The Journal of Phytopharmacology 2014; 3(1): 44-51
Online at: www.phytopharmajournal.com

Research Article

ISSN 2230-480X
JPHYTO 2014; 3(1): 44-51
January- February

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Antimicrobial effects of a crude plant lectin isolated from the stem of *Tinospora tomentosa*

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Abstract

Crude plant lectins were isolated from the stem of *Tinospora tomentosa* and found its antibacterial and antifungal effects. Lectins were isolated by ammonium sulphate precipitation method. Presence of carbohydrate and proteins were investigated by thin layer chromatography and infrared spectroscopy techniques. Lectin was characterized by its binding affinity with carbohydrates and human erythrocytes by hemagglutination inhibition assay and SDS-page gel electrophoresis. The amount of proteins was quantitatively measured by Lowry method. Antibacterial and antifungal activities were investigated by disk diffusion assay. Minimum inhibitory concentrations of bacteria and fungus were determined from their dose-response curve. *Salmonella* induced hemagglutination activity was performed to investigate its binding affinity with bacterial cell surface. Isolated lectin contained carbohydrates and protein residues in its structure. Its molecular weight was about 32 kD and seemed as a monomeric. It showed binding affinities to lactose sugar and bacterial cell surfaces and inhibited hemagglutination. It showed a dose-response relationship in its antibacterial and antifungal activities. The stem of *Tinospora tomentosa* may be considered as an important medicinal plant for antimicrobial therapeutics.

Keywords: Lectin, Antibacterial, Antifungal, Hemagglutination, Protein, Carbohydrate.

Introduction

Stem of *Tinospora tomentosa* Miers. (Family: Menispermaceae) are used in Indian system of medicine. The plant is a large deciduous climbing shrub widely found in Bangladesh and almost throughout India. As per traditional use, the different parts like stems, leaves and roots of the plant are used as stomachic, bitter-tonic, anti-periodic, mild diuretic, emetic, anti-purgative, antipyretic, analgesic, anti-inflammatory, anti-diabetic, anti-leprotic, anti-gout).[1-3] The extracts of this plant were found to be non-toxic orally in doses up to 3.5 g/kg in both mice and rats).[4] Some activities of some plants of this genus have been reported. *T. cardifolia* showed anti-inflammatory, hypoglycemic, and antidiabetic activity).[5] Lectins are carbohydrate-binding proteins, which are highly variable in their amino acid sequences, widely distributed in microorganisms, viruses, animals and higher plants, and with different functions, structures, tissue localizations and carbohydrate-binding specificities).[6] Lectins are a class of ubiquitous glycoproteins that are abundantly found in seeds and fruit pulp, agglutinate erythrocytes, and interact with sugar moieties of glycoconjugates).[7, 8] Lectins mainly affected leukocyte vesicle infiltration, by competitive blockage of

glucosylated (mannose-glucose) selectin binding sites by showing anti-inflammatory[9] and antibacterial properties[10]. Hence, in this present study we made an attempt to isolate lectins with potential medicinal application. Thus in this present study we report, the isolation and partial characterization of a lectin from the stem of *Tinospora tomentosa* Miers Lectin (TTML). We have also investigated the inhibitory activity of TTML against bacterial hemagglutination, and bacterial growth.

Materials and Methods

Isolation of Lectin

Tinospora tomentosa Miers plants were collected from the district of Barisal, Bangladesh. The stem was dried, chopped, and grinded. Lectins were isolated as previously described method with some modification.[11] Stems of *Tinospora tomentosa* Miers was ground to a powder in an electric mill and filtered through 80 mesh grit. The powder (5 g) was mixed with 0.15M NaCl (1:8, w/v) for 48 h at 4°C, and filtered through 80 mesh grid. Subsequently, the filtrate was centrifuged at 10000×g for 30 minutes, and the supernatant was fractionally precipitated with ammonium sulfate at 40%, 50%, 60%, and 70% saturation, respectively. The four pellets were combined, dissolved in a minimal volume of water, and separated using ultra centrifugation at at 10000×g for 30 minutes at 4°C.

Determination of protein and carbohydrate concentration

The presence of carbohydrate and protein was detected using TLC method as previously described method with little modifications.[12, 13] For detection of proteins, TLC tank was saturated with mobile phase having the composition of 1-butanol, glacial acetic acid and water in the ratio of 4:1:1 respectively. To the saturated tank the previously spotted plate was placed and the mobile phase was allowed to run through the spots until the solvent line was reached. The plate was then taken out of the tank, dried and then visualized under UV light in dark room. After marking the florescent compounds, the plate was sprayed with 2% ninhydrine in ethanol solution, dried and then heated using heat gun to make the protein or amino acid component spots visible. For detection of carbohydrate, the TLC tank was saturated with mobile phase having the composition of 1-butanol, acetone & phosphate buffer in the ratio of 4:5:1 respectively. To the saturated tank the previously spotted plate was placed and the mobile phase was allowed to run through the spots until the solvent line was reached. The plate was then taken out of the tank, dried and then visualized under UV light in dark room. After marking the florescent compounds, the plate was sprayed with mixture of anisaldehyde with 0.5% sulphuric acid, dried and then heated using heat gun to make the carbohydrate component spots visible.

Lowry's method[14] was used for protein quantification, using bovine serum albumin (BSA) as the standard. The relative protein concentration of the eluted fractions was determined by measuring the absorbance at 280 nm. Phenol suplhuric acid method was used for measurement of total carbohydrate in TTML as previously described method.[15] Fourier transforms infrared (FT-IR) spectrometer (Shimadzu, Japan) with a spectrum range of 4000–400 cm^{-1}, and scanning accumulative limitation of 32 times, was used for the analysis as previously described.[16] About 1.5 mg of dried TTML was taken and grinded with 300 mg KBr under IR light till evenly mixed. Then the mixture was crushed in a mechanical mould to form a tablet with a diameter of 3 mm and a thickness of 0.6 mm. Finally, the spectrum of an extract could be gained by scanning the sample tablet immediately.

Hemagglutination inhibition assay

Hemagglutination activity was measured as previously described[11] with little modification. Briefly, serial two-fold dilutions of the lectin solution in microtiter v-plates (25 µL) was mixed with 25 µL 0.5% human red blood cell suspension in saline (pH 7.2). Readings were recorded after about 30 minutes at room temperature, when the blank had fully sedimented. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was treated as one hemagglutination unit. Specific activity was expressed as the number of hemagglutination units per mg protein.

SDS-PAGE gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in accordance with the method of Laemmli[17], using a 15% separating and a 5% stacking gel.

Bacterial cultures

Bacteria were cultured in 10 g/l Bacto tryptone, 10 g/l NaCl, pH 7.0 in petri dishes or glass slant bottles. Bacterial

cells which were used as test microorganism were the following, the gram negative: *Escherichia coli*, the gram-positive: *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The bacterial cultures were prepared by transferring 2 to 3 colonies into bacteria growth medium and incubated at 37°C for 14 hrs before use.

TLC bioautography

TLC bioautography assay was performed by agar overlay bioautography technique. TTML was applied 1 cm from the base of the silica plate. After drying, the plates were developed using solvent chloroform: methanol (8.2: 1.8) and chloroform: hexane (5.4: 6.6). Developed TLC plates were carefully dried for complete removal of solvents. Aliquot of 50 ml of nutrient agar was prepared by adding *E. coli* bacterial inoculum. Now, the dried TLC plate was overlaid on inoculum containing agar under aseptic condition in laminar airflow. The plates were incubated at 37°C and examined for the zone of inhibition

Antibacterial assay

TTML was dissolved in water to a final concentration of 100 mg/ml. Disk diffusion assay method was performed as previously described method.[18] The bacterial or fungal strains were cultured in a nutriment broth for 24 hours. Then, previously prepared 1ml of suspension bacteria or fungus containing 3X1010 colony forming unit (CFU/ml) was spread on nutrient broth agar. Disks were made by using a sterile filter paper and were loaded with 20 µl of each sample extract. Methanol was used as negative control and azithromycin (30 µg/disk) as positive reference standard. All the plates were incubated at 37°C for 24 hours. Antibacterial activity was evaluated by measuring the zone of inhibition in millimetres. All experiments were done in triplicates. No inhibition zone indicated a disk diffusion zone of 6 mm, i.e., growth up to the edge of a 6-mm disk.

Minimum inhibitory concentration determination

In order to determine the minimum inhibitory concentration (MIC) 660 CFU/ml of cultured solutions were used. To investigate Salmonella induced antibacterial activities *S. typhi* and for Aspergillus induced antifungal activities *A. niger* cultured solutions were used. Various concentrations of TTML (0-550 mg/ml) were incubated with either bacterial or fungal solutions for 0.5 h at room temperature. Then the solutions were mixed with nutrient agar medium and cool them until solidify. Then the plates were incubated at 37°C for 24 h. Then bacterial colonies were counted and graph was prepared.

Bacteria induced hemagglutinin inhibition assay

Hemagglutination studies of the isolated lectin were carried out using human erythrocytes in a 96-well microtiter plate. The purified protein solution [50 µl (1 mg/ml)] was placed in the first well and serially diluted into the successive wells with phosphate buffered saline, pH 7.4. Then, 100 µL of 0.5% human erythrocyte suspension was added to all the wells. Hemagglutination was visualized in the plate after 30 min. of incubation at 37ºC. Hemagglutination inhibition assays with the purified lectin were performed as follows: 50 µL of different sugar solutions (0.1M) was placed in the plate and serially diluted. Then, 50 µL of the purified lectin (1 mg/mL) was added to each well and incubated for 30 min at 37ºC. Later, 100 µL of 0.5% erythrocyte suspension was added and the plate was incubated for 30 min at 37ºC. Hemagglutination inhibition titer was scored visually according to Benevides et al.[19] Hemagglutinating activity was expressed as a titer (per mg of lectin), namely, the reciprocal of the highest dilution that showed positive results.[20]

Results

Qualitative and quantitative determination of carbohydrate and protein

We separated the carbohydrate like molecules and protein like molecules present in TTML by TLC as described in the method section. Then, we examine the plates under ultra-violet light and normal light. For detection of carbohydrate the dried plates were sprayed with anisaldehyde with 0.5% sulphuric acid and heated. As shown in Figure 1A the similar retention factors (Rf value) was found in the separated fractions of TTML and other sugars used in the experiments as a control. Such a result indicates the presence of carbohydrate like residue in TTML. Charring the dried plates with 0.2% ninhydrin solution formed a brown spot on the TLC plate (Figure 1B) indicates the presence of protein in TTML. It was found in this study that the spectral region between 1500 and 800 cm^{-1} would cover most of characteristic absorption bands relevant to major sugars. Absorption peaks of glucose include 902, 1036, 1360, and 1431 cm^{-1} with a key peak at 1032 cm^{-1} has been shown in Figure 1C. Absorption bands of fructose includes 923, 978, 1067,

1250, 1346, and 1418 cm^{-1} with a key peak at 1053 cm^{-1} which corresponds to C-O bending and C-OH stretching.[21] Therefore TTML showed the presence of carbohydrate residues. On the other hand, the absorption associated with the Amide I band leads to stretching vibrations of the C=O bond of the amide, absorption associated with the Amide II band leads primarily to bending vibrations of the N—H bond.[22] Peak at 2349 cm^{-1} was found due to the asymmetric stretching of O=C=O and peak at 667 cm^{-1} was found due to the deformation of O=C=O in the amide structure. Peak at 1510 cm^{-1} and 1580 cm^{-1} was found due to the presence of N-H bending of the peptide bond.

500 µg and 200 µg of TTML contained 1.6 µg and 1.3 µg of protein calculated from the standard curve prepared as described in the method section. Therefore, TTML contained about 0.5% of protein. We also found that TTML contained about 0.09% of carbohydrate residues.

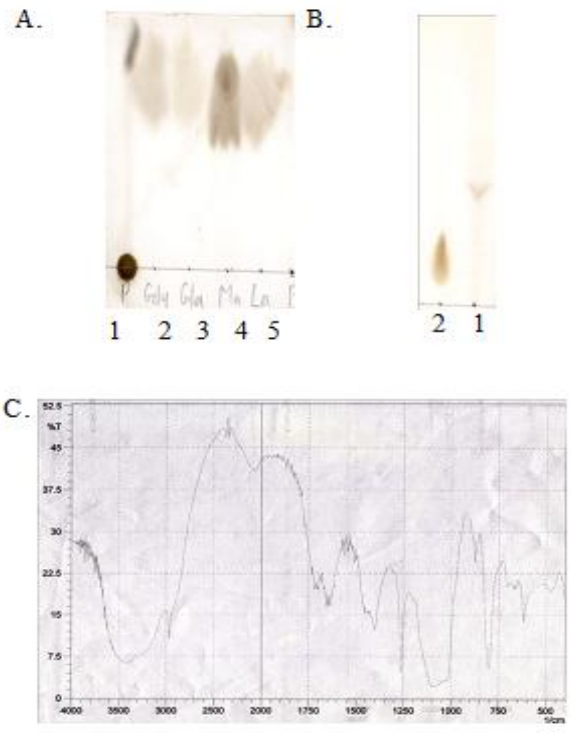

Figure 1: Detection of carbohydrate (1A) and protein (1B) in TTML after charring the TLC plates. FT-IR spectrum of TTML (1C)

Hemagglutination inhibition assay

In this study we made an attempt to isolate and characterise lectin from *Tinospora tomentosa* Miers. Preliminary experiments indicated using crude extract from the stem of *Tinospora tomentosa* Miers has hemagglutinating activity on blood-group-A. *Tinospora tomentosa* Miers stem showed hemagglutinating activity on blood-group-AB, B, A, and O at 2.65, 1.32, 0.66, and 1.32 mg/well respectively (Figure 2). Therefore, TTML showed the activity unlike the bryony lectin, which is not a blood-group-specific and Eranthis hyemalis lectin with group-O erythrocytes specificity. Crude extract from *Tinospora tomentosa* Miers preferentially agglutinates group-A erythrocytes, thus exhibiting presence of lectins.[23, 24]

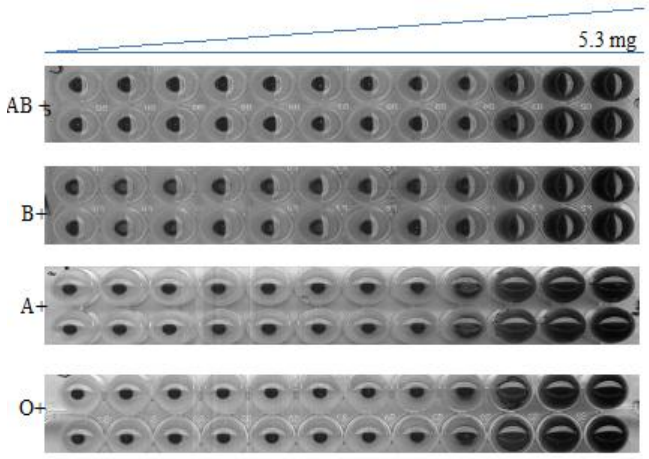

Figure 2: Hemagglutination inhibition activities of TTML

SDS PAGE protein analysis

Reducing SDS PAGE of TTML showed a single band with a molecular weight of approximately 32 kDa was seen (Figure 3). Therefore, TTML resembles some of plant lectin with respect to sugar binding specificity and some structural aspects, namely that from bryony (Bryonia ioica) root stocks and Eranthis hyemalis lectin (which is also an Nacetylgalactosamine- specific lectin composed of two non-identical subunits of MW 30 and 32 kDa held together by disulphide bridges.[24, 25]

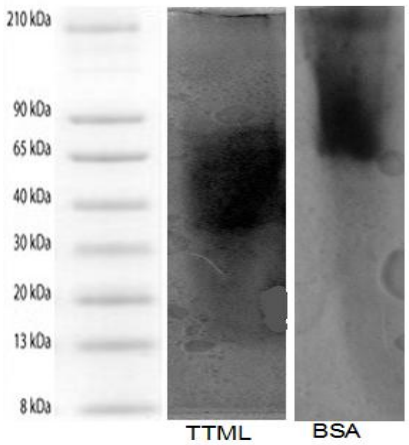

Figure 3: SDS-PAGE analysis of TTML

Determination of carbohydrate binding specificity

The carbohydrate binding specificity of TTML was determined by hapten inhibition tests with a series of simple sugars such as galactose, lactose, and maltose. As shown in Figure 1 alike agglutinins from seeds of *Lotus tetragonobulus* (Asparagus pea)[24] and *Ulex europeus* (gorse)[25] which exhibits specificity to more than one sugar, TTML only shows the specificity to lactose (data not shown).

Salmonella induced heamagglutination inhibition assay

Some plant lectins have been studied for their interactions with bacteria.[26] Some studies with *Araucaria angustifolia* lectin showed its antibacterial activity against C. michiganensis subsp.[27] Binding of lectins to muramic acid and N-acetylmuramicacid, carbohydrates present in the bacterial cell wall, has been reported earlier.[28, 29] Almost all microorganisms express surface-exposed carbohydrates which may be covalently bound, as in glycosylated teichoic acids to peptidoglycan, or non-covalently bound, as in capsular polysaccharides.[30] Every surface-exposed carbohydrate is a potential lectin-reactive site. Ability of lectins to form complexes with microbial glycoconjugates can be exploited as potential drug targets. Hence, we investigated the inhibitory activity of TTML against bacterial hemagglutination, colony formation and bacterial growth of *Salmonella typhi*. Agglutination of blood - group-A specific erythrocyte by Shigella dysenteriae implicates its virulence.[31] Hence, we analysed the effect of TTML on hemagglutination activity of *Salmonells typhi* showed hemagglutinating activity on blood-group-A and O erythrocytes exhibiting its virulence nature. But a decrease in hemagglutinating activity was observed in lectin pre-incubated *Salmonella* at different concentrations as shown in Figure 4. Hemagglutinating activity of *S. typhi* was found to be 1.25 mg. TTML inhibited this hemagglutinating activity of *S. typhi* at same concentrations in blood group A and O. These results clearly exemplifies that TTML inhibited Salmonella induced hemagglutination, due to masking of adhesion sites. Thus, lectin protects against Salmonella induced erythrocyte damage.

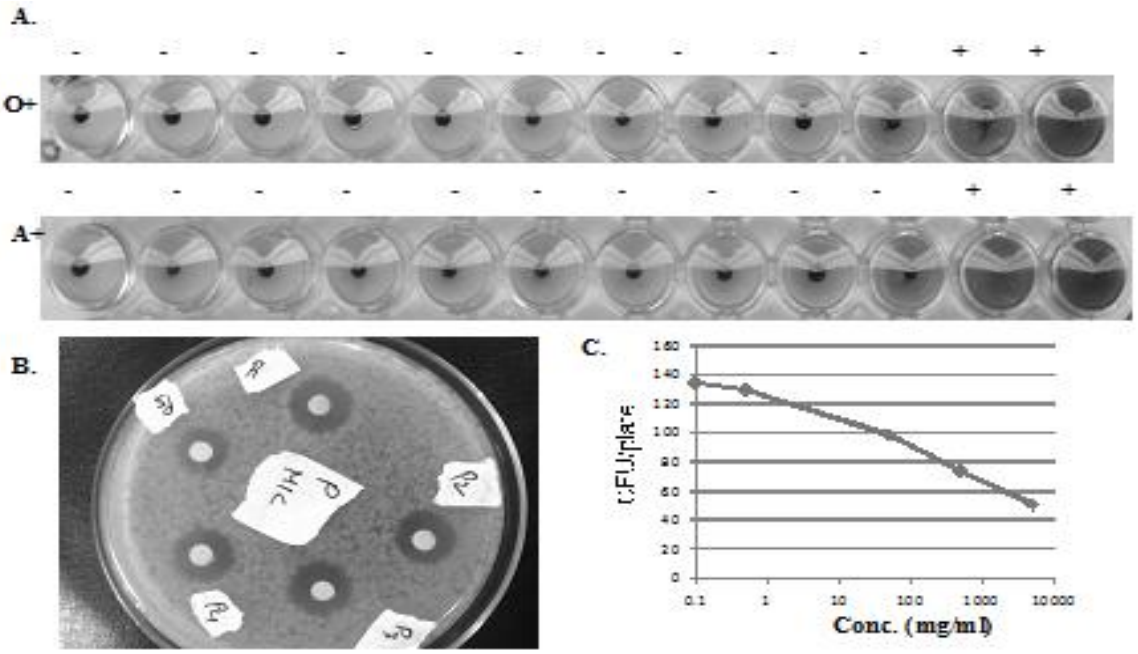

Figure 4: Effect of TTML on *Salmonella typhi*. Salmonella induced hemagglutination inhibition activities of TTML (4A). Bacterial inhibition activities of TTML in disk diffusion assay (4B). Inhibition of bacterial colony number in a dose response curve (4C)

Antibacterial activity

We investigated the sensitivity test using disk diffusion assay of TTML on various strains of bacteria including *Vibrio mimicus*, *Staphylococcus aureus*, *Bacillus sereus*, *Salmonella typhi*, *Shigella dysentery*. 4 mg/ disk of TTML showed inhibition of bacterial growth on each bacterial strain in agar as shown in Table 1.

Table 1: Antibacterial activity of different TTML against different strains

Name of microorganisms	Negative control	TTML 4000 µg/disc	Azithromycin 30 µg/disc
	Zone of inhibition in mm		
V. mimicus	—	17	22
S. typhi	—	9	21
S. dysentry	—	20	23
S. aureus	—	8	25
B. sereus	6	8	23

The minimum inhibitory concentrations of *V. mimicus* were also measured using disk diffusion assay as shown in Figure 5B. The minimum inhibitory concentration was detected as 0.2 µg. In TLC autoradiography the separated fraction of TTML on TLC plate also inhibited bacterial growth of *E. coli* strains on McCony agar. Therefore a clear zone of inhibition was formed on agar plate (Figure 5A).

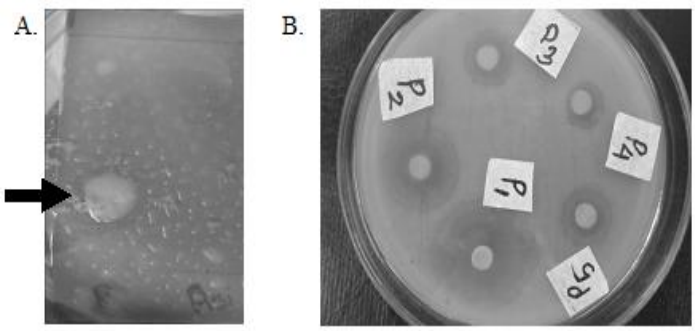

Figure 5: Antibacterial activities of TTML. TLC autography of *E. coli* (3A) and disk diffusion activity of *V. mimicus* (3C)

Antifungal activity

TTML also inhibited fungal growth of the strain of *Aspergillus niger*. The dose-response curve shows the clear reduction of fungal growth according to the concentration of dose (Figure 6). TTML also showed a clear inhibition of the growth of another fungal strains *S. cerevasecis*. Therefore, TTML showed broad spectrum antifungal activities.

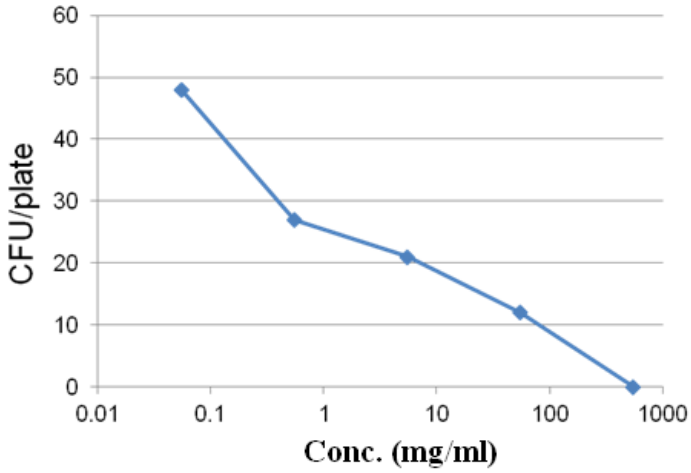

Figure 6: Antifungal activities of TTML on *A. niger*

Discussion

Qualitative and quantitative determination of carbohydrate and protein

TLC analysis of TTML showed the presence of carbohydrate and protein molecules in the isolated crude lectin. IR analysis also showed the presence of sugar residues and amino acid molecules in TTML. Further, the quantitative analysis of protein indicated the amount of protein present in TTML. These results confirmed that TTML is a sample of lectin and it carries the characteristics of a plant lectin.

Hemagglutination inhibition assay

Tinospora tomentosa Miers stem showed hemagglutinating activity on blood-group-AB, B, A, and O at different concentarions. However, it showed more potent activities against A group erythrocytes indicates the specificity of TTML.

SDS PAGE protein analysis

TTML showed a monomeric protein and its MW was about 32 kDa. The result also indicates the possible binding site of TTML.

Salmonella induced heamagglutination inhibition assay

TTML inhibited *Salmonella* induced RBC damage indicated that it may have a specific binding site with bacterial cell surface receptors and it may show antibacterial activities.

Antibacterial and antifungal activities

TTML showed weak antibacterial and antifungal activities. These activities probably due to the binding specificity of TTML on microbial cell surface receptors. However, further studies are required to establish the binding site of TTML on the cell surface receptor sites.

Conclusion

In conclusion, *Tinospora tomentosa* Miers plant may be considered as a medicinal plant having antimicrobial potentiality. Its stem contain a specific types of lectins like molecules those may have many pharmacological potentialities.

Acknowldgement

The work has been supported by the grant from the pharmacy department of East West University, Dhaka, Bangladesh. Grant was used for Spring and Fall semester, 2011 research (EWU: PHRM: Spring & Fall: 2011).

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