Advances in life science research

Physicochemical properties of Lectin from the medicinal plant *Calycopteris floribunda* (Roxb.) Lam that agglutinate microbial cells

Surya P H[a]|Gajala Parween[a]|Elyas K K[a*]

[a]Department of Biotechnology , University of Calicut, Kerala
[*]Corresponding author Email: kkelyas@yahoo.com,
Phone: +91-9846156890

ABSTRACT

Calycopteris floribunda (Roxb) Lamleaf extract showed high lectin activity. The lectin was precipitated at high percentage saturation of ammonium sulphate to give a titre value 3277 haemagglutination units per mg of protein. Partial purification of the precipitated fraction on DEAE anion exchange chromatography gave only 0.63 fold purification. Among the tested sugars, mannitol was found to be more inhibitory to hemagglutination activity at a concentration of 11.7 mM.Lectin activity was found to be stable upto 80 ˚C beyond which there is a complete loss of agglutination. The lectin was found to be more stable at pH range, 8 to10. Denaturing agents showed a decrease in lectin activity. The purified lectin could agglutinate both Gram negative and Gram positive bacteria but it was unable to inhibit bacterial growth as determined by disc diffusion assay. The agglutination could be inhibited by either mannitol or glucose or both confirming that the lectin is binding to these sugars or their structural analogs on the bacterial surface.

INTRODUCTION

They are proteins of non-immune origin having at least one non catalytic domain that can bind to glycan residues in a reversible manner (Hamid et al., 2013). This specificity to sugars accounts for their ability to precipitate glycoconjugates in solution and to agglutinate cells including cancer cells. The potential of lectin to agglutinate erythrocytes is widely exploited as a screening criterion to detect their presence in plant extracts. The specific sugar involved in the reaction could be identified by analyzing the ability of different sugars to inhibit lectin mediated agglutination.

Based on the overall structure and carbohydrate binding domains, lectins have been classified as merolectins, hololectins, superlectins and chimerolectins. Merolectins are monovalent in nature and hence they are incapable of precipitating glycoconjugates or agglutinating cells. Hololectins possess atleast two identical or very similar sugar binding sites. Superlectins on contrast, have different binding domains that bind structurally different sugars. Chimerolectins possess atleast one sugar binding domain fused to an unrelated domain with a biological activity (Peumans and Van Damme, 1995). The sugar binding potential of lectin is the key to their various biological functions including sugar transport or storage in plants, symbiotic association with rhizobia in nodule formation and in the defense mechanisms to combat microbial infections (Kumar et al., 2012). They are widely used to elucidate changes on cell surfaces during infections, cancer development and their toxicity towards cancer cells, microbial cells, viral particles and insect pests are also widely reported (Lis and Sharon, 1981; Selitrennikoff 2001; Keyaerts et al., 2007; Souza et al. 2013).

This research paper discusses about the physicochemical characterization of lectin activity from leaf extract of the medicinal plant *Calycopteris floribunda* (Roxb.) Lam that agglutinate microbial cells. The plant is a large climbing shrub of 5-10 meter long, with vine which are about 5-10 cm in diameter. The stem and leaves of the plant possess medicinal properties. The leaves are useful in intestinal worms, Colic, leprosy, malarial fever, dysentery, ulcer and vomiting. The fruits are used to treat jaundice and skin diseases. Aqueous extract of the plant was used in this study for the purification of lectin.

MATERIALS AND METHODS

Materials

The leaves of *Calycopteris floribunda* (Roxb.) Lam was collected from Calicut university campus. All the fine sugars used for the haemagglutination inhibition were purchased from Sisco Research Laboratories (SRL), India. DEAE cellulose was obtained from Himedia labs, India. All the other reagents and chemicals used for the study were of analytical grade.

Preparation of plant extract

Fresh leaves of the plant were collected, cleaned and soaked overnight in Phosphate Buffered Saline (PBS) in the ratio of 1:4. Homogenate was filtered using a strainer. Supernatant was collected by centrifuging the homogenate at 8000 rpm for 30 minutes which served as the crude plant extract.

Purification of lectin

The crude extract was subjected to ammonium sulphate fractionation at two different percentage saturations of ammonium sulphate; 0-50% and 50-100%. The protein fractions obtained were dialyzed against 0.1X PBS for 24 hours with minimum of 4 buffer changes. Protein concentration in each fraction was estimated following Bradford assay (Bradford, 1976) with Bovine Serum Albumin (BSA) as standard. The fraction with high lectin activity was subjected to DEAE cellulose anion exchange chromatography. Phosphate buffered saline containing 0.5M-2M Sodium chloride was used for elution. The fractions collected were dialyzed against PBS and haemagglutination potential was monitored as described below. Specific activity was expressed as the number of haemagglutination units per milligram of protein.

Haemagglutination assay

Haemagglutination assay was carried out according to Ynalvezand coworkers (Ynalvez et al., 2015). 50 µL of the lectin fraction was serially diluted in 96-well U bottom microtitre plate with PBS as the dilution buffer. 50 µL of 2% erythrocyte suspension was added to all these wells and incubated for 30 minutes at room temperature. Mat like formation of erythrocytes on titre plate wells is positive for haemagglutination whereas button like appearance is negative for agglutination. Haemagglutination activity of lectin was expressed in titre which is the inverse of the highest dilution of lectin that shows visible haemagglutination.

Haemagglutination inhibition assay

Various sugars will be tested here to monitor their ability to inhibit lectin mediated haemagglutination (Pla et al. 2003). 25 µL of each of the sugars (1M stock) was serially diluted in 96-well U bottom microtitre plate with PBS as the dilution buffer. These dilutions were incubated for 1 hour at room temperature with 25 µL of lectin dilution having 4 haemagglutination units. 50 µL of 2% erythrocyte suspension was added to all these wells and incubated for a further period of 30 minutes at room temperature. The specific sugar will bind and block lectin binding sites and there by inhibit agglutination. The well without any sugar served as negative control. The sugars used for the study were D(+) Arabitol, N-Acetyl-D-glucosamine, D(+) Fucose, D(+) Melizitose, D- Raffinose, D- Sorbitol, D-Fructose, D- Arabinose, D-Mannitol, D- Galactose, D-Mannose, D- Glucose, L- Rhamnose, D-Xylose, D- Maltose, Lactose, Sucrose and D- Trehalose.

Effect of temperature and pH on the activity of lectin

Temperature stability of the lectin was analyzed according to a modified protocol from Tomohiro and coworkers(Laija et al., 2010). 50 µL of lectin were incubated at various temperatures-20˚C, 40˚C, 60 ˚C, 80 ˚C and 100 ˚C for one hour and haemagglutination activity was determined. Effect of pH on lectin was analyzed by incubating 10µl of lectin with 90µL of different buffers with various pH for two hours at 4˚C and haemagglutination activity was monitored. Maximum activity was taken as 100 %. The buffers used for the study were acetate buffer pH- 3 – 5, citrate buffer pH- 3 – 6, phosphate buffer pH-6 – 8, tris-HCl pH – 8 – 9 and gly-NaOH pH – 9 – 10.

Effect of denaturing agents on the activity of lectin

Urea and Guanidine hydrochloride (0.0625M-0.5M) were used as denaturing agents. 50µL of lectin was incubated with an equal volume of denaturing agents at 4˚C for two hours and haemagglutination activity was analysed (Osman et al., 2016). Activity of the lectin sample (control) without denaturing agents was taken as 100 %.

Effect of EDTA and metal ions on the activity of lectin

Effect of EDTA and metal ions were studied according to the method of Osman and coworkers (Osman et al., 2016). 50µL of lectin was treated with EDTA at a final concentration of 100mM and kept overnight at 4˚C. EDTA was then removed by dialysis and an aliquot of the same was treated with various cations (final concentration of 100mM) for two hours at 4˚C and haemagglutination activity was observed. Activity of the lectin sample (control) without any treatment was taken as 100%.

Anti-bacterial property of lectin

Agglutination of bacterial by the lectin was studied by the method of Kabir and coworkers(Kabir et al., 2015). Both Gram-positive (*Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* -clinical isolate, *Staphylococcus aureus* ATCC 29213)and Gram-negative (*Klebsiella pneumoniae* ATCC 700603, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) cells were used for the experiment. Overnight cultures of bacteria were pelleted by centrifugation and re-suspended in 1X PBS to adjust absorbance to 2.0 at 630 nm. Serially diluted lectin was then incubated with an equal volume of the cell suspension and kept overnight at 37^0C. Agglutination was analysed and expressed in titre values. For determining the inhibition of bacterial agglutination by specific sugars, Serial 10 fold dilutions of the sugars were prepared in a U bottom microtitre plate and incubated with an equal volume of lectin (4 hemagglutination unit) for 1 hour at room temperature. The mixture was then treated overnight at 37^0C with an equal volume of bacterial suspension having absorbance of 2.0 at 630nm and observed for inhibition of agglutination.

Conventional disc diffusion assay was used to monitor the inhibition of bacterial growth by lectin. Chloramphenicol at 60 µg per disc was used as positive control. The lectin sample was used at a concentration of 100µg per disc. Sterile PBS (20µl per disc) served as negative control.

RESULTS AND DISCUSSION

The crude plant extract of the plant was prepared in PBS as described previously. The haemagglutination titre and specific activity of the two ammonium sulphate fractions are represented in Figure 1 and Table 1

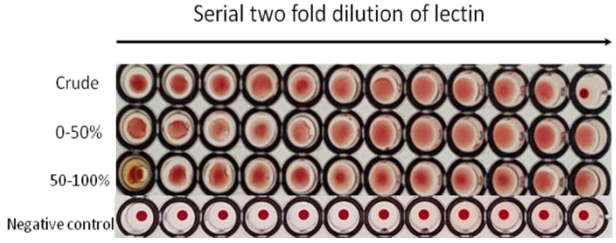

Figure 1 Haemagglutination potential of lectin fractions.

The crude plant extract and the protein fractions precipitated by 0-50% and 50-100% ammonium sulphate were serially diluted in PBS, incubated with an equal volume of 2% RBCs for 30 minutes at room temperature. U-bottomed 96 well plate was used for this purpose. The wells with red button like settlement of erythrocytes is negative for agglutination whereas spreaded mat like appearance indicates haemagglutination potential of lectin. The wells containing PBS and RBC alone served as negative control.

Table1: Haemagglutination titre and specific activity of lectin fractions from *C. floribunda*

Sample	Titre (HU)	Specific activity (HU/mg)
Crude	10240 ±8689	6921 ±5872
Fraction 0-50%	4096 ±0	2731 ±0
Fraction 50-100%	8192 ±0	3277 ±0

Data represents mean ± SD

Note: Titre is the inverse of the highest dilution positive for haemagglutination and is treated as one haemagglutination unit (HU). Specific activity is expressed as the number of haemagglutination units per mg of protein.

The protein fraction precipitated by 50-100% ammonium sulphate showed more specific activity and was then subjected to anion exchange column chromatography. The fraction resolved as three major peaks (Figure 2) and the haemagglutination potential was shown only by the first fraction. Purification profile of the fractions is outlined in Table

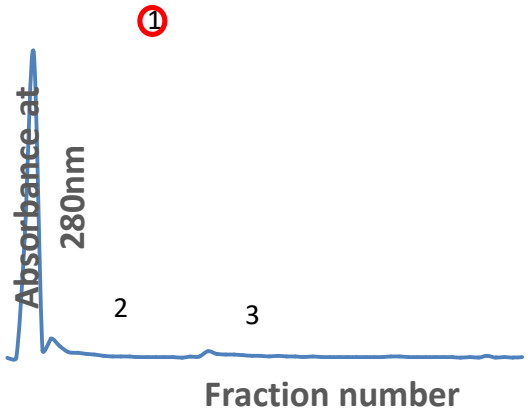

Figure 2 Elution profile of *C. Floribunda* lectin from DEAE anion exchange chromatography.

Phosphate buffered saline (pH 7.4) was used as the equilibration buffer. 0.5 M-2M Sodium chloride in PBS was used for elution. Flow rate was maintained at 2 mL/minute. Only the peak 1 fraction showed haemagglutination potential.

Table 2 Purification profile of *C. Floribunda* lectin

Sample	Concentration	Titre (HU)	Specific activity	Yield of protein	Total activity	Fold of purification	Total protein Content (mg)	Yield of Activity
Crude	1.5	10240 ±8689	6921 ±5872	100*	2560770	1*	370 ±47.57	100*
50-100% fraction	2.5	8192 ±0	3277 ±0	9.46	1146 95	0.47	35 ±0	4.48
DEAE fraction (Peak 1)	0.7	3072 ±1448	4389 ±2069	0.38	6145	0.63	1.4	0.24

*Values taken arbitrarily , Data represents mean ± SD

The sugar specificity of the lectin was evaluated by checking the ability of various sugars to inhibit lectin induced haemagglutination. Out of the various sugars tested, many sugars were found to inhibit agglutination. This suggests the multivalent nature of lectin with carbohydrate recognition domains (CRDs) of different sugar specificities. For oligomeric lectin, each subunit has a single CRD. Due to the their dimeric or tetrameric nature, most plant lectins are multivalent in nature. This multivalency results in ligand crosslinking with increased avidity (Cummings et al., 2017). The same recognition domain will be able to bind structurally similar sugars as in case of D-glucose and D-mannose which are C2 epimers. The CRD of lectin is comprised of about 120-160 amino acids that can bind more than one simple sugars due to the similarity in their structure. Wheat germ agglutinin is an example of that which can bind both N-acetyl glucosamine and N-acetyl neuraminic acid (Monsigny et al., 1980). Here, D-mannitol, D-fructose and D-glucose were found to be the best sugar inhibitors with minimum haemagglutination inhibitory concentrations of 11.72 ± 5.52, 15.62 ± 0 and 18.48 ± 18.05 respectively. The various sugars tested and their minimum haemagglutination inhibitory concentrations are given in Table 3.

Table 3 Sugar specificity of the *C. floribunda* lectin

Sl No	Sugars	Minimum Haemagglutination Inhibitory Concentration (MIC) (mM)
1	D(+) Arabitol	35.16 ±38.67
2	N-Acetyl-D-glucosamine	125.0 ± 0
3	D(+) Fucose	NI
4	D(+) Melizitose	35.16 ±38.67
5	D- Raffinose	125.0 ± 0
6	D- Sorbitol	70.31 ± 77.34
7	D- Fructose	15.62 ± 0
8	D- Arabinose	125.0 ± 0
9	D-Mannitol	11.72 ± 5.52
10	D- Galactose	35.16 ± 38.67
11	D- Mannose	31.25 ± 0
12	D- Glucose	18.48 ± 18.05
13	L- Rhamnose	NI
14	D-Xylose	70.31± 11.05
15	D- Maltose	31.25 ± 31.25
16	Lactose	NI
17	Sucrose	23.43 ± 11.05
18	D- Trehalose	31.25 ± 0

NI – No inhibition, Data represents mean ± SD

It is reported that the thermal inactivation of some lectins is associated with the unfolding of protein. However, many lectins are generally resistant to heat at 70 ˚C for more than 30 minutes (Rocca, 2004). The *C. floribunda* lectin was stable at a wide range of temperatures upto 80 ˚C and about 40% of haemagglutination potential was retained after one hour treatment at 100 ˚C (Figure 3). Some lectins have a broad pH optimum between 5-12 (Zuo et al., 2012). *Erythrina senegalensis* seed lectin was sensitive to acidic pH 3-6. However, its activity was unaffected between pH 6-10 (Kuku et al., 2012). The *C. floribunda* lectin incubated at alkaline pH of 8-10 showed highest haemagglutination potential and the

activity differed among different buffers of same pH (Figure 4). This accounts for the ionic dependency of lectin. Some of the ions tend to enhance lectin activity while others will not. In the present study, EDTA could decrease lectin activity to 6.25% confirming the requirement of divalent ions. But none of the ions tested could increase the activity further, whereas the ions Na^{+}, Ba^{2+} , K^{+}, and Mg^{2+} showed an inhibitory effect on agglutination potential (Figure 5). The ionic requirements of lectin differ from species to species. The activity of *Tamarindus indica* seed lectin was completely abolished upon treatment with EDTA and a 3 fold increase in activity was observed on treatment with divalent ions such as Mg_2+, Mn_2+, Zn_2+ and Ca_2+ (Osman et al., 2016).

High concentration of denaturing agents allow water molecules to disrupt the hydrophobic interactions in the interior of lectin (Singh and Saxena, 2013). Urea acts by allowing salvation of the hydrophobic residues (Bennion and Daggett, 2003) whereas guanidine HCl is reported to mask the positively and negatively charged aminoacid side chains in protein thus eliminating the electrostatic interactions (Monera et al., 1994). Treatment of *C. floribunda* lectin with urea and guanidine hydrochloride decreased the haemagglutination potential. Urea at 0.0625M lowered lectin activity to less than 60% whereas guanidine hydrochloride treatment diminished the activity to less than 20%. This suggests that guanidine hydrochloride is a potent inhibitor than urea on the haemagglutination potential of *C. floribunda* lectin.

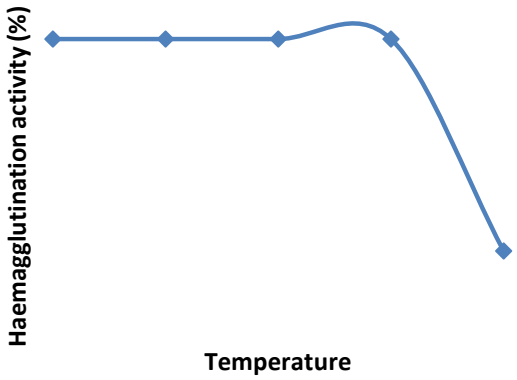

Figure 3 Temperature stability of purifiedlectin: 50 µl of lectin were incubated at various temperatures of 20˚C, 40˚C, 60 ˚C, 80 ˚C and 100 ˚C for one hour and hemagglutination activity was determined. Maximum activity was taken as 100 %. The lectin was found to be stable upto 80 ˚C.

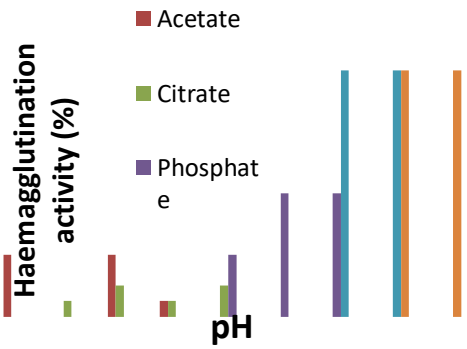

Figure 4 Effect of pH on lectin activity: 10µl Lectin was incubated with 90µl of different buffers with various pH for two hours at 4˚C and hemagglutination activity was analysed. Maximum activity obtained was taken as 100%. Highest activity of lectin was observed in the alkaline pH of 8-10. The buffers used were acetate buffer pH- 3 – 5, citrate buffer pH- 3 – 6, phosphate buffer pH- 6 – 8, tris-HCl pH – 8 – 9 and Gly-NaOH pH – 9 – 10.

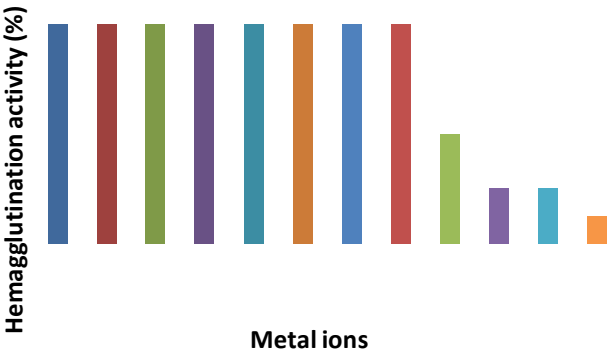

Figure 5 Effect of metal ions on lectin. 50µl of lectin was treated with EDTA at a final concentration of 100mM and kept overnight at 4˚C. EDTA was then removed by dialysis and an aliquot of the same was treated with various metal ions (final concentration of 100mM) for two hours at 4˚C and hemagglutination activity was observed. Activity of the lectin sample (control) without any treatment was taken as 100 % . EDTA could reduce lectin activity to 6.25% confirming the requirement of divalent ions for lectin activity. But none of the ions tested could increase the activity further, whereas the ions Na^{+}, Ba^{2+} , K^{+}, and Mg^{2+} showed an inhibitory effect.

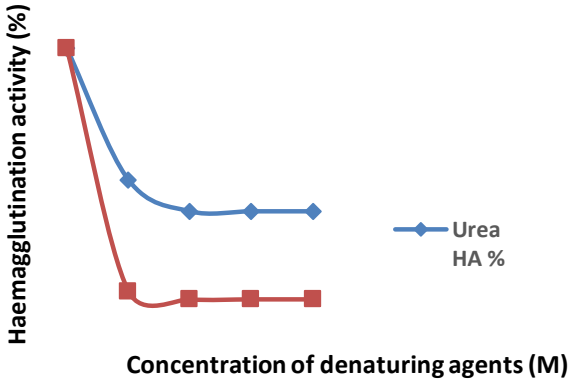

Figure 6 Effect of denaturing agent on lectin: Urea and Guanidine hydrochloride was used as denaturing agents. 50µl of lectin was incubated with an equal volume of denaturing agents at 4˚C for two hours and haemagglutination activity was analysed. Haemagglutination potential was decreased on treatment with both the denaturing agents. Activity of the lectin sample (control) without denaturing agents was taken as 100 %.

The denaturing agents used were urea and guanidine hydrochloride of 0.0625M – 0.5 M.

All the tested bacterial cells that included both Gram positive and Gram negative species were agglutinated by *C. floribunda* lectin. The tested organisms were *Enterococcus faecalis, Listeria monocytogenes, Staphylococcus aureus*(Gram-positive) *Klebsiella pneumoniae, Escherichia coli and Pseudomonas aeruginosa* (Gram-negative). A titre of 16 was obtained for *Escherichia coli* andfor all other bacteria a titre of 32 was obtained. Most of the tested bacteria could be well inhibited by either glucose or mannitol or both. The agglutination pattern and titre values are represented in Figure 7 and Table 4. The *Apuleialeiocarpa* seed lectin (ApulSL) was bacteriostatic against the Gram-positive bacteria *S. aureus*, *Streptococcus pyogenes*, *Enterococcus faecalis* and on the Gram-negative bacteria *Klebsiella pneumoniae*, *E. coli*, and *P. aeruginosa*. (Carvalho et al., 2015).Lectins can interact with the N-acetylglucosamine, teichoic acid, teichuronic acid, N-acetylmuramic acid and tetrapeptides linked to N-acetylmuramic acid present in the cell wall of Gram positive bacteria thus exerting their Anti-bacterial effects. In case of Gram negative bacteria, binding is to the cell wall lipopolysaccharides (Ayouba et al., 1994). These interactions results in crosslinking of bacterial cells and thereby agglutination (Velayutham et al., 2017).

However, none of the bacteria mentioned above could be inhibited by lectin sample at a concentration of 100µg per disc. Chloramphenicol at 60 µg per disc was used as positive control whereas PBS at 20µl per disc served as negative control.

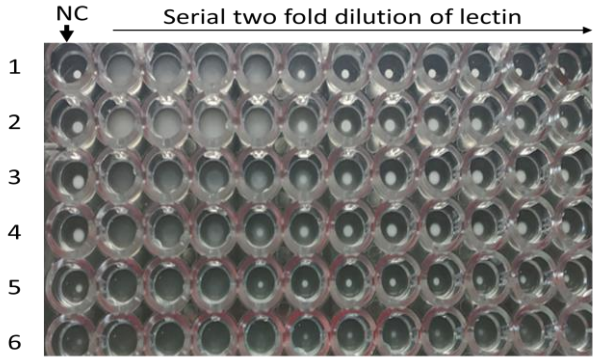

Figure 7 Agglutination of bacteria by the purified lectin: Overnight cultures of bacteria and Yeast were pelleted by centrifugation and re-suspended in 1x PBS to adjust absorbance to 2.0 at 630 nm. Serially diluted lectin was then incubated with an equal volume of the cell suspension and kept overnight at 370C. Agglutination was analysed and expressed in titre values. The purified lectin could agglutinate all the tested bacteria.

Table 4 Titre and minimum agglutination inhibitory concentration of sugar associated with bacterial agglutination induced by *C. Floribunda* lectin

S.No.	Test culture	Average titre	Minimum agglutination Inhibitory Concentration (MIC) of sugar (mM)	
			Mannitol	Glucose
1.	*Enterococcus faecalis* (ATCC 29212)	32 ±0	2.5	2.5
2.	*Listeria monocytogenes* (clinical isolate)	32 ±0	25	NI
3.	*Klebsiella pneumoniae* (ATCC 700603)	32 ±0	2.5	2.5
4.	*Staphylococcus aureus* (ATCC 29213)	32 ±0	2.5	0.25
5.	*Escherichia coli* (ATCC 25922)	16 ±0	0.25	2.5
6.	*Pseudomonas aeruginosa* (ATCC 27853)	32 ±0	NI	25

Data represents mean ±SD

CONCLUSION

The study could effectively precipitate a lectin from the leaves of *Calycopteris floribunda* that agglutinated human erythrocytes as well as bacterial cells. The specific sugar involved in these interactions could also be revealed which in the future could be used to tag cell surfaces during disease conditions including cancer. Future studies have to be done to develop the lectin as cytotoxic agents during microbial infections and cancer development. How-ever this would be the first report of a bacterial agglutinating lectin from this plant.

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Authors contribution

Surya P H- Conceptualization, design of the work, data collection, data analysis and writing the original draft
GajalaParween- Data collection, data analysis and revision
Elyas K K- Supervision, Data analysis and Proof reading

Conflicts of interests

None

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