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# Antiviral Activity of Euphorbia Lectin Against Herpes Simplex Virus 1 and its Antiproliferative Activity Against Human Cancer Cell-Line

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## Abstract

Lectin is a protein that specifically bound and cross-linked with carbohydrates and is involved in the activation of the lectin pathway which is considered a part of both innate and adaptive immunity. Lectin also has a role in plant defense system. In this study, lectin was isolated from Euphorbia and purified through aqueous extraction, ammonium sulfate precipitation, dialysis, filtration on superdex 75, ion exchange chromatography on SP-sepharose gel.

The study showed that lectin exhibits hemagglutination activity towards rabbit erythrocytes on concentration 50 µg/ml. This hemagglutination activity was retained by lectin till 60°C. Effect of pH, and denaturalizing agent on lectin was also studied, and found to have a low pH range (6-8). Molecular weight was determined for lectin using SDS gel-electrophoresis and found to be 30 KD, and determined by gel filtration to be 60 KD, indicating that lectin is homodimer in the plant.

Cytotoxicity assay of Euphorbia on the vero cell lines using MTT, was also studied and revealed that using a concentration till 100 µg/ml of Euphorbia was within the safe limit. Also the determination of antiviral activity of the Euphorbia lectin against HSV1 on vero cell lines was investigated using plaque reduction assay and MTT, showing 76% of virus inhibition. Mechanism of action of the effect of lectin on HSV1 was also investigated using MTT, and RT-PCR.

The study also showed that lectin affected the virus entry and attachment phase. This inhibition of the virus was totally diminished when the lectin was combined with EDTA before infection with HSV1.

Antiproliferative activity of Euphorbia was also studied against human cancer cell line by MTT assay.

The gene(s) encoding the lectin was isolated by PCR, which revealed that lectin is actually encoded by multi gene family.

**Keywords:** HSV; Antiviral; Antiproliferative; Lectin; *Euphorbia*

## Introduction

Although, Herpes Simplex Virus (HSV) infection is benign or asymptomatic in immune-competent individuals in general, such benign infections can be serious and even life-threatening in patients with weak immune system [1,2]. HSV infection has been treated using nucleoside analogues, such as acyclovir; however, the increasing in clinical use of this type of antiviral agents has been linked with the emergence of drug-resistant herpes virus strains [3]. Therefore, the development of anti-HSV agents with different modes of action is a must.

Being the second most common cause of morbidity and mortality in the world, Cancer related research is heavily focused on understanding the molecular biology and mechanism of cancer development suggesting that it is a systemic disease. Therapies like chemotherapy is known to be the most common and probably the most promising therapy reducing the risk of cancer propagation and recurrence, but it is known to be the most costly one too. Hence, searching for safe, effective, and economic anti-cancer drugs from natural plants became an interesting topic in anti-cancer research.

The study of medicinal plants can lead to the identification of novel bioactive plant compounds which are suitable for drug discovery and development.

Lectins, on the other hand, are a group of proteins found in all types of living organisms, either in soluble or in membrane-bound form that recognize specific carbohydrate structures and thereby agglutinate cells by binding to cell-surface glycoproteins and glycoconjugates [4]. In general, they are structurally complex molecules with one or more carbohydrate recognition domains [5]. Plant lectins have different defensive roles such as insecticidal, anti-fungal, anti-microbial as well as being toxic to birds and mammals [6].

Recently, lectins have been widely used in studies of biochemistry, cell biology, immunology, glycobiology and have widespread applications in biomedical researches [7], as one of the major forces driving the progress of glycoscience.

Finally, Euphorbiaceae is among large flowering plant families consisting of a wide variety of vegetative forms, some of which are plants of great importance, which their classification and chemistry have been subjects of interest recently, possibly because of the wide variety of chemical composition of its members many of which are poisonous but useful [8]. Others are ornamental due to their attractiveness such as *Euphorbia tirucalli*, and *Euphorbia pulcherrima*. This is indicating

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that the Euphorbiaceae is an important source of herbal medicine for humans, veterinary, and agricultural importance [8].

Different *Euphorbia* species are subjected to environmental conditions like stress [9] or herbivore [10] or microbial attack [11], which regulate genetic and enzymatic processes leading to the production of secondary metabolites in leaves and other plant parts to and a response to a stimuli in their original habitat [12] and manufacturing of lectin as a constituents part of plant's defense system [13].

*Euphorbia pulcherrima* is a popular ornamental plant grown for its red leafy bracts [14]. It is a nontoxic plant although some species of the family Euphorbiaceae that produce latex are toxic.

*Euphorbia tirucalli* is a large shrub or a small tree with erect branches. *E. tirucalli* is used in traditional medicine, ornamental, and as a source of rubber [15].

For all the Euphorbia species studied so far, the antiviral, or antimicrobial, and antiproflerative substances were isolated in the form of diterpenes [16,17] triterpenes [18], alkaloids [19,20] for tannins and flavonoids. In this study however, different components like lectins have been investigated for their antiviral and antiproflerative activity.

## Materials and Methods

### Plant material, chemicals and reagents

*E. pulcherima* and *E. tirucalli* were acquired from the ornamental plant store (USA). Whole plant or stem were collected and thoroughly washed with 2% sodium hypochlorite solution and subsequently with sterial distilled water. After sterilization, the stems were dried and grounded to powder [21].

Bovine serum albumin (BSA), Acyclovir, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Dimethylsulphoxide (DMSO) most monosaccharides, polysaccharides were purchased from Sigma (St. Louis, USA). Standard molecular weight markers (gel-filtration and SDS-PAGE protein markers) and pH range 1-14 were procured from Amersham Pharmacia (New Jersey, USA). RNA and DNA Extraction Kit and primers were purchased from Takara Biotechnology, China. All the other reagents were of analytical grade.

### Cell line, cell culture, and virus

The human cancer cell line employed, Hela (cervix), was purchased from the cell bank of Shanghai institute of cell biology (China). The cell lines were selected in such a way that almost all the cell line grow on a single growth medium (PRMI-1640) in tissue culture flask and the mass doubling time was such that enough cells were obtained for screening. Used cells were free from bacteria, yeast, mold, mycoplasma and from viruses at all the stages. If contamination appeared at any stage, the stock in which it occurred was discarded immediately. Cell lines were maintained in RPMI 1640 medium (Gibco, USA) with 10% FBS (Gibco, USA), 10 U/ml penicillin and 100 µg/ml streptomycin at 37°C, in humidified atmosphere (90% relative humidity and 10% $CO_2$) in $CO_2$ incubator. Herpes simplex virus type 1, African green monkey kidney cells (Vero) ATCC-81 was obtained from American type culture collection.

For the anti HSV-1 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% $CO_2$ atmosphere. The HSV-1 was propagated in Vero cells, while stock viruses were prepared as previously described. After three cycles of freezing/thawing, the fluids were titrated on the basis of PFU count as previously described and stored at -80°C until use. The virus titre expressed by ($TCID_{50\%}$) was determined by cytopathic effect in cell culture.

### Purification method

**Preparation of *lectin* extracts and purification:** The whole plants were rinsed, chopped, and homogenized in three volumes of extraction buffer (50 mM Tris–Cl, pH 6.8, containing 0.2 M NaCl, 10 mM ascorbic acid, 1 mM phenylmethylsulfonyl fluoride, and 1% (w/v) polyvinylpyrrolidone). The homogenate was stirred for 12 h and centrifuged at 8000 x**g** for 20 min. The supernatant was lyophilized and resuspended in deionized water (final concentration of 20 mg/ml). Ammonium sulfate was slowly added to a final concentration of 85%, stirred for 2 h, and centrifuged at 8000 x**g** for 15 min. The precipitate was resuspended in deionized water. Both fraction 2 and the supernatant were dialyzed against water for 2 days. The dialysate was tested for the hemaglutenation activity. The active fraction causing hemaglutenation was collected and adjusted to 20 mM sodium phosphate (pH 6.8) containing 150 mM NaCl and loaded onto a Superdex 75 column (120 × 2.0 cm; GE) which was previously equilibrated with same buffer by using a AKTA-fast protein liquid chromatography (FPLC) system (Pharmacia Amersham Biotech, Uppasala, Sweden). The proteins were eluted with a gradient of 20 mM sodium phosphate (pH 6.8) containing 150 mM NaCl, at a flow rate of 0.2 ml/min. Each fraction (2ml) was monitored at 280 nm and analyzed for hemaglutenation activity. The active fractions were then pooled, and subjected to column chromatography by using a SP Sepharose column (13 × 2.6 cm; GE). Equilibration, loading, and washing were carried out in 20 mM sodium phosphate (pH 6.8) and elution was performed with a linear NaCl gradient at a flow rate of 5 ml/min at 4°C. After removal of unabsorbed material, the column was eluted consecutively with 0.1 M NaCl, 0.2 M NaCl, and 1.0 M NaCl. The absorbance at 280 nm of each fraction was measured. Each fraction was collected, concentrated, and desalted using 10 kDa ultra-filtration devices (Millipore, Bedford, USA), and tested for anti-HSV-1 activity by using plaque reduction assay.

**Lectin concentration**: The lectin content of the samples obtained during the purification process was determined by the method of Lowry et al. [22] using bovine serum albumin as the standard. Readings at 280 nm were also used to determine the protein content of the column eluates.

### Assay of hemagglutinating activity

In the assay for lectin (hemagglutinating) activity, a serial twofold dilution of the lectin solution in microtiter plates (50 $\mu$L) was mixed with 50 $\mu$L of a 2% suspension of rabbit red blood cells [23] in phosphate-buffered saline (pH 7.2) at 20°C. The results were read after about 1 hour, when the blank had fully sedimented. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, is considered as one hemagglutination unit. Specific activity is the number of hemagglutination units per mg protein [24].

### Physical characterizations of lectin-induced hemagglutination

**Effect of temperature:** The effect of temperature on hemagglutinating activity of the lectin was examined as previously described [25]. A solution of the lectin with concentration 50 µg/ml was incubated at various temperatures for 30 minutes: 0°C, 10°C, 20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, 90°C, and 100°C. The tubes were then put on ice, and assay of hemagglutinating activity was then carried out.

**Effect of pH:** The pH stability of the lectin was determined by incubation of the lectin (50 µg/mL) in buffers of different pH values ranging from pH 1.0–14.0 for 60 minutes. The pH of the lectin solution was adjusted to 7.0 by the addition of 0.1 N HCl or 0.1 N NaOH before hemagglutination activity was determined.

**Effect of denaturating agent:** The purified lectin (50 µg/mL) was incubated for 10 h with 10, 50 and 100 mmol.$L^{-1}$ of Ethylene diamine tetraacetic acid (EDTA) chelating agent for 18 h at 4°C, followed by incubation with rabbit erythrocytes with continuous shaking. The lectin sample was dialyzed exhaustively against 0.145 M NaCl, and the hemagglutinating activity was assessed, also addition of effective concentration of EDTA on lectin and then added to vero cells at the same time of virus infection to determine if the antiviral activity of lectin will be dimensioned with EDTA or not .

**Inhibition of lectin-induced hemagglutination by carbohydrates:** The sugar specificity of Euphorbia lectin was tested by inhibiting the hemagglutinating activity using simple sugars by using 1ml from different concentrations (10, 30, and 50 mM) of the following (D-glucose, D-glactose, D-mannose, D-lactose, and sucrose) was added to 1ml of lectin and allowed to stand for 30 mins at 25°C. An erythrocytes suspension (1 ml) was then added and the mixture was left for 30 mins with a non-sugar containing solution (blank). The hemaglutnation activity was observed microscopically. Results were expressed as the minimal concentration of carbohydrates with well-defined structures which effectively inhibited four hemagglutinating dose units of the lectin.

## Determination of molecular mass of Euphorbia lectin by gel-filtration and SDS-PAGE

The molecular mass of Euphorbia lectin (EL) was estimated by gel filtration chromatograph on the same superdex G-75 column as mentioned previously following the procedure of P. Andrews [26]. The column was calibrated with soybean trypsin inhibitor (30.2 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa) and cytochrome C (12.5 kDa). Gelfiltration purified lectin was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), pH 8.3, using 11% (w/v) acrylamide slab gel for subunit molecular mass determination as described by Laemmli [27]. A molecular weight calibration curve was prepared using gel filtration based on the protein standards (markers).

Elution time was plotted against the molecular weight and the molecular weight of the lectin was estimated from the calibration curve.

## Evaluation of cytotoxicity and antiviral activity against HSV1

**Virus titration (Cytopathogenic effect):** Confluent monolayers in 96-well plates were overlaid with equal volume of serial appropriately diluted virus suspension. After 1 h of adsorption, the unabsorbed virus was washed by PBS and fresh culture medium was added to each well. The morphology of Vero cells was inspected for microscopically detectable alterations, i.e. loss of monolayer, rounding, shrinking of cells, granulation, and vacuolization in the cytoplasm. After 72 h of incubation at 37°C in a 5% $CO_2$ incubator, the value of TCID50 (tissue culture infection dose) was calculated [28].

**Evaluation of cytotoxicity by MTT:** It was performed by MTT [3-(4,5- dimethylthiazol-2,5-diphenyl tetrazolium bromide] assay, with minor modifications [29]. To assess the cytotoxic effects of the Euphorbia lectin on uninfected Vero cells ($5 \times 10^3$ cells per well) seeded onto 96-well culture plates, 100 µL of their dilutions ranging from 0 to 300 µg/mL were added to confluent cell monolayers. As cell controls, only 100 µL of MEM were added to the cells. After 72 h at 37°C, the medium was removed and 50 µL of MTT solution prepared in MEM (1 mg/mL; Sigma) were added to each well and the plates incubated for 4 h. The MTT solution was removed, 100 µL of DMSO (Sigma, USA) were added to each well to dissolve formazan crystals, and the plates have been gently shaken, whereby crystals were completely dissolved. The absorbencies were read on a multi-well spectrophotometer (BioTEK, USA) at 540 nm, each concentration will be repeated at least three times. The 50% cytotoxic concentration (CC50) was defined as the concentration of the test substances that reduced cell viability by 50% when compared to untreated controls [30].

**Antiviral activity by viral plaque formation number reduction assay:** This assay followed the procedures previously described [31], with minor modifications. Vero cells ($3 \times 10^5$ cells per well) were seeded onto 24-well culture plates, and after 24 h, the cells were infected with 100 PFU of HSV-1. After 1h adsorption at 37°C, the plates were washed and overlaid with MEM plus 1.5% carboxymethylcellulose (CMC, Sigma) containing or not different concentrations of the lectin. After 72 h, the cells were fixed and stained with crystal violet (Sigma) and viral plaque formation was counted. The 50% inhibitory concentration (IC50) was defined as the concentration that inhibited 50% of viral plaque formation when compared to untreated controls. Acyclovir (Sigma®) was used as a positive control (10 µg/ mL), since it completely inhibited the viral replication. Its stock solution was prepared in DMSO (Merck) at the final concentration of 1000 µg/mL. Results were expressed as 50% cytotoxic (CC50) and 50% inhibitory (IC50) concentrations, respectively, in order to calculate the selectivity index (SI=CC50/ IC50) of each extract [32]. The antiviral activity of E. lectin was determined by the following formula:

Percentage Inhibition = [1 – (number of plaques tested/number of plaques in control)] × 100

**Antiviral assay by MTT:** The antiviral activity of EL against HSV-I was also assessed using MTT method [30]. In short, confluent monolayers in 96-well plates were pre-incubated with 20 µl of 100 times diluted virus suspension (100 PFU/100 µL) for 1 h at 37°C. Then 0.2 ml of maintenance medium containing appropriate serially diluted concentrations of the test sample was added. Acyclovir was used as the positive control for HSV-I. Cell control and virus control were run simultaneously. After 3 days of incubation, the absorbance OD 540 nm was determined by multiwell spectrophotometer BioTEK, USA. The percentage of viable treated cells was calculated in relation to untreated controls and was defined as 50% effective concentration ($IC_{50}$), which was determined from the dose dependent curve. The therapeutic index (TI) was calculated from the ratio $CC_{50}/IC_{50}$ [30].

## Effect of lectin on HSV 1 life cycle

4.8.1 **Virucidal assay:** The virucidal activity of lectin against HSV-I was assessed using plaque reduction assays with monolayers of Vero cells grown in 6-well plates. The assays were performed by adding the compound (0, 10, or 100 µg/mL) to an equal volume to HSV-I (100 PFU/100 µL). For 1 h at 37°C, the mixtures were added to Vero cells for 1 h at room temperature. Thereafter, the cells were washed three times with PBS, and medium containing 0.6% agar and 0.001% trypsin (PFU assays) was added. Monolayers were fixed with methanol:acetone after incubation for 72 h at 37°C and 5% $CO_2$ and stained with 1% crystal violet in an incubator, after which plaques were counted.

**Time of addition assay:** Vero cell monolayers were infected with 100 PFUs of HSV-I. E. lectin was added at a concentration of 100 µg/mL at different times of infection: 1 hour before infection, at the same time

of infection, 15, 30 mins, and 1, 2, 4, and 6 h post infection. Thereafter, for each treatment, cells were incubated with lectin for 1 h and then washed three times with PBS and medium containing 0.6% agar and 0.001% trypsin (PFU assays) was added. Monolayers were fixed with methanol:acetone after incubation for 48 or 72 h at 37°C and 5% $CO_2$ and stained with 1% crystal violet; subsequently, plaques were counted.

**Reverse-transcriptase polymerase chain reaction (RT-PCR):** Total RNA was isolated from Vero cells infected with HSV-1 for the indicated times, using the Qiagen RNeasy kit. The RNA was treated with DNase (Roche diagnostics) to remove contaminating DNA, for 10 min at 65°C, RNA templates were reverse transcribed using one step RT-PCR kit (Qiagen) according to the manufacturer's instructions. cDNAs obtained from reverse transcription reactions were amplified by PCR using taq (Invitrogen USA) and by using DNA polymerase primers according to Tachikawa et al. [33]. (Forward: GCTCGAGTGAAAAAAACGTTC) and (Reverse: TGCGGTTGATAAACGCAGT). The reactions were performed using the light cycler PCR system (Roche Switzerland) with the following conditions: an initial denaturation step at 95°C for 2 mins, followed by 40 cycles at 95°C for 2 seconds, 58°C for 15 seconds and 72°C for 15 seconds, with a final extension at 40°C for 30 seconds.

### Assay of antiproliferative activity on tumor cell line

American type culture collection cancer cell line in logarithmic growth phase, Hela cells was suspended in RPMI medium and at concentration $2 \times 10^4$ cells/mL. A 100 µL aliquot of this cell suspension was seeded to a well of a 96-well plate, followed by incubation for 24 hours. Different concentrations of the lectin in 0, 50, 100, 150, 200, 250, and 300 µg/ml complete RPMI medium were then added to the wells followed by incubation for 24 h. After 24 h, 50 µL of a 5 mg/mL solution of 3-(4, 5-dimethylthiazol-2- yl)-2, 5-diphenyltetrazolium bromide (MTT) in phosphate buffered saline was spiked into each well, and the plates were incubated for 2 hours. The plates were then centrifuged at 2500 rpm for 5 minutes. The supernatant was carefully removed, and 150 $\mu$L of dimethyl sulfoxide was added in each well to dissolve the MTT (formazan) at the bottom of the wells. The absorbance at 590 nm was then measured with a microplate reader within 10 minutes. Inhibition percentage of cells by lectin was calculated by [(OD590nm of the control – OD590nm of a culture exposed to a particular lectin concentration) / OD590nm of the control] × 100

### Isolation of gene(s) coding *E. pulcherima* lectin by PCR

DNeasy Plant MiniKit (Qiagen Inc., cat. No. 69104) was used for DNA isolation from Euphorbia pulcherima as described in the manufacturer manual. Specific primers were used as follows [34]:

Forward: 5` ATGGCTGATCTGTATGT 3`and Reverse: 5` TACTTGAATCTGGCTGC 3`

PCR technique was conducted to ensure the presence of the lectin coding gene(s), using the above mentioned primers.

Reaction conditions were optimized and PCR reagent mixtures were prepared as follows:

50 µl total volume containing: 2.5 µl dNTPs (2.0 mM), 2.5 µl 10x buffer, 2.0 µl forward primer (2 µM), 2.0 µl reverse primer (2 µM), 3.0 µl template DNA (50 ng/µl), 0.2 µl Taq (5 U/µl), $ddH_2O$ up to 25 µl. Amplification was carried out in thermal cycler programmed as follows: 95°C for 15 min; followed by 40 cycles at 95°C for 1 min; 45°C for 1 min; 72°C for 2 min; and then 72°C for 5 min; and finally 4°C (infinitive). Agarose gels (1.2%), used for resolving the PCR products, were prepared and run as described by Maniatis et al. [35]. The gel was stained with ethidium bromide and evaluated under UV light. The sizes of PCR products were estimated according to the migration pattern of a 100bp DNA ladder.

## Results and Discussion

### Purification of Euphorbia lectin from *Euphorbia* species

The extraction and purification of lectin from whole plant of *E. pulcherima*, and *E. tirucalli* has been done in two steps: the first step is the precipitation of lectin fraction from the saline extract using 85% ammonium sulphate followed by purification using gel filtration column chromatography on superdex 75 equilibrated with the saline phosphate buffer (SPB). Three peaks of protein were detected using this step of purification, as shown in Figure 1 below. Heamagglutinating activity (HA) was found only in the first peak. Maximum activity was detected at fraction number 80 with OD=0.35 in case of *E. pulcherima* and OD=0.3 in case of *E. tirucalli*. The fraction that gave the highest agglutination activity, peak (1), was then purified on SP sepharose columns, and the resulted peaks were further examined for hemagglutination.

Hemaglutination activity of sp-sepharose purified *E. pulcherima* and *E. tirucalli* lectins are summarized in Table 1 below. 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 in the lectins from the two plants.

The purification procedure of lectin from Euphorbia is summarized in Table 2 below. Saline, $(NH_4)_2SO_4$, and purified lectin obtained from superdex 75, and sp-sepharose were used for determining the agglutination activity using trypsinized rabbit erythrocytes. The higher agglutination activity was observed in purified lectin from sp-sepharose (Figure 2). The result of the purification procedure employed in E. lectin summarized in Table 2 as shown. The purification fold achieved in $(NH_4)_2SO_4$ was higher than that achieved by gel chromatography. One possible explanation for the low purification fold achieved is the

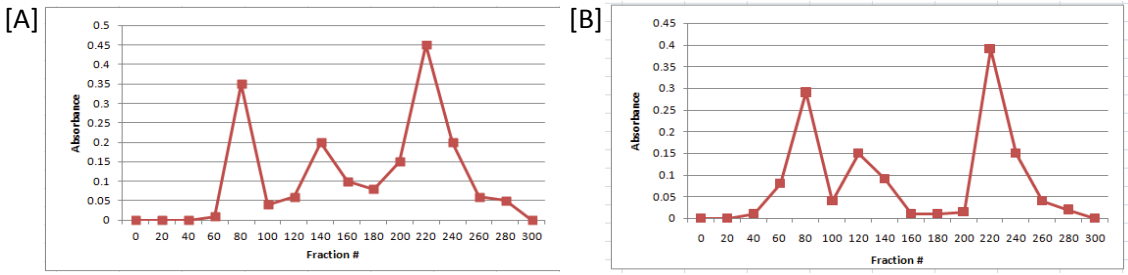



**Figure 1:** Elution profile of (A) *E. pulcherrima* lectin, and (B) *E. tirucalli* lectin purification. Gel filtration chromatography of 45% ammonium sulphate precipitate on superdex-75 column

| Dilution | 1:1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 | 1:1024 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Concentration | 12.4 mg/ml | 6.2 mg/ml | 3.1 mg/ml | 1.55 mg/ml | 0.775 mg/ml | 0.387 mg/ml | 0.193 mg/ml | 100 µg/ml | 50 µg/ml | 25 µg/ml | 12.5 µg/ml |
| Presence of Agglutination | + | + | + | + | + | + | + | + | + | - | - |

**Table 1A:** Calculation of hemagglutination activity of sp-sepharose purified *E. pulcherrima* Lectin

| Dilution | 1:1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 | 1:1024 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Concentration | 11.02 mg/ml | 5.51 mg/ml | 2.75 mg/ml | 1.37 mg/ml | 0.688 mg/ml | 0.344 mg/ml | 0.172 mg/ml | 86 µg/ml | 43 µg/ml | 21.5 µg/ml | 10.75 µg/ml |
| Presence of Agglutination | + | + | + | + | + | + | + | + | - | - | - |

**Table 1B:** Calculation of hemagglutination activity of sp-sepharose purified *E. tirucalli* Lectin

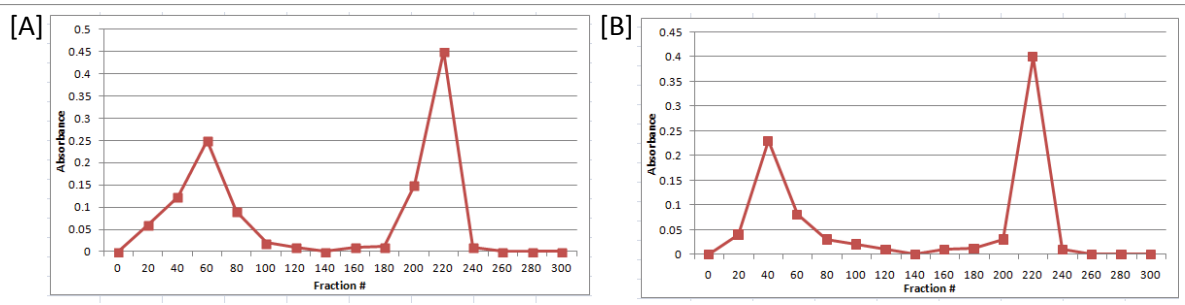



**Figure 2:** Elution profile of (A) *E. pulcherrima* lectin, and (B) *E. tirucalli* lectin purification. Gel filtration chromatography of 85% ammonium sulphate precipitate on SP-Sepharose column

adsorption. We noted that the adsorption of lectin on cross linked dextran gels like sephadex G50, furthermore, the relatively lower activity observed after the gel filtration was due to the co-precipitation of the lectin with the inhibitor protein resulting in a lower concentration. Similar results were obtained by Agrawal and Godstein [36].

Specific activity is expressed as titer; the reciprocal of maximum dilution of protein that gives viable agglutination with 2% trypsinized erythrocytes. The least concentration of lectin causes visible agglutination 50 µg/ml for *E. pulcherima* and 86 µg/ml for *E. tirucalli*. The specific hemaglutinating activity is expressed as the number of hemgalutination units per mg protein.

## Cytotoxicity of *E. pulcherima* lectin and *E. tirucalli* lectin by MTT

To determine the cytotoxicity of *Euphorbia pulcherrima* and *Euphoriba tirucalli*, MTT assays were performed. For both the purified lectins, the number of viable cell decreased with increasing concentrations of the extract in a dose-responsive manner. The highest concentration of plant extract that did not significantly decrease the number of viable cells relative to the control was used for all subsequent analysis.

No significant cytotoxicity was detected for *E. pulcherrima* at concentrations up to 100 µg/mL in Vero cells, and a 50% cytotoxic concentration ($CC_{50}$) > 225 µg/mL was obtained. The antiviral assays were performed at concentrations below or equal to 100 µg/mL. The percentage of cell viability for *E. pulcherrima* was found to be 100% at 100 µg/mL for all the extracts. The anti-HSV activity was therefore determined at concentration of 100 µg/mL or lower for the E. Lectin extracts (Figure 3A).

On the other hand, the percentage of cell viability for *E. tirucalli* was found to be 100% at 10 µg/mL. The cytotoxicity increases as the concentration increases. The anti-HSV activity was therefore determined at concentration of 10 µg/mL or lower for the E. Lectin extracts, and $CC_{50}$=60 µg/ml (Figure 3B).

## Anti HSV-I assay of *E. pulcherima* lectin and *E. tirucalli* lectin detected by MTT

The TCID50 of HSV-I against Vero cells, determined by cytopathogenic effect (CPE), was 10−4 in the virus titration assay. The amount of surviving cells after incubation with sample solutions at different concentrations was estimated by MTT. The antiviral activity was determined as the % of inhibition of the HSV-I as calculated by the absorbance OD 540 nm and the $IC_{50}$ was calculated for the lectin in both the two Euphorbia plants. The *E. pulcherima* lectin caused a max inhibition of around 78% and an $IC_{50}$ of 37µg/ml, whereas the *E. tirucalli* gave comparable results of a max inhibition around 84% and an $IC_{50}$ of 40 µg/ml (Figure 4). To select a potentially good drug a ratio called the selectivity index (SI) needs to be calculated by dividing the $CC_{50}$ over the $IC_{50}$ and the objective is to find the bigger ratio which means that the extract is stronger in killing the virus than killing the host itself.

SI for *E. pulcherima*=225/37=6.08, and SI for *E. tirucalli*=60/40=1.5. Hence, it's clear that *E. pulcherima* lectin is much better than *E. tirucalli* as a potential drug, therefore the rest of the experiments in this study will be conducted on *E. pulcherima* lectin alone, and will be referred to as E. lectin.

## Determination of molecular mass of E. Lectin by gel-filtration and SDS-PAGE

Upon SDS-PAGE in the presence and 2-mercaptoethanol, E. Lectin migrated as an apparent single band of about 30 kDa (Figure 5), indicating the homogeneity of the lectin. These results suggested the dimeric nature of EL in which subunits are not held by disulphide linkages but joined together by noncovalent bonds. This finding is in good agreement with most of lectins which have no disulphide linkage between subunits [37,38].

## Effect of temperature, pH and denaturants

When subjected to heat treatment, E. Lectin is thermally stable over

| Steps of Purification | Protein Yield (mg/ml) | HA (titre)$^{-1}$ | Specific Activity (Hu/mg) | Purification (fold) |
|---|---|---|---|---|
| Saline Solution | 340.4 | 16 | 0.047 | 1 |
| $NH_4SO_4$ | 139.4 | 32 | 0.229 | 4.87 |
| Superdex 75 | 49.5 | 128 | 2.58 | 54.89 |
| SP-Sepharose | 12.4 | 256 | 20.6 | 438.297 |

**Table 2A:** Summary of the purification steps of the *E. pulcherrima* Lectin

| Steps of Purification | Protein Yield (mg/ml) | HA (titre)$^{-1}$ | Specific Activity (Hu/mg) | Purification (fold) |
|---|---|---|---|---|
| Saline Solution | 460.0 | 16 | 0.034 | 1 |
| $NH_4SO_4$ | 160.2 | 32 | 0.2 | 5.88 |
| Superdex 75 | 86 | 128 | 1.48 | 43.5 |
| SP-Sepharose | 11.02 | 128 | 11.61 | 341.47 |

**Table 2B:** Summary of the purification steps of the *E. tirucalli* Lectin

a wide range of temperatures from 20 to 60°C. However, the activity starts to decline very rapidly afterwards, and is completely abolished after 10 min of exposure at 90°C and 100°C, same results obtained by Chan, et al. [37]. E. Lectin was quite stable from pH 6 to 8, and still retains 50% and 60% agglutination activity at pH 4 and 9 respectively, suggesting the pH stability of the protein. Addition of metal chelating agent like EDTA to E. Lectin and tested for antiviral activity on HSV-I, revealed that some or all antiviral activity of E. Lectin was diminished (disappeared after addition of EDTA) indicating that the lectin was a metaloprotein that needed both $Ca^{+2}$ and $Mn^{+2}$ ions for its activity. Similar results (Figure 6) were obtained by Konozy et al. [39]. Also addition of EDTA diminished antiviral activity of E. Lectin when it is applied to vero cells infected with HSV-1.

## Carbohydrate specificity

The hemmaglutinating activity was inhibited by D. galactose and D. galactose driving sugars especially lactose. In the carbohydrates specificity test for E Lectin, it was found that the lectin did not interact with glucose, mannose, and sucrose. Comparable results were obtained by Chan et al. [37]. The minimal concentration of glactose and lactose required for reduction of E Lectin hemaglutinating activity was 20 µM, and 10 µM respectively. Similar results were obtained by Silva et al. and Al-saman et al. [40,41] who reported that lectin is a non-immune carbohydrate-binding protein which is very specific for sugar moieties, agglutination of cells precipitation of the polysaccharide (Figure 7).

## Anti HSV-I assay of E. lectin detected by plaque reduction assay

The Vero cells were incubated with E. lectin at different concentrations and then estimated by cytopathoic effect in the plaque reduction assay. The toxicity of most concentrations tested was relatively low. After addition of different concentrations of E. Lectin on HSV-I a significant reduction >50% in plaque number was observed for concentration of 30 µg/ml. While at higher concentration, a significant reduction of plaques was observed at concentration 100 µg/ml indicating a high percentage of inhibition (Figure 8). No cytotoxic effect on cell multiplication at concentration that achieve antiviral activity [42].

## Assay of *in vitro* anti-proliferative potential on human cancer cell line

The *in vitro* anti-proliferative activity of E. lectin was evaluated against human cancer Hela cell line representing cervix adenocarcinoma 0, 50, 100, 150, 200, 250, and 300 µg/ml, after a treatment period of 48 hours, cytotoxicity was carried out using MTT assay. Cytotoxicity was considered when more than 50% reduction on cell survival was observed.

Figure 9 below show that purified lectin can inhibit the profilration of Hela cell lines in a dose-dependent manner at the concentration range of 50-300 µg/ml, in comparison less inhibitory effect was observed in concentration less than 150 µg/ml. The highest growth inhibition rate was observed at the concentration of 200 µg/ml. The importance of E. lectin as an anti-tumor substances comes from the view of lectin with hemaglutinating activity are devoid of anti-tumor activity, Sharma et al. [43], and others can be used as anti-cancer drugs [44].

Some lectins having potent anti-proliferative activities have also been reported earlier [45,46]. However, the exact molecular mechanism of these action is poorly understood, but evidence is now emerging that lectins are dynamic contributors to tumor cell recognition, cell adhesion and localization, signal transduction across membranes, mitogenic stimulation, host immune defense augmentation, cytotoxicity and apoptosis [47-49]. Saccharide determinants or glycoconjugates present on tumor cell surface serve as important binding sites for specific lectins.

## Virucidal activity

Results demonstrated that E. lectin has an effect on viral infectivity at concentrations less than 40 µg/ml. Higher concentrations of E. lectin on the other hand (>40 µg/ml) showed significant virus inhibition (Figure 10). For that reason, concentration >40 µg/ml was selected and applied in all the following mode of action studies. Comparable results were reported by Cheng et al. [50].

Lectins can counter-act viral infection in various ways: they may directly neutralize virus by destabilizing or aggregating virions, interfere with crucial steps in the viral infection process (e.g. entry), and/or opsonize virus to facilitate uptake and degradation. Upon virus binding, some soluble lectins can trigger complement deposition on

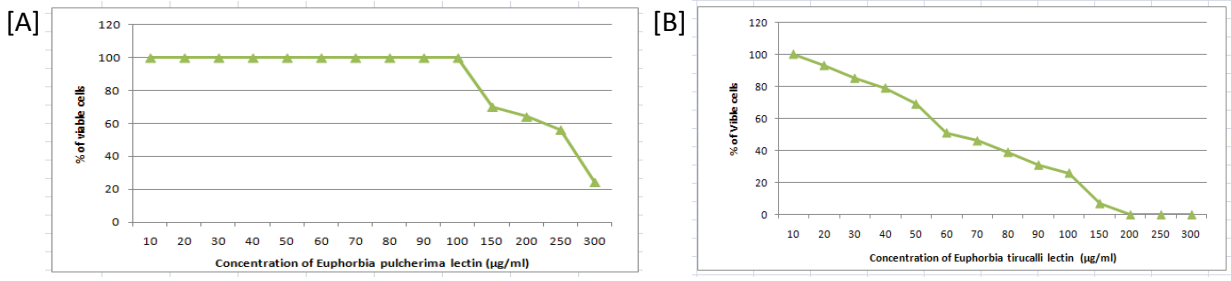



**Figure 3:** Determination of cytotoxicity activity of *E. lectin* from (A) *E. pulcherima* and (B) *E .tirucalli* on Vero cells by MTT assay

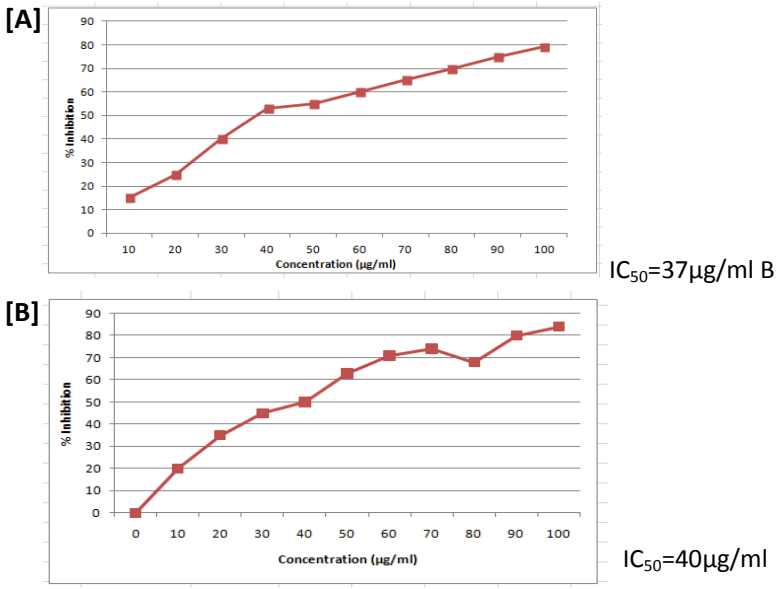



**Figure 4:** Concentration effect of (A) *E. pulcherrima* Lectin, and (B) *E. tirucalli* Lectin on HSV-1 replication in Vero cells The $IC_{50}$ was calculated using regression line. Data were expressed as mean ± SD from three independent experiments.

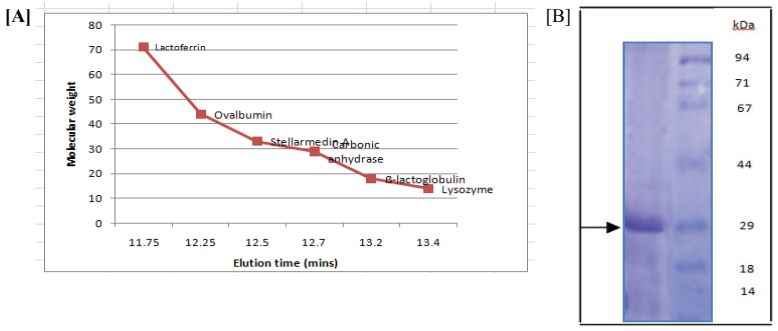



**Figure 5:** Native molecular mass estimation by standard plot on gel-filtration chromatography column. Standards used for gel-filtration analysis were lactoferrin (71 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18 kDa), and lysozyme (14 kDa) were used as standard markers. (A) a curve plotting the molecular mass versus the elution time; (B) the SDS gel representing the *E. lectin* band on the left lane on 30 kDa represented by the arrow and the marker on the right lane.

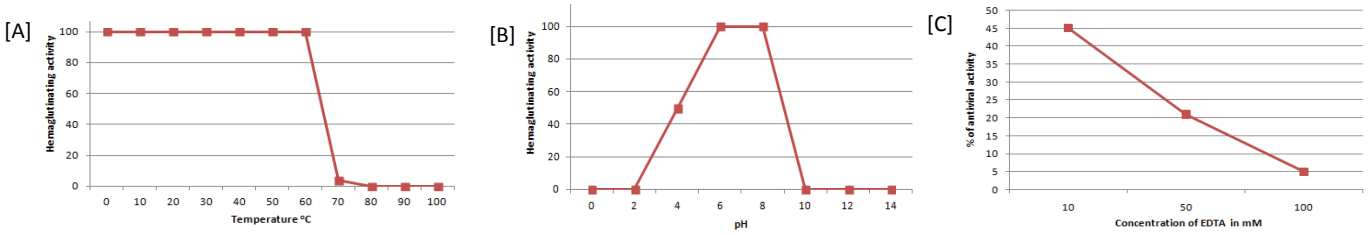



**Figure 6:** Effect of (A)Tempertaure; (B) pH on hemagglutinating activity of Lectin; and (C) concentration of EDTA on % of antiviral activity

the virus, which may inhibit viral infection, enhance viral uptake via complement receptors and subsequent degradation in immune cells, and/or cause virolysis.

## Effect of E. Lectin on viral infection as determined by time of addition assays

To determine which step of the HSV-I cycle was targeted by E. Lectin, "time of addition" experiments were performed in Vero cells infected with HSV-I and exposed to E. Lectin at different times of infection (1 hour before infection, at the same time of infection, 15, 30mins, and 1, 2, 4, and 6 hours post infection) as shown in Figure 11.

From the results obtained due to addition of E. Lectin at concentration that cause the maximum % inhibition (100 µg/ml) one hour before virus infection, showed considerable decrease in activity of virus so maybe lectin compete with the virus on receptor cites, indicating that E. Lectin can prevent adsorption of virus on the cell surfaces (references) while addition of E. Lectin that cause the

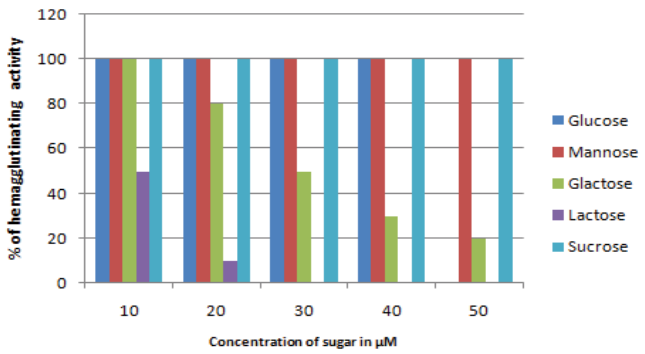
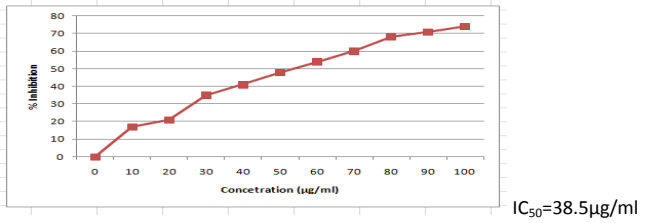



**Figure 7:** Effect of specific carbohydrates on hemagglutinating activity of E. Lectin




$IC_{50}$=38.5µg/ml

**Figure 8:** Concentration effect of E. Lectin on HSV-1 replication in Vero cells The $IC_{50}$ was calculated using regression line. Data were expressed as mean ± SD from three independent experiments.

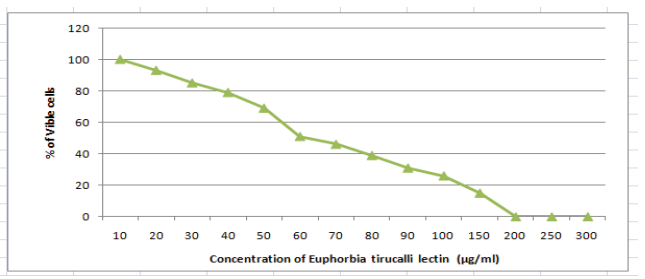



**Figure 9:** Effect of concentration of E. lectin on the cell viability of Hela cell line

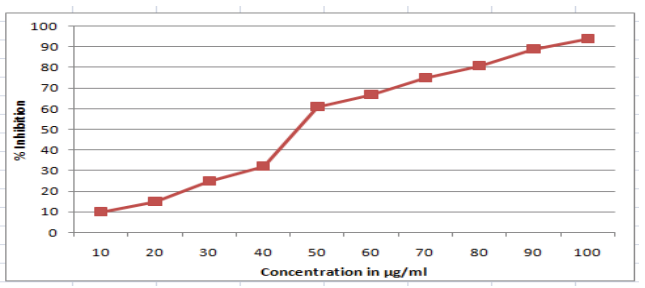



**Figure 10:** Effect of E. lectin concentrations on viral infectivity

maximum inhibition for the virus concurrently with the HSV-I cause considerable decrease in viral plaques, which indicates that E. Lectin can compete with the virus for the receptor cites on the vero cells, preventing the attachment and binding, and viral penetration or viral DNA entry to the nucleus. Similar results were reported by Hidari et al. [51]. Also, from the result of post infection, addition of E. Lectin showed that there is still an amount of virus inhibition during the first hour of virus infection indicating that E. Lectin can affect the virus entry and the early event of virus replication [52].

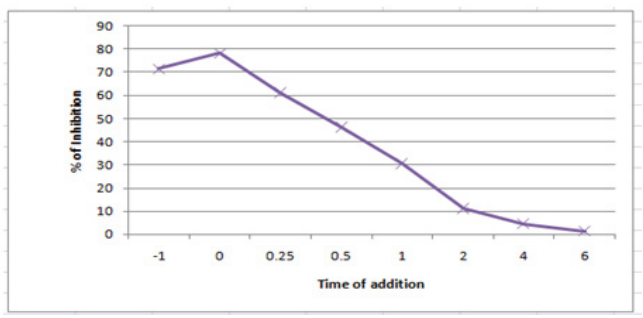



**Figure 11:** Effect of time of addition of ELectin on HSV-I life cycle using MTT

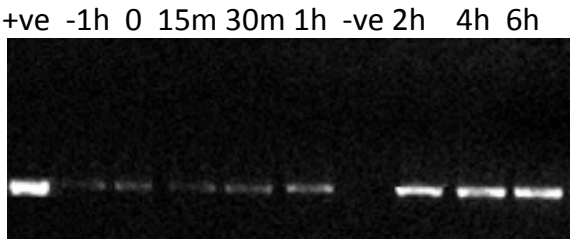



**Figure 12:** RT-PCR for detection of HSV-1 polymerase gene after treatment with E. Lectin at different times

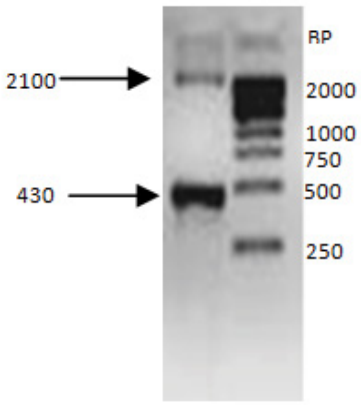



**Figure 13:** DNA electrophoresis of the genomic DNA of *E. pulcherima*, showing two bands at 430 and 1950 bp

Our findings indicated that the extracts were most effective when added either simultaneously with the initiation of virus infection or post infection as well when given pre-infection, suggesting the extract may act at the early stage of infection such as during viral attachment and entry as well as viral replication.

## Reverse transcriptase polymerase chain reaction assay for the determination, and mechanistic action, of E. Lectin on the different stages of HSV-I viral replication cycle

In order to study at which stage the lectin was exerting its effect on the virus life cycle via its effect on the viral DNA polymerase gene, which is an essential step in the viral DNA replication [53] as indicated in the pretreatment, simultaneous treatment, post treatment at 15, 30 mins, 1, 2, 4, and 6 h respectively, by using RT-PCR approach. Figure 12 summarized the effect of antiviral activity of lectin at concentration 100 µg/ml which was found to be most effective in inhibiting HSV-I viral replication by inhibiting viral DNA polymerase gene as indicated in

pre, simultaneous and also for post infection especially during the first hour, no more than that, suggesting that the E. Lectin might affect the attachment, binding, entry, and the early event for the viral replication. Same results were obtained by Weller et al., and Tan et al. [42,52].

The role of lectin in the deposition of a virus may interfere directly with crucial steps in the viral infection process (e.g. receptor binding), but can also trigger complement receptor-mediated uptake of the pathogen into immune cells. In addition, for enveloped viruses, complement activation may result in membrane attack complex (MAC) formation on the viral envelope and subsequent virolysis [54].

### Isolation of gene(s) encoding *E. pulcherima* lectin by PCR

Results showed that electrophoretic analysis of PCR product on 1% agarose gel for the genomic DNA of *E. pulcherima* (Figure 13) using the primers mentioned previously, detected two different sized genes in the PCR amplified product with size lengths 430, and 2100 respectively, indicating that the gene encoding E. lectin belongs to multi gene family.

## Clinical Implications

The biological research on the Euphorbia species has supported the use of some plants in the traditional medicines as well as creating new medicines. In addition to the observed biological activities of euphorbia species noted from previous research, include anti-inflammatory, antiseptic, analgesic, anti-microbial activities [55-58], this study found out that they also have anti-cancer and anti-viral activities, and given the experiments used in this study lectin can be extracted, and purified from euphorbia, and then according to its physical characterizations studied here be used to form a drug.

## Limitation of the Study

This research studied the antiviral and antiproliferative activity of the Euphorbia lectin against HSV1 and cancer cell line correspondingly, using methods such as cytotoxicity and so forth, to prepare and test this lectin to be used as a drug though it has to be tested on many cancer cell-lines.

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