Published in final edited form as:
Phytother Res. 2010 August ; 24(8): 1132–1140. doi:10.1002/ptr.3085.

In vitro antiviral activity of neem (*Azardirachta indica* L.) bark extract against herpes simplex virus type-1 infection

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Abstract

Herpes simplex virus type-1 (HSV-1) causes significant health problems from periodical skin and corneal lesions to encephalitis. We report here that an aqueous extract preparation from the barks of neem plant *Azardirachta indica* acts as a potent entry inhibitor against HSV-1 infection into natural target cells. The extract from neem bark (NBE) significantly blocked HSV-1 entry into cells at concentrations ranging from 50 to 100 μg/ml. The blocking activity of NBE was observed when the extract was pre-incubated with the virus but not with the target cells suggesting a direct anti-HSV-1 property of the neem bark. Further, virions treated with NBE failed to bind the cells which implicate a role of NBE as an attachment step blocker. Cells treated with NBE also inhibited HSV-1 glycoprotein mediated cell to cell fusion and polykaryocytes formation suggesting an additional role of NBE at the viral fusion step. These finding open a potential new avenue for the development of NBE as a novel anti-herpetic microbicide.

Keywords

Azardirachta indica; neem; plant antiviral; herpes simplex type-1; virus-cell interaction

INTRODUCTION

Herpes simplex virus (HSV) infections are extremely widespread in the human population. The virus causes a broad range of diseases ranging from labial herpes, ocular keratitis, genital disease and encephalitis (Liesegang *et al.*, 1989; Roizman and Sears, 1996; Whitley *et al.*, 1998; Whitely and Kimberlin, 1997). The herpetic infection is a major cause of morbidity especially in immunocompromised patients. Following initial infection in epithelial cells, HSV establishes latency in the host sensory nerve ganglia (Knickelbein *et al.*, 2008; Snyder *et al.*, 2008; Spear, 1993). Over 90 % of the trigeminal ganglia examined in a post-mortem study of the American population contained HSV type-1 (HSV-1) (Hill *et al.*, 2008). The virus emerges sporadically from latency and causes lesions on mucosal epithelium, skin, and the cornea, among other locations. Prolonged or multiple recurrent episodes of corneal infections can result in vision impairment or blindness, due to the development of herpetic stromal keratitis (HSK) (Kaye *et al.*, 2000). HSK accounts for 20–48% of all recurrent ocular HSV infections leading to significant vision loss (Liesegang *et*

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al., 1989; Liesegang, 2001). HSV infection may also lead to other diseases including retinitis, meningitis, and encephalitis.

HSV entry into host cells initiates the primary infection. It is a multi-step process that is initiated by specific interaction of viral envelope glycoproteins and host cell surface receptors (Spear, 1993; Spear *et al*., 2000). Both HSV-1 and highly related HSV-2 use envelope glycoproteins B and C (gB and gC, respectively) to mediate their initial attachment to cell surface heparan sulfate proteoglycans (HSPG) (WuDunn and Spear, 1989; Shieh *et al*., 1992; Herold *et al*., 1991). The initial binding of HSV to HSPG likely precedes a conformational change that brings viral glycoprotein D (gD) to the binding domain of host cell surface gD receptors (Whitbeck *et al*., 1999; Krummenacher *et al*., 1998, 1999, 2000). Thereafter, a concerted action involving gD, its receptor, three additional HSV glycoproteins; gB, gH, and gL, and possibly an additional gH co-receptor trigger fusion of the viral envelope with the plasma membrane of host cells (Scanlan *et al*., 2003; Spear and Longnecker, 2003; Perez-Romero *et al*., 2005). Subsequently viral capsids and tegument proteins are released into the cytoplasm of the host cell.

HSV gD receptors are cell-surface molecules derived from three structurally unrelated families. These include a member of the tumor necrosis factor (TNF) receptor family, two members of the nectin family of receptors, and the product of certain 3-*O sulfotransferases (3-O*STs) called 3-*O*-sulfated heparan sulfate (3-*O*S HS) (Shukla *et al*., 1999; Shukla and Spear, 2001; Spear and Longnecker, 2003). Herpesvirus entry mediator (HVEM or TNFRSF14) principally mediates the entry of HSV-1 and HSV-2 (Montgomery *et al*., 1996; Marsters *et al*., 1997; Kwon *et al*., 1997) into human T lymphocytes and is expressed in many fetal and adult human tissues including the lung, liver, kidney, and lymphoid tissues (Montgomery *et al*., 1996). We recently reported the requirement of HVEM receptor for HSV-2 entry in ocular cells of human origin (Tiwari *et al*., 2007). Nectin-1 and nectin-2, also known as herpesvirus entry proteins C and B (HveC and HveB) respectively are cell-adhesion molecules that belong to the immunoglobulin superfamily (Cocchi *et al*., 1998; Milne *et al*., 2001; Shukla *et al*., 2000). Both nectin-1 and nectin-2 mediate entry of HSV-1 and HSV-2, but only nectin-1 mediates bovineherpesvirus-1 (BHV-1) entry (Martinez and Spear, 2002; Warner *et al*., 1998). HSV-1 entry mediated activity of nectin-2 is limited to some mutant strains only (Warner *et al*., 1998; Lopez *et al*., 2000). Nectin-1 is extensively expressed in human cells of epithelial and neuronal origin (Richart *et al*., 2003; Tiwari *et al*., 2008), while nectin-2 is widely expressed in many human tissues, but only with limited expression in neuronal cells and keratinocytes. The non-protein receptor, 3-*O*S HS, is expressed in multiple human cell lines (e.g. neuronal, endothelial cells and human corneal fibroblasts) and mediates entry of HSV-1, but not HSV-2 (Shukla *et al*., 1999; Shukla and Spear, 2001; Tiwari *et al*., 2004; Tiwari *et al*., 2006; Tiwari *et al*., 2007).

The bark of neem plant [*Azardirachata indica* Linn (Meliaceae)] has been widely used as a traditional medicine for many centuries in tropical countries. Earlier studies have indicated that stem bark of neem contains some substance with strong anti-inflammatory activity (Murthy *et al*., 1978). Various preparations of neem obtained from its different parts have been found to exert antibacterial, antiviral, ant malarial, antioxidant, antifungal, anti mutagenic, anticarcinogenic, contraceptive and antiulcer activity (Subapryya and Nagini, 2005). The previous reports have documented that neem extracts significantly inhibited the polio virus, HIV, coxackie B group virus, and dengue virus at early step of viral genome replication (Badam *et al*., 1999; Parida *et al*., 2002; Rai and Sethi, 1972, Rao *et al*., 1969, Reddy and Sethi, 1974; Upadhyay *et al*., 1993; Sai Ram *et al*., 2000). Additionally, neem extract act as a vriucidal agent against coxsackiervirus virus B-4 as suggested by virus inactivation and yield reduction assay besides interfering at an early event of its replication cycle (Badam *et al*., 1999). Similarly, virus inhibition and RT-PCR assays confirmed

inhibitory potential of neem extract at virus replication step for Dengue virus type-2 (Parida *et al.*, 2002).

Inhibition of HSV-1 at the stage of viral entry provides a unique opportunity for therapeutic intervention. In this present study we report *in vitro* anti HSV-1 activity of neem bark extract (NBE) in the natural target cells. The pre-incubation of HSV-1 virus with NBE significantly blocked viral attachment and entry process. In addition, NBE further blocked the viral spread during cell-to-cell fusion assay and polykaryocytes formation. This finding opens a potential new avenue for the development of NBE as anti-herpetic microbicide.

MATERIALS AND METHOS

Plant extract

The stock (1 mg/ml) of neem bark powder (Thera Neem; Organi X South Inc. FL) was dissolved in 1× dilution of phosphate buffer saline (PBS) in a 50 ml tube and kept on a rotator shaker at 4°C for overnight. Red brown supernatant obtained after centrifugation at 3000 rpm (10 min) was filtered through 0.22 μm membrane filter (Millipore, US) and stored at −20°C until used.

Cells, plasmids and viruses

Wild-type Chinese hamster ovary (CHO-K1) cells provided by P. G. Spear (Northwestern University, Chicago) were grown in Ham's F12 (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), while African green monkey kidney (Vero) cells were grown in Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen) supplemented with 5% FBS. CHO-IEβ-8 cells, obtained by stable transfection of CHO-K1 cells with pMLP01, express the *Escherichia coli lacZ* gene under control of the HSV-1 ICP4 promoter (Montgomery *et al.*, 1996). Cultures of HeLa and retinal pigment epithelial (RPE) cells were grown in L-glutamine containing DMEM (Invitrogen Corp.) supplemented with 10% FBS. As previously described, cultures of human corneal fibroblasts (CF; provided by Dr. Beatrice Yue, University of Illinois at Chicago) were grown in DMEM media supplemented with 10% FBS and 5% calf serum (CS) (Tiwari et al., 2006). Recombinant β-galactosidase-expressing HSV-1(KOS) gL86 were used. Dr. P.G. Spear also provided additional strains of HSV-1 viruses (F, MP, 17; Dean *et al.*, 1994) as well as the plasmids expressing HVEM (pBec10), nectin-1 (pBG38) and 3-OST-3 (pDS43) used throughout this study. GFP expressing HSV-1 (K26GFP) was provided by P. Desai (Johns Hopkins University, Baltimore). The viral stocks were propagated at low multiplicity of infection (MOI) in complementing cell lines, tittered on Vero cells and stored at −80°C.

Cytotoxic assay

The cytotoxicity level of NBE to Vero cells was evaluated to ensure that it showed no cytotoix effect on cell viability at concentration that blocked HSV-1 infection. The assay was performed using 96-well microtiter plates. Increasing concentration of (10–6400 μg/ml) of the NBE were added to monolayers of Vero cells in triplicate and the plates were incubated at 37°C in a 5% CO_2. After 6 hrs, MTT (3-[4,-5-dimethylthiazol-2-yl]-2-diphenyltetrazolium bromide) was added as previously described. The absorbance at 570 nm was measured using a 96-well plate ELISA reader. The 50% cytotoxic concentration (CC_{50}) of NBE was calculated as previously described (Weislow *et al.*, 1989).

Viral entry assay

Viral entry assays were based on quantitation of β-galactosidase expressed from the viral genome in which β-galactosidase expression is inducible by HSV infection. Cells were transiently transfected in 6-well tissue culture dishes, using Lipofectamine 2000 (Invitrogen)

with plasmids expressing HSV-1 entry receptors (necitn-1, HVEM and 3-*O*ST-3 expression plasmids) at 1.5 μg per well in 1 ml. At 24 hr post-transfection, cells were re-plated in 96-well tissue culture dishes (2×10^4 cells per well) at least 16 hr prior to infection. Cells were washed and exposed to serially diluted pre-incubated virus with NBE or with 1 × PBS at two fold dilutions for 2 hr at room temperature. In parallel experiments the cells were also pre-incubated with NBE for 2 hr at room temperature before infected with virus. Later the cells were washed with 1 × PBS and allowed 6 hr at 37 °C before solubilization in 100 μl of PBS containing 0.5% NP-40 and the β-galactosidase substrate, o-nitro-phenyl-β-D-galactopyranoside (ONPG; ImmunoPure, PIERCE, Rockford, IL, 3 mg/ml). The enzymatic activity was monitored at 410 nm by spectrophotometry (Molecular Devices spectra MAX 190, Sunnyvale, CA) at several time points after the addition of ONPG in order to define the interval over which the generation of the product was linear with time. Inhibitory effect of NBE on HSV-1 entry in cells were also confirmed by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining. The cells were grown in Lab-Tek chamber slides. After 6 hr of infection with reporter virus treated with NBE or left untreated with1 × PBS, cells were washed with PBS and fixed with 2% formaldehyde and 0.2% glutaradehyde at room temperature for 15 min. The cells were then washed with PBS and permeabilized with 2 mM $MgCl_2$, 0.01% deoxycholate and 0.02% Nonidet NP-40 for 15 min. After rinsing with PBS, 1.5 ml of 1.0 mg/ml X-gal in ferricyanide buffer was added to each well and the blue color developed in the cells was examined. Microscopy was performed using 20 × objective on an inverted microscope (Zeiss, Axiovert 100M). The slide book version 3.0 was used for images. All experiments were repeated a minimum of three times unless otherwise noted.

Viral binding assay

Purified green fluorescent protein (GFP)-expressing HSV-1 (K26-GFP)pre-incubated with NBE (50–100 μg/ml) or with 1 × PBS for 90 min at room temperature were challenged to the gD receptor expressing CHO-K1 cells or naturally susceptible cells (HeLa, Vero and human CF) grown in 96-well assay plates (BD Falcon). All cells were incubated at 4 °C for 1 hr, washed five times to remove unbound virus, and finally replaced with warm medium for further incubation. Viral binding measured as relative fluorescence units (RFU) per treatment was determined using GENios Pro plate reader (TECAN) at 480-nm excitation and 520-nm emission spectrum. Measurements of three replicates of NBE treated and untreated samples were performed. Data were expressed as mean ± SD.

Viral glycoprotein induced cell-to-cell fusion assay

In this experiment, the CHO-K1 cells (grown in F-12 Ham, Invitrogen) designated “effector” cells were co-transfected with plasmids expressing four HSV-1(KOS) glycoproteins, pPEP98 (gB), pPEP99 (gD), pPEP100 (gH) and pPEP101 (gL), along with the plasmid pT7EMCLuc that expresses firefly luciferase gene under the T7 promoter (Pertel *et al.*, 2001). Wild-type CHO-K1 cells express cell surface HS but lack functional gD receptors, therefore transiently transfected with HSV-1 entry receptors. Wild type CHO-K1 cultured cells expressing HSV-1 entry receptors (nectin-1, HVEM and 3-*O*ST-3) considered as “target cells” were co-transfected with pCAGT7 that expresses T7 RNA polymerase using chicken actin promoter and CMV enhancer. The untreated effector cells expressing pT7EMCLuc and HSV-1 essential glycoproteins and the target cells expressing gD receptors transfected with T7 RNA polymerase were used as the positive control. Effector cells pre-incubated with NBE (50–100 μg/ml) were used for the test. For fusion, at 18 hr post transfection, the target and the effector cells were mixed together (1:1 ratio) and co-cultivated in 24- well dishes. The activation of the reporter luciferase gene as a measure of cell fusion was examined using reporter lysis assay (Promega) at 24 hr post mixing.

Quantification of polykaryocytes

In this experiment CHO-K1 effector cells expressing four HSV-1(KOS) glycoproteins (gB, gD, gH-gL) along with the plasmid pT7EMCLuc that expresses firefly luciferase gene under the T7 promoter were either pre-incubated with NBE (50 μg/ml) or with 1 × PBS for 90 min. This incubation was followed by co-culture of effector cells with target cells expressing gD receptor (nectin-1, HVEM and 3-*O*ST-3) with pCAGT7 that expresses T7 RNA polymerase using chicken actin promoter and CMV enhancer. Both populations of effector and target cells were cultured in 1:1 ratio for 24 hrs. The cells were then fixed and Gimesa stained for microscopic visualization at 40 × magnification. A group of multinucleate cells (10–15 joint cells) were scored positive for polykaryocytes formation as previously described (Tiwari et al., 2007).

RESULTS

Neem bark extracts (NBE) significantly blocks HSV-1 entry into glycoprotein D (gD) receptor expressing CHO-K1 cells

First, to assay for cytotoxic effects of NBE, Vero cells were incubated with increasing amounts (10–6400 μg/ml) of the plant extract. The viability of the treated cultures was investigated using the MTT method. The result indicated that extract concentrations up to 1000 μg/ml did not impair at all the cell viability (Fig. 1). Next, we determined the effect of NBE on HSV-1 entry into target cells. Chinese hamster ovary (CHO-K1) cells expressing gD receptors (nectin-1, HVEM or 3-*O*ST-3) were infected with β-galactosidase expressing HSV-1 reporter virus (gL86) pretreated with NBE or mock treated. As shown in Fig. 2, pre-treatment with NBE significantly blocked HSV-1 entry in a dose dependent manner in gD receptor expressing CHO-K1 cells. In contrast, the control cells treated with 1× PBS (untreated) showed HSV-1 entry. The blocking activity of NBE was pronounced even at lower concentrations (0.1 mg/ml or 100 μg/ml) and as our results indicate, effective with all gD receptors used.

NBE significantly blocks HSV-1 entry into naturally susceptible cells

To confirm blocking activity of NBE on HSV-1 entry, we used natural susceptible cells such as HeLa, Vero and retinal pigment epithelial (RPE) cells. Recently we showed that RPE cells express gD receptor nectin-1 to allow HSV-1 entry (Tiwari *et al.*, 2008). As shown in Fig. 2 (panel B) compared to the mock treated cells, the HSV-1 treated with NBE (0.1 mg/ml or 100 μg/ml) or even lower dilutions showed significant blocking of HSV-1 entry in all cell types tested. These results were consistent with the previous observations made with CHO-K1 cells expressing gD receptors. We further confirmed the above results by X-gal assay. In this experiment we used both CHO-K1 cells expressing nectin-1, HeLa cells and primary cultures of human corneal fiborbalsts (CF). HeLa cells express all known gD receptors while CF exclusively express HVEM and 3-*O*ST-3 (Tiwari *et al.*, 2005; Tiwari *et al.*, 2006; Tiwari *et al.*, 2008). As demonstrated in Figure 3, the HSV-1 treatment with NBE significantly reduced the number of blue cells in CHO-K1 cells expressing nectin-1, HeLa and CF cells (panels A1–C1). In contrast, mock-treated virions were able to infect fine as 100% cells turned blue (panels A–C). Taken together, the results highlight the HSV-1 entry blocking ability of NBE, which appears to be receptor independent.

Anti-HSV-1 entry activity of NBE is not viral strain specific

The next question was to evaluate the broader significance of NBE as an anti-HSV agent. We therefore, tested the ability of NBE to block viral entry in different but more virulent strains of HSV-1 (F, G, MP and 17 strains) (Dean *et al.*, 1994). Here we used nectin-1 expressing CHO Ig8 cells that express β-galactosidase upon viral entry (Montgomery *et al.*,

1996). The high virulence HSV-1 strains were pre-incubated with NBE and were subsequently used to infect the cells. The results again showed that NBE blocked entry of different HSV-1 strains in a dosage dependent manner as evident by the ONPG-based entry assay (Fig. 4; panel A). Similarly, X-gal assay supported the above results as the untreated virions were able to infect virtually all the cells while the NBE treated F-strain HSV-1 virions were unable to infect cells (Fig. 4; panel B).

NBE interacts with HSV-1 envelope glycoproteins

We next determined whether the inhibitory activity of NBE on HSV-1 entry was attributed to target cells or viral particles. CHO-K1 cells expressing nectin-1 receptor and the natural target HeLa cells were pre-incubated with NBE or mock treated with 1 × PBS for 90 min followed by HSV-1 infection at 25 pfu/cell. In parallel we used NBE treated (100 μg/ml) HSV-1 (KOS) gL86 to infect CHO-nectin-1 and HeLa cells. Interestingly, as shown in Figure 5, pre-incubation of CHO-K1 expressing nectin-1 (panel A) and HeLa cells (panel B) with NBE had a minor or no effect on HSV-1 entry, while virus incubation with NBE significantly blocked HSV-1 entry into both CHO-K1 cells expressing nectin-1 (panel A) and HeLa cells (panel B). This data suggesting that anti-HSV-1 activity of NBE is due to the effect on viral particles.

NBE significantly affects HSV-1 binding to the cells

Because NBE blocked HSV-1 entry, we next tested its ability to affect viral binding to the cells. To determine the difference between NBE (100 μg/ml) treated viruses versus untreated ones on viral attachment or binding we used GFP-expressing HSV-1 (K26GFP). As shown in Fig. 6 the GFP signal on cell surface was significantly weaker when virus was treated with NBE in both gD expressing CHO-K1 cells (panel A) and natural target cells (panel B). The untreated virus had very high viral binding read out in the cell types. This data indicated that NBE significantly affected viral entry at the attachment step. In a parallel experiment similar findings were obtained using flow cytometry (Fig. 6; panel C).

NBE treatment inhibits HSV-1 glycoprotein mediated cell to cell fusion and polykaryocytyes formation

Finally, we tested the effect of NBE during HSV-1 glycoproteins mediated cell to cell fusion. Cell to cell fusion has been studied to demonstrate the viral and cellular requirements during virus-cell interactions and also as means of viral spread. We sought to determine whether interaction between NBE and HSV-1 envelope glycoproteins that are essential for viral entry affects cell to cell fusion. Surprisingly, effector cells expressing HSV-1 glycoproteins treated with NBE (100 μg/ml) impaired the cell to cell fusion in both CHO-K1 cells expressing gD specific receptors (Fig. 7; white bars in panel A). In parallel the control untreated effector cells co-cultured with target cells had high fusion (Fig. 7; black bars in panel A). This response was further observed when polykaryocytes formation was estimated. Again NBE treated-effector cells failed to form polykaryons when co- cultured with target cells (Fig. 7; panel B; right panel), while in control untreated effector cells efficiently showed larger polykaryons (Fig. 7; panel B; left panel). Similar counts in polykaryons were noticed in NBE-treated cells (Fig. 7; panel C; white bars) versus mock treated cells (Fig. 7; panel C; black bars). Fusion of the membrane between virion envelop and plasma membrane of the target cell requires glycoproteins (gB, gD, gH-gL) or in combination of all of them (Roizman and Sears, 1996; Spear, 1993). Our results indicate that the presence of NBE significantly reduced the viral attachment and penetration. We therefore propose that NBE possibly interferes with viral envelope glycoproteins thereby preventing the binding and fusion process.

DISCUSSION

Our understanding on the molecular mechanisms of viral entry into cell provides a unique opportunity to generate novel ways of preventing viral cell interactions for novel therapeutic interventions (Dimitrov, 2004; Sieczkarski and Whittaker, 2005). We used an aqueous extract from neem bark (NBE) from *A. indica* to test its ability to block HSV-1 entry. Plants have been used as folk remedies, and many are now being collected and examined in an attempt to identify possible source of antiviral agents. It has been suggested that natural products are preferable to synthetic compounds (Vanden Berghe *et al.*, 1986; Vlietinck and Vanden Berghe, 1991). A large number of small molecules, like phenolics, polyphenols, terpenes, flavinoids, and sugar containing compounds from plants have been reported against herpes simplex viruses (Khan *et al.*, 2005). NBE is widely used to prepare a number of skin and personal hygiene products. It is a multi functional as well as multi utility natural product and without any side effects. The bark contains 3.43% protein, 0.68% alkaloids and 4.16% minerals. Polysaccharides in NBE possess anti-tumor as well as anti-inflammatory properties.

This study identifies a potent anti-HSV activity in aqueous extracts (NBE) from dried bark of neem plant. NBE significantly inhibited HSV-1 entry and viral glycoprotein mediated cell-to-cell fusion in a cell culture model. Further, the anti-HSV-1 activity of NBE was not limited to particular receptor or the viral strain tested. Our results showed that HSV-1 entry was significantly blocked in CHO-K1 cells expressing either protein receptors (nectin-1 and or HVEM) or a sugar receptor (3-*O*ST-3 modified 3*O*S HS). Similar blocking was also observed in a natural target CF cells isolated from human cornea which expresses 3*O*S HS (Tiwari *et al.*, 2006). HSV-1 is involved in several ocular diseases (Liesegang, 2001) including herpes stromal kertatitis which is leading cause of corneal blindness. Therefore, anti-HSV plant extract have significant relevance in infectious epithelial keratitis, stromal keratitis and endothelitis.

Interestingly, the blocking activity of NBE was only seen when HSV-1 virions were pre-incubated with the extract as compared to pre-incubation with target cells. This result suggested two important points; 1, the tested concentrations of NBE was not cytotoxic to the cells as the viral entry was quantified when NBE was pre-incubated with the target cells, and 2, the inhibitory effect of NBE targets the virions as virucidal effect was seen when the extract was incubated with the virus (Fig. 5). Future studies will be needed to isolate, detect and characterize the active molecule present in NBE which is responsible for anti-herpes activity. Whether the activity of *A. indica* is against all herpesvirus infections or is specific to HSV-1 needs to be tested. In addition, NBE-anti herpes activity may be evaluated against individual HSV-1 glycoprotein. Further characterization of the molecule and its toxicity will aid in the development of the therapeutic agents against herpes infection.

Acknowledgments

We thank Dr. P.G. Spear (Northwestern University, Chicago) for providing reagents. An institutional grant support from Western University of Health Sciences (WUHS) to V.T. is acknowledged. D.S. is a recipient of Lew Wasserman Merit award from Research to Prevent Blindness, USA.

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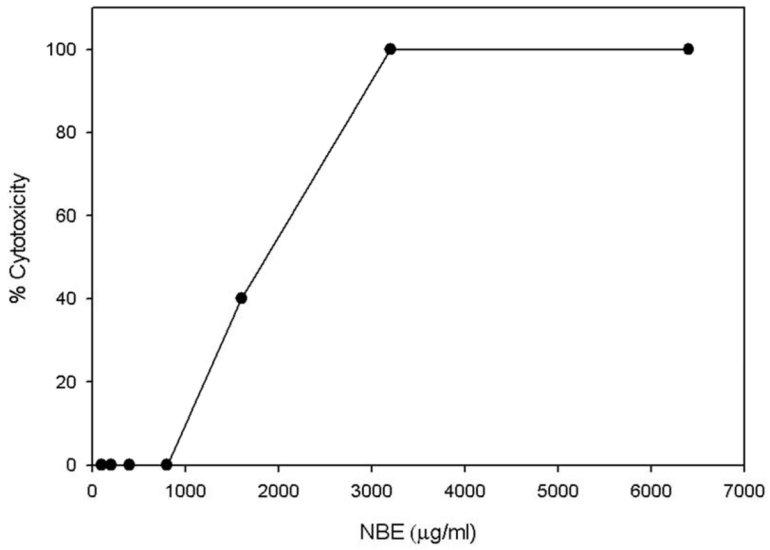

Figure 1. Cytotoxicity of neem bark extract (NBE) from *A. indica* on Vero cells monlayers
Data are expressed as percent cytotoxicity (OD_{570} test/control) ×100. The NBE concentration at or below 1000 µg/ml was not significantly toxic to cells.

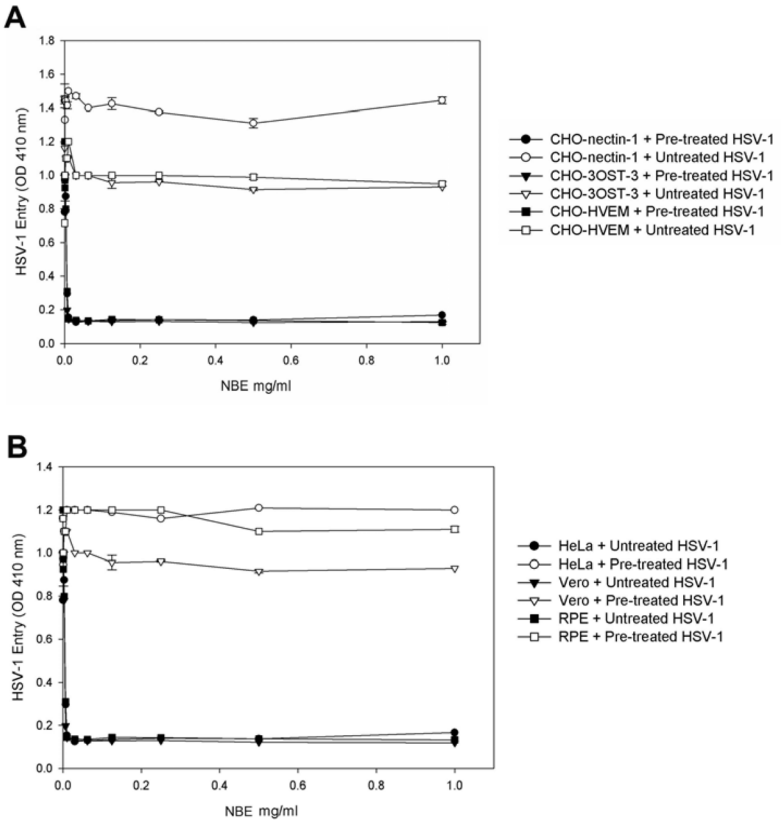

Figure 2. Neem bark extract (NBE) significantly blocks herpes simplex virus type-1 (HSV-1) entry into Chinese hamster ovary (CHO-K1) cells expressing glycoprotein D (gD) receptors

(A). In this experiment, β-galactosidase-expressing recombinant virus HSV-1 (KOS) gL86 (25 pfu/cell) was pre-incubated for 90 min at room temperature with NBE at indicated concentrations or mock-incubated with 1 × phosphate buffer saline (PBS). Subsequently the virus was incubated with CHO-K1 cells expressing gD receptors: nectin-1, HVEM and 3-*O*ST-3 expressing cells. After 6 hr, ONPG assay was performed. In this and other figures each value shown is the mean of three or more determinations (± SD). HSV-1 treated with 1 ×PBS was used as a control.

(B). Evaluation of HSV-1 entry blocking activity of NBE into natural target cells. Naturally susceptible cells (HeLa, Vero and retinal pigment epithelial; RPE) were used in this experiment. The β-galactosidase-expressing recombinant virus HSV-1 (KOS) gL86 (25 pfu/cell) was pre-incubated for 90 min at room temperature with NBE at indicated concentrations or mock treated with 1 × phosphate buffer saline (PBS). The virus was then incubated with HeLa, Vero and RPE cells. After 6 hr, ONPG assay was performed.

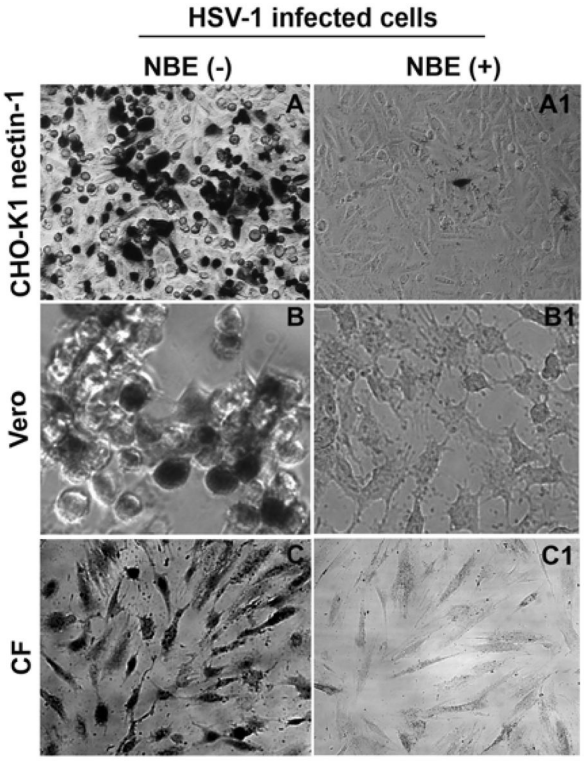

Figure 3. Confirmation of entry inhibition by X-gal (1.0 mg/ml) staining

Infection of nectin-1-expressing CHO-K1, Vero and primary corneal fibroblasts (CF) with HSV-1 (KOS gL86 at 25 pfu/cell) virus pre-incubated (50 μg/ml) for 90 min with NBE (panels A1, B1, and C1) is shown (panels A1, B1and C1). Cells infected with identical dosage of virus incubated with 1 × PBS was used as control (panels A, B and C). Dark (blue) cells represent viral entry. Microscopy was performed using a 20 × objective on Zeiss Axiovert 100. The slide book version 3.0 was used for image processing.

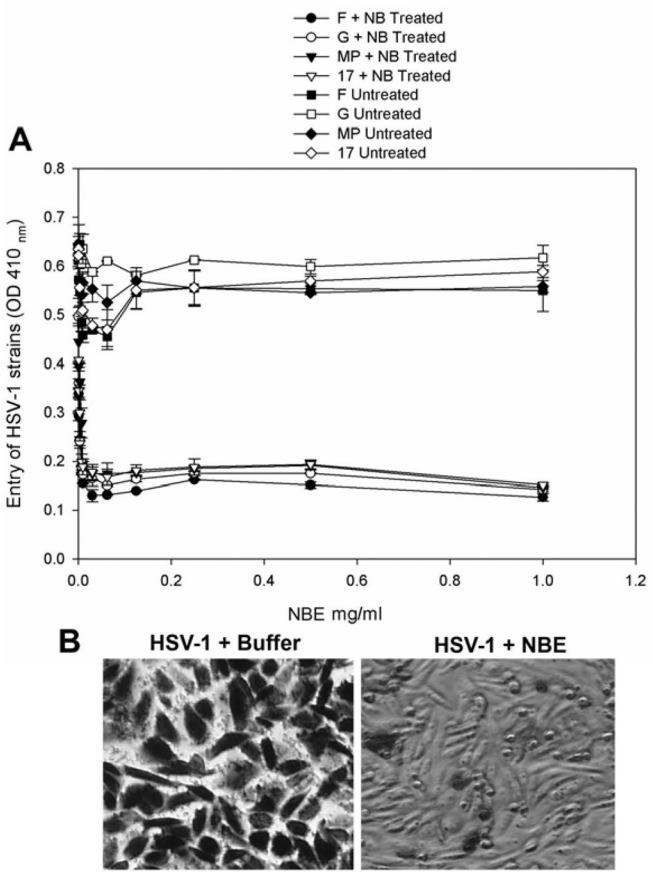

Figure 4. HSV-1 entry blocking activity of NBE is not viral-strain specific

Different strains of HSV-1 (F, G, MP and 17 strains of HSV-1 at 25 pfu/cell) were either pre-incubated with 1 × PBS (control) or with NBE at indicated concentrations for 90 min at room temperature. The two pools of viruses were incubated on CHO Ig8 cells that express β-galactosidase upon viral entry. The viral entry blocking was measured by ONPG assay (panel A) or by X-gal assay (panel B) as previously described. In X-gal assay (panel B), F-strain of HSV-1 is shown.

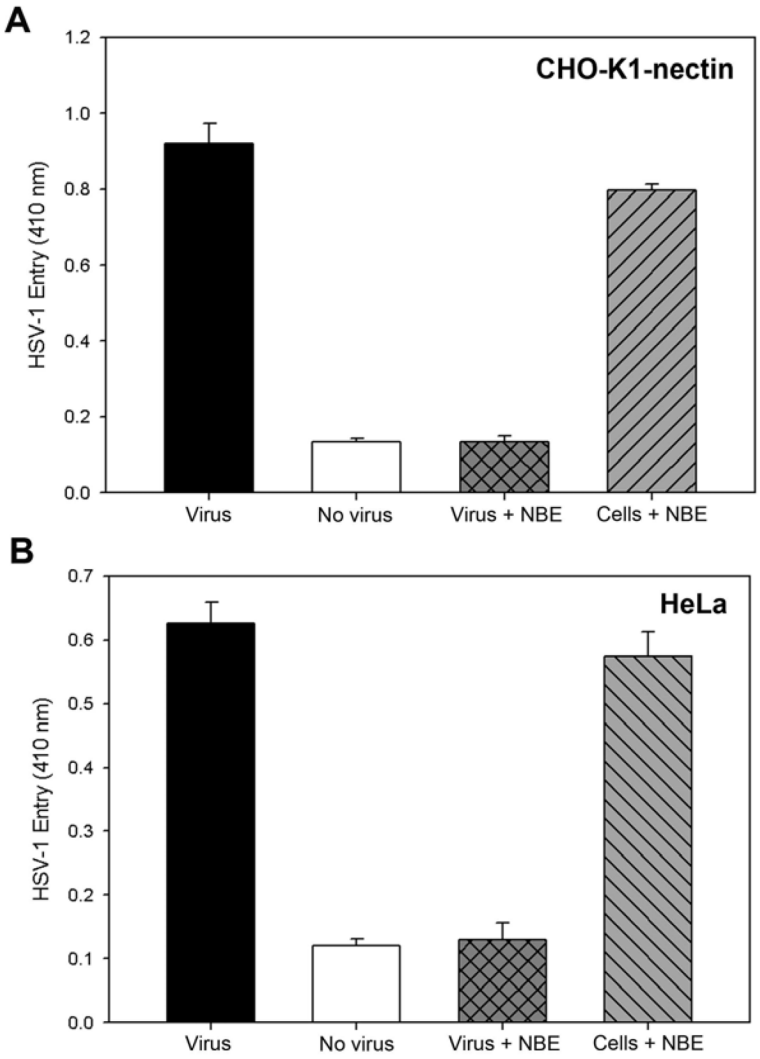

Figure 5. Pre-incubation of NBE with HSV-1 virions but not with the target cells significantly blocks HSV-1 entry

In this experiment, target CHO-K1 cell expressing HSV-1 gD receptor nectin-1 (panel A) or a natural target HeLa cells (panel B) were either pre-incubated for 90 min with 50 μg/ml NBE or treated with 1 × phosphate buffer saline (PBS as a control) before viral infection. In parallel experiment β-galactosidase-expressing recombinant virus HSV-1 (KOS) gL86 (25 pfu/cell) was similarly pre-incubated with NBE. The four sets of combinations (virus alone, no virus, pre-incubated virus with NBE and pre-incubated target cells with NBE) were used to infect CHO-K1 cells expressing nectin-1 (**A**) or naturally target HeLa cells (**B**) for 2 hrs at room temperature followed by washing of cells with PBS. After 4 hr, ONPG assay was performed. Each bar represents the value of three independent experiments.

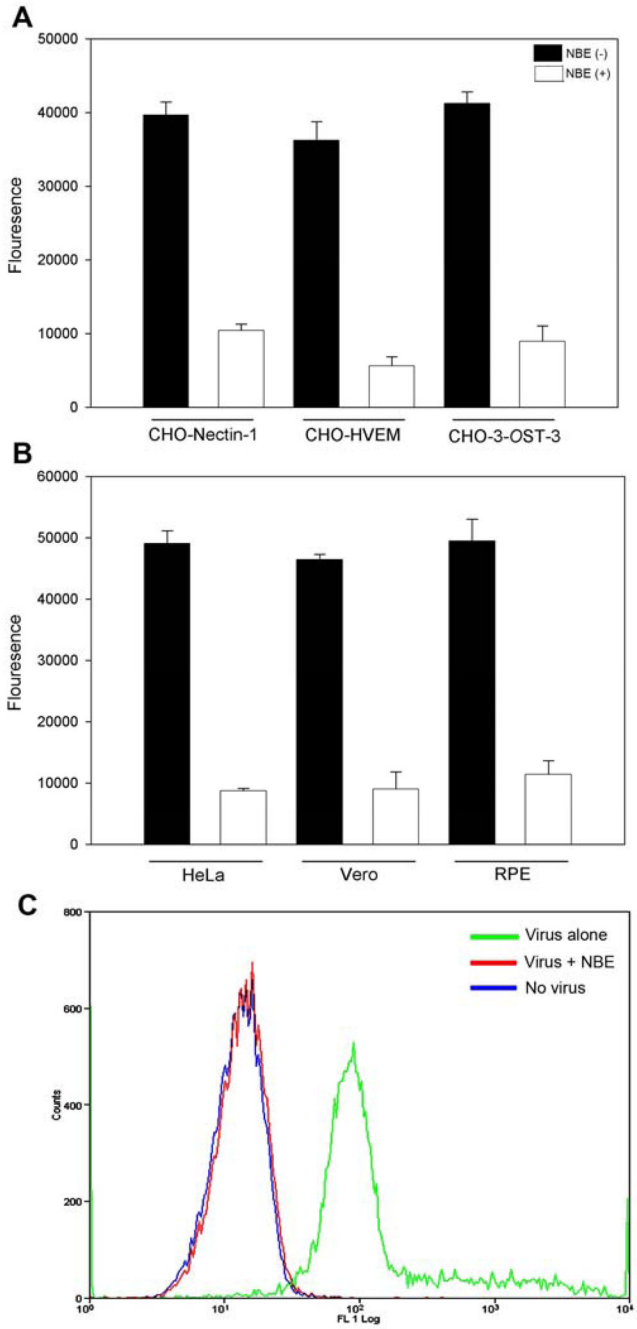

Figure 6. NBE significantly inhibits HSV-1 binding to the target cells (A–C)

Green fluorescent protein (GFP) expressing HSV-1 (K26 GFP at 25 pfu/cell) was pre-incubated with NBE (50 μg/ml; represent as white bar) or with 1 × PBS (represent as black bar) for 90 min at room temperature. The mixture was allowed to incubate with CHO-K1 cells expressing gD receptors; nectin-1, HVEM and 3-*O*ST-3 (panel A) or natural target cells; HeLa, Vero and RPE cells (panel B) for 1 hr at room temperature followed by a quick citrate buffer treatment to remove unbound viruses. The fluorescent output as a result of viral binding to the cells was recorded using Tecan spectrophotometer is presented. Each value shown is the mean of three or more determinations (± SD). GFP-expressing HSV-1 pre-incubated with 1 × PBS was used as a control. **(C).** HSV-1 binding to cells in presence

of NBE was quantified using flow cytometry analysis. GFP expressing HSV-1 virions (25 pfu/cell) pre-incubated with NBE (50 μg/ml) or treated with 1 ×PBS were cold bound to HeLa cells at 4°C. Uninfected RPE cells were used as a negative control. Cells were examined by fluorescence-activated cell sorter (FACS) analysis after 35 min of incubation with GFP-expressing HSV-1. Note that the NBE-pretreatment to GFP virions significantly impaired the ability to bind the cells (Red). Mock treated GFP-HSV-1 virions were able to bind cells (Green). Uninfected HeLa cells were used as background control (blue).

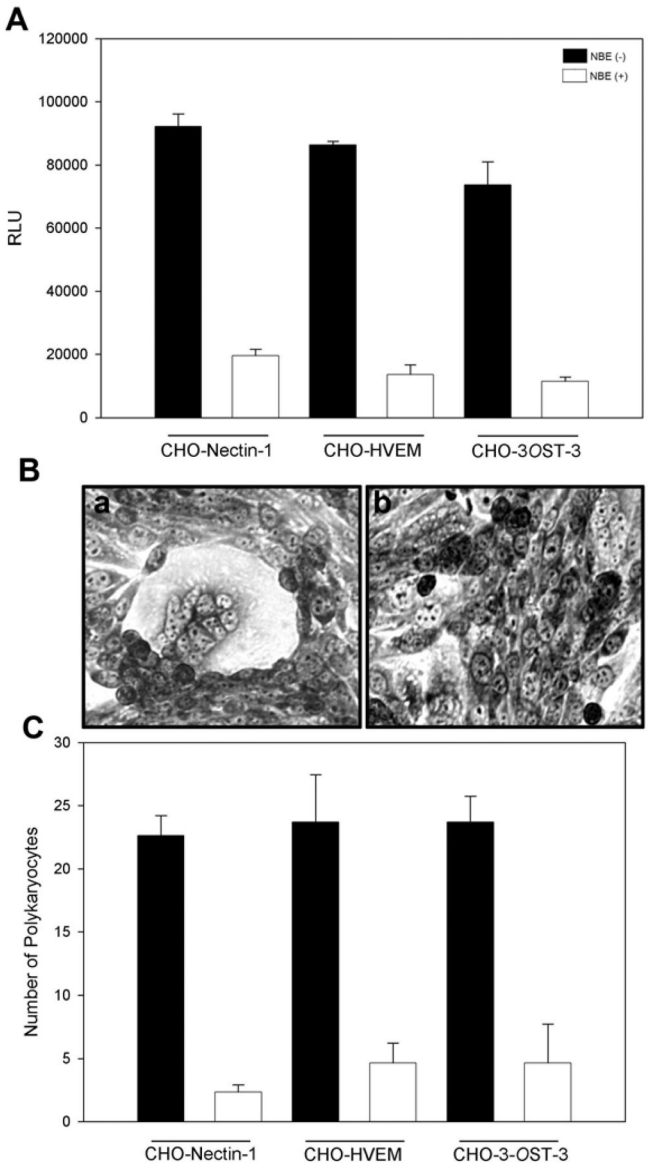

Figure 7. NBE significantly impairs HSV-1 glycoproteins induced cell to cell fusion and polykaryocytes formation

(A). The "effector CHO-K1 cells" expressing HSV-1 glycoproteins (gB, gD, gH-gL) along with T7 plasmid were pre-incubated with 100 μg/ml NBE or 1 × PBS for 90 min. The two pools of effector cells (NBE treated and PBS treated) were mixed with CHO-K1 cells expressing luciferase gene along with specific gD receptors; nectin-1, HVEM and 3-*O*ST-3. Membrane fusion as a means of viral spread was detected by monitoring the luciferase activity. Black bars and white bars represent 1× PBS treated and NBE treated cells respectively. Error bars represent standard deviations. * P< 0.05, one way ANOVA. **(B).** Microscopic visualization of polykaryocyte impairments by NBE. The effector CHO-K1 cells expressing four essential HSV-1 glycoproteins (gB, gD, gH-gL) were pre-incubated with either NBE or 1 × PBS for 90 min before they were co-cultured in 1:1 ratio for 24 hrs. The cells were fixed for 20 min. and then stained with Gimesa stain (Fluka) for 20 min. Shown are photographs of representative cells (Zeiss Axiovert 200) pictured under microscope at 40 × objective. The left panel shows cell fusion and polykaryocytes formation

in the absence of NBE, the right panel shows significant inhibition of polykaryocytes formation in the presence of NBE.

(C). In parallel polykaryocytes formation was also quantified by counting polykaryons (group of 15 cells). The panel shows number of polykaryons in mock-treated cells (black bars) versus NBE treated cells (white bars). The values shown were from one representative experiment performed in triplicate.