Chapter-5

In vitro antiviral activity
5.1. INTRODUCTION

_In vitro_ assays are the preliminary step towards the identification of any bioactivity including antiviral activity of a plant extract or compound. These assays measure the ability of a virus to infect and replicate in specific cell lines and the response of a particular extract towards the relevant virus infection. The cell culture system provides a rapid and less cumbersome method to grow viruses at higher titers, testing of cytotoxicity and antiviral efficacy, maintenance of cultures, and genetic manipulations. This chapter deals with _in vitro_ antiviral activity of the test extract and isolated compound of _P. tithymaloides_.

5.2. MATERIAL AND METHODS

5.2.1. Materials

Dulbecco modified Eagle medium (DMEM), RPMI 1640, phosphate buffer saline (PBS), fetal calf serum (FCS), fetal bovine serum (FBS), Penicillin and streptomycin were obtained from Gibco BRL (Grand Island, NY, USA) while, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was obtained from Sigma Chemical Co. (St Louis, MO, USA).

5.2.2. Viruses and the cell line:

African green monkey kidney cells (Vero cells, CCL-81) were grown and maintained in Eagle's minimum essential medium (EMEM), supplemented with 5-10% fetal bovine serum (FBS) (Mindel, 1998). The HSV-1 and HSV-2 isolates (VU 04, VU 06, VU07 and VU 11), isolated from clinical specimens were obtained from ICMR Virus Unit repository and the quality control wild type strain of HSV-1F (ATCC-733) and HSV-2G (ATCC-734) were purchased from the American type culture collection (ATCC), USA. For isolation of viral strains, fluids from patients’ blisters was collected with a tuberculin (1ml) syringe and transported in a transport medium (m-4, contains gelatin, vancomycin, amphotericin B, colistin) and kept at -20°C in our laboratory coded but without identifiers, as outlined in our institutional ethical Committee protocol. The samples were processed within 2-3 h and inoculated in semi-confluent Vero cell monolayer and incubated at 37°C at 5% CO₂ for 2-3 days for cytopathic effect. The viruses isolated from clinical specimens were identified by fluorescein-conjugated monoclonal
antibodies and polymerase chain reaction (PCR) amplification. After plaque purification, the virus was grown and the virus stocks were stored at -80°C for further use (Hsiang et al., 2001). When required the virus stocks were grown to determine their titers on Vero cells for subsequent studies.

5.2.3. Determination of cytotoxicity by MTT assay
Cell toxicity was monitored by determining the effect of the test extract and isolated compounds on cell morphology. Vero cells monolayers were grown onto 96 well plates at 1.0 x10^5 cells/ml. After incubation at 37°C in 5% CO₂ for 6 h, different concentrations of test extracts (1-1000 µg/ml) and compounds (1-300 µg/ml) were added to each culture wells at a final volume of 100 µl, in triplicate. The DMSO (0.1%) and acyclovir was used as a negative and positive control respectively. The drugs treated cells were incubated at 37°C with 5% CO₂ for 2 days, and 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide or MTT reagent was added to each well. After further 4 h incubation (37°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 (Biorad, USA). Data were calculated as the percentage (%) of cell viability by the formula: \[
\frac{(\text{sample absorbance} - \text{cell free sample blank})/\text{mean media control absorbance})}{100%}.
\]
The 50% cytotoxic concentration (CC₅₀) causing visible morphological changes in 50% of Vero cells with respect to cell control were determined (Bag et al., 2014; Ojha et al., 2013).

5.2.4. Antiviral assay using MTT assay
The antiviral activity of extracts and isolated compounds of P. tithymaloides against HSV-1 and HSV-2 was evaluated by MTT assay (Mosmann et al., 1983), a sensitive and accurate method for rapid screening of antiviral agents. Vero cells were seeded onto 96 well plates with a concentration of 1.0x10⁵ cells/ml. After incubation at 37°C in 5% CO₂ for 6h, the virus at (multiplicity of infection; MOI: 1) was added and incubated for 1h. Different concentrations of test drugs were added to culture wells in triplicate at a final volume was 100µl in each well. The maximum concentration of DMSO (0.1%) serve as a negative control and acyclovir as a positive control for HSV assay. After incubation at 37°C with 5% CO₂ for 3 days, the MTT test was carried out as described above. Viral inhibition rate was calculated as: \[
\frac{(A_{tv}-A_{cv})}{A_{cd}}\]
Acv)/100%. Where Acv indicates the absorbance of the extract/isolated compounds in virus infected cells. Acv and Acd indicate the absorbance of the virus control and the absorbance of the cell control. The antiviral concentration of 50% effectiveness (EC50) was defined as the concentration which achieved 50% inhibition of virus-induced cytopathic effects (Chattopadhyay et al., 2009).

5.2.5. Antiviral plaque assay

Vero cells monolayers seeded in 6-well plates (2 x 10^6 cells per well) were treated with serial dilutions of the the test extract or isolated compounds luteolin and tetradecanediol of P. tithymaloides for 1h at 37°C and then separately challenged with HSV-1 and HSV-2 (MOI: 1) for 1 h. The inocula and drugs were subsequently removed from the wells, and the cells were washed with PBS twice and added again with different dilutions of the extract or compound. After further incubation for 72 h at 37°C in 5% CO2, 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) in test/(number of plaques) in control] X 100%, where “(number of plaques) test” indicates the plaque counts from virus infection with the test extract or isolated compounds luteolin and tetradecanediol treatment and “(number of plaques) control” indicates the number of plaques derived from virus-infected cells with DMSO treatment (Bag et al., 2013; 2014). The 50% effective concentration (EC50) for antiviral activity was defined as the concentration of the compound that produced 50% inhibition of the virus induced plaque formation (Chattopadhyay et al., 2009; Bag et al., 2012).

5.2.6. Statistical analysis

Results were expressed as Standard Error Mean (SEM) (n = 6) and the statistical analyses were performed with one-way analysis of variance (ANOVA), followed by Dunnett’s test. This analysis indicated that differences among means of treatment columns (methanol extract and or isolated compounds luteolin) were found to be statistically significant then DMSO control column (P < 0.05; P < 0.001). A value of p < 0.05 was considered to be statistically significant, compared with the respective control.
5.3. RESULTS

5.3.1. Assessment of Cytotoxicity by MTT Assay on Vero cell
The MTT assay was used to determine the toxicity of the test agents. The results revealed that the test extract and isolated luteolin exhibited a cytotoxic effect on Vero cells at concentrations higher than their EC50 while the isolated tetradecanediol have no activity. Results presented in Table 3 revealed that the CC50 of the test extract, and isolated luteolin were 436.5 µg/ml and 278.6 µg/ml respectively. The cell morphology at cytotoxic and non-cytotoxic concentration of the test extract or luteolin was presented in Fig. 3.1.

5.3.2. Inhibition of HSV infection
The antiviral activity of the test extract, isolated luteolin and tetradecanediol was determined against HSV-1F and HSV-2G, four clinical isolates (VU 04, VU 06, VU 07 and VU 11), and one TK-deficient stain of HSV-2 (100 PFU) using MTT and PRA in the presence of acyclovir, and DMSO (0.1%) as control. The result of MTT assay and PRA demonstrated that the test extract at 43.6-52.6 µg/ml and luteolin at 19.2-27.5 µg/ml inhibited all the strains tested, which was far below their CC50 concentration (Table 3), but tetradecanediol had no anti-HSV activity. Further, nearly complete (EC99) inhibition of viral growth was achieved at 79.6 and 86.5 µg/ml of the test extract or 36.4 and 40.2 µg/ml of luteolin against HSV-1F and HSV-2G respectively. The virus infected cell morphology at EC50 and EC99 concentration of the test extract or luteolin were presented in Fig. 3.2.
**Figure 3.1.** (A) Cell control; (B) cytotoxic concentration; (C) non cytotoxic concentration of the test extract (200 µg/ml); (D) Cell control; (E) cytotoxic concentration ; (F) non cytotoxic concentration of luteolin (100 µg/ml).
**Figure 3.2.** (A) Cell control, (B) Virus control, (C) EC$_{50}$ concentration; (D) EC$_{99}$ concentration of the test extract; (E) EC$_{50}$ concentration; (F) EC$_{99}$ concentration of luteolin.

**Table 3.** Assessment of cytotoxicity and ant-HSV activity of the test extract and Luteolin.

<table>
<thead>
<tr>
<th>Virus</th>
<th>The test Extract</th>
<th></th>
<th>Luteolin</th>
<th></th>
<th>Acyclovir</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC$_{50}$</td>
<td>EC$_{50}$</td>
<td>SI$^c$</td>
<td>CC$_{50}$</td>
<td>EC$_{50}$</td>
<td>SI$^c$</td>
</tr>
<tr>
<td>HSV-1F</td>
<td>436.5</td>
<td>43.6 ± 1.84</td>
<td>10.01</td>
<td>278.6</td>
<td>19.2 ± 2.33</td>
<td>14.51</td>
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<tr>
<td>HSV-1 CI 1 (VU 04)</td>
<td>± 2.84</td>
<td>47.4 ± 1.66</td>
<td>9.20</td>
<td>± 3.56</td>
<td>21.5 ± 1.56</td>
<td>12.95</td>
</tr>
<tr>
<td>HSV-1 CI 2 (VU 07)</td>
<td>44.2 ± 1.52</td>
<td>9.87</td>
<td></td>
<td>20.8 ± 1.38</td>
<td>13.39</td>
<td></td>
</tr>
<tr>
<td>HSV-2G</td>
<td>48.5 ± 1.24</td>
<td>9.00</td>
<td></td>
<td>22.4 ± 2.56</td>
<td>12.43</td>
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<tr>
<td>HSV-2 CI 1 (VU 06)</td>
<td>49.2 ± 1.88</td>
<td>8.87</td>
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<td>26.8 ± 1.66</td>
<td>10.39</td>
<td></td>
</tr>
<tr>
<td>HSV-2 CI 2 (VU 11)</td>
<td>52.6 ± 1.48</td>
<td>8.29</td>
<td></td>
<td>23.8 ± 1.44</td>
<td>11.70</td>
<td></td>
</tr>
<tr>
<td>TK$^{-}$ strain</td>
<td>50.8 ± 1.33</td>
<td>8.59</td>
<td></td>
<td>27.5 ± 1.76</td>
<td>10.13</td>
<td></td>
</tr>
</tbody>
</table>

CI, Clinical Isolates of HSV-1 and HSV-2; TK$^{-}$, Strain, Thymidine Kinase deficient strain of HSV-2;

$^a$CC$_{50}$, 50% cytotoxic concentration for Vero cells in µg/ml;

$^b$EC$_{50}$, Concentration (µg/ml) producing 50% inhibition of virus-induced plaques in three separate experiments; $^c$SI, Selectivity index (CC$_{50}$/EC$_{50}$).