1. INTRODUCTION

Herpes simplex viruses (HSV) are a common human pathogen that causes herpes labiles, herpes genitalis, keratitis and encephalitis. The HSV infection caused by type-1 (HSV-1) and type-2 (HSV-2) is mainly transmitted by close personal contact, and the virus can establishes lifelong latent infection in sensory neurons with recurrent lesions [Barton, 2005]. Herpes genitalis, usually caused by HSV-2, spread silently through sex, wreaks enormous financial and emotional damage due to its silent epidemic potential, and can cause life threatening infection in immunocompromised people and neonates [Fatahzadeh and Schwartz, 2007]. Moreover, HSV-2 is a high risk factor for acquisition of HIV infection [Cowan et al., 2003; Safrin et al., 1991] and there is a synergistic relationship between HIV and HSV [White et al., 2003; Wald, 2004; Mbopi-Keou et al., 2000]. A recent study showed that HSV-suppressive therapy greatly reduced genital and plasma HIV-1 RNA levels in co-infected patients [Nagot et al., 2007]. Hence, the risk of acquiring or transmitting HIV infection can be greatly decreased by reducing the spread of genital herpes.

Extensive and long term clinical use of anti-herpesvirus agents like acyclovir, and its derivatives ganciclovir, foscarnet results severe side effects and drug-resistant viruses [Morfin et al., 2003; Reyes et al., 2003; Miserocchi et al., 2007]. Further, acyclovir is reported to incorporate into the cellular DNA, yielding adverse drug reactions and thus, unsuitable for pregnant women [Narayana, 2008] and neonates [Brigden et al., 1982; Sawyer et al., 1988]. Moreover, the major determinants of effective immunity against HSV infection is not yet identified [Stanberry, 2004], and animal efficacy has not predicted success in humans [Koelle, 2006]. Furthermore, the therapeutic vaccines failed to induce antibody-specific responses to protect recipients from recurrences [Stanberry, 2004]. Therefore, there is an unmated and urgent need for cheap, readily available, less toxic alternate agents to control and prevent HSV infection and its transmission.

Ethnomedicinal plants offer a potential alternative because of their wide use in folklore medicine and some have promising therapeutic potential [Chattopadhyay and Khan, 2008].

Pedilanthus tithymaloides (L.) Poit. is a low tropical shrub of Euphorbiaceae, and is used for diverse ailments including antiviral, antibacterial, anti-inflammatory [Renne, 1996],
anticancer and anti-inflammatory [Bunyapraphatsara and Chokchaichareonporn, 2000]. In folklore medicine, *P. tithymaloides* leaves has been used in mouth ulcers, and venereal disease; while its sap for ringworm, skin cancer, and warts [Nellis, 1997] and the aerial part for skin disorder [Kumar and Chaturvedi, 2010], and against inflammation [Abreu et al., 2006]. The plant is reported to contain several bioactive principles including long-chain alcohol, triterpenes [Misra and Khastgir, 1969; Mukherjee et al., 1989; Mukherjee et al., 1992], anticancer diterpene pedilstatin [Pettit et al., 2002], antiinflammatory pedulantha [Dhar et al., 1988] and a galactose-specific lectin with mitogenic [Seshagirirao, 1995] antidiabetic [Nagda and Deshmukh, 1998] and anti-tubercular [Ankush et al., 2003] activity. Moreover, leaves and stems yielded antioxidant kaempferol 3-O-β-D-glucopyranoside-6-(3-hydroxy-3-methylglutarate), quercitrin, isoquercitrin and scopoletin [Abreu et al., 2008]. However, we have demonstrated that methanol extract of *P. tithymaloides* leaves and its isolated compounds promote wound healing in rats [Ghosh et al., 2012] and others report of its antimicrobial activity [Vidottia et al., 2006]. Based on the traditional use in skin infections we have also evaluated, for the first time, the *in vitro* anti-HSV activity of crude methanolic extract and its isolated compound(s).

2. EXPERIMENTAL

2.1. Plant materials

The methanol extract (ME) and isolated bioactive compounds luteolin (compound-5) and 1,2-tetradecanediol, 1-(hydrogen sulfate), Na salt (compound-6) was used as described in Chapter 2.

2.2. Viruses and the cell line

The standard strains of HSV-1F (purchased from the ATCC, Manassas, VA, USA) was grown in African green monkey kidney cells (Vero cells, ATCC, Manassas, VA, USA) in Eagle's minimum essential medium (EMEM), supplemented with 5-10% foetal bovine serum (FBS). After plaque purification, the virus was grown in EMEM and the virus stocks were stored at -80°C for future use, and whenever required the virus stock was grown on Vero cells to determine the titters and used for further study.
2.3. MTT assay of cytotoxicity

Cell toxicity was monitored by determining the effect of the extract and two bioactive compounds on Vero cell morphology. Vero cells were cultured onto 96 well plates at 1.0 \times 10^5 \text{cells/well}. Different concentrations of ME (0-400 \, \mu g/ml) and isolated compounds 5 and 6 (0-300 \, \mu g/ml) were added to each culture wells at a final volume of 100\,\mu l, in triplicate. The DMSO (0.1\%) and acyclovir (0-200 \, \mu g/ml) was used as a negative and positive control respectively. The drug treated cells were incubated at 37\degree C with 5\% CO_2 for 2 days, and then the MTT reagent (10\,\mu l) was added to each well. After further 4h of incubation (37\degree C), the formazan was solubilized by adding diluted HCl (0.04N) in isopropanol, and the absorbance was determined at 570 \, nm with a reference wavelength of 690 \, nm by an ELISA reader [Zhang et al., 2007]. Data was calculated as the percentage of cell viability by the formula: \[(\text{sample absorbance} - \text{cell free sample blank})/\text{mean media control absorbance})\]/100\%. The 50\% cytotoxic concentration (CC_{50}) causing visible morphological changes in 50\% of Vero cells with respect to cell control were determined [Zhang et al., 2007].

2.3. Antiviral plaque assay

Vero cells seeded in 12-well plates (5 \times 10^5 \text{cells per well}) were treated with serial dilutions of ME and or test compounds 5 and 6 (0-100 \, \mu g/ml) for 15 min at 37\degree C and then challenged with HSV-1 (100 CFU/well) for 1 h. The inoculum and drugs were subsequently removed from the wells, and the cells were washed with PBS twice and overlaid again with different dilutions of the ME and compounds. After further incubation for 72 h, the supernatant was removed, and the wells were fixed with methanol and stained with Giemsa (Sigma). Viral inhibition (%) was calculated as follows: [1 - (number of plaques)_{\text{exp}}/(number of plaques)_{\text{control}}] \times 100\%; where “(number of plaques)_{\text{exp}}” indicates the plaque counts from virus infection with test extract or compound treatment and “(number of plaques) control” indicates the number of plaques derived from virus-infected cells with control (HSV-1 with DMSO only) [Cheng et al., 2003]. The 50\% effective concentration (EC_{50}) for antiviral activity was defined as the concentration of antiviral compound(s) that produced 50\% inhibition of the virus induced plaque formation [Chattopadhyay et al., 2009; Bag et al., 2012].
2.4. Drug dose-response analysis
For dose-response assay, Vero cells seeded in 96-well plates were infected with HSV-1 (multiplicity of infection or MOI: 1) in the presence or absence of the isolated compounds at various concentrations (0, 5, 10, 20, 30, 60 and 100 µg/ml). After 2 days MTT assay was carried out to determine the inhibition of infection, as described previously. Values were obtained from three independent experiments with each sample assay performed in triplicate [Cheng et al., 2004].

2.5. Assays for effect of ME and compound 1 treatment at different times
The effect of drug addition over time was assessed according to a previously published method [Madan et al., 2007] with some modifications. To assess the effect of pre-treating cells with ME and isolated compound-5, Vero cell monolayers seeded in 96 well plates and were treated with compound-5 (30 µg/ml) and extract (100 µg/ml) for 24 h (long term) or 1 h (short term) and then washed with PBS before challenge with HSV-1 (at MOI of 1.0) in EMEM containing 2% FCS. To study the effect of adding compound and virus concurrently, Vero cells were treated simultaneously with HSV-1 (MOI 1) and compound-5. After incubation for 1 h at 37°C, the virus-drug mixture was removed, and cells were washed prior to overlay with media. To evaluate whether the extract or compound had any effects after viral entry, Vero cells were challenged with HSV-1 (MOI 1) for 1 h, and after removal of the virus inoculum, infected cells were washed and subsequently overlaid with media containing the test compound. For continuous drug treatment, cells were pretreated for 1 h with the compound-5, then challenged with HSV-1 in the presence of the compound, and overlaid with media containing the test compound after viral entry. After 2 days MTT assay was carried out to determine the inhibition of infection, as described previously. DMSO (0.1%) was included as control in each condition.

2.6. Statistical analysis
The selective index (SI), a marker of antiviral activity, was determined as the ratio of CC$_{50}$ to EC$_{50}$. The statistically different effects of crude methanolic extract or isolated compound and acyclovir on the inhibition of HSV were compared with the control group
using Student’s $t$-test. While the dose dependent effect of antiviral activity was
determined by linear regression

3. RESULTS

3.1. Inhibition of HSV-1 infection

Our results indicated that the ME and isolated compound 5 and 6 did not have apparent
cytotoxic effects below 100 µg/ml in vero cells, while a dose-dependent cytotoxic effect
was observed at 100 µg/ml. The 50% cellular toxicity indices (CC$_{50}$) of methanol extract
as well as compound 5 and 6 were 236.5, 178.6, and 146.4, respectively (Table 7.1).
We then evaluated the antiviral effects of the two natural compounds against HSV-1
infection by using a plaque assay. Acyclovir was used as positive controls, while DMSO
(0.1%) was included as a negative control. The results revealed that both ME and
compound-5 could inhibit viral plaque formation, after inoculation of 100 PFU, in a dose-
dependent manner, and their 50% effective concentration (EC$_{50}$) were 42.4 and 14.8
µg/ml respectively (Table 7.1). The selectivity index (SI), which measures the
preferential antiviral activity of a drug in relation to its cytotoxicity, was calculated
according to their CC$_{50}$ and EC$_{50}$. The SIs of ME, compound 5 and 6 were 5.58, 12.07
and 1.17, respectively (Table 7.1). Out of the two isolated compounds tested, the SI
value of compound 1 was >12 and thus, luteolin was chosen for subsequent analyses.
To obtain a more accurate dose-response curve for ME and compound-5, Vero cells were
infected with HSV-1F (MOI: 1) in the presence of ME, and compound-5 and tested by
MTT assay. Both extract and compound-5 displayed anti-HSV-1 activity in a dose-
dependent manner (Fig. 7.1), and compound-5 at 30 µg/ml and ME at 100 µg/ml
provided near complete protection against the infection at an MOI of 5, were chosen for
all subsequent experiments. Here compound-6 used as negative control.

3.2. Time dependent antiviral activities of compound-5

To understand the antiviral mode of action and the stage of HSV-1 infection affected by
ME and compound-5, we added the test samples at different times of the virus life cycle
(pre-entry, entry, and post-entry). In order to study pre-entry, i.e., the effect of the compound on Vero cell itself, prior to virus addition, Vero cells were pretreated with ME and compound-5 for 24 h (long) or 1 h (short) periods and then washed prior to HSV-1 infection. For the effects of test samples on the viral entry, virus and the drugs were simultaneously applied to the cells. To investigate events after virus entry, vero cells were first infected with HSV-1 for 1 h and then treated with the compound. For comparison, the ME and compound-5 were also maintained throughout the experimental period. Pretreating Vero cells with extract and compound-5 (both long and short term) did not protect the Vero cell against HSV-1 infection. Both extract and compound-5 were effective in preventing cell distraction when added during virus adsorption, immediately after viral entry, and throughout multiple cycles of virus replication (Fig. 7.2). The results indicate that HSV-1 infection is severely impaired only if the drug(s) was present at the time of infection or during viral spread and that it is unlikely that the antiviral activity is due to its direct effects on the cells (such as masking cellular receptors or entry factors for HSV-1).

Table 7.1. Assessment of Anti-HSV activity by MTT assay

<table>
<thead>
<tr>
<th>Test drug</th>
<th>CC₅₀ ±σ</th>
<th>HSV-1F at MOI: 0.5</th>
<th>Antiviral activity (EC₅₀ ±σ)</th>
<th>Selectivity index (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME</td>
<td>236.5±4.84</td>
<td>42.4±3.76</td>
<td>5.58</td>
<td></td>
</tr>
<tr>
<td>Compound-5</td>
<td>178.6±3.56</td>
<td>14.8 ± 1.24</td>
<td>12.07</td>
<td></td>
</tr>
<tr>
<td>Compound-6</td>
<td>146.4±6.33</td>
<td>124.6 ± 0.32</td>
<td>1.17</td>
<td></td>
</tr>
<tr>
<td>Acyclovir</td>
<td>128.8±3.44</td>
<td>2.2 ± 0.1</td>
<td>58.55</td>
<td></td>
</tr>
</tbody>
</table>

*a* The 50% cytotoxic concentration for Vero cells in µg/ml; *b* Concentration of compound (µg/ml) producing 50% inhibition of virus induced CPE of three separate experiments; *c* Selectivity index (SI) = CC₅₀/EC₅₀
**Fig. 7.1.** Dose dependent anti-HSV-1 activity induced by ME and isolated compound.

**Fig. 7.2.** Effect of time of addition of ME and isolated compounds on plaque formation by HSV-1. After 3 days, inhibition was evaluated by MTT assay and expressed as the inhibition rate.
4. DISCUSSION

The present study for the first time, demonstrated the anti-HSV activity of crude methanolic extract of *P. tithymaloides* leaf as well as its isolated phytoconstituents. The antiviral activity of the crude methanolic extract (ME) was significant, probably due to the higher concentration of bioactive compounds within the fraction whereas petroleum ether and chloroform extract do not show any anti-HSV activity, hence not included in further study. Phytochemical study reveals that ME contains 5 major fractions, out of which two fractions are found to be active in several pharmacological tests including antimicrobial, antiinflammatory and wound healing activities. These, two bioactive fractions yielded compound 5 and 6. Methanol extract (ME) along with compound 5 and 6 are subjected to antiviral testing against a common human pathogen Herpes simplex viruses (HSV) type-1 (HSV-1) and type-2 (HSV-2).

HSV manifest a number of human infections like herpes labiles, herpes genitalis, keratitis and encephalitis etc and transmitted silently by close personal contact and sexual intercourse and can cause enormous financial and emotional damage as well as life threatening infection in immunocompromised people and neonates [Fatahzadeh and Schwartz, 2007]. Extensive and long term clinical use of anti-herpesvirus agents like acyclovir, and its derivatives ganciclovir, foscarnet results severe side effects and drug-resistant viruses [Morfin et al., 2003; Reyes et al., 2003; Miserocchi et al., 2007; Brigden et al., 1982; Sawyer et al., 1988] and till date there is no effective vaccines, thus a new alternatives agent to control and prevent HSV infection, particularly from ethnomedicinal plants is an urgent need. Thus, as an ongoing effort of identifying a new antiviral lead from natural products we have tested the efficacy of ME and the isolated compounds from *P. tithymaloides*.

Antiviral activity of crude ME was weak compared to isolated compound-5 probably due to the low concentration of compound-5 in crude extract; while compound-6 isolated from ME was less active or inactive, compared to compound-5.

Cytotoxicity study revealed that ME and its isolated compound-5 had different CC$_{50}$ due to variable concentration of bioactive compounds and antiviral activity was found to be fur less than CC$_{50}$. Further study revealed that ME and compound-5 had a dose-
dependent antiviral activity and infection of Vero cells by HSV-1 was significantly prevented by compound-5 compared to ME with higher SI value. However, the CC\textsubscript{50} and EC\textsubscript{50} of compound-6 are close given an insignificant SI.

The dose and time response analysis was performed to understand the inhibitory potential and to know the exact time of viral inhibition. Interestingly, ME and compound-5 was found to inhibit HSV-1 in dose-dependent manner with EC\textsubscript{50} of 42.4 and 14.8 µg/ml. However, 100% inhibition was achieved at 30 µg/ml of compound-5 but 100 µg/ml of ME. Results of time course study between 0 to 24 h post infections revealed that both ME and compound-5 have a similar inhibitory effect, and both are effective in prevention cell distraction when added between viral adsorption (within 1 h of infection), immediately after entry and perhaps upto the multiple cycle of viral replication (Fig. 7.2). The results also pointed out that the HSV-1 infection is severely impaired if the compound-5 or ME was added at the time of infection or during viral spread, thus, neither ME nor compound-5 have any direct effect on Vero cells. However, real antiviral mechanism of compound-5 remains to be further elucidated.

Contemporary research revealed that a wide variety of active phytochemicals, including the flavonoids, terpenoids, organosulfur compounds, limonoids, lignans, sulfides, polyphenolics, coumarins, saponins, chlorophyllins, furyl compounds, alkaloids, polyines, thiophenes, have probable therapeutic applications against different genetically and functionally diverse viruses [Chattopadhyay, 2006; Chattopadhyay and Naik, 2007; Chattopadhyay and Bhattacharya, 2008]. The antiviral mechanism of these agents may be explained on basis of their antioxidant activities, scavenging capacities, inhibiting DNA, RNA synthesis, inhibition of the viral entry, or viral reproduction and so on [Chattopadhyay and Bhattacharya, 2008; Christopher and Wong, 2006].

The isolated luteolin (a flavone) is a yellow crystalline powder, sparingly soluble in water, reported to have anti-inflammatory [Jang \textit{et al.}, 2008], antioxidant [Lemanska \textit{et al.}, 2004; Odontuya \textit{et al.}, 2005], antimicrobial [Liu and Matsuzaki, 1995; Tsuchiya \textit{et al.}, 1996] and immunomodulation activities. The structure activity relationship study showed that the luteolin exerts its anti-inflammatory activity by inhibiting thromboxane and leukotriene enzyme of arachidonic acid pathway, along with scavenging of hydrogen peroxide, due to \textit{ortho}-dihydroxy groups at its B ring and OH substitution at C-5 position.
It is known that luteolin had bacteriostatic activity against *Staphylococcus aureus* [Liu and Matsuzaki, 1995; Tsuchiya *et al*., 1996], *Helicobacter pylori*, and *Neisseria gonorrhoeae* due to the inhibition of arylamine N-acetyltransferase [Tsou *et al*., 2001]. Moreover, the ability of luteolin to inhibit IL-6 [Jang *et al*., 2008], phosphodiesterase [Yu *et al*., 2010], and multiple sclerosis [Theoharides, 2009], neuroprotection through rebalancing of pro-oxidant-antioxidant level [Zhao *et al*., 2012] and the activation of monoamine transporter [Zhao *et al*., 2010] may contribute to the faster wound healing potential of this plant. Modulation of ROS, inhibition of topoisomerases I and II, reduction of NFkB, stabilization of p53, and inhibition of PI3K, STAT3, IGF1R, and HER2 are possible mechanisms for the putative bioactivities of luteolin [Lopez-Lazaro, 2009].

### 5. CONCLUDING REMARK

In conclusion, our results of clearly demonstrated that ME of PT leaves containing luteolin (compound-5) as one of the major compound might be a potential therapeutic candidate against HSV infections, and can be further studied for its potential against an alternative anti-HSV candidate, through mechanistic and steriochemical studies.