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**RESEARCH ARTICLE**

# Antiviral activity of Indigofera heterantha Wall. ex Brandis against Herpes Simplex Virus - type 2 (HSV-2)

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## *Abstract*

*Indigofera heterantha* Wallich ex Brandis is a commonly distributed plant species in the western Himalayan region. Here, we report for the first time, the presence of an antiviral activity in this plant species. The activity was found to reside in the root extracts of the plant as evaluated by the *in vitro* screening assays, was specific to HSV-2 virus and did not show any cross-reactivity against HSV-1 virus. Results generated from the cytopathic effect inhibition and MTT assays, demonstrated that this root extract suppressed HSV-2 multiplication in a dose-dependent fashion. The extract was evaluated in a murine genital infection model against HSV-2 as a topical formulation, using acyclovir as the comparator molecule. The plant extract exhibited potent therapeutic effect against a lethal HSV-2 challenge. It also demonstrated efficacy in a prophylactic manner and was able to protect mice when applied at least 4 hours prior to viral challenge. In comparison, acyclovir acts at the replication stage of the HSV-2 life cycle and was efficacious only when treated in the therapeutic mode. Mice treated with the extract showed significant reduction in mortality as well as low or no genital lesions as compared to placebo treated and untreated control animals. In conclusion, due to its ability to act both therapeutically and prophylactically, the extract of *I. heterantha* can be developed as a topical microbicide or condom coating for treatment of genital herpes infection.



## INTRODUCTION

Herpes viruses are enveloped viruses with approximately 150,000 base pair, linear double-stranded DNA and a virion size of about 150-200 nm (Thapa and Carr, 2008). Herpes simplex virus (HSV) types 1 and 2 are alpha herpes viruses that infect humans and are known to establish latent infections in the neuronal cells. HSV-2, the causative agent of genital ulcer worldwide is a significant co-factor in the transmission and acquisition of human immunodeficiency virus (HIV) and can result in meningitis and encephalitis especially in neonates and immunocompromised individuals. The antiviral drugs used for treatment of genital herpes infection are guanosine analogues such as acyclovir (standard of care), valaciclovir, cidofovir and famciclovir. However, it has been observed that repeated use of this class of drugs do not result in permanent clearance or long-term control of the infection (Greco et al., 2007). This reduction in the ability of the currently available synthetic drugs to effectively treat a range of viral diseases, drug related toxicity issues due to repeated usage and the concern regarding emergence of drug resistant viral strains has spurred the need for newer antivirals. Attempts have also been made to

develop vaccines, for genital herpes infection but have met with little or no success (Shestakov et al., 2012). An alternate approach would be the exploration of medicinal plants - the mainstay of traditional medicine, for their potential therapeutic benefits in alleviating various ailments, primarily due to the belief that they are safe and reliable.

A recent World Health Organization (WHO) report has estimated that 80% of the inhabitants of the world rely on traditional medicines (Abonyi et al., 2009). Plants, exhibit the ability to defend themselves in an ecological setting against insects, microbes, and others by synthesizing a rich variety of secondary metabolites such as polyphenols that may be further exploited for their antimicrobial and antiviral benefits (Yarmolinsky et al., 2009). Considerable efforts are being directed towards plants as a source of novel treatment modalities and positive results have been obtained (Docherty et al., 2005). The discovery of Veregen, the first US FDA approved drug for the topical treatment of genital warts is a defined mixture of catechins obtained from green tea. Another example from microbes is Fumagillin, an endothelial cell proliferation inhibitor isolated from *Aspergillus fumigatus* for the treatment of intestinal microsporidiosis (Mishra and Tiwari, 2011).

In our search for new antiviral compounds we identified the plant *Indigofera heterantha* from our in- house natural products plant extracts library. This plant species exhibited potent antiviral activity against HSV-2 virus. The activity was found to be present in the hydro-methanolic extract of the roots of this plant, as determined by both *in vitro* and *in vivo* efficacy studies. *Indigofera heterantha* Wall. ex Brandis (family- *Fabaceae*), the Himalayan Indigo, is one of the 700 species belonging to the genus *Indigofera* in the world and are amongst 60 species and 10 varieties in India (Shastry et al., 1995). The plant possesses antiulcerogenic, antioxidant and antibacterial activities as well as inhibitory effect against enzymes such as lipoxygenase and dehydrogenase (Uddin et al., 2011).
Some species of *Indigofera* such as *Indigofera tinctoria* (Kavimani et al., 2000) has been reported to be active against the replication of human immunodeficiency virus type 1 (strain HTLV-$III_B$LAI) and human immunodeficiency virus type 2 (strain LAV-2ROD) in HIV sensitive MT4 (metallothionein) cells. Rajkapoor et al. (2007) reported cytoprotection in infected HEL cell cultures (human embryonic lung derived cell line) and HeLa cells (human epithelial cells) with alcohol extracts from the stems of *Indigofera aspalathoides* against HSV-1, HSV-2, Vaccinia, Vesicular Stomatitis, Coxsackie and Respiratory Syncytial viruses.

## 2. Materials and methods

*2.1 Plant material*

The whole plant of *I. heterantha* or parts of the plant such as roots, twigs, stem, leaves and inflorescence were collected from the hills of Uttarakhand, India. The freshly collected plants or parts of the plant were air-dried to achieve moisture content below 10%. For taxonomic characterization, voucher specimens (AS 01788; May 17, 2008) during flowering and fruiting season were collected, identified and deposited in the departmental herbarium of Piramal Healthcare Limited, Mumbai, India.

*2.2 Preparation of hydro-methanolic root extracts*

The chopped and dried roots of *Indigofera heterantha* were pulverized with mesh size # 6-8. The coarse material (100 g) was soaked in 0.6 L of methanol and water at a ratio of 3:1, with constant stirring for 3 hours in a heating mantle at 40°C ± 5°C. The extract obtained was filtered using a Whatman No. 1 filter paper. This marc was further extracted two more times using the process of extraction described above. The three filtrates thus obtained, were pooled and concentrated using a rotary evaporator at 45°C ± 5°C under vacuum, and freeze dried to obtain the desired powdered extract. The extracts so prepared were dissolved in dimethyl sulfoxide (DMSO) (Sigma, St.Louis, USA) to a concentration of 20 mg/ml and stored at 4°C until use.

*2.3 Acyclovir*

For the *in vitro* studies, acyclovir (ACV) USP, a white crystalline powder, was purchased from Matrix Laboratories Limited, Hyderabad, India and a stock solution of 10 mg/ml in DMSO was prepared fresh before each assay.
For the *in vivo* efficacy studies, ACV cream (5% w/w), was purchased from Cipla, India and used at the desired dose per volume.

*2.4 Topical formulation*

A topical cream formulation of the *I. heterantha* hydro-methanolic root extract was prepared using GRAS ("generally recognized as safe") excipients such as white soft paraffin, beeswax, propylene glycol, glyceryl monostearate, methyl paraben, propyl paraben and purified water. *In vivo* therapeutic efficacy was evaluated with

the cream formulations at 1500, 750 and 375 mg/kg doses and prophylactic efficacy at 500 and 125 mg/kg doses respectively.

*2.5 Cells and viruses*

African green monkey kidney cells (Vero) (ATCC CCL-81) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen; Gibco, Carlbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen; Gibco, Carlbad, CA), 100X antibiotic-antimycotic liquid solution (Invitrogen; Gibco, Carlbad, CA) which contained 10,000 units of penicillin (base), 10,000 µg of streptomycin (base) and 25 µg of amphotericin B/ml utilizing penicillin G (sodium salt), streptomycin sulfate and amphotericin B as Fungizone® antimycotic in 0.85% saline. The cells were incubated at 37°C in a 5% $CO_2$ incubator.

Herpes simplex virus 2 (HSV-2) G strain (ATCC VR-734) was propagated in Vero cells. Briefly, Vero cells were infected with HSV-2 for 1 hour and harvested at 48 hrs post infection (p.i.) by centrifugation at 1000 rpm and 4°C for 10 mins. The supernatant was collected and stored at –80˚C for use. Viral titer was determined by cytopathic effect inhibition assay (CPE) and was expressed as tissue culture infectious dose 50 ($TCID_{50}$) as per the protocol followed by Lindenbach, 2004.

*2.6 In vitro* antiviral activity assays

*2.6.1 Cytopathic effect (CPE) inhibition assay*

The antiviral effect of the extracts was evaluated by the standard method of Moore et al., 1981 and Vijayan et al., 2004, with a few modifications. Vero cells were seeded into 96 well flat-bottomed tissue culture plates, at a density of 1 x $10^4$ cells/well in DMEM supplemented with 10% FBS. After incubation for 24 hrs at 37°C in a 5% $CO_2$ incubator, 100 µl of two-fold serially diluted *I.heterantha* extracts in increasing concentrations from 3.125-400 µg/ml were added in triplicates, with ACV as a positive control. After one hour, Vero cells were infected with 100 $TCID_{50}$ dose of HSV-2 per well. The extract and virus dilutions were prepared in 2% FBS-DMEM maintenance medium. Appropriate controls were included such as Vero cells alone (cell control) and Vero cells with virus (virus control). The infected cells were incubated for 48 hrs following which medium was aspirated, cell monolayer washed with 0.85% saline and stained with 0.13% crystal violet (Sigma, St.Louis, USA) solution for 30 minutes. The 96-well plates were allowed to air dry at room temperature for 24 hours, CPE was evaluated and graded (Table A.1), both visually and microscopically.

*2.6.2 Antiviral assay*

The MTT 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide assay protocol was originally described by Mosmann in 1983. The antiviral activity and cytotoxicity of the *I.heterantha* extracts was quantitatively evaluated, by this assay. Briefly, confluent monolayer of Vero cells in 96-well flat-bottomed plates were treated with different dilutions of *I. heterantha* extracts prepared in 2% FBS-DMEM, for 1 hr, with ACV as a positive control. The cells were infected with $10^3$ $TCID_{50}$ of HSV-2 virus and incubated for 48 hrs at 37˚C. 100 µl of MTT reagent (Trevigen, Inc., MD, USA) diluted in maintenance medium was added to the plates and incubated for a period of 4 hours. 100 µl detergent (Trevigen, Inc., MD, USA) was then added to the wells and left overnight at 37˚C and 5% $CO_2$. The absorbance in each well was measured at 570 nm, and the data was analyzed as antiviral activity according to the formula described by Cheng et al., 2002:

The percent antiviral activity may be expressed as $[(O.D_{TEST/SAMPLE} - O.D_{VIRUS\ CONTROL})/ (O.D_{CELL\ CONTROL} - O.D_{VIRUS\ CONTROL})] \times 100$

Where, ($OD_{TEST/SAMPLE}$) is the absorbance measured at a concentration of extract/compound in HSV infected cells, ($OD_{VIRUS\ CONTROL}$) is the absorbance measured for the control untreated HSV-infected cells, and ($OD_{CELL\ CONTROL}$) is the absorbance measured for control untreated cells without virus and the compound. The minimal concentration of extract/pure compound required to inhibit 50% HSV-2 growth ($IC_{50}$) was calculated from the data obtained by plotting a graph of concentration (µg/ml) vs. percent inhibition of CPE formation by the extract.

*2.6.3 Cytotoxicity assay*

The cell cytotoxic effect of *I.heterantha* extracts and ACV on Vero cell viability was evaluated by the MTT method. The assay was performed according to the procedure described above, without virus addition. Toxic effects of the extracts were calculated as a percentage of the reduction of viable cells in the presence of plant extract / ACV, as compared to viable cells observed in their absence. The following formulae for evaluating percent viability and toxicity (Cheng et al., 2002), were used:

% Viability = $(O.D_{TEST/SAMPLE}/ O.D_{CELL\ CONTROL})$ x 100,

% Toxicity = 100 - % viability

The 50% cell cytotoxic concentration ($CC_{50}$) was calculated from this data. To determine if each compound has sufficient antiviral activity that exceeds its level of toxicity, a selectivity index (SI) was evaluated as the ratio of ($CC_{50}$ / $IC_{50}$).

*2.7 In vivo* efficacy studies

*2.7.1 Therapeutic efficacy of the extract in a murine genital infection model against HSV-2*

Six to eight weeks old female Balb/C mice were used for vaginal inoculation with HSV-2 virus. Five days prior to intravaginal challenge, mice were injected subcutaneously (s.c) with 2 mg of progesterone (Depo-Provera®; Pfizer, Belgium) in their upper back, using a 29-gauge needle. This was done to synchronize the estrous cycle of all the study animals, prior to viral challenge. The mice were kept under pathogen-free conditions in the animal facility at the Department of Pharmacology, Piramal Healthcare Limited. The studies were approved by the Institutional Animal Ethical Committee for animal experiments. On the day of challenge, the vaginal area of each mouse was cleaned with a cotton swab soaked in 0.1N NaOH. Mice were infected with 2 x $10^4$ PFU (plaque forming units) of HSV-2 virus (ATCC, VR-734). The hydro-methanolic root extract was formulated as a topical cream and its antiviral efficacy was evaluated therapeutically. Animals were dosed 30 min. post infection (p.i.) with placebo cream 25mg/kg per day or the plant extract topical cream formulation at 375, 750 and 1500 mg/kg per day or acyclovir at 225 mg/kg per day, by topical application, at the genital area, thrice a day, for 5 days. The animals were assessed daily for extravaginal disease signs and survival for a period of 21 days p.i. The severity of the disease was quantified using a well-established lesion score scale (Table A.2). Extravaginal signs of disease were recorded daily for each animal and scored on a four-point scale (Zak and Sande, 1999; Carlucci et al., 2004).

*2.7.2 Prophylactic efficacy of the extract in a murine genital infection model against HSV-2*

A similar protocol as described above was followed with a few modifications. Briefly, on the day of challenge, mice were infected intravaginally with 6.84 x $10^4$ PFU/mouse. All the animals being dosed with plant extract topical cream formulation received topical administration of the extract (500 and 125 mg/kg) at 1, 2 and 4 hrs prior to viral infection. Animals belonging to ACV (75 mg/kg) group were dosed only once viz. 4 hrs prior to infection. A dosing volume of 25 mg of the extract formulation and 30 mg of ACV cream were topically applied on the vaginal area at the designated times using a sterile applicator stick. One infection control group was included to monitor the progress of disease, which did not receive any treatment. This control group was common to both study groups. The animals were assessed daily for extravaginal disease signs and survival for a period of 21 days post infection and scored in accordance with the scale described above.

*2.8* Statistical analysis

In the *in vitro* studies, the $IC_{50}$ and $CC_{50}$ values were determined using the PRISM software (PRISM 5.0®GraphPad Software Inc., San Diego,CA). This statistical analysis software was also used in the *in vivo* efficacy studies to determine the rate of survival and mean lesion scores within the study groups.

## 3. Result

*3.1 Antiviral and cytotoxic effects of the root extracts of Indigofera heterantha*

Primary screening of 1050 hydro-methanolic extracts from the in-house plant extract library was performed at 100 µg/ml by the standard cytopathic effect inhibition (CPE) assay. The CPE assay was carried out to evaluate the antiviral potential of these plant extracts against both HSV-1 and HSV-2 viruses. The extracts were added to Vero cells an hour prior to virus infection and positive hits were identified based on CPE gradation (Table A.1) and those with '+++'/ '++++' gradation were taken up for further evaluation. Our primary results identified the plant extract, *Indigofera heterantha* as having potent antiviral activity specifically against HSV-2 and not HSV-1 virus (data not shown). Further, fresh collections of different parts of the plant such as the root, twig, stem and inflorescence were

collected and their hydro-methanolic extracts prepared. Amongst these the hydro-methanolic root extract was found to possess the most potent antiviral activity against HSV-2 (Table A.3).

The activity and cytotoxicity of the root extract was further evaluated by the MTT dye reduction method in a dose dependent manner from 3.125-400 µg/ml. The cytotoxicity of this extract was determined to ensure that the viability of Vero cells in presence of the extract alone is not affected at the concentrations at which potent antiviral activity was observed. Fig.B.1. represents the rate of inhibition of HSV-2 replication and the related cytotoxicity with the hydro-methanolic root extract of *I. heterantha*. Potent anti HSV-2 activity was observed at 50 µg/ml and 25 µg/ml; 97.36% and 91.52%, respectively. The cytotoxicity of the extract at these concentrations was negligible. The $IC_{50}$, $CC_{50}$ and SI values were 21.93 µg/ml, 284.2 µg/ml and 13.3, respectively suggesting that the root extracts of *I. heterantha* indeed possess potent anti HSV-2 activity.

*3.2 In vivo efficacy in a murine genital infection model against HSV-2*

Based on the results obtained in our *in vitro* studies, the hydro-methanolic root extract of *I. heterantha* was explored for its *in vivo* efficacy in a therapeutic and prophylactic mode.

*3.2.1 Therapeutic efficacy study*

Topical cream formulations were prepared from the hydro-methanolic root extract of *I. heterantha* and evaluated in the HSV-2 vaginal infection model in Balb/c mice. The formulation concentrations tested were 1500, 750 and 375 mg/kg/day. Animals were assessed daily for clinical signs and symptoms, and the survival was determined by the Kaplan Meier plot. Mice treated with 1500 and 375 mg/kg/day of the extract formulation showed 100% protection against a lethal HSV-2 challenge and the dose of 750 mg/kg/day demonstrated 87.5% survival. The ACV treated animals showed 100% survival and animals from control groups such as placebo treated and infection alone showed 100% mortality between days 10 and 13 post-infection (Fig.B.4a).

The earliest sign of extravaginal infection appeared on day 7 in the placebo and infection control groups and their lesion scores continued to remain high throughout the study. The animals that were dosed with 1500 mg/kg/day of the extract formulation did not exhibit extravaginal disease or any other clinical symptom throughout entire course of the study (21 days). However, lesions and symptoms were observed in animals that were dosed with 750 and 375 mg/kg/day of the formulation, on days 8 and 13, respectively and these continued until the study was terminated on day 21. The ACV treated animals demonstrated no clinical signs of extravaginal disease (Fig.B.4b).

*3.2.2 Prophylactic efficacy study*

The *I. heterantha* root extract was evaluated in a prophylactic mode in the murine genital infection model of HSV-2. The extract was formulated into a topical cream formulation and evaluated at two doses *viz.* 500 and 125 mg/kg, at 1, 2 and 4 hrs prior to intravaginal infection with HSV-2 virus. Animals were dosed with ACV at one time-point, 4 hrs, prior to viral challenge. The mice were assessed daily for clinical signs and symptoms and survival.

The animals dosed with 500 and 125 mg/kg of the extract formulation at 1, 2 and 4 hrs prior to a lethal HSV-2 challenge showed 100% survival. Significant reduction in clinical lesions and mortality was also observed at these dose concentrations as compared to infection control. Positive control acyclovir showed only 33.3% survival at 75 mg/kg dose and an increased number of lesions and symptoms were observed between days 7 and 14 (Table A.4 and Figs.B.5a and b).

## APPENDIX A

**Table A.1.** Gradation chart for percentage antiviral activity by the cytopathic effect inhibition assay

| **Grade** | **% Antiviral activity** |
|---|---|
| - | 0-10% |
| + | 11-25% |
| ++ | 26-50% |
| +++ | 51-75% |

++++ 76-100%

**Table A.2:** Lesion score scale for *in vivo* efficacy studies

| **Score scale** | **Lesion characteristics** |
|---|---|
| 0 | No infection |
| 1 | Few isolated papules and slight redness of extravaginal tissue |
| 2 | Few isolated papules, ulcers, and/or eschar and/or swelling and redness of extravaginal tissue |
| 3 | Multiple fused ulcers/eschars, moderate swelling and redness of extravaginal tissue with extention to surrounding tissue; wetting of peritoneum cavity |
| 4 | Ulceration with severe redness and swelling of extravaginal tissue with extension to surrounding tissue, rear leg paralysis. |

**Table A.3**. *In vitro* anti HSV-2 activity of different parts of the plant *I. heterantha*

| **Plant name** | **Plant part** | **Anti HSV-2 activity (% CPE) at 100 µg/ml[a]** |
|---|---|---|
| ***Indigofera heterantha*** **Root** | **++++** | |
| *Indigofera heterantha* | Twig | +++ |
| *Indigofera heterantha* | Stem | - |
| *Indigofera heterantha* | Inflorescence | +++ |

[a] The antiviral activity is expressed as the following gradation: - ; 0 – 10%, +; 11 – 25%, ++; 26 – 50%, +++; 51 – 75%, ++++; 76 – 100%

**Table A.4**: Efficacy of prophylactic administration of the topical cream formulation of the *I. heterantha* hydro-methanolic root extract

| **Groups** | **Time of prophylactic treatment** | **Dose (mg/kg)** | **Survival observed** |
|---|---|---|---|
| **Acyclovir cream (5% w/w)** | 4 hours | 75 | 33.3% |
| | 4 hours | 500 | 100% |
| ***I. heterantha*** **topical cream formulation** | 2 hours | 125 | 100% |
| | | 500 | 100% |
| | 1 hour | 125 | 100% |
| | | 500 | 100% |
| | | 125 | 66.7% |

**APPENDIX B**

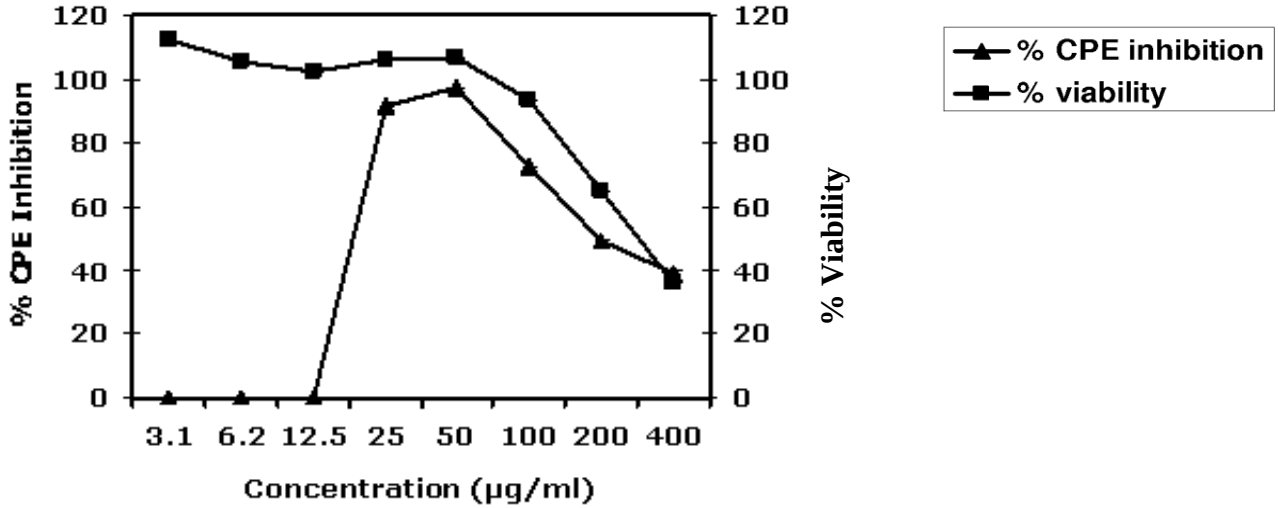



**Fig. A.1**. Effect of hydro-methanolic root extract of *I. heterantha* on HSV-2 infectivity and its corresponding cytotoxic effect on Vero cells after 48 hrs.

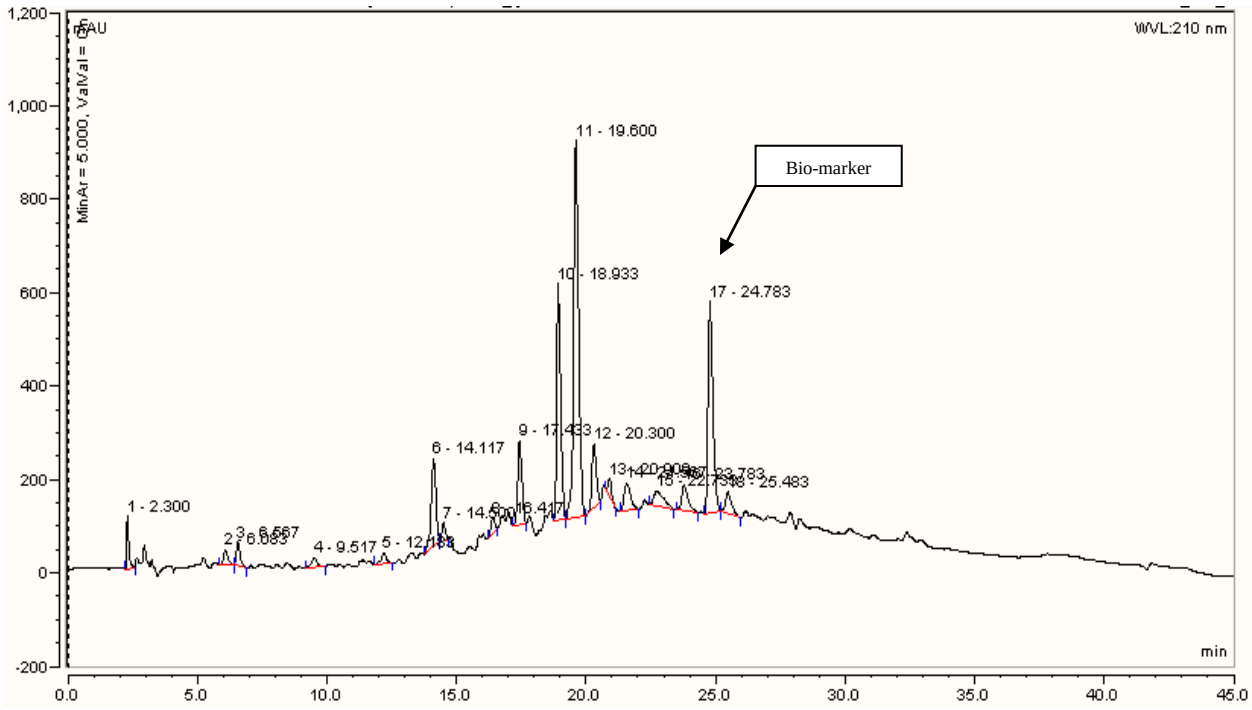



**Fig.B.2**. HPLC chromatogram of the concentrated hydro-methanolic root extract of the plant *Indigofera heterantha*

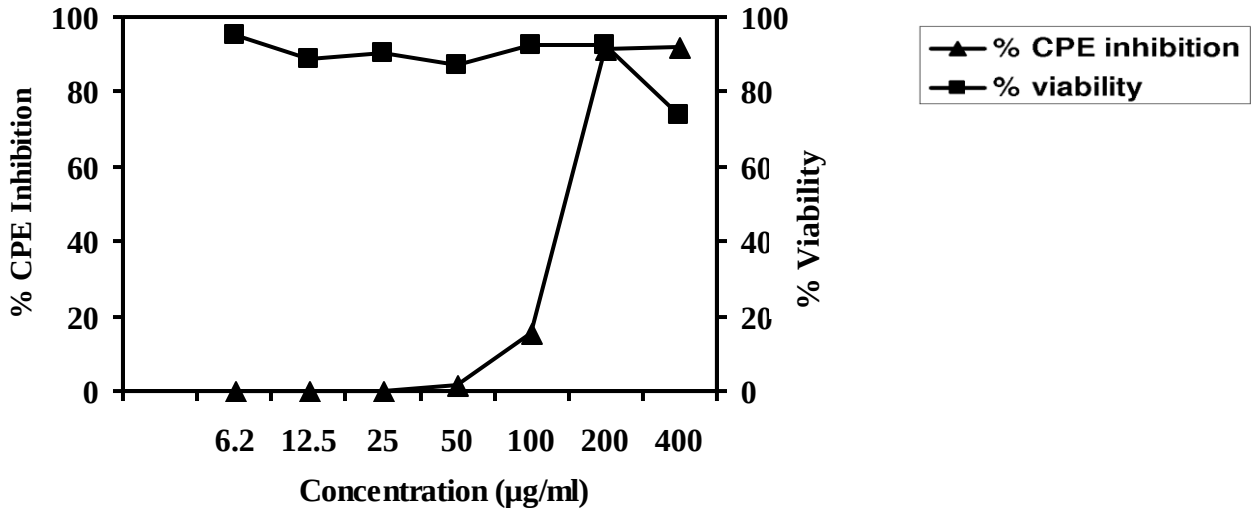



**Fig.B.3**. Graph showing the anti HSV-2 activity and cytotoxicity for the isolated biomarker peak at the retention time of 24.7 minutes.

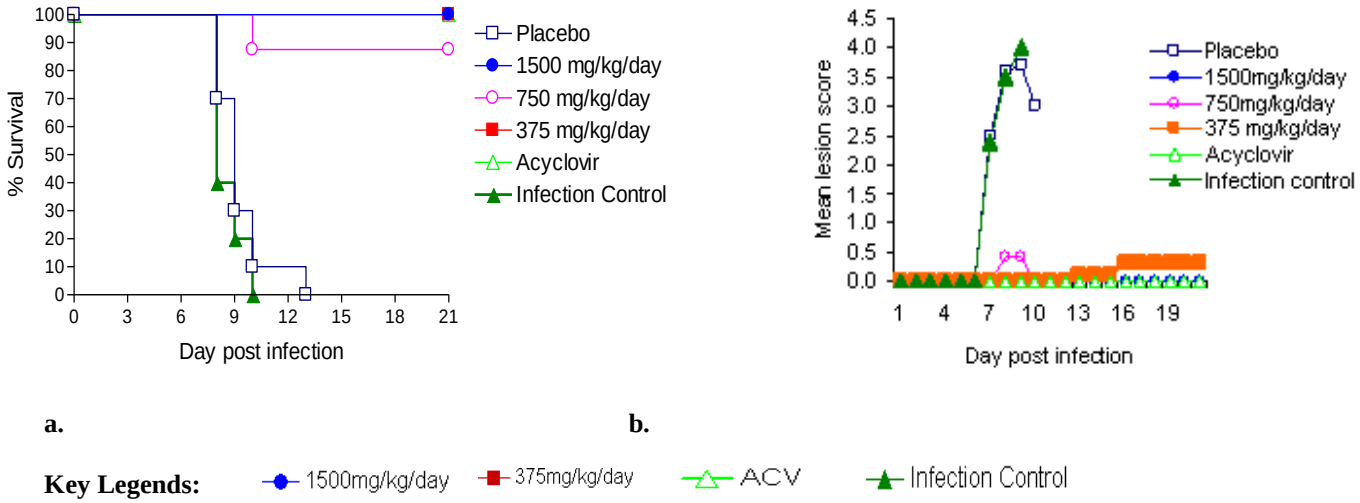
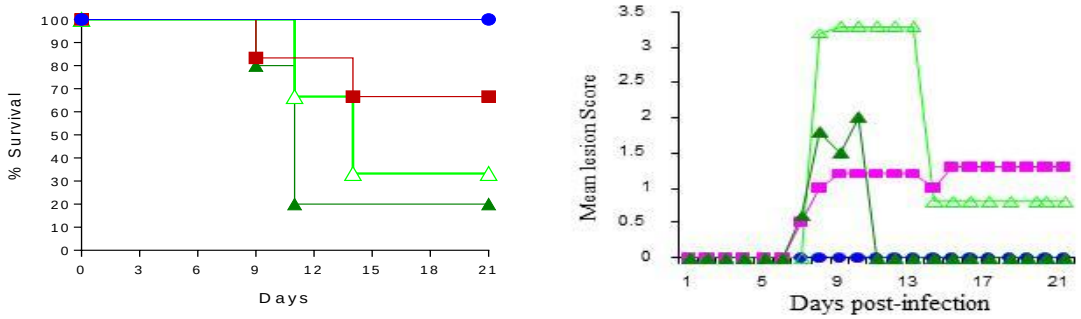



**Fig.B.4**: Therapeutic efficacy study: **a.** Percent survival and **b.** mean lesion score of a topical cream formulation of the *I. heterantha* hydro-methanolic root extract in a murine genital infection model of HSV-2.

Prophylactic efficacy of *I. heterantha:*1hour



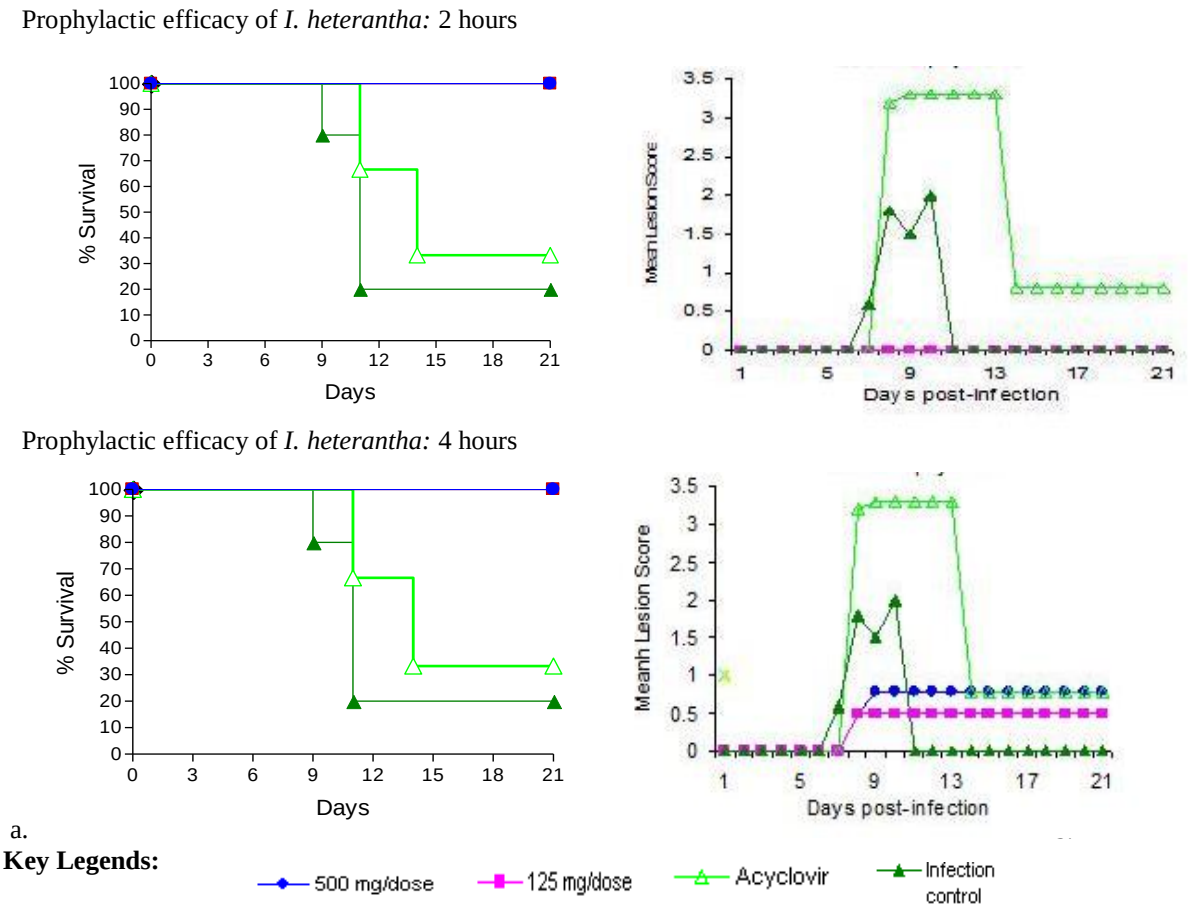



**Fig.B.5**: Prophylactic efficacy study at 1, 2 and 4 hrs prior to infection: **a.** Percent survival and **b.** mean lesion score of a topical cream formulation of the *I. heterantha* hydro-methanolic root extract in a murine genital infection model against HSV-2.

## 4. Discussion

The work presented here describes for the first time an antiviral activity from the root of *I. heterantha* against HSV-2, G strain when examined and evaluated by both *in vitro* and *in vivo* experiments. The *in vitro* data suggests that, the hydro-methanolic root extract exhibited potent anti HSV-2 activity as evident from the obtained $IC_{50}$, $CC_{50}$ and SI values of 21.93 µg/ml, 284.2 µg/ml and 13.3, respectively. The window between potent antiviral effect of the extract and its resultant cytotoxic effect on Vero cells was wide; indicating that the observed antiviral activity is not influenced by the presence of the extract alone on Vero cells but rather due to its underlying mechanism of action on HSV-2 virus replication.

As stated by Kurokawa et al. (2010), *in vivo* studies form an integral part in demonstrating the prophylactic and/or therapeutic efficacy of a potential antiviral drug when administered at the appropriate dosage in animals. In the *in vivo* studies, we employed an HSV-2 Balb/c murine genital infection model which as observed from literature is a routinely used animal model for the purpose of screening and validation of potential anti HSV-2 agents, as they closely mimic human disease conditions. BALB/c mice are known to respond well to drug therapy and the test compound required is minimum quantity (Zak and Sande, 1999). In the therapeutic efficacy study, the topical formulation containing the hydro-methanolic root extract was able to cause protection of mice, even when administered topically, 30 mins post HSV-2 virus challenge up to the lowest dose of 375 mg/kg/day. This activity was also comparable with the 100% protection observed with positive control ACV, in the study. Similarly, in the

prophylactic efficacy study, the extract containing topical formulation was efficacious against HSV-2 infection when applied 1, 2 and 4 hrs prior to infection. In contrast, positive control ACV was ineffective prophylactically with a survival rate of 33.3%. The ability of the extract to inhibit HSV-2 virus related genital infection due to pre-treatment immediately upon contact implies that the extract may act as an inhibitor of the initial entry steps of the virus into host cells (mice epithelial mucosa). According to Greco et al. (2007), a potential antiviral drug with a mechanism of action targeting viral entry is advantageous as it is easier to facilitate the development of such a drug which need not enter the cell to be effective. The *I. heterantha* root extract may thus act on the initial HSV-2 virus attachment and adsorption stages either by blocking the viral binding sites present on host cell surface receptors, thus preventing virus-cell interactions or by direct inactivation of HSV-2 virus upon contact. In addition, the therapeutic potential of the extract suggests that it also acts on the later stages of infection post viral entry.

Another factor responsible for the potent antiviral activity of the root extract could be due to the synergistic effects of multiple compounds in the extract which is a general phenomenon observed with plant extracts. In an attempt to isolate such compounds that may be present in our plant extract, we successfully isolated and identified the active pure compound- Procyanidin A2. Proanthocyanidins are polyphenols which belong to the class of non-hydrolyzable tannins. They are flavonoid containing oligomers or polymers known for their antiviral activity (catechins and epicatechins) particularly at the entry stage of HSV into host cells (Gescher et al., 2011). Thus, in combination, this ability of the extract to act both prophylactically and therapeutically warrants its development as a topical agent, for example, as a cream or microbicide.

Topical microbicides are agents used for the prevention of sexually transmitted diseases (STD's) by means of application to the genital tract or rectum prior to sexual contact. An ideal microbicide should be i) non-toxic even upon repeated application ii) protective immediately upon application with the ability to retain its antiviral effect up to a significant period and iii) able to withstand the unique physiological conditions of the vagina (Gong et al., 2005). In fact, one such successfully developed vaginal microbicide, is VivaGel® (SPL7013 Gel) by Starpharma Holdings Limited. VivaGel® has been shown to prevent transmission of both HIV and genital herpes. In addition it has also been licensed for development as an antiviral condom coating (http://www.starpharma.com). Thus to follow by example, our extract, too can be envisaged as a potential microbicide or condom coating as it was efficacious up to 4 hrs prior to infection. This indicates that as a potential HSV-2 virus entry (into host cells) inhibitor, presence of the extract prior to sexual contact, irrespective of a symptomatic or asymptomatic sexual partner, sexual transmission of the virus may be averted. It has been rightly asserted that in cases where women are unable to negotiate the use of a condom with their partner, a microbicide by itself is beneficial. However, a microbicide in combination with a condom can aid in providing additional protection against transmission of infection (Rupp et al., 2007).

In conclusion, we have demonstrated here for the first time the antiviral potential of the root of the plant *Indigofera heterantha*. The root derived extract showed *in vitro* antiviral activity against HSV-2 when the extract was present on Vero cells before being exposed to the virus. The antiviral potential of the plant was further evaluated *in vivo* in a mouse genital infection model. The extract was found to be efficacious when administered both prophylactically and therapeutically. The promising results obtained in both the *in vitro* and *in vivo* studies merits exploration of this *Indigofera heterantha* hydro-methanolic root extract for development as a potential topical microbicide and/or condom coating. It would be interesting to determine if the hydro-methanolic extract of *I. heterantha* is targeting one or more stages of the HSV-2 life cycle. This may be elucidated by carrying out studies which look at deciphering the mechanism of action of the *Indigofera heterantha* hydro-methanolic root extract in inhibiting HSV-2 replication.

## 5. Acknowledgement

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