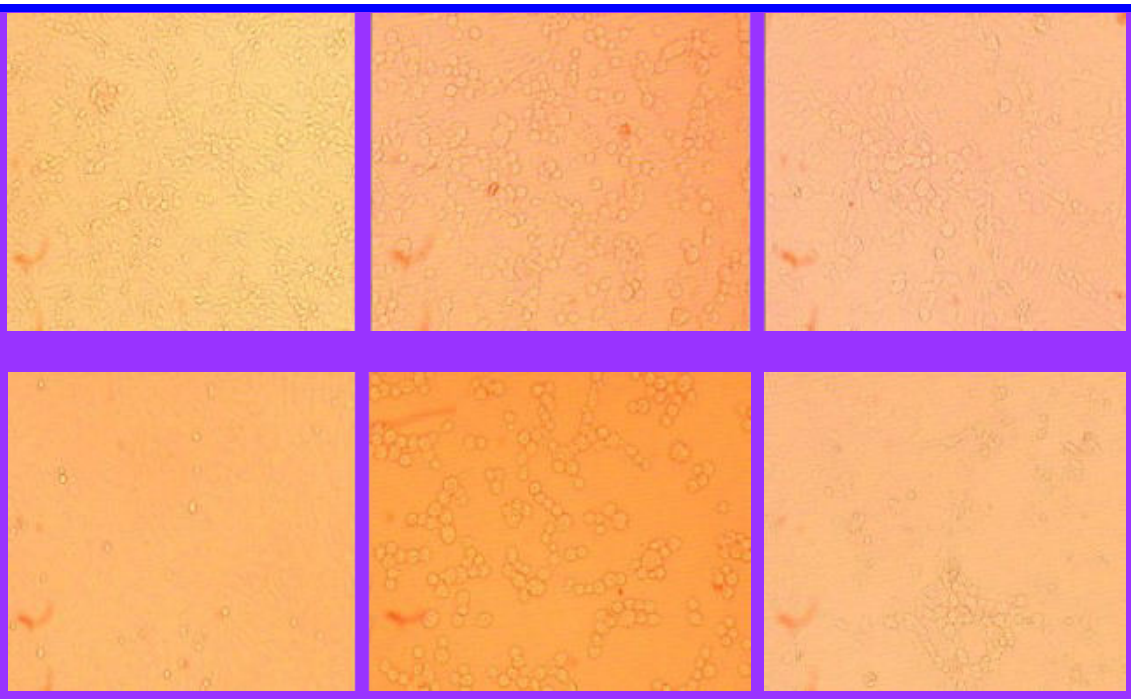

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In vitro antiviral activity of BanLec against herpes simplex viruses type 1 and 2

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Abstract

The present study evaluates the antiviral activity of banana lectin (BanLec) against herpes simplex virus type 1 and 2 (HSV-1 and HSV-2). Lectin was isolated from the ripen pulp of bananas (*Musa paradisiaca*). The study showed that lectin exhibited hemagglutination activity towards human erythrocytes A, B, AB and O group. The molecular weight of BanLec using SDS gel-electrophoresis was found to be 14,000-30,000 Da. Cytotoxicity of BanLec on the Vero cell lines showed an inhibitory concentration of 172.7 µg/mL. BanLec was virucidal and showed no cytotoxicity at the concentration tested. The lectin showed a dose-dependent antiviral activities, inhibiting HSV-1 by 16.0 µg/mL with selectivity index 10.8 and HSV-2 inhibition by 67.7 µg/mL with selectivity index 2.6. These results corroborate that BanLec could be a rich source of potential antiviral compound for HSV-1 when compared to HSV-2.

Introduction

Lectins are a unique and heterologous class of proteins with the ability to recognize and reversibly bind a variety of sugar structures present on the cell surface (Santos et al., 2014). They are found in a wide range of organisms, from viruses and bacteria to animals, plants, and humans (Mitchell et al., 2017). They have important biological functions in the organisms, including cell-cell interaction, protection from pathogens, cell adhesion, and intracellular translocation of glycoproteins, and they also act as storage proteins (Yamashita et al., 1999; Jiang et al., 2006; Wang et al., 2007). At present, they are being widely used in studies of biochemistry, cell biology, immunology, glycobiology and have wide-spread applications in biomedical researches (Sharon and Lis, 1989). Due to its fine specificity, most plant lectins have been employed for various applications including cancer therapy and virus research.

Banana lectin (BanLec) was first isolated from *Musa paradisiaca* (Koshte et al., 1990). It is a homodimeric protein that binds mannose and mannose-containing oligosaccharides and functions as a potent T-cell mitogen (Meagher et al., 2005; Koshte et al., 1992).

Herpes simplex virus (HSV) is a DNA-containing enveloped virus, which brings commonly viral infections in humans causing a variety of diseases. HSV-1 and HSV-2 can be distinguished based on clinical manifestations, biochemical and serological characteristics. However, in patients with an immature or weak immune system, such infections can be serious and even life-threatening (Naesens and De Clercq 2001; Whitley and Roizman 2001). The current investigation was undertaken to test the BanLec for their antiviral activity against HSV-1 and HSV-2. The lectin was found to possess various *in vitro* activity towards HSV strains and potent anti-viral response against HSV-1 at low concentration far below the cytotoxicity threshold.

Materials and Methods

Collection of plant material

Ripe banana fruits were purchased from the local market. It was kept for 2 days at room temperature allowing it to be slightly over ripen. The ripened banana pulp was washed thoroughly using tap water and wiped using a clean cloth. The succulent parts of the banana pulp were cut into pieces.

Isolation and purification of lectin

The pulps of the bananas were blended in distilled water using a warning blender. To the supernatant obtained after centrifugation (15000 x g, 30 min) of the homogenate, ammonium sulfate was added to achieve 10% saturation. The mixture was centrifuged again (15000 x g, 30 min) and ammonium sulfate was added to attain 80% saturation. The supernatant obtained after centrifugation was then dialyzed against distilled water at 4°C to get rid of ammonium sulfate. Tris–HCl buffer (pH 7.8) was added to the dialyzed supernatant until the concentration of Tris attained 10 mM. The supernatant was then loaded on a 3 × 10 cm column of DEAE-cellulose (Sigma) in 10 mM Tris–HCl buffer (pH 7.8). After removal of the unadsorbed fraction (D1), adsorbed fractions (D2 and D3) were eluted with starting buffer containing 0.2 and 1M NaCl, respectively. After examination of the hemagglutinating effect of fractions, the active fraction causing hemagglutination was collected and tested for antiviral activity (Cheung et al., 2009).

Lectin concentration

The lectin content of the samples obtained during the purification process was determined by the method of (Lowry et al., 1951) using bovine serum albumin as the standard. Readings at 280 nm were also used to determine the protein content of the column eluates.

Molecular weight determination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out following the procedure of Laemmli, 1970 using a 10% resolving gel and a 5% stacking gel. At the end of electrophoresis, the gel was stained with Coomassie brilliant blue. After destaining, the electrophoretic mobilities of the marker proteins and the purified lectin were determined.

Virus and virus titration

For the anti-HSV-1 and HSV-2 activity screening, African green monkey kidney cells (Vero) were grown in minimum essential medium supplemented with 10% Fetal bovine serum, penicillin (100 U/mL), streptomycin (100 µg/mL) and amphotericin B (25 µg/mL). Cell cultures were maintained at 37°C in a humidified 5% CO_2. HSV-1 and HSV-2 strain were propagated in Vero cells. The virus was divided into aliquots and stored at -80°C until use. Virus titers were calculated as 50%tissue culture infectious dose ($TCID_{50}$) by cytopathic effect assay. HSV-1 and HSV-2 titers were obtained by the limit-dilution method and expressed atmosphere as 50% tissue culture infectious dose per mL ($TCID_{50}$/mL) (Reed and Muench, 1938).

Determination of mitochondrial synthesis by MTT assay

Cytotoxicity of BanLec was assessed by MTT **(**(3-[4,5-16 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay using Vero cells in 96-well plates according to the methodology proposed (Francis and Rita, 1986). The percentage of cell viability was calculated and the

Box 1: Hemagglutination Test

Principle

A method for titering viruses based on their ability to attach to molecules present on the surface of red blood cells. The hemagglutination titer is a simple number of the highest dilution factor that produced a positive reading.

Requirements

BanLec; Centrifuge machine; Biological safety cabinet; Conical tubes (15 mL); Disposable pipettes (1, 5, and 10 mL); Human erythrocyte; Inverted microscope; 96-well round-bottom microtiter plate; Micropipette; Phosphate buffer solution; Sterile disposable aerosol resistant tips (160 µL)

Procedure

Step 1: Phosphate buffer solution (50 µL) was added to each well

Step 2: Banana lectin (50 µL) was added in the first column

Step 3: Mix each well and transfer 50 µL to the next well on its right. Repeat mixing and transferring 50 µL down the length of the plate. Discard 50 µL from the last well into a bleach solution

Step 4: Add 50 µL of 0.5% red blood cell working solution to each well. Mix gently

Step 5: Leave at room temperature for 30-60 min to develop. Negative results appeared as dots in the center of round-bottomed plates. Positive results formed an uniform reddish color across the well

Step 6: The hemagglutination titer was a simple number of the highest dilution factor that produced a positive reading

Note

A round-bottomed 96-well dish is preferred. Flat-bottomed plates will also work, but need to be placed at an incline to develop

References (video)

Babu et al., 2016

concentration of drug or test samples needed to inhibit cell growth by 50% values were generated from the dose-response curves for each line.

In vitro antiviral activity by MTT assay

A rapid and sensitive procedure to evaluate antiviral compounds *in vitro* is based on spectrophotometrical assessment for the viability of virus-infected and mock-infected cells via *in situ* reductions of a tetrazolium dye by MTT as per the method described (Kurokawa et al., 2016).

Data analysis

The 50% inhibitory concentration (IC_{50}) and 50% effective (EC_{50}) concentrations were calculated from concentration-effect curves after linear regression analysis. The results represent the mean ± standard error of the mean values of three different experiments.

Results

Purification of BanLec from M. Paradisiaca

Four peaks of protein were detected using this step of purification, as shown (Figure 1A). Hemagglutination activity (HA) was found only in the third peak. Maximum activity was detected at fraction number 8 with total lectin content of 78 mg/mL. The fraction that gave the highest agglutination activity, peak (3) lectin was taken for the entire study.

Determination of molecular weight by SDS-PAGE

Multiple bands could be seen in the BanLec when comparing with the marker. Examining the SDS-PAGE result, the lectin sample showed a concentrated band at the low molecular weight. However, the lectin sample had a band at around 14-30 kDa (Figure 1B).

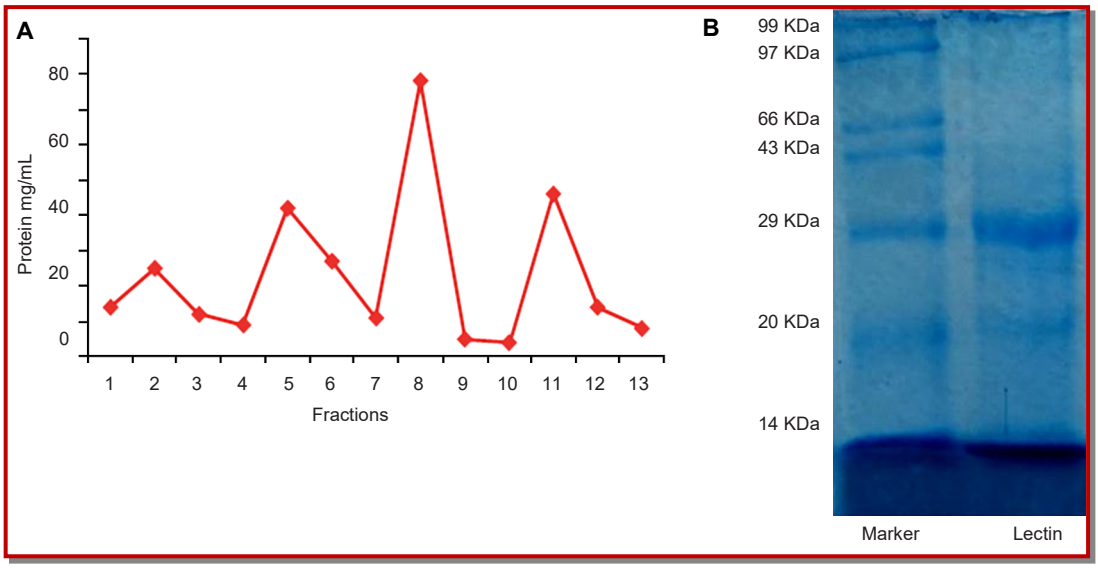

Figure 1: Elution profile of BanLec purification, with 80% ammonium sulfate precipitate on DEAE cellulose column (A); SDS-PAGE representing the molecular weight of BanLec (B). Left lane contained marker proteins and the right lane contained 10 µg of BanLec

Table I

Calculation of hemagglutination activity of DEAE cellulose purified BanLec

Dilution	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
Lectin concentration (µg/mL)	100	50	25	12.5	6.25	3.125	1.56	0.78	0.39	0.19	0.09
A	+	+	+	+	+	+	+	+	+	-	-
B	+	+	+	+	+	+	+	+	+	-	-
AB	+	+	+	+	+	+	+	+	-	-	-
O	+	+	+	+	+	+	+	-	-	-	-

"+"agglutination; "-" no agglutination

Table II

Summary of the purification steps of the BanLec

Purification stages	Protein (mg/mL)	Protein yield (mg)	Blood Groups	HA (titre)*	Specific activity (HU/mg)	Purification fold
Crude extract (100 mL)	0.83	83	A	6,400	77.1	-
			B	3,200	36.0	-
			AB	1,600	19.3	-
			O	1,600	19.3	-
Ammonium sulfate (50 mL)	0.74	74	A	12,800	173	2.2
			B	6,400	86.5	2.4
			AB	1,600	22.0	1.1
			O	1,600	22.0	1.1
DEAE cellulose column (50 mL)	0.78	78	A	25,600	328.2	4.3
			B	25,600	328.2	4.3
			AB	12,800	164.1	8.5
			O	6,400	82.05	4.3

Hemagglutination activity

BanLec had the ability to hemagglutinate human red blood cell types A, B, AB and O (Table I). The ability of the sample to hemagglutinate was determined by its ability to suspend red blood cells in a buffer, done through their ability to bind to two red blood cells and to form a lectin–erythrocyte–lectin matrix, leading to their clumping. When agglutination occurs, crosslinked red blood cells form a network that prevents the red blood cells from sedimenting to the bottom of the well. They appear like a carpet that covers the whole microtiter plate well. When there was no agglutination, the red blood cells sedimented and formed a button on the bottom of the well.

The isolated fraction showed the agglutination activity for various human erythrocyte suspensions namely (A, B, AB and O) at 328.2, 328.2, 164.1, 82.1 HU/mg for BanLec respectively. The least concentration of lectin causes visible agglutination in blood cells A and B are 0.4 µg/mL followed by the least concentration of agglutination in group AB and O are 0.8 µg/mL and 1.6 µg/mL respectively.

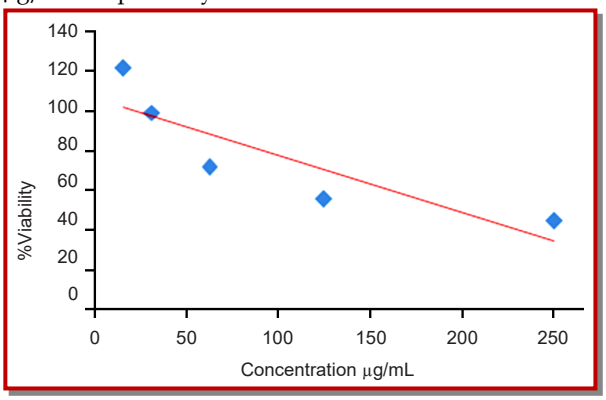

Figure 2: Concentration effect of BanLec on Vero cells. The IC_{50} was calculated using regression line. Data's were expressed as mean ± SD from three independent experiments

The maximum concentration (lowest dilution) where there is agglutination that disappears, if further dilution happens is the specified titer that will be used in the purification process. This is 1:256 for both groups A and B, 1:128 and 1:64 for groups AB and O. Here, PC acts as a positive control where all the wells agglutinated and NC as negative control were all the red blood cells have sedimented to the bottom of wells and formed a small button. Hemagglutinating activity of BanLec was stable at 0–80°C for 30 min, but was abolished after exposure to 90°C for 30 min. The hemagglutinating activity was stable over the pH range of 1–13. Although the agglutination assay was used qualitatively to provide evidence for the presence of lectin in isolated fractions, the results from multiple tests were combined to produce an arithmetic average of the highest dilutions that produced agglutination. The highest dilution that was positive for agglutination and the minimum concentration of the extract that produced agglutination (HU) for the different blood cells has been summarized in the (Table II).

Cytotoxicity of BanLec by determination of mitochondrial synthesis by MTT assay

For the purified lectin, the number of viable cells decreased with increasing concentration of the lectin in a dose-responsive manner (Figure 2). The highest concentration of BanLec significantly decreases the number of viable cells relative to the control. No significant cytotoxicity was detected for BanLec at concentrations up to 250 µg/mL in cultured Vero cells. Therefore, it can be concluded that the IC_{50} (the concentration which causes 50% cytotoxic effect) was more than 100 µg/mL and a 50% inhibitory concentration (IC_{50}) >172.7 µg/mL was obtained. Hence from this conclusion, the antiviral assays were performed at a concentration below or equal to 100 µg/mL. IC_{50} values obtained by microscopic evaluation of cell morphology was significantly different from EC_{50} values obtained by

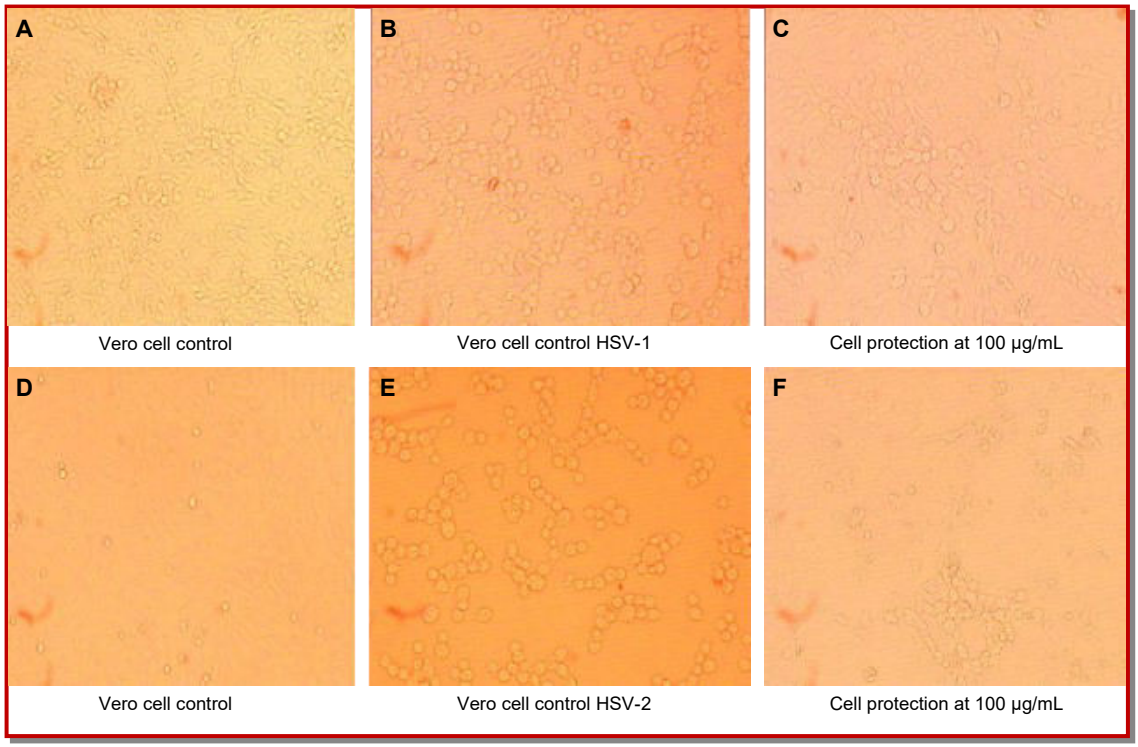

Figure 3: Antiviral activity of BanLec against HSV-I (A-C) and HSV-2 (D-F) and its morphological changes in the Vero cells were examined under a phase contrast microscopy

the MTT assay. Cells appeared almost normal when observed microscopically.

Effects of BanLec on HSV-1 in vitro

The result indicated the $TCID_{50}$ of HSV-1 was different in all groups treated with various concentrations of lectin (12.5, 25, 50, 100 µg/mL) and showed a cytopathic effect on HSV-I. The assessment of the viability was depending on the morphological changes and condensation of insoluble formazan particles as shown in (Figure 3). Firstly, cultured Vero cells were treated with 100 µg/mL of BanLec. After 48 hours, the MTT assay was applied for the assessment of any toxic effect on cell viability. This pre-step was done for the optimization of BanLec. Therefore, all subsequent studies were done for BanLec at concentrations of ≤100 µg/mL.

HSV-1 infected Vero cells that were treated with 12.5 µg/mL of lectin showed inhibition percentage of about 77.4% with cell protection of 22.7% of virus growth. The activity was elevated subsequently. Therefore, lectin exhibits protective activity against the HSV-1. Results shown in (Figure 4) revealed the lectin effects. HSV-1 growth and its expression to its protective activity on Vero cells by 66.3% of cell protection and inhibition of 33.7% of virus growth at 25 µg/mL concentration were observed. This inhibition percentage decreased gradually with subsequent increase in BanLec concentration to reach 27.2% and 16.1% in inhibition percentage with cell protection of 72.8% and 83.9% at 50 µg/mL and 100 µg/mL respectively.

By evaluating the cell protection from $10TCID_{50}$, the higher antiviral activity of BanLec was shown at a higher concentration of 100 µg/mL with 83.9% cell protection in virus growth with inhibition of 16.1%. From the data obtained from MTT assay for antiviral action of BanLec against the HSV-1 strain, the dose that inhibited viral infection by 50% (EC_{50}), the effective concentration required to inhibit 50% virus infection with 12.5, 25, 50, and 100 µg/mL of lectin was determined by plotting the graph against the inhibition of the virus yield versus the concentration of lectin by GravPad Prism (Figure 4).

From the graph, the EC_{50} and IC_{50} values of BanLec on HSV-1 were 16.0 ± 2.5 and 172.7 µg/mL respectively, and from the results obtained there was a significant difference between the values. Thus, BanLec has anti-HSV-1 activity *in vitro* (Table III).

Effects of BanLec on HSV-2 in vitro

When cells were infected with HSV-2, the cells were killed by its cytotoxic effect. By the addition of increasing concentrations of BanLec, the viability of cells decreases. The antiviral effects of the drug of various concentrations against 10 $TCID_{50}$ titer of HSV-2 were compared morphologically and biochemically with those of the control group, they were found to inhibit viral reproduction. Cytopathic changes indicative of viral proliferation were found morphologically

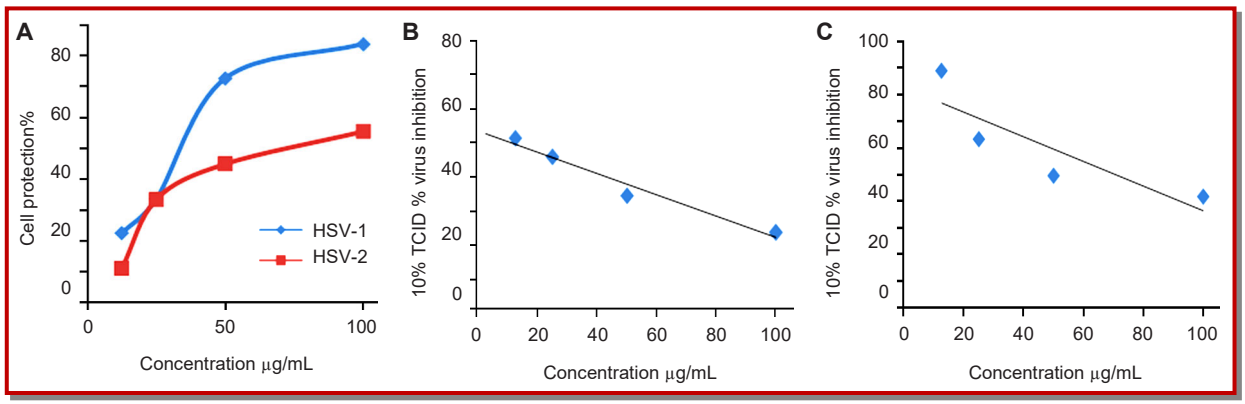

Figure 4: MTT antiviral assay on BanLec and the cell protection against HSV-1 and HSV-2 (A). Antiviral activity of BanLec against HSV-1 (B) and HSV-2 (C) virus at 10 $TCID_{50}$ (50% Tissue culture infectivity dose). The EC_{50} was calculated using regression line. Data's were expressed as mean ± SD from three independent experiments

(Figure 3). All four concentrations of BanLec revealed varying degrees of inhibitory effect against the HSV-2 with various cell protection. In the cytopathic effect inhibition assay, the BanLec showed less protection against the HSV-2 virus challenge dose of 10 $TCID_{50}$.

According to MTT assay, the inhibition percentage gradually decreases with an increase in the concentration of BanLec to reach 55.6%, 45.1%, 33.6% and 11.2% of cell protection of virus growth (Figure 4). As for antiviral assay, 44.4% and 55.0% inhibition were observed when the concentration of BanLec was at 100 µg/mL and 50 µg/mL respectively for HSV-2 and 66.5% and 88.9% inhibition was observed at 25 µg/mL and 12.5 µg/mL. The 50% effective concentration (EC_{50}) obtained was comparatively low as 67.7 ± 2.4 with inhibitory concentration IC_{50} of 172.7 µg/mL (Figure 4). The selectivity index (SI), calculated from the ratio IC_{50}/EC_{50} was 2.6 µg/mL suggesting moderate to no activity of lectin on HSV- 2 (Table III).

Table III

Anti-HSV activity of BanLec by cytopathic inhibition assay

	Concentration
Anti-HSV1 activity	
IC_{50}	172.7 µg/mL
EC_{50}	16.0 ± 2.5 µg/mL
Selectivity index	10.8
Anti-HSV 2 activity	
IC_{50}	172.7 µg/mL
EC_{50}	67.7 ± 2.4 µg/mL
Selectivity index	2.55

IC_{50} means 50% inhibition concentration, defined as a drug concentration that induced 50% inhibition of cultured Vero cells; EC_{50} means concentration that inhibits 50% cytopathogenic effect, as compared to the untreated culture; Selectivity index is the ratio of IC_{50} to EC_{50}

Discussion

From the current findings, the BanLec inhibits the HSV replication of virus infected cells by evaluating the cytopathogenic effect. No significant cytotoxicity was detected for BanLec at concentration up to 250 µg/mL. Further, using phase contrast microscopy cellular morphology was investigated in order to identify the effect of BanLec on Vero cells and it was found that the cells appeared almost normal. The results of MTT assay suggest that BanLec has association with antiviral activity and protective rule against HSV-1 infections.

Carbohydrate-binding agents such as lectins are prime candidate drugs for preventing sexually-transmitted viral infections (i.e. HIV, HBV, HCV, Herpes viruses), its oral bioavailability of such drugs is not required, and it can be an advantage to have poor absorption through cell layers in order to avoid some undesired systemic side effects (Balzarini, 2007). The mechanism of the antiviral activity of carbohydrate-binding proteins has recently been proposed as the interruption of virus fusion with its target cell and it can be mediated either by direct binding to the glycans moiety present on the virus envelope, similar to that of virus/cell-cell interaction and hence preventing further interaction with the co-receptors (Balzarini, 2006). According to (Swanson et al., 2010) it has been reported that BanLec-1 could bind with human immunodeficiency virus (HIV) coat protein gp120 and prevent HIV infection. Previous studies showed that the lectins from different resources could interact with the coat or envelope proteins of different viruses. (Jin et al., 2004; Sun et al., 2003, Takebe et al., 2013). Lectin of the *Galanthus nivalis* agglutinin (GNA)-related lectin family it exhibit significant anti-human immunodeficiency virus (HIV) and anti-herpes simplex virus (HSV) properties that are closely related to their carbohydrate-binding activities (Yang et al., 2011; Ding et al., 2010). Many plant lectins such as *Canavalia ensiformis agglutinin*, *Concanavalin A* (Okada and Kim, 1972), *Soybean*

agglutinin, Wisteria floribunda agglutinin, Narcissus pseudonarcissus agglutinin, Bauhinia purpurea agglutinin and *Eranthishyemalis agglutinin* (Marchetti et al., 1995) possess inhibitory activity towards the infection by Herpes simplex virus. A mannose binding lectin from *Typhonium divaricatum* (L) Decne (family *Araceae*) displays antiviral effects against HSV-II (Luo et al., 2007) and similar effect on Jackfruit lectin (JFL) from *Artocarpus heterophyllus* (Wetprasit et al., 2000).

Virus replication is often detected by the morphological changes, or cytopathic effects that are seen in infected cell cultures. In the present investigation, the cytopathic effect of BanLec was dose-dependent with the most effective antiviral concentrations assumed to be from 12.5 µg/mL to <100 µg/mL. The higher antiviral activity of BanLec was shown at higher concentration 100 µg/mL. As the BanLec concentration increased there was a gradual decrease in the inhibition percentage resulting in increase in the cell protection. The cytopathic effect of BanLec on HSV-1 and HSV-2 treated Vero cells characterized by morphological changes like ballooning of the infected cells or their nuclei when compared to the Vero cell control. To confirm the antiviral potential of the BanLec, the (Selectivity Index) SI was calculated. A high selectivity index suggests that the BanLec would have good antiviral properties. In this study BanLec had high (selectivity index) SI of 10.8 with an effective concentration (EC_{50}) 16.0 µg/mL against HSV-1 showing good antiviral activity and had a least selectivity index (SI) 2.6 with an (EC_{50}) 67.7 µg/mL towards HSV-2 showing a very less antiviral activity towards the BanLec. Based on these results, BanLec is most appropriate to be developed into therapeutic compound for viral diseases.

Conclusion

BanLec displays inhibitory activity against HSV-1 but less potent towards HSV-2.

Conflict of Interest

The authors declare no conflict of interest to this study.

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