The present study aimed to evaluate the antiviral activity of a flavonoid rich plant extract and its isolated compound against Herpes Simplex virus (HSV) infection, *in vitro* and *in vivo*, and its molecular mechanism of action. The flavonoid rich extract was previously separated from an ethnomedicinal shrub *Pedilanthus tithymaloides* L. Poit of the family Euphorbiaceae, commonly used for antiviral, antibacterial, anti-inflammatory and related ailments (Bunyapraphatsara & Chokchaichareonporn, 2000; Ghosh *et al*., 2013). As an ongoing effort to identify natural molecules with drug-like property a number of ethnomedicinal plants used by diverse tribal communities of India including Onge, Nicobarese, Shompen, Birhore, Kattabhai, Santal etc was evaluated by this group (Chattopadhyay *et al*., 2015; Ojha *et al*., 2015). Our previous study with methanol extract of *Pedilanthus tithymaloides* leaves showed promising wound healing (Ghosh *et al*., 2012), anti-inflammatory (Ghosh *et al*., 2013) and moderate antiviral activity; while the aqueous part was nearly inactive with respect to the above studies. Thus, out of several use of *P. tithymaloides* we aimed to validate its anti-infective and related pharmacological activity, specifically antiviral potential against the common DNA virus, HSV. The chromatographic separation of bioactive methanol extract revealed a flavonoid rich part with better activity than its counterpart. Thus, following a bioactivity guided fractionation we attempt to isolate the active compound and evaluate the antiviral activity of both flavonoid rich extract and its isolated compound(s) against the wild type and clinically isolated strains of HSV-1 and HSV-2.

The preliminary cytotoxicity and antiviral activity study showed that the antiviral activity (EC$_{50}$) of flavonoid rich extract against HSV-1 and HSV-2 (43.6-52.6 µg/ml) was ten times less than its cytotoxic concentration (CC$_{50}$ of 436.5 µg/ml), compared to the standard antiherpes drug acyclovir (CC$_{50}$ 128.8 and EC$_{50}$ 2.3-2.9 µg/ml), indicated that the test extract has potential anti-HSV activity, which was also supported by its therapeutic or selectivity index (SI) and the extract need to be evaluated further for its antiviral spectrum against both wild type and clinical isolates of HSV-1 and HSV-2.

Following bioactivity guided fractionation the antivirally active flavonoid-rich extract yielded 200 sub-fractions, which were re-chromatographed with different solvent system, and the similar spots on TLC were combined (70-100), re-chromatographed and collected into 1-92 fractions of which 21-37 were combined, chromatographed and crystallized into Compound-1. While sub-fractions
128-141 were re-chromatographed, combined and crystallized to obtain **Compound-2**. The spectroscopic data, molecular weight, melting point and the existing literature indicated that **Compound-1** is a bioflavonoid chemically known as 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-chromenone, or luteolin (Hirobe *et al.*, 1997; Chiruvella *et al.*, 2007). While, the **Compound-2** was 1,2-tetradecanediol, 1-(hydrogen sulfate), sodium salt (Piepmeyer, 1966; Cahn *et al.*, 1966), first isolated from this plant.

Further, cytopathic effect (CPE) reduction, MTT and Plaque reduction assay revealed that the isolated luteolin inhibit both the virus strains (EC\textsubscript{50}) at 19.2- 26.8 µg/ml with CC\textsubscript{50} of 278.6 µg/ml, indicating its better activity spectrum then the parent extract. Additionally, both the flavonoid-rich extract and isolated luteolin inhibit a thymidine kinase deficient (TK\textsuperscript{−}) acyclovir (ACV) resistance HSV-2 isolate. Moreover, the SI value (10-14) indicated that this compound needs further study for its potential antiviral nature. On the other hand, the second compound 1,2-tetradecanediol-1-hydrogen sulfate Na-salt (Compound-2) with CC\textsubscript{50} 146.4, EC\textsubscript{50} of 124.6 µg/ml and SI of 1.2 indicated less or no potential, particularly against the test viruses and thus, was not studied further. Furthermore, the evaluation of *in vitro* antiviral activity on wild type HSV-1F and HSV-2G, clinical isolates of HSV-1 and HSV-2, and a TK\textsuperscript{−} ACV-resistant HSV-2 isolate by plaque reduction assay revealed that the test agents do have potent anti-HSV activity against all the isolates, far less than their CC\textsubscript{50} and perhaps with different mode of action than ACV. Then to find out the possible stage of viral life cycle affected by the test agents, we perform time-of-addition and removal assay. The life cycle of HSV comprises of six different stages including attachment, fusion and penetration (entry), transport, replication, protein synthesis, assembly and release. Thus, we attempted to study each step separately. The time kinetic study revealed that the inhibitory effect of test agents was significant when the extract or luteolin were added 2 h prior, during (co-infection) or 4 h post infection that correlates with the entry and immediate early replication of HSV infection cycle, as well as prior to infection.

Thus, our next aim is to study the antiviral mode of action of the test agents on uninfected and virus-infected Vero cells. As the time-of-addition assay revealed that the test agents are active 2h prior to viral infection and continued during infection upto 4 h post-infection, so we tested the effect of the test agents on immediate early infection as well as on host-modulation. For this we have
examined the entry stages of HSV life cycle in virus-infected Vero cells, untreated or treated with the test agents, particularly the attachment and penetration of the virus to the host cell, as well as the host cell modulatory events. When test extract or luteolin was incubated with HSV-1 or HSV-2 for 3 h at 4 °C, and then subjected to plaque reduction assay we found that the test extract was unable to interact with the HSV virion significantly, but irreversibly prevent the infection at three time higher dose (60 µg/ml) of EC₅₀ concentration (Figure 4.3A). Simultaneously to detect the bound viruses on the adherent Vero cells we conducted the indirect Immunofluorescence assay (IFA), and observed that only luteolin inhibit viral attachment to the Vero cell surface at a concentration of 60 µg/ml (Figure 4.3.B). Thus, collectively our results suggest that luteolin might interact with the viral glycoprotein and/or cellular receptor during its attachment to the Vero cell at higher dose, and probably the test agents also interfered during infection and prior to infection to modulate the host defense. Thus, we have also examined the immuno-regulatory events of the host cell prior to virus infection as well as during and upto 4 h post-infection in presence or absence of the test agents.

As the efficient replication of HSV requires NF-κB activation (Gregory et al., 2004) and HSV-infection selectively promotes MAPK phosphorylation (Zhang et al., 2010; Chenoweth et al., 2012), so we have examined whether the test agents could block NF-κB, MAPK and JNK1/2 pathways during infection. The immunoblotting assay of HSV-infected Vero cells, untreated or treated with the test agents (79.4-86.5 of extract or 36.4-40.2 µg/ml of luteolin) for 4 h, detect p65 at relatively low level in uninfected cells; whereas in HSV-infected cell p65 activation was significantly induced at 24 h post-infection, indicating that the test extract or luteolin treatment abated HSV-induced p65 nuclear translocation, and the test agents might blocked HSV induced NF-κB activation, but not MAPK phosphorylation (Fig. 4.4, 4.5). Moreover, the treatment of test agents demonstrated significantly down-regulated expression of viral ICP0 and ICP27, the two major immediate early proteins, which lead to the inhibition of HSV replication (Fig. 4.6). Additionally we found that the inhibition of IκB-α degradation was induced by HSV infection from the same samples. Together, our data suggest that the test agents block HSV infections through NF-κB inhibition, which is consistent with the earlier report that compounds which block NF-κB also have antiviral activity (Chen et al., 2011).
As the time-of-addition assay suggests that our test agents prevent HSV infection during the viral entry up to 4h post infection, so we have evaluated the effect of test agents on HSV-2 mRNA expression up to 12 h post-infection, to address whether this is due to the interference on viral replication or not. When the total cellular RNA extracted from the HSV-2 infected Vero cells, treated or untreated for 12 h post-infection, at various time and subjected to cDNA synthesis and amplification by RT-PCR, we observed significantly down-regulated expression of ICP0 and ICP27 in the test extract and luteolin treated cells (Fig. 4.6), indicating that the test agents interact with the immediate early replication of HSV.

Moreover, HSV infection elicits production of various chemokines and cytokines, including TNF-α, IL-1β, IL-6, RANTES, and IFN-γ (Swiecki et al., 2013; Dai et al., 2008; Mukherjee et al., 2013). Here, we observed that HSV infection of murine macrophage leads to significantly increased production of TNF-α, IL-1β, IL-6, IFN-γ, NO and iNOS expression. In contrast, test extract- or luteolin-treated groups exhibited significantly down-regulated production of TNF-α, IL-1β, IL-6 and IFN-γ expression (Fig. 4.7 and 4.8). Moreover, the NO and iNOS expression were suppressed in both the test extract and luteolin treated virus infected macrophage, compared with infected and untreated cell (Fig. 4.9).

It is known that the HSV infection is influenced by cellular signaling pathways and transcription factors, including the activation of MAPK that promotes viral replication (Zachos et al., 1999; McLean et al., 1999). Furthermore, HSV also activates IκB kinase to triggers IκB-degradation in the early stage of infection that result remarkable and unrelenting activation of NF-κB in epithelial, neuronal and lymphoid cells (Amici et al., 2001; Amici et al., 2006; Song et al., 2014), and the secondary plant metabolites that inhibit NF-κB or MAPK activation can inhibit HSV infection (Behbahani et al., 2013). Our results revealed that the test extract and luteolin can inhibit replication of the wild-type, clinical isolates and TK-deficient ACV-resistant strains of HSV-2, accompanied with NF-κB, which is in accordance with the above finding (Behbahani et al., 2013). However, In contrast to the previous reports that HSV-2 activates MAPK (Zhang et al., 2010; Chenoweth et al., 2012), we observed that neither MEPT nor luteolin affects HSV-2 induced MAPK activation. During early infection HSV activates NFkB by activating IKK, and the activated NFkB induces enhanced viral gene expression (Amici et al., 2001). It has been shown that the A and J type
cyclopentenone prostaglandins (cyPG) can block TNF-α induced NF-κB activation through direct inhibition and modification of the IKKβ subunit of IKK (Amici et al., 2006) leading to the potent antiviral activity of cyPG. While anti-HSV activity of berberine is due to the down-regulation of NF-κB and cellular c-Jun N-terminal protein kinase activation (Song et al., 2014). The curcumin, an inhibitor of NF-κB activation (Shishodia et al., 2005) has been shown to inhibit HSV immediate early gene expression and replication (Kutluay et al., 2008), but resveratrol, a natural stilbenoid, suppresses HSV-induced NF-κB activation, resulting in reduced expression of HSV genes and viral DNA synthesis (Faith et al., 2006; Chen et al., 2011). Altogether, these findings support our observation and reveal that the compounds targeting signaling pathways may exert selective activity against a varied group of viral pathogens (Ludwig, 2009; Baba, 2006).

Then we have tested the safety profile of the test agents in Balb/C mice. Oral (p.o.) treatment upto 14 days showed no manifestation of toxic effects or death in the treated animals, indicating that the test agent’s posse’s good safety profile. The oral sub-acute toxicity study for 28 days did not induce mortality or clinical toxicity and histopathological of kidney, liver and spleen was almost normal in test agents treated animals (Fig. 5.1) at 500 and 1000 mg/kg of test extract and 250 and 500 mg/kg of luteolin. Additionally, all the tested biochemical (SGPT, Alkaline Phosphatase, Cholesterol, Urea and Blood glucose) and hematological tests (Hb, RBC, WBC and clotting time) were within the normal range. Moreover, the dermal toxicity study showed that the test extract and luteolin did not induce any erythema and edema upto 72 h or reveal any histopathological changes in skin tissue, established the safety profile of the test agents.

The efficacy study showed that the virus yields in the skin and brain of infected mice on day 2, 4, 6 and 8 after infection were significantly reduced by the test extract (200 mg/kg) and luteolin (50 mg/kg) treatment. Further, the histopathology of skin and brain tissues of treated animals revealed that uninfected skin had intact epithelium without any inflammation and infection, while the HSV-1 infected skin tissues showed acute inflammation and leukocytic infiltration with extensive infection, but the infected animals treated with luteolin (50 mg/kg) and ACV (10 mg/kg) demonstrated limited infection. Therapeutic efficacy study of the test extract and luteolin examined on genital herpes model showed that the virus yields in the vaginal and brain tissue of infected mice on day 2, 4, 6 and 8 after infection were also reduce (57.12 -85.71% from day 2 to 8 post infection) significantly,
compared to virus infected and drug control group at doses of 200 and 50 mg/kg of test extract and luteolin. While in the brain, the reduction was 52.32-78.57% from day 4-8 post infection, but it was 26.37% in vaginal mucosa and 62.79% in brain of acyclovir treated group. Collectively our result showed that luteolin exhibited significant anti-HSV activity by reducing virus load in both the vaginal mucosa and the brain (p<0.01).

Next, to determine the immunomodulatory role of test agents we used HSV-1 infected drug treated Trigeminal Ganglia and found significantly down-regulated level of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IFN-γ compared to infection control (Fig. 6.3), indicating that the test extract and luteolin indeed had immunomodulatory potential to clear the virus. Further, the HSV-1 infected mice, untreated or treated with the test agents when subjected to test for NF-κB nuclear translocation we found that the test agents abated HSV-induced p65 nuclear translocation by the extract (200 mg/kg) or luteolin (50 mg/kg) in 8th day, suggesting that the test agents might block HSV induced NF-κB activation, but not MAPK phosphorylation (Fig. 6.4) and thereby prevent HSV-1 infection.

Moreover, as it was reported that HSV infection can induce TLR3 expression (Zhang et al., 2007; Chenoweth et al., 2012) so we determined the effects of the test extract or luteolin on TLR3 signaling pathway in HSV infected animals. The results showed significantly high TLR3 mRNA expression in infected mice but reduced in test agents treated group, compared to the controls (Fig. 6.5). Western blot analysis further illustrated a luteolin-mediated inhibition of TRIF expression in HSV-infected mice compared to the untreated virus-infected animals (Fig. 6.5), indicating that the flavonoid-rich extract or luteolin could reduce TLR3 mRNA levels, and inhibit TRIF expression following HSV-infection.

Further, we observed that HSV infection leads to significantly increased production of TNF-α, IL-1β, IFN-γ with increased expression of iNOS; while the test extract or luteolin-treated groups exhibited significantly down-regulated production of TNF-α, IL-1β, IL-6, IFN-γ and expression of iNOS. It is known that the Toll-like receptors (TLRs) represent an innate immune antigen recognition system to detect molecular patterns associated with the respective pathogens; and the ligation of the respective TLRs with their ligands in turn activates immune responses resulting in
pathogen clearance (Zang et al., 2011). These important innate immune molecules are necessary for the initiation of effective immunity as well as implicated in the early activation of cellular immune responses to generate chemokines at the site of infection. Out of several TLRs involved in viral infections, TLR2 (Kurt-Jones et al., 2004), TLR3 (Reinert et al., 2012), and TLR9 (Lund et al., 2003; Krug et al., 2004) have definite role in HSV infection. The replication of HSV requires dsRNA that in turn is recognized by TLR3, resulting in the activation of host immune responses; and the activation of TLR3 in epithelial cells, following exposure to HSV, leads to the significant production of IL-6, RANTES, and IFN-inducible protein 10 (IP-10), indicating that the TLR3 activation mediated inflammatory cytokine and chemokine production induced inflammation in HSV-infected epithelial cells (Li et al., 2012). Previously it has been reported that the activation of TLR3 during immune response in epithelial cell, promote a Th1-type response to reduce infection resulting in diminished pathology (Hayashi et al., 2012). Interestingly, Polyinosinic- polycytidylic acid (poly (I:C)), a potent inducer of different Type IFN, suppresses HSV replication by increased activation of an antiviral state stimulated by TLR-3 (Cherpes et al., 2013). Here, we found that the TLR3 expression was increased during HSV infection; while the test extract and luteolin treatment decreased TLR3 expression and inhibited the upregulation of TRIF expression, suggesting that the test extract and luteolin can alter HSV infections via a TLR3 signaling pathway. This is consistent with the earlier report that luteolin suppressed NF-kB activation and COX-2 expression by inhibiting TRIF signaling associated with TLR3 and TLR4 MyD88-independent pathway by targeting TANK-binding kinase 1 and RIP1 of TRIF complex (Lee et al., 2009). Based on the above data, we hypothesized that HSV-induced TLR3 expression reduced IFN-γ production, and our study showed that luteolin suppressed the NF-kB activation by inhibiting TRIF signaling, and demonstrated that luteolin exerts its anti-HSV activity by reduced IFN-γ production, related to TLR3 signaling. Interestingly, luteolin treatment decreased TLR3 mRNA expression with down-regulation of TRIF and NF-kB expression, following HSV infection. Taken together our data suggested that luteolin do have partially direct anti-HSV activity and capable to alter the HSV-induced inflammation, via TLR3 signaling pathway.