5 mL of epichlorohydrin (12.4 M) during 24 hours at 40 °C, and subsequently, the reaction was stopped by increasing the temperature to 70 °C for 12 hours.

Spectroscopy In The Infrared (FT-IR).
The spectroscopy information in the infrared region (FT-IR) was obtained through the Spectrometer Machine FTLA 2000 ABB Bomem (Department of Organic and Inorganic Chemistry - UFC). Samples of xyloglucan T indica crosslinked and uncrosslinked were analyzed using KBr tablet. Spectra were obtained in the range of 400-4000 cm^{-1} (Kurt and Kahyaoglu, 2014).

Affinity Chromatography.
The crosslinked columns were equilibrated with 150 mM NaCl, crude extracts were centrifuged at (10,000 x g, 4 °C, 20 min.) and then filtered and immediately applied (50 ml) in the column. The elution of the non-retained fraction was performed with balance buffer, and the retained fraction was eluted with 150 mM NaCl and 200 mM galactose. Elution was performed under a constant flow (0.5ml / min) and the absorbance of the fractions was monitored at 280 nm as described by Moreira et al (1998).

Evaluation Of Anomeric Recognition Of Lectins.
The suspension of each of the β-galactose binding lectin was made under equilibrium conditions with NaCl (150 mM), and then applied 5 ml of protein suspended (1mg/mL) in the galactomannan column and in the xyloglucan column obtained from treatment 9. Elution of the unretained fraction was done with balance buffer, and the retained fraction was eluted with 200 mM galactose in NaCl 150 mM solution. The elution was performed under a constant flow (0.5 ml/min), and the absorbance of the fractions was monitored at 280 nm.

Identification Of Lectins By Mass Spectrometry.
50 mcg of each protein digested were isolated in 50 μL of 50 mM ammonium bicarbonate containing trypsin (Promega) 1:50 (w/w) ratio of enzyme/substrate at 37 ° C overnight. The peptides were concentrated and injected into the system nanoACQUITY connected to the source of an electrospray mass spectrometer (SYNAPT HDMS system, Waters Corp.). The sample was applied to a C18 chromatography column (75 mm x 100 mm) and eluted with 85% acetonitrile gradient containing 0.1% formic acid. The mass spectrometer was configured in positive mode, using a source temperature of 90 °C and 3.0 kV capillary voltage. The LC-MS / MS experiment was performed with the DDA function selection (data-dependent acquisition) for MS / MS experiments. The data were processed and analyzed with a Proteinlynx v2.4 (Waters) using the fingerprint of the peptide mass (FPP) and the pattern of peptide fragmentation.

Results And Discussion

In the spectral analysis of the infrared region (Fig 1), we can observe and classify some bands concerning the vibration of the binding of the functional groups present in the polysaccharides. Both spectra exhibit a broad and robust band between 3000 and 3600 cm^{-1} that can be assigned to the vibration stretch of the group O-H in water and alcohols (Yuen et al, 2009). It was also noted a decrease in the intensity of the crosslinked band of xyloglucan compared with non-crosslinked suggesting that crosslinking occurred between groups (-OH) of xyloglucan per share of epichlorohydrin under alkaline conditions.

There were also evidenced two medium-strength absorption bands in the range 2923-2934 cm^{-1}, which could be attributed to the symmetric and asymmetric vibration of (C-H) bonds of methylene (–CH2) and methyl groups (-CH3); however, it seems that in the gridded xyloglucan there was a decrease in the intensity of these absorption bands confirming the results obtained by Nep and Conway (2011) and Tavares et al(2011).

The region between 1350 to 1450 cm $^{-1}$ is attributed to the symmetric deformation of CH_2 and C-OH groups, while the band at 1160 cm $^{-1}$ could be assigned to the link of the angular vibrational module δ (C-O) assigned to the stretching vibration of the pyranose ring (Figueiró et al, 2004; Prashanth et al, 2006).

The bands at 820 cm^{-1} refer to the crosslinking of the anomeric conformation of β-D-galactopironose (Cui et al, 2007). These results were verified through FT-IR spectra for the band xyloglucan 820 cm $^{-1}$ and 870 cm^{-1} which was assigned to the stretching of the α-linked D-xylose and galactose β-D conformations of the xyloglucan. The profile between 800 and 1200 cm^{-1} represents the region of "finger point" for this type of xyloglucan (Cui et al, 2007; Fillippove, 1992).

Ten matrices obtained from different treatments specified in Table 1 were evaluated to determine the best treatment to isolate lectins of Arachis hypogaea and Ricinus communis. Treatment 9 was efficient to isolate the two lectins (Table 2); this column was selected to perform the assessments of anomeric recognition of galactose ligand lectins, and the chromatographic profiles obtained can be seen in Figure 2.

The amount of protein fraction retained from each chromatography performed in xyloglucan crosslinked matrices is shown in Table 2. The matrices that best strain retained lectins were treatments 2 and 9. Treatment 9 showed a lower concentration of sodium hydroxide in the preparation; maybe this condition increases the exposure of β-galactose residues in the crosslinked matrix.

The best xyloglucan crosslinking condition for a chromatographic matrix to isolate β-galactose ligands lectins exposed in this work differs from the conditions proposed by Garros-Rosa et al (2006), Lima et al (2002), Seshagirirao et al (2005), Moreira et al (1998), Braga et al (2011), and Teixeira et al (2007), the last author isolated lectins with other crosslinked hemicellulose. The results show that finding ideal conditions to extract lectins implies evaluate the intrinsic characteristics of each hemicellulose, since these vary deeply among different kinds of organisms.

In this sense, developing the experimental design to evaluate each of the conditions proposed here (Table 1) is relevant. The column of condition 9 was adequate to isolate β-galactose ligand lectins, with excellent yields for ricin and PNA, as shown in Table 2. Lotan et al (1986) reported that per 100 gr of extract of A. hypogaea, it is possible to isolate 150 mg of PNA, which corresponds to a yield of 0.15%. In this study, it was possible, with column 9, to isolate 7.0 mg of PNA from 5 gr of extract, which corresponds to a yield of 0.14%, confirming the reliability of the methodology described.

Anomeric Recognition Assay

To test the hypothesis: lectins would recognize differentially alpha and beta exposed galactosidic residues present in hemicelluloses, we proceeded to submit lectins in the hemicelluloses chosen, finding that both, frutalin and jacalin, did not show retained fraction in the chromatography performed with xyloglucan matrix (Figure 2). It has been described that lectins interact with residues of galactomannans possessing α-D-galactosides exposed (Braga et al, 2011; Moreira et al, 1998).

According to Jeyaprakash et al (2003), in a complex with a monosaccharide, the primary binding site of jacalin is occupied by galactose or N-acetyl-galactosamine. With α-substituted residues, this arrangement is not damaged as α-substituted sugars interact primarily in a hydrophobic way with the recognition site, which has variable geometry; in the presence of the α-linked group, the chain of Tyr122 moves to accommodate the linking, this involves the side chains of Tyr78 and Tyr122, and the α-link group is aligned with the side chain of Tyr78, Tyr122 and Trp123 (Jeyaprakash et al, 2003).

On the other hand, β-substituted residues lead to an unacceptable steric hindrance with the protein. In β-substituted disaccharides, the reducing sugar binds to the primary site with the nonreducing residue exposed. Then, it can be affirmed that the affinity of jacalin is in many orders of magnitude higher for α-linked residues than for β-bound residues (Jeyaprakash et al, 2003), and this explains its low affinity for xyloglucan matrices as shown by not retained fraction chromatograms (Figure 2), which leads to conclude that this lectin structure is linked to anomeric recognition. About frutalin, it has structural similarities with jacalin (Campana et al, 2002), which suggests a similar

steric frutalin behavior, as confirmed by the absence of fraction retained in the xyloglucan chromatography matrix (Figure 2).

To assess the interaction of PNA with galactomannan and xyloglucan, we initially confirmed the isolation of the protein recognizing peptides typically found in a single peak of the fraction retained throughout affinity chromatography on a column of nine xyloglucan conditions using mass spectrometry. Subsequently, affinity chromatography was performed in matrices of galactomannan and xyloglucan (Figure 3); and later, it was performed on column 9 and the crosslinked galactomannan according to Brag et al (2011), which describes the best condition of galactomannan crosslinking for isolatingα-galactose binding lectins.

The amount of protein retained in the xyloglucan chromatographic matrix and galactomannan was 0.48 mg and 2.63 mg, respectively. These results expressed in (Table 3) suggest that lectin from A. hypogaea interacts weakly with the galactomannan chromatographic matrix. However, it interacts strongly with the xyloglucan one, as demonstrated in chromatographic profiles (Figure 3).

Lotan et al (1986), showed that for this lectin, since no affinity for glucose, the configuration of C-4 and the free hydroxyl at C-6, are crucial for recognition; it has been demonstrated that it recognized residues of α-galactose. Besides, it has also been proved that the best affinity for disaccharide Gal β-(1→3) is with the GalNAc residue; therefore, it´s presence causes a potent inhibition of hemagglutination activity compared with that obtained in the presence of galactose, suggesting that the lectin has an extended binding site (Lotan et al, 1986). The chromatographic profiles obtained suggest that this lectin prefers structures with β-galactosidic residues, according to Safina et al (2011), who defines PNA as β-galactose binding lectin after studies of surface plasmon resonance.

To evaluate the interaction of ricin with galactomannan and xyloglucan, we used the same methodologic approach as PNA (Figure 4). The amount of retained protein was 2.1 mg and 2.8 mg, respectively. The information in the Table 3 shows that lectins interact with the chromatographic matrices of xyloglucan and galactomannan.

The lectin domain of ricin prefers β-D-galactose residues recognizing the non-reducing terminal, in the next affinity order: galactose β (1 → 4), galactose β (1 → 3), galactose β- (1→6) (Wu et al, 2006). Saccharide residues recognition found in this work is concordant with the present literature, however, the use of chromatrography to differential anomeric recognition has not been reported yet (Table 3), despite of lectin recognition activity for residues α-galactosidic had already been reported (Appukutan et al, 1977; Nicolson et al, 1974; Roberts et al, 1985; Tanemura et al, 2002).

The amount of protein retained on the affinity chromatography is shown in (Table 2). PNA lectin (Tanemura et al, 2002) and ricin (Wu et al, 2006) are reported as β-galactose binding lectins, although the difference between the amounts of protein retained by the column galactomannan for both lectins are significant; PNA has a preference for β-galactosides residues, but ricin has mixed anomeric recognition. This phenomenon is due to their different structures in the binding site, which implies differences in anomeric affinities.

The results shown here suggest that the strategy proposed can be significant for studying anomeric recognition by lectins in clinical applications. Furthermore, studies using chromatographic profiles, or the amount of protein retained in the hemicellulose matrix, as an information source for the study of anomeric recognition by galactose-binding lectins, have not been reported yet.

Depending on recognition, lectins are classified according to the recognition pattern of the carbons 3 and 4 (Mákela, 1957). Galactose ligands lectins recognize a variation in the C4 configuration of monosaccharides who interacts with. They can differentiate epimers; some are able to distinguish between galactose and glucose by recognizing the axial and equatorial configuration of the hydroxyl in C4 of galactose and glucose, respectively.

In subsequent classifications, they were grouped according to carbohydrate recognition. However, they have galactose ligands lectins which also bind to GalNAc, thus tolerating the replacement of the functional group in the pyranose ring C2, which justifies the classification of lectins in one group (Sharon, 2007). However, none of these clasifications consider the anomeric recognition that undoubtedly has a fundamental role in recognizing carbohydrates, as shown in this work. Accordingly, studies of this type are an essential source of information for future classifications.

Conclusion

There is an anomeric differential recognition by galactose-binding lectins for residues exposed in hemicelluloses after crosslinking. This differential recognition can be evidenced by evaluating the chromatographic profiles of xyloglucan and galactomannan crosslinked matrices.

Table 1:- Experimental conditions applied to obtain the xyloglucan chromatography matrix of *T. indica* for 0.5 g of polysaccharide.**M**= $g.mol^{-1}$

Treatment	NaOH (mL)	NaOH (M)	ECH (mL)	Matrix (g/m)	NaOH (M)	ECH (M)	NaOH/ECH (M/M)
1	4	3	0,25	0,10	2,8	1,4	2
2	4	3	0,5	0,12	2,7	2,8	0,9
3	4	3	1	0,10	2,4	5,0	0.5
4	4	3	2	0,11	2	8,2	0,25
5	1.5	3	0,5	0,33	2,2	6,2	0,35
6	3	3	0,5	0,14	2,6	2,9	0,9
7	5	3	0,5	0,09	2,7	2,2	1,22
8	4	1	0,5	0,10	0,9	2,8	0,3
9	4	2	0,5	0,10	1,8	2,8	0,64
10	4	4	0,5	0,09	3,6	2,8	1.3

Table 2:- Quantity in mg of lectins isolated using the crosslinked xyloglucan matrix after applying 50 ml of crude extract.

Treatments	PNA (mg)	Ricina (mg)
1	2.1	15.9
2	5.0	35.6
3	1.8	11.4
4	0.8	9.0
5	-	-
6	0.7	7,28
7	1.7	23
8	3.0	29.5
9	**7.0**	**39.8**
10	1.5	11.8

Table 3:- Amount of isolated lectin using the xyloglucan crosslinked 9.

Lectin/Hemicellulose	Galactomanana	Xiloglucana
PNA(mg)	0,5	3.4
Ricina (mg)	2.3	3.2

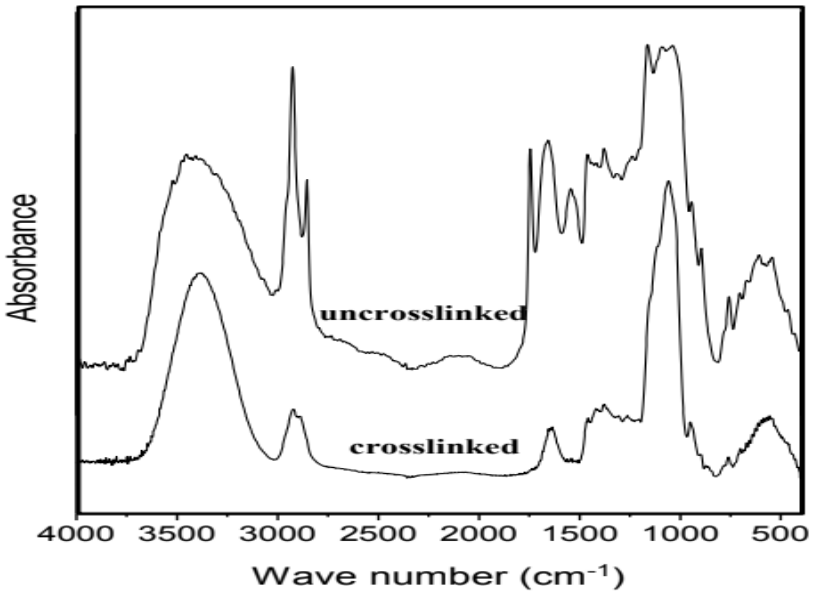

Fig1:- The Spectra of xyloglucan infrared indicates non-crosslinked and crosslinked with epichlorohydrin.

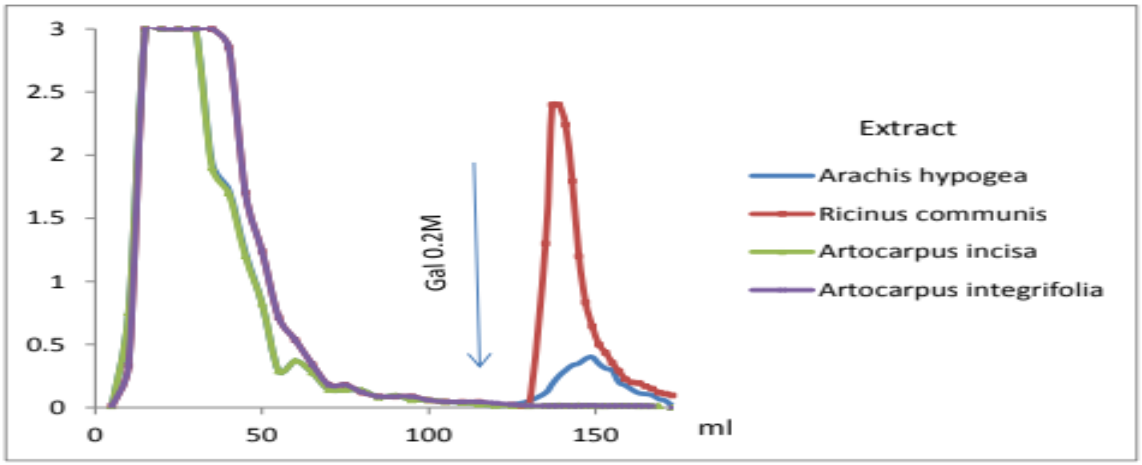

Fig 2:- Affinity chromatographic profiles obtained after applying 50 ml of each seed specie extracts tested containing galactose binding lectins about xyloglucan matrix.

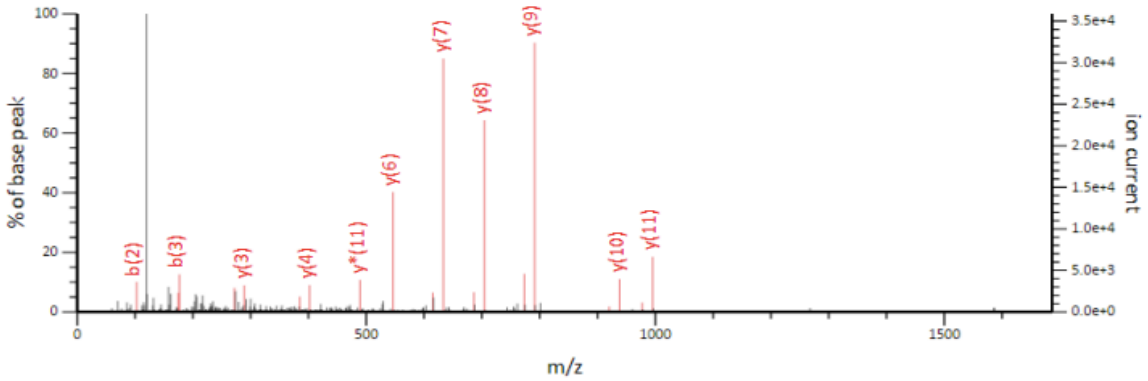

Fig 3:- Chromatograms obtained from 5 mg of PNA about crosslinked hemicelluloses A) galactomannan of *C. pulcherrima* B) xyloglucan of *T. Indica. Xyloglucan.*

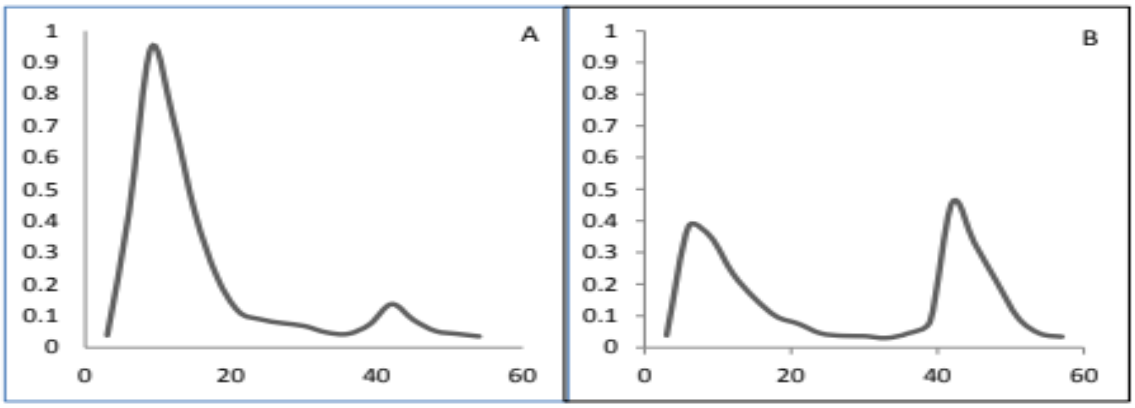

Figure 4:- Chromatograms obtained from 5 mg of ricin about hemicelluloses crosslinked. A) Galactomannan of *C. pulcherrima.* B) Xyloglucan of *T. Indica*.

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