Chapter 6

Studies on the mechanism of action
6.1. INTRODUCTION

The idea behind modern antiviral drug design is to identify the exact mode and target site of action on the viral proteins, nucleic acid, or its components, that can be disabled by the investigating drug and those "targets" should not be the host proteins, nucleic acid or its parts, to reduce the likelihood of side effects. The targets should also be common across many strains of a virus, or even among different species of virus in the same family, so a single drug will have broad effectiveness. For example, a drug may target a critical enzyme synthesized by the virus, but not the patient, which is common across strains, and observed what can be done to interfere with its operation. Once targets are identified, candidate drugs with appropriate effect can be selected.

Phytochemicals or other compounds can also be evaluated for their antiviral activity when challenged with different amounts to different viruses, ranging from very low to very high MOI. One of the well known studies to identify the mode of action of a compound on viral system is the Time-of-addition and time-of-removal assay, where test compounds are added or removed from drug treated growing cultures at various time points like: pre-infection, co-infection and post-infection. By comparison with other known herpesvirus inhibitors, this allows to determine the relative point in the virus life cycle that is being inhibited (immediate early, early, late gene expression, or DNA polymerization, etc.). This standard technique typically used early during the process of determining mode of action as it allows a smaller target window of activity for further experimentation. It also act as an easy way to determine the uniqueness or novelty of a compound compared to other known inhibitors. Furthermore, time of removal studies help to determine the reversibility of a compounds activity.

This chapter deals with the determination of mode and mechanism of antiviral action of a well known ethnomedicine *P. tithymaloides* and its isolated compound, using the dose dependent (Dose response), time of addition or removal (Time response) assay, attachment and penetration assays and the drug induced viral reduction through indirect immunofluorescence assay (where the infected viruses were tagged with specific antibodies and stained with fluorescence dye).
Moreover, the extract and isolated compound have also been assayed to find out whether the test agent/compound(s) are acting in the same events of viral life cycle.

6.2. MATERIAL AND METHODS

6.2.1. Materials
DMEM, RPMI 1640, PBS, FBS, Penicillin and streptomycin were obtained from Gibco BRL (Grand Island, NY, USA) while, the ELISA antibody kits (IL-1α, IL-6, IL-12 and TNF-α) were purchased from BD Biosciences (San Diego, CA); and antibodies against IkB-α, p-IkB-α NF-κB/p65, p38, p-p38, JNK1/2, p-JNK1/2, GAPDH and ECL kit were from Abcam (Cambridge, MA, USA). The FITC-labelled anti-HSV-1 mouse monoclonal antibodies were procured from Santa Cruz Biotechnology (Santa Cruz, CA). Primers for semi-quantitative PCR were obtained from IDT, California, USA. Other chemicals are obtained from either Sigma (USA) or Merck (Germany).

6.2.2. Dose-response assay
A study was conducted to analyse the dose-dependent effect of the test agents on the HSV infected Vero cell. Vero cells monolayers at 1.0 x 10^5 cells/ml was grown onto 96 well plates. After incubation at 37°C in 5% CO₂ for 6 h, the virus (at 1.0 MOI) was added and incubated for 1 h for viral adsorption. Different concentrations (0, 5, 10, 20, 30, 40, 60, 80 and 100 µg/ml) of the test extract and isolated luteolin were added to culture wells in triplicate at the final volume of 100 µl, using DMSO (0.1%) as negative and acyclovir (0-5 µg/ml) as positive control. After 3 days of incubation at 37°C in 5% CO₂, the MTT assay was carried out as described earlier (Mukherjee et al., 2013).

6.2.3. Time-of-addition assay
The effect of drug addition over time was performed to determine the possible step(s) of viral life cycle targeted by luteolin. Following three different approaches Vero cells were exposed to the test extract or luteolin before, after and during infection with the virus (100 PFU). For pre-infection, cells were treated with the test extract or luteolin for 2 h and then infected with HSV,
after washing with PBS. For co-infection, the cells were treated simultaneously with HSV and the test extract or luteolin. After 1 h the virus-drug mixture was removed and the treated cells were subjected to PRA. While for post-infection (p.i) the cells were first infected with HSV for 1 h, washed with PBS, and then treated with the test extract or luteolin at intervals of 4, 8, and 12 h and finally the cells were harvested after 24 h for PRA (Bag et al., 2013).

6.2.3. Viral attachment assay by Plaque reduction and Immunofluorescence
HSV-1 and HSV-2 (10^4 PFU/ml) was mixed separately with the the test extract or luteolin at different concentration (10-80 µg/ml) and then incubated at 37°C for 1 h. The the test extract or luteolin–virus mixture was then diluted 100-fold (final inoculum, 100 PFU per well) with DMEM containing 2% FBS to yield a sub therapeutic concentration, and the virus inoculars were subsequently added to Vero cells monolayers seeded in 6-well plates for subjected to PRA and indirect immunofluorescence (IF) assay (Bag et al., 2013; Ojha et al., 2013). Infected cell monolayers were incubated 4°C for 3 h to allow viral adsorption. At the end of the incubation period, extracellular virus was inactivated by citrate buffer (pH 3.0) for 1 min, and then the cells were washed with PBS. The cells were then fixed with paraformaldehyde (4%) and blocked with 1% BSA in 0.1% PBS-Triton X100 solution. The cells were further washed with PBS, then permeabilized with 0.1% Triton X100 in PBS, and incubated either overnight at 4°C or 1 h at room temperature with FITC-labelled anti-HSV-1 mouse monoclonal antibodies (Bag et al., 2012). After washing with PBS, secondary rabbit polyclonal antibodies and DAPI were mixed to visualize both the virus and cell nucleus under Axio Imager M1 (Carl Zeiss, USA) inverted epifluorescence microscope (Bag et al., 2013).

6.2.4. Viral penetration assay
The viral penetration assay was performed according to Lin et al., (2011) with minor modifications. Vero cells grown in 12-well plates were prechilled (4°C) for 1 h and subsequently incubated with HSV-1 (MOI 1.0) for 3 h at 4°C for viral adsorption. The infected cell monolayers were then incubated in the presence or absence of the test extract (80 µg/ml) or luteolin (30 µg/ml), or DMSO (0.1%) for an additional 20 min at 37°C to facilitate virus penetration. At the end of the incubation period, extracellular virus was inactivated by citrate
buffer (pH 3.0) for 1 min, and then the cells were washed with PBS and overlaid with DMEM containing 2% FCS. After 48 h of incubation at 37°C, viral plaques were stained with crystal violet to count the plaque number.

6.2.5. Preparation of mouse peritoneal macrophages

Male BALB/c mice (18-20 gm), acclimatized for 7-10 days with standard food and water *ad libitum*, housed in polypropylene cages in Animal House facility were used for the experiment in accordance with the OECD guidelines accepted as per the approval of the Institutional Animal Care and Use Committee (IACUC, Approval No: 367/01/C/CPCSEA). When required, the surgical procedures were performed under Ketamine hydrochloride (100 mg/kg i.m.) anesthesia, and all efforts were made to minimize the suffering. The mice were intraperitoneally injected with 1 ml of 4% thioglycolate, and after 5 days the animals were subjected to Ketamine hydrochloride (100 mg/kg i.m.) anesthesia to minimize the sufferings, and euthanized by cervical dislocation. The peritoneal cells were harvested by ice-cold PBS, and centrifuged at 1200 rpm in 4 °C for 5 min. The cell pellet was suspended in RPMI-1640 supplemented with 10% FCS, and the cells were counted on Neubaur’s chamber. Then the cells were cultured at 37 °C in 5% CO₂ for 6 h, washed with PBS to remove the non-adherent cells, and further incubated for 24 h (Mukherjee *et al.*, 2013; Ojha *et al.*, 2013).

6.2.6. Western blot analysis

This study was conducted to know how the virus was inactivated by the test agent(s) on infected cell. The HSV-1 and HSV-2 (MOI= 10) infected Vero cells were treated with the test extract (80 µg/ml) or isolated luteolin (30 µg/ml) and after 4 h of treatment at 37°C at 5% CO₂, equal amounts of protein (40 µg/sample) extract from whole cell were harvested in buffer (200 µl/well) containing 20 mM Tris (pH 7±0.5), 50 mM NaCl, 5% NP-40 and 0.05% DOC. The soluble fraction was then separated by centrifugation (16000 g for 10 min) at 4 °C, subjected to SDS-PAGE and blotted to pre-equilibrated PVDF membrane (Thermo Scientific, USA). The membrane was then blocked in 5% NFDM in 1X TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Tween 20), rinsed and incubated with specific antibody in 5% BSA at 4 °C overnight.
Immunoblotting was performed with peroxidase-labelled specific antibodies and visualized by ECL Western blot detection kit (Millipore, USA) (Wang et al., 2005).

### 6.2.7. ELISA

Macrophages isolated from Balb/C mice peritoneum were plated in 24 well ELISA plate at a density of 1x10^6 cells/well with RPMI1640 media at 37°C at 5% CO2 incubator, and incubated (1 h) for attachment, and then infected with HSV-1 and/or HSV-2 (MOI= 10), and subsequently treated with the extract at 80 µg/ml or luteolin at 30 µg/ml for 24 h. The supernatant was collected for determining the level of TNF-α, IL-1β, IL-6 and IFN-γ by commercial ELISA kits (BD Biosciences, CA).

### 6.2.8. Isolation of RNA and Semi-quantitative RT- PCR

Semi-quantitative RT- PCR

Virus (5 moi) infected Vero /macrophage cells were treated with the test extract or luteolin for 12 h p.i, and the RNA was isolated using RNeasy Mini kit (Qiagen, Germany). The total RNA in RNase-free water was mixed in 20 µl of RT mix (containing 5X VILO Reaction Mix, 10X SuperScript Enzyme Mix and DEPC treated water) and subjected to cDNA synthesis using the GeneAmp PCR System 9600 (Bio-Rad MJ Mini, Hercules, CA, USA). The cDNA (10%) was subjected to standard PCR amplification using the primers for ICP0, DNA pol genes, and iNOS with GAPDH as internal standard. The sequences of the forward and reverse primer were listed in Table 4.

**Table 4.** Primers used in Real time-PCR assays.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>ICP0</td>
<td>5’-GATCGGATCCGGCGCTGGGGAGAGAGAAACC-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GATCGTCGACCCCGAGTGTTAGCTCCCCCTACTCC-3’</td>
</tr>
<tr>
<td>DNA pol</td>
<td>5’-CAGAACTTCTACAAACCCCCCA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-TAGATGATGCGCATGGAGTA-3’</td>
</tr>
<tr>
<td>iNOS</td>
<td>5’-CCCTTCCGAAGTTTTCTGGCAGCAGC-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GGCTGTACAGAGCCTCGTGGAAGTG-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-CAAGGCTGTGGGCAAGGTC-3’</td>
</tr>
<tr>
<td></td>
<td>5’-AGGTGGA AGAGTGGGAGTTGCTG-3’</td>
</tr>
</tbody>
</table>
6.2.9. Densitometry analysis
The respective RNA and proteins bands were analysed using a model GS-700 Imaging Densitometer and Molecular Analyst software (version 1.5; Bio-Rad Laboratories, CA, USA) (Ojha et al., 2015).

6.2.10. 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 ‘t’ test. A value of \(p < 0.05\) was considered to be statistically significant, compared with the respective control.

6.3. RESULTS

6.3.1. Dose-dependent effect of the test extract and isolated luteolin
To determine the effect of different doses of the test extract and or isolated luteolin on HSV post-infection, we exposed the virus (HSV-1 and HSV-2) infected Vero cells to the the test extract and or isolated compound-luteolin at two fold concentrations (0, 10, 20, 40, 60, 80 and 100 µg/ml) for 3 days. The results presented in Fig. 4.1.A showed that the test extract  at 79.6 and µg/ml and luteolin  at 36.4 and µg/ml exhibited greater than 99% inhibition of HSV-1. On the otherhand, similar effect was noticed with the test extract at 86.5 µg/ml and luteolin at 40.2 µg/ml on HSV-2 (Fig. 4.1.B), indicating a high correlation between drug concentration and its inhibition rate.
Figure 4.1. Dose dependent antiviral effect of the test extract (A) and luteolin (B) on HSV-1. Here MEPT, The test extract of *P. tithymaloides* leaves. Each bar represents the mean ± S.E.M of three independent experiments (**, P<0.001; *, P<0.05 compare with control).
6.3.2. Effect of the test extract or isolated luteolin at different time prior or after infection

To identify the possible phase of viral infection imparted by the test extract or luteolin, we performed the time-of-addition assay. The results revealed that the inhibitory effect was significant when the test extract or luteolin were added 2 h prior, during or 4 h post infection, which correlates with the entry and early stages of both HSV-1 and HSV-2 (Fig. 4.2) infection cycle.

Figure 4.2. Inhibitory effect of the test extract or isolated luteolin at different time using time-of-addition assays on HSV-1 (A) and HSV-2 (B). Inhibitory effect of the test extract leaves, Luteolin or ACV at various time points: Pre-infection (-2 h), Co-infection (0 h) and Post-infection (1–12 h) with
HSV-2G (100 PFU/well), determined by a plaque reduction assay. Here, DMSO, Di-Methyl Sulfoxide; ACV, Acyclovir; MEPT, The test extract of *P. tithymaloides* leaves. Each bar represents the mean ± S.E.M of three independent experiments (*, P<0.001, compare with control).

### 6.3.3. Virus entry (attachment and penetration) assay

To evaluate the antiviral mode of action we have studied the test extract or isolated luteolin treated virus infected Vero cell, investigated its effect on virus entry, particularly the attachment and penetration steps of HSV-1 life cycle. In this assay, when test extract or isolated luteolin was incubated with HSV-1 for 3 h at 4 °C in presence of test extract or luteolin and subjected to PRA, we found that test extract was not interacted with the virion significantly. On the otherhand luteolin interacts with the virion irreversibly to prevent infection at 2-3 time higher dose (60 µg/ml) of EC50 (Fig. 4.3.A). In another experiment to detect the bound virus on the adherent cells by indirect Immunofluorescence assay (IFA), we found that luteolin inhibit HSV-1 attachment to the Vero cell surface (Fig. 4.3.B) at 60 µg/ml. Together, these results suggest that luteolin might interact with the viral glycoprotein and/or cellular receptor during its attachment to the host cell at 2-3 time higher dose of EC50.
Figure 4.3. Inhibition of HSV-1 entry in presence of test extract (MEPT) and luteolin by inactivating viral particles and thereby preventing virus binding and internalization into Vero cells. (a) Viral inactivation assay by plaque reduction assay. The data shown are means ± SEM of three independent experiments with each treatment performed in duplicate. (b) Viral inactivation assay by IFA at 60 µg/ml of luteolin. (i) Cell control, (iii) Virus control, (iii) At 60 µg/ml concentration of luteolin.

6.3.4. Effect of test extract or luteolin on HSV induced NF-κB, MAPK, JNK1/2 and IκBα activation

As efficient virus (HSV-1 and HSV-2) replication requires NF-κB activation (Gregory et al., 2004), and HSV-infection selectively promotes MAPK phosphorylation (Zhang et al., 2010; Chenoweth et al., 2012), we examined whether test extract or isolated luteolin would block NF-κB, MAPK and JNK1/2 pathways during infection. The HSV-1 and/or HSV-2 infected Vero cells, untreated or treated with extract (79.4 and 86.5 µg/ml respectively) or isolated luteolin (36.4 and 40.2 µg/ml respectively) for 4 h, were used to determine the NF-κB nuclear
translocation by immunoblotting. In uninfected cells, p65 was detected at relatively low levels; whereas, in HSV infected cell p65 activation was significantly induced at 24h p.i. The test extract or luteolin treatment abated HSV-induced p65 nuclear translocation, suggesting that the test extract or isolated luteolin blocked HSV induced NF-κB activation, but not MAPK phosphorylation (Fig. 4.4 and 4.5). As shown in Fig. 4.6, the test extract or luteolin treatment significantly blocked viral ICP0 and ICP27 expression, implying the inhibition of HSV replication. Inhibition of IκB-α degradation induced by HSV infection, was also detected in these samples. Together, our data suggest that test extract/isolated luteolin block HSV-1 and HSV-2 infections through NF-κB inhibition.

Figure 4.4. Effect of the test extract and isolated Luteolin on HSV-1-induced MAPK, JNK1/2, NF-κB and IκBα activation. Expression of MAPK (A), JNK (B), NF-κB (C) and IκBα (D) were
determined by the Western blot, using GAPDH as the internal control. The average expression of NF-kB was significantly higher in the HSV-1 induced macrophage, as compared to the control and the test extract (MEPT) or luteolin co-treated group (*, P< 0.001). Here, lane 1, cell control; lane 2, cell + HSV-1; lane 3, cell + HSV-1 + the test extract (MEPT); lane 4, cell + HSV-1 + luteolin.

**Figure 4.5.** Effect of the test extract and Luteolin on HSV-2-induced MAPK, JNK1/2, NF-kB and IκBα activation. Expression of MAPK (A), JNK (B), NF-kB (C) and IκBα (D) were determined by the Western blot, using GAPDH as the internal control. The average expression of NF-kB was significantly higher in the HSV-2-induced macrophage, as compared to the control
and test extract (MEPT) or luteolin co-treated group (*, \( P<0.001 \)). Here, lane 1, cell control; lane 2, cell + HSV-2; lane 3, cell + HSV-2 + the test extract (MEPT); lane 4, cell + HSV-2 + luteolin.

6.3.5. Effect of the test extract and luteolin on HSV RNA expression

The time-of-addition assay suggests that the test extract and luteolin prevent HSV-2 infection when the treatment was carried out during virus entry into the Vero cell. To address whether this is due to the interference of the test agents on viral replication, we evaluated the effects of test drugs on HSV-1 mRNA expression after 12 h post infection. Vero cells were infected with HSV-1 for 1 h and extracellular virus particles were inactivated, washed and subsequently added with the test extract or luteolin. Total cellular RNA isolated from all samples at various time after infection, was subjected to cDNA synthesis and amplification by RT-PCR. Our results demonstrated that the test extract and luteolin blocked viral ICP0 and ICP27 expression, implying inhibition of HSV-2 replication (Fig. 4.6).

Figure 4.6. Effect of test extract and Luteolin on HSV-2-infected ICP0 and DNA polymerase gene expression. The HSV-2 infected Vero cells were treated with the test extract or Luteolin, incubated for 12 h, following which RNA was isolated and subjected to the RT–PCR analysis for the expression of ICP0 and DNA pol mRNA. Here, lane 1, cell control; lane 2, cell +HSV-2; lane 3, cell + HSV-2 + test extract (MEPT); lane 4, cell + HSV-2 + luteolin.
6.3.6. Test extract and isolated luteolin reduces HSV-induced cytokine and NO production

As NF-κB regulates the production of many inflammatory cytokines during HSV-infection (Mukherjee et al., 2013), we examined HSV-1 and HSV-2 induced cytokines, NO and iNOS gene expression in mouse peritoneal macrophages treated with the test extract or luteolin. We observed a significant down-regulation of TNF-α, IL-1β, IL-6 and IFN-γ in the test extract and luteolin treated HSV-1 (Fig. 4.7) and HSV-2 (Fig. 4.8) infected macrophage, compared with the infection control, by ELISA. Moreover, the NO and iNOS expression were suppressed in both the test extract and luteolin treated HSV-1 and HSV-2 infected macrophage, compared with virus infected untreated cell (Fig. 4.9).

Figure 4.7. Effect of the test extract and Luteolin on pro-inflammatory cytokine release in HSV-1-infected peritoneal macrophages by sandwich ELISA. The ELISA data are expressed as Mean ± SD from triplicate experiments, yielding similar results. Asterisks (*) indicate statistically
significant (*, P< 0.001) induction of TNF-α (A), IL-1β (B), IL-6 (C) and IFN-γ (D) release, compared to the infected macrophages.

Figure 4.8. Effect of the test extract (MEPT) and Luteolin on pro-inflammatory cytokine release in HSV-2-infected peritoneal macrophages by sandwich ELISA. The ELISA data are expressed as Mean ± SD from triplicate experiments, yielding similar results. Asterisks indicate statistically significant (*, P< 0.05; **P< 0.001) induction of TNF-α (A), IL-1β (B), IL-6 (C) and IFN-γ (D) release, compared to the infected macrophages.
Figure 4.9. Effect of the test extract (MEPT) and Luteolin on NO production and iNOS expression in HSV-1 and HSV-2 infected murine macrophages. (A) and (C) Macrophages (1X10^6 cells/well) were infected with HSV-1 and HSV-2 treated with the test extract (79.4 and 86.5 µg/ml respectively) or Luteolin (36.4 and 40.2 µg/ml respectively), and incubated for 24 h. The supernatant was removed and the concentration of NO was determined by Griess reagent. Data were expressed as Mean ± SD from triplicate experiments, yielding similar results (M, moles of nitrite). Asterisks indicate a statistically significant increase (**, P< 0.001; *, P<0.05) in nitrite generation, compared to the infected macrophages. (B) and (D) HSV-1F and HSV-2G induced macrophages were treated with the test extract or Luteolin, incubated for 12 h, following which RNA was isolated and subjected to the RT–PCR analysis for the iNOS2 mRNA expression. The data were expressed as Mean ± SD from triplicate experiments yielding similar results. The asterisk (*) indicates a statistically significant increase (*, P< 0.001) in iNOS2 expression, compared to the infected macrophage.