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SCREENING OF AGGLUTINATION ACTIVITIES OF COMMON VEGETABLES IN BANGLADESH

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Abstract

Lectins are capable of cell agglutination by interacting with the cell surface exposed glycans. Lectins that agglutinate erythrocytes are called hemagglutinin. These compounds occur in various organisms including plants. In this study, twenty edible vegetables were subjected to screen for haemagglutination and lectin activity. Lectin activity was assessed by measuring the inhibition ability of haemagglutination by different sugars. PBS (Phosphate Buffer Saline) treated homogenate was tested for haemagglutination against erythrocytes suspension, and inhibition of agglutination was determined by serially diluted sugar solution. The results of this study demonstrated that one homogenate among the homogenates of 20 vegetables was found to be capable of human erythrocyte agglutination. The Ipomoea aquatica homogenate shown positive haemagglutination reaction against red blood cells (RBCs) with HA titer value of 4 HA-U/25uL, and four out of seven sugars were able to inhibit RBCs agglutination. Among the four sugars, two are potent inhibitors of agglutination. The neutralization of haemagglutination by four monosaccharides provides a suggestion that the lectin present in I. aquatica may be multivalent carbohydrate binding hemagglutinin. This is the first report to identify that Ipomoea aquatica contains lectin protein which is a polyvalent sugar binding lectin and specific for glucose, galactose, *mannose and N-Acetyl-D-Glucosamine.*

Keywords: Vegetables, haemagglutination, lectin, erythrocytes, sugar specificity

Introduction

'Hemagglutinin' or 'lectin' named by Boyd and Shyleigh, was discovered for the first time by Stillmark in 1888 (Boyd, 1963). Lectins that can agglutinate RBCs are called hemagglutinins. However, this is an oversimplification because all hemagglutinins cannot agglutinate erythrocytes. Reversible binding of cell surface sugar is exclusive for lectins (Hassan et al., 2015). A well-established haemagglutination inhibition assay was utilized in this research for the purpose of screening hemagglutinins (Hirst, 1942). Plant lectins have been sectioned, conferring their specificity of carbohydrate binding. Based on this specificity, mannose, mannose/glucose, mannose/maltose, Gal/GalNAc, GlcNAc/ (GlcNAc)n, fucose, and sialic acid binding lectins have been distinguished (Goldstein & Poretz, 1986; Van Damme et al., 1998). Rapid progress in structural analysis of lectins and detailed sequence information of lectins provides a tool for determining lectins in seven families with legume lectins, monocot mannose-binding lectins, chitin-binding lectins, type 2 rip, and related lectins, jacalin-related lectins, amaranthine lectin, and Cucurbitaceae phloem lectins husking (Damme et al., 1998). Each family of lectins has some physiologic role. Legume lectins (LL) acts like plant defense proteins when insects invade them (Damme et al., 1998). Similarly, jacalin related lectins (JRL), Amaranthin lectins (AL), and Cucurbitaceae phloem lectins (CPL) family are also involved in plant defense (Damme et al., 1998). Hemagglutinin or lectin has garnered attention in medical research and therapy because lectin-based approaches are becoming more popular in novel cancer biomarker research. Lectins are a valuable tool for investigating different cellular processes of cell signaling using the fluorescent-protein biosensors technique (Giuliano & Taylor, 1998). Lectins, for their carbohydrate-binding affinity, can inhibit enveloped viruses from attachment and fusion with the target cell membrane. Lectins also play a role in the study of immunomodulatory effects. *Agaricus bisporus* lectins (ABL) has shown potential action against autoimmune inflammatory pathologies (Ditamo et al., 2016). Lectins could be used

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as carrier molecules to target drugs specifically to different cells and tissues, as other cell types express different glycan arrays, particularly in diseased cells. Several lectins are now used in lectin-mediated drugs targeting in lung, blood brain barrier (BBB), and eye (Bies et al., 2004).

Bangladesh is an agriculture-based country. Most of the selected plants are used traditionally for the management of different diseases including pain, splenic disorders, cholera, dysentery, asthma, cough, bronchitis, hypertension and no report are available on HA activity of the selected plants except seeds of *Amaranthus spinosus, Piper longum L, Moringa oleifera* and *Cucurbita pepo* (Hussain et al., 2010; Kumar et al., 2012; Kumar et al., 2014; Mandelker et al., 2009; Meira et al., 2012; Metha & Belemkar, 2014; Ratnam et al., 2017; Shavandi et al., 2012; Tanmoy et al., 2014; Tripathi et al., 1999). Previous studies reported pharmacological activity including antitumor, antioxidant, anti-inflammatory, antifungal, antibacterial, cytotoxic, hepatoprotective, anti-cancer, anthelmintic, anti-proliferative activity of multiple plants utilized in this research (Ali et al., 2012; Bezerra et al., 2013; Bhatia & Jain, 2004; Huang et al., 2005; Hussain et al., 2011; Kolya et al., 2015; Maeda et al., 2008; Samojlik et al., 2010; Sannigrahi et al., 2010; Shokrzadeh et al., 2010). As these plants possess multiple pharmacological activities, the selected plants are an interesting candidate for the present screening study of haemagglutination activity (HA) or lectin activity, as lectins also possess antitumor, antiproliferative and immunomodulatory activities (Hassan et al., 2015), anthelmintic (Ríos-de Álvarez et al., 2012), antibacterial, antifungal, antiviral (Paiva et al., 2010; Peumans & Van Damme, 1995), antioxidant, anticancer, antitumor (Dhuna et al., 2005; Saha et al., 2014; Zhang et al., 2015), cytotoxic activity (Ribéreau-Gayon et al., 1995).

The aim of this paper is to dive deep into understanding if regular vegetables do possess lectin activity or not because their traditional uses indicate that they might. Documented research to back up these traditional reports can pave the way for future utilization of these plants in a more informed and formal manner. Lectins were previously reported to have benefits of ameliorating cardiovascular diseases, type 2 diabetes, and other detrimental health complications (Kolb et al., 1986; Pągowska-Klimek & Cedzyński, 2014). Thus, findings of these papers can also promote utilizing these highly available food sources for overall positive physiological outcomes.

Materials and Method

Validation of method

After setting a working protocol by reviewing several literatures, validation of the working method was done to ensure that the active protocol was suitable for driving the thesis. *Lens culinaris* was used as a working standard control for running HA assay and HA inhibition assay. It was chosen as the standard because lens culinaris agglutinin (LCA) agglutinates human erythrocytes of all ABO types equally well (Heritage, 1973). Mannose sugar was used for inhibition assay (Yamamoto et al., 2000).

Collection of samples

In this investigation, selected 20 vegetables (*Spinacia oleracea, Ipomoea aquatica, Typhonium trilobatum L., Amaranthus spinosus, Alternanthera Sessilis R. Br., Piper longum L., Amaranthus gangeticus, Amaranthus viridis L, Eryngium foetidum, Cucurbita pepo, Bacopa monnieri, Desmodium triflorum, Moringa oleifera, Enhydra fluctuans, Coriandrum sativum L, Hygrophila auriculata, Rumex acetosa, Trianthema portulacastrum, Alternanthera philoxeroides and Cinnamomum tamala*) were collected from local market of Khulna, Bangladesh to screen them for possible haemagglutination or lectin activity. These samples are from fourteen different families, including Acanthaceae, Aizoaceae, Amaranthaceae, Apiaceae, Araceae, Asteraceae, Convolvulaceae, Cucurbitaceae, Lauraceae, Leguminoseae, Moringaceae, Piperaceae, Plantaginaceae, Polygonaceae. All samples were collected prior to the test with a view to confirming that fresh vegetables were used. Each sample was stored at frozen condition before analysis. For this screening study, leaves, stems and leafy parts of all selected plants were used.

Preparation of PBS

PBS (Phosphate Buffer Saline) was prepared with a pH of 7.4 (Rouf et al., 2011) and stored in the refrigerator. Before using PBS, pH was checked by hana pH meter.

Sample Homogenization

Immediately after defrosting 6-10g of sample was homogenized on ice in PBS of pH with 7.4 and 2mL PBS was used for per gram of sample to be homogenized (Rouf et al., 2011). After 25-30 minutes homogenization the homogenate was subjected to blending in jaipan heavy duty blender machine for 5-10 minutes. The homogenate was left for

overnight homogenization on a magnetic stirrer, ensuring cool temperature condition and was filtered at the following day using cotton gauge. After centrifuging the filtrate at 1500 RCF for 20 minutes at 4°C temperature, the supernatant was collected, and centrifugation was done once again at 10000 RCF at the same temperature condition for the same duration. The supernatant was collected in 15 falcon tubes and was made air-tight using paraffin. The supernatant sample was stored in deep freeze until an assay was performed (Rouf et al., 2011).

Preparation of RBC suspension

This study was conducted under the as per disciplines human ethical protocol no. KU/PHARM/HEC/16/007/01. From some students at Khulna University, blood samples were collected. RBCs were washed at 4°C temperature at 600 RCF using PBS with pH value of 7.4. Washing was done for 3 times and 2% RBC suspension was prepared. With a view to marking off the suppositional lectin activity of *I. aquatica* homogenate, the HA inhibition capability of specific sugar had been used. Seven sugars were used including Glucose (G), Galactose (Gl), Maltose (Ml), Arabinose (Ar), Ribose (R), Mannose (Mn) and GlcNAc/N-Acetyl-D-Glucosamine (Gln).

Haemagglutination assay

After the preparation of 2-fold serially diluted homogenate (PBS: supernatant=1:1), an equal volume (25uL) of PBS was mixed in a U-bottom 96-well plate. Subsequently, 50uL of 2% RBC suspension was mixed and left at room temperature. The result was recorded after one hour when the negative control was sedimented completely. The result was determined by taking the reciprocal of the highest dilution that showed haemagglutination and expressed as HA Unit (HA-U) (Rouf et al., 2011). Next, serially diluted sugar solutions (D-Glucose, Galactose, Maltose, L-Arabinose, D-Ribose, D-Mannose and N-Acetyl-D-Glucosamine each 25uL) were taken in 96-well plate as inhibition chemicals and the maximum dilution of homogenate (25uL) that possessed hemagglutinin was mixed into the U-bottom 96-well. Following that 50uL of 2% RBC suspension was added and left at room temperature for some time. Result was recorded one hour later, and complete sedimentation indicates the presence of lectin. The data were represented as images of whether lectin was present (mean complete sedimentation) or not (mean no sedimentation) without any statistical analysis.

Table 1. Selected sample with their family, distribution and collection period

Scientific name	Family	Collection date	Distribution
Spinacia oleracea	Amaranthaceae	01/02/2018	All over Bangladesh
Ipomoea aquatica	Convolvulaceae	16/07/2018	Bangladesh
Typhonium trilobatum (L.)	Araceae	15/09/2018	Indian Subcontinent
Amaranthus spinosus	Amaranthaceae	23/09/2018	All warmest continents
Alternanthera Sessilis R. Br.	Amaranthaceae	23/09/2018	Tropical and subtropical regions
Piper longum l.	Piperaceae	01/10/2018	India, Bangladesh
Amaranthus gangeticus	Amaranthaceae	01/10/2018	Bangladesh
Amaranthus viridis L	Amaranthaceae	09/10/2018	Asia, Africa, America and Europe
Eryngium foetidum	Apiaceae	09/10/2018	Grow worldwide
Cucurbita pepo	Cucurbitaceae	09/10/2018	Grow in South Asia
Bacopa monnieri	Plantaginaceae	09/10/2018	Asia, Australia
Desmodium triflorum	Leguminoseae	22/10/2018	Bangladesh
Moringa oleifera	Moringaceae	22/10/2018	Found worldwide
Enhydra fluctuans	Asteraceae	22/10/2018	Bangladesh
Coriandrum sativum L	Apiaceae	22/10/2018	Tropical and subtropical regions
Hygrophila auriculata	Acanthaceae	29/10/2018	Native to tropical Asia & Africa.
Rumex acetosa	Polygonaceae	29/10/2018	Cultivated in Bangladesh
Trianthema portulacastrum	Aizoaceae	29/10/2018	Grown in Bangladesh
Alternanthera philoxeroides	Amaranthaceae	29/10/2018	Introduced to tropical Asia

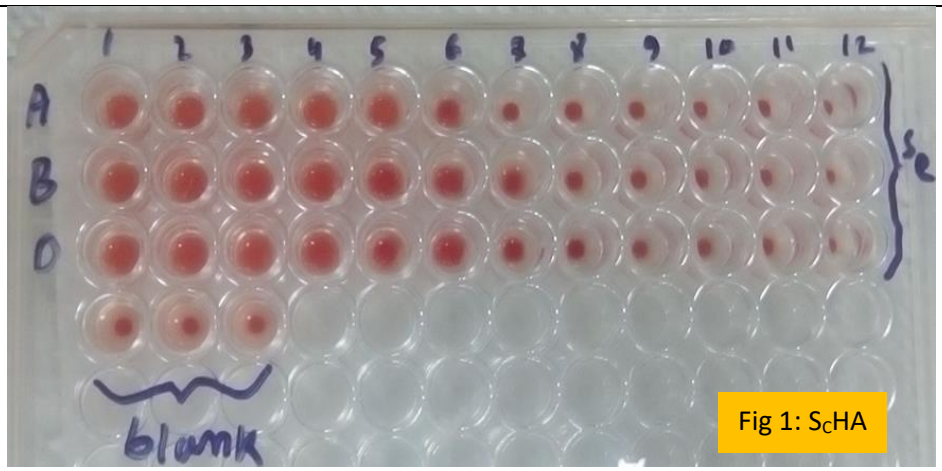
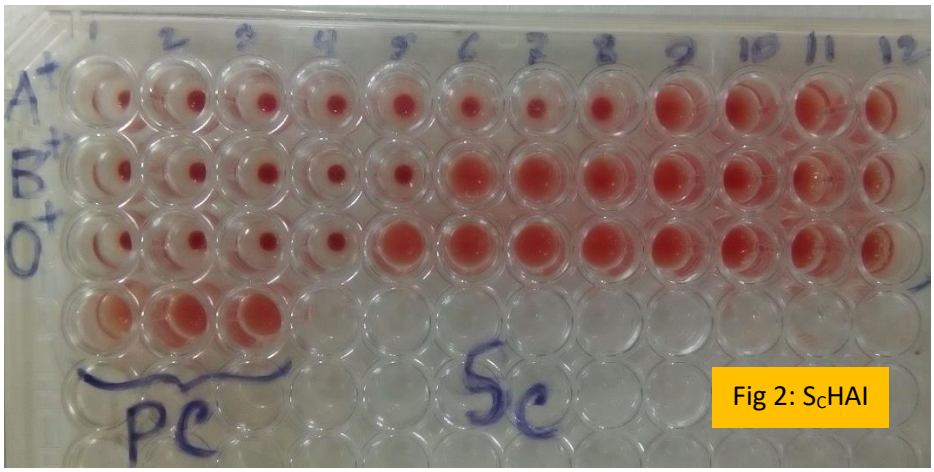
Results

The distribution and collection dates of 20 samples with their botanical profile have been summarized in Table 1. Among twenty vegetables, one was found to be active in haemagglutination titer. The titer results are represented by Figure 1 in Table 2. The plant active in HA titer was *Ipomoea aquatica* from the Convolvulaceae family. Extract of *I.*

aquatica was able to agglutinate 2% RBC suspension of B (+)ve blood type with a HA titer value of 4 HA-U/25uL (Table 3). This agglutination was observed at 2nd well of 6th row of 96-well containing 1:4 dilution of homogenate (Figure 1 of Table 2).

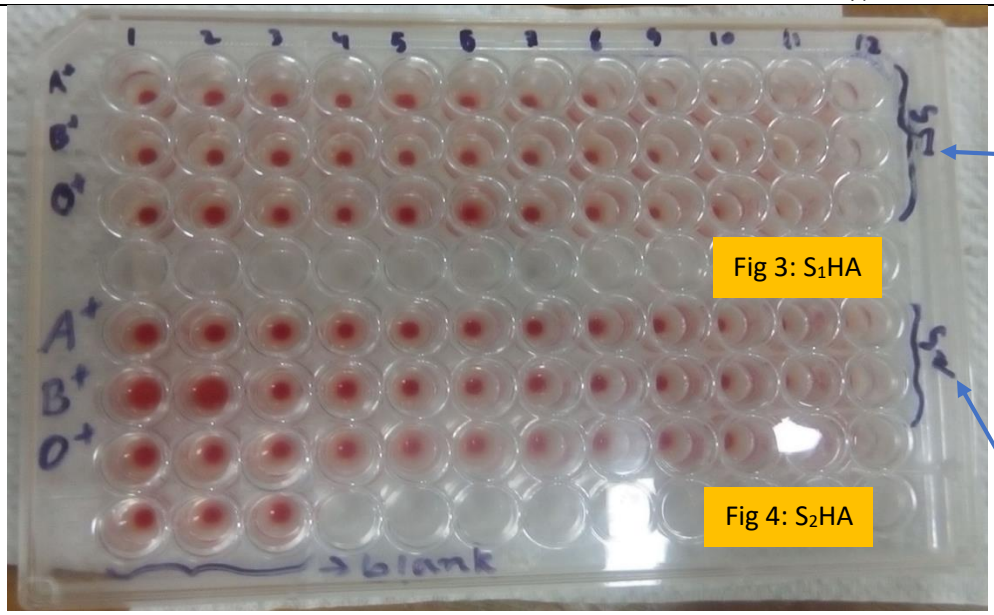
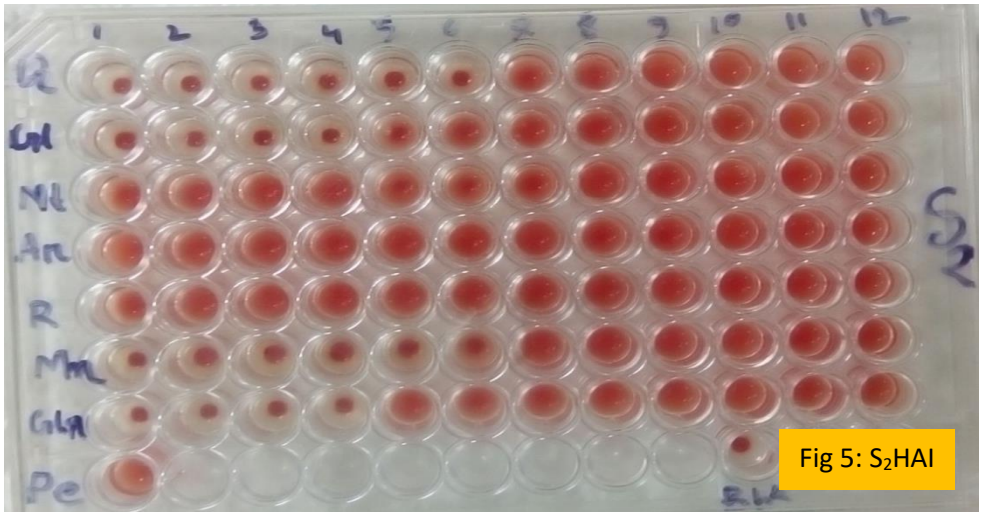
Next, 7 sugars were tested for haemagglutination inhibition assay. Three of the tested sugars are present in human RBC as cell surface molecule, i.e. Glucose, Galactose, and N-Acetyl-D-Glucosamine (GlcNAc). As the homogenate of *I. aquatica* agglutinated the B type RBCs, B type 2% blood suspension was used for HA inhibition, and 200mM stock solution of each sugar was used after serial dilution for finding out the minimum inhibitory concentration (MIC). The HA of RBC by the homogenate was opposed by glucose (G), galactose (Gl), mannose (Mn) and GlcNAc (Gln). The MIC of glucose and mannose was equal (6.25mM). Galactose and GlcNAc had MIC of 12.5mM and 25mM, respectively. Maltose, L-Arabinose, D-Ribose did not show any inhibitory activity. These data are compiled in Table 4. A picture of HA inhibition is displayed by Figure 2 in Table 2.

Table 2. Images HA and HA inhibition of this investigation.

Sample code	Image 1:2 1:4 1:8 1:16 1:32 1:64 1:128 1:256 1:512 1:1024 1:2048 1:4096	Result status
		P
		LA

a. SC=Standard control (Lens culinaris); PC=Positive Control; P=Positive; LA=Lectin active
b. SCHA=Standard control hemagglutination; SCHAI=Standard control inhibition

Table 2. Images HA and HA inhibition of this investigation (continued).

Sample Code	Images												Result Status
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	
S_1 and S_2													N P
													LA

S1= Spinacia oleracea; S2= Ipomoea aquatica; G=Glucose; Gl=Galactose; Ml=Maltose; Ar=Arabinose; R=Ribose; Mn=Manose; Gln=GlcNAc or N-Acetyl-D-Glucosamine; PC=Positive control; P=Positive; LA=Lectin active.; b. S1HA= Hemagglutination of Sample 1; S2HA= Hemagglutination of Sample 2; S2HAI= Hemagglutination inhibition of Sample 2; N=Negative

Table 3.Sample found to have haemagglutination activity against human ABO type erythrocytes.

Sample Name	HA activity against PBS treated 2% RBC suspension (HA-U/25µL)[a]			Part used[b]
	A (+)ve	B(+)ve	O(+)ve	
Spinacia oleracea	N	N	N	L
Ipomoea aquatica	N	4	N	LEP
Typhonium trilobatum (L.)	N	N	N	L, T
Amaranthus spinosus	N	N	N	L
Alternanthera Sessilis R. Br.	N	N	N	L
Piper longum l.	N	N	N	L
Amaranthus gangeticus	N	N	N	L
Amaranthus viridis L.	N	N	N	L
Eryngium foetidum	N	N	N	L,T
Cucurbita pepo	N	N	N	L
Bacopa monnieri	N	N	N	LEP
Desmodium triflorum	N	N	N	LEP
Moringa oleifera	N	N	N	L
Enhydra fluctuans	N	N	N	LEP
Coriandrum sativum L.	N	N	N	LEP
Hygrophila auriculata	N	N	N	L,T
Rumex acetosa	N	N	N	LEP
Trianthema portulacastrum.	N	N	N	L,T
Alternanthera philoxeroides	N	N	N	LEP
Cinnamomum tamala	N	N	N	L

a. HA-U; Haemagglutination Unit, N; Negative, A(+)ve; A positive erythrocytes, B(+)ve; B positive erythrocytes, O(+)ve; O positive erythrocytes.
b. L; Leaf, LEP; Leafy Edible Part, T; Twig

Table 4. The profile of HA activity of I. aquatica homogenate against human RBCs

Type of 2% erythrocytes suspension	Sugar used for HAI assay[a]	MIC (mM)*
B positive	D-Glucose;	6.25
B positive	Galactose	12.5
B positive	Maltose	-
B positive	L-Arabinose	-
B positive	D-Ribose	-
B positive	D-Mannose	6.25
B positive	N-Acetyl-D-Glucosamine	25

a. HAI; Haemagglutination Inhibition
b. * The minimum sugar concentration able to inhibit HA activity, which was evaluated when RBCs were fully sedimented, was used to determine the minimum inhibitory concentration (MIC), mM; Millimole

Discussion

Lectins being polyvalent or univalent carbohydrate-binding oligomeric proteins are enable to interact with diverse type of cell, including normal or modified cell of human, animal as well as microorganisms, as every cell is distinct from each other by expressing different glycan arrays on their cell surface (Bies et al., 2004; Vishweswaraiah et al., 2022). In this study, human red blood cells have been used to try out the interaction with lectins using 20 vegetables and the seed of *Lens culinaris* has been used as a standard control for its potent lectin activity against all ABO type blood of humans as attested in (Heritage, 1973; Makela, 1957). Among the investigated sample, the extract of *I. aquatica* had been found to cover HA activity, and inhibited by four sugars, namely Glc, Gal, Man, and GlcNAc (Table 2), interestingly furnishing a notification of strong inhibition by Glc and Man in comparison with another two sugars. Strong inhibition of agglutination reaction by mannose reverted that D-mannose was the most powerful inhibitor of lectin due to its nifty affinity for lectin (Heritage, 1973; Stein et al., 1971). On the contrary, in this study, inhibition of HA activity by Gal is two times lower than that of the two mentioned above, while inhibition by GlcNAc is four times

lower in comparison with Glc and Man. For multiple binding specificities of *I. aquatica* lectin, it's going to be done to make some hypothetical comments. Since only human RBC suspension was used in this investigation, it would not be evident that *I. aquatica* lectin has only interaction with human RBCs but may also interact with cells of other organisms. Because various cells of several microorganisms are available in the universe have one or more of above mentioned four sugar encoding cell surfaces, i.e. GlcNAc in the peptidoglycan of the gram-positive and gram-negative bacterial cell wall (Vollmer & Höltje, 2004; Weidel & Pelzer, 1964), Chitin, a polymer of GlcNAc in the fungal cell wall (Garcia-Rubio et al., 2020). The sialoglycoprotein of monkey erythrocyte membranes, glycophorin MK may provide a binding site for *I. aquatica* lectin due to being composed of NeuGc, Gal, and GalNAc (Aoki, 2017; Murayama et al., 1989). This lectin may also have interaction with erythrocytes of horse, rat, rabbit, pig, chicken and carp, as galactose is present in their erythrocytes membrane (Aoki, 2017; Fukuda et al., 1980; Krotkiewski, 1988).

Interaction between surface sialic acid of urinary bladder epithelial cells and pathogenic *E. coli* modulate the adherence independently of P and type 1 fimbria adhesion (Sakarya et al., 2003), and causes urinary tract infection (UTI) (Lewis et al., 2016). The mannose specific lectin from *I. aquatica* may have interaction with bladder epithelial cells, as mannose-containing glycoprotein receptors uroplakin 1a is present in differentiated bladder umbrella cells (Wu et al., 2009; Zhou et al., 2001), and may be used as drug career for UTI, because the fim-H of type-I pili of UPEC do bind to mannose-containing glycoprotein receptors UP1a (Zhou et al., 2001). To be a GlcNAc specific lectin, this may interfere with the synthesis and/or deposition of chitin in the fungal cell wall as like as UDA (Van Damme et al., 1998; Van Parijs et al., 1992). Mannose/glucose specific lectin are potent anti-tumor cytotoxins due to their inhibitory effect on protein synthesis (Barre et al., 2019), and might be used for developing receptor specific cytotoxin, i.e. conjugation of cytotoxic plant lectin to monoclonal anti-tumor antibody (Mody et al., 1995). According to the result of this study, the *I. aquatica* lectin has multiple specificities against carbohydrates, and after the purification of the lectin might be used for glycan research in the different fields of medical science.

Other 19 plants used in this probe did not show any HA or lectin activity against *Homo sapiens* blood. No positive HA activity of the 19 plants does not mean that these plants do not possess any lectin. It may be found to possess lectin activity or HA activity against blood of other animal or cell surface glycan of an organism (Rouf et al., 2011). Several reports has disclosed that, plant lectin can interact with surface-exposed carbohydrates of microbes and can agglutinate them (Gaidamashvili & Van Staden, 2002; Nachbar & Oppenheim, 1980; Ratanapo et al., 2001). Over and above, these plants have some reasonable pharmacological activity to be worth lectin activity accordingly (Ali et al., 2012; Bezerra et al., 2013; Bhatia & Jain, 2004; Huang et al., 2005; Hussain et al., 2011; Kolya et al., 2015; Maeda et al., 2008; Samojlik et al., 2010; Sannigrahi et al., 2010; Shokrzadeh et al., 2010). Plants with antioxidant, antimicrobial, antifungal, antibacterial, antitumor, anticancer, cytotoxic property may contain lectin, as the pronounced properties are also possessed by lectins (Dhuna et al., 2005; Hassan et al., 2015). Three plants from Convolvulaceae have already been reported to contain lectin, including *Calystegia sepium*, *Convolvulus arvensis,* and *Ipomoea batatas* (Van Parijs et al., 1992) and in this study, it has been found to have lectin in *Ipomoea aquatica* which is also a plant Convolvulaceae family.

According to this study, B-type erythrocyte is agglutinated by *I. aquatica* homogenate and show specificity towards four sugar, and further studies may reveal the lectin activity or HA activity against erythrocytes of another animal or cell of another organism. The lectin activity found from this screening investigation would be an interesting subject for purification, lectinological research as well.

Conclusion

Ipomoea aquatica has lectin protein, a polyvalent sugar binding lectin that is specific for glucose, galactose, mannose, and N-Acetyl-D-Glucosamine. Four monosaccharides' ability to neutralize haemagglutination suggests that the lectin in *I. aquatica* might be a multivalent hemagglutinin that binds carbohydrates. This is the first publication to document this. Future research can explore to evaluate these in-vitro results in-vivo and utilize this haemagglutination property of *I. aquatica* for clinical research in health care.

Funding

There was no external funding available for this research.

Abbreviations

ABL; Agaricus bisporus lectin, AL; Amaranthin Lectins, BBB; Blood brain barrier, CBL; Chitin-binding lectins, ConA; Concanavalin A, CPL; Cucurbitaceae Phloem Lectins, Gal; Galactose, Glc; Glucose, GlcNAc; N-acetyl-D-glucosamine, HA ;Haemagglutination, HAI; Haemagglutination Inhibition, HA-U; Hemagglutination Unit, JRL; Jacalin-Related Lectins, LCA; Lens culinaris agglutinin, LL; Legume lectins, LTL; Lotus tetragonolobus Lectin, Man

;Mannose, MMBL; Monocot Mannose-Binding Lectins, NeuGc; N-glycolylneuraminic acid, PBS; Phosphate Buffer Saline, RBC; Red Blood Cell, RCA; Ricinus communis Agglutinin, RCF; Rotational centrifugal force, RIP; Ribosome-inactivating proteins, UDA; Urtica dioica agglutinin, UP1a;Uroplakin 1a, UPEC; Uro-pathogenic E. coli, UTI; Urinary Tract Infection, WGA; Wheat Germ Agglutinin

Conflict of Interest

The authors declare no conflict of interest.

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