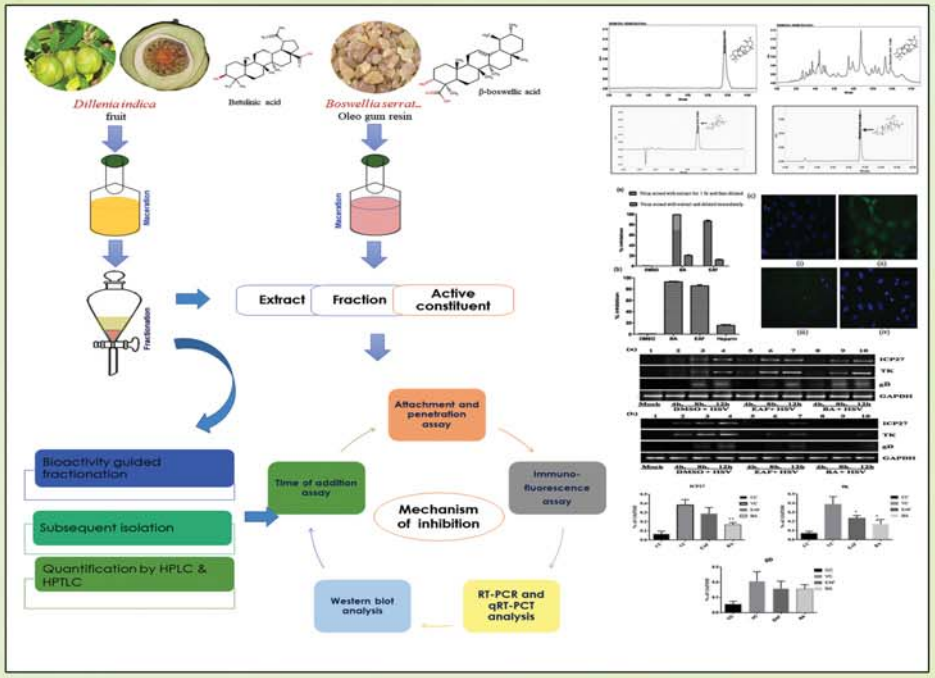
HSV infection is globally prevalent in individuals of all age groups, and a matter of serious concern as 60-90% of global population are infected with this virus. Currently available antiviral drugs acyclovir and its related agents successfully managed the infection but neither able to eliminate the virus from the host not can prevent recurrent infections. Moreover, frequent development of HSV resistance to anti-herpetic drugs is considered a serious problem. Thus, identification of new anti-herpetic lead is the need of the hour. Medicinal plants are a good source of antivirals, particularly as HSV inhibitors. Several medicinal plants are reported to inhibit and prevent the HSV infection in human. In this context, this study was designed to evaluate the HSV-1 inhibitory potential of two Indian medicinal plants along with their mode and molecular mechanism of inhibition to develop potential anti-HSV leads.



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Ph.D Thesis

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1. Screening of ethnomedicinal plants of diverse culture for antiviral potentials. Indian Journal of Traditional Knowledge (CSIR-NISCAIR, India) 2016, 15 (3), 474-481.

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3. *Boswellia serrata* oleo-gum-resin and β-boswellic acid inhibits HSV-1 infection *in vitro* through modulation of NF-κB and p38 MAP kinase signaling. Phytomedicine (Elsevier Science, USA) 2018, 51, 94-103.

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Certificate

This is to certify that the thesis entitled "Lead finding from Indian medicinal plants for antiviral potentials" submitted by Mr. Debayan Goswami, who got his name registered on 12.09.2012 for the award of Ph. D (Pharmacy) degree of Jadavpur University is absolutely based upon his own work under the supervision of Prof. Pulok K. Mukherjee and Dr. Debprasad Chattopadhyay that neither this thesis nor any part of the thesis has been submitted for any degree/diploma or any other academic award anywhere before.

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Declaration

I hereby declare that my research work embodied in this Ph.D. thesis entitled **"Lead finding from Indian medicinal plants for antiviral potentials"** have been carried out by me in the School of Natural Product Studies, Dept. of Pharm. Tech., Jadavpur University, West Bengal, Kolkata, India and in ICMR Virus Unit, ID & BG Hospital, Beliaghata, Kolkata, India under the direct supervision of Prof. Pulok K. Mukherjee, Director, School of Natural Product Studies, Dept. of Pharm. Tech., Jadavpur University, West Bengal, Kolkata, India and co-supervision of Dr. Debprasad Chattopadhyay, Scientist-G, ICMR Virus Unit, ID & BG Hospital, Beliaghata, Kolkata, India. I also confirm that this work is original and has not been submitted partially or in full for any other degree or diploma to this or other University or Institute.

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Content

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Debayan Goswami

List of Figures

List of Tables

Abbreviations Used

Abb. form	Explanation	Abb. form	Explanation
ACV	Acyclovir	HSV	Herpes Simplex Virus
AF	Aqueous fraction	VZV	Varicella zoster virus
AIDS	Acquired Immune Deficiency Syndrome	IL	Interleukin
ANOVA	Analysis of variance	ICP	Infected cell protein
ATCC	American type culture collection	PRA	Paque reduction assay
BA	B-boswellic acid	IE	Immediate early
BSA	Bovine serum albumin	IF	Indirect immunofluorescence
BSE	*Boswellia serrata* extract	IFA	Indirect Immunofluorescence Assay
BT	Betulinic acid	JKN	c-Jun N-terminal Kinases
CC50	Cytotoxic concentration that is toxic to 50% cells	LAT	Latency associated transcript
CNS	Central Nerves System	ME	Methanol extract
CO2	Carbondioxide	MOI	Multiplicity of infection
CPE	Cytopathic effect	MeOH	Methanol
DAPI	(4',6-diamidino-2-phenylindole)	$MgCl_2$	Magnesium chloride
DMEM	Dulbecco's minimum essential medium	NBF	n-butyl fraction
DMSO	Dimethyl sulfoxide	MAPK	Mitogen-activated protein kinases
DNA	Deoxiribo Nucleic Acid	MS	Mass spectrometry
EAF	Ethyl acetate fraction	MTT	(3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
EBV	Epstein-Barr virus	NF-κB	Nucler Factor Kappa B
EC50	Effective concentration required to achieve 50% protection	OECD	Organisation for Economic Cooperation and Development
ELISA	Enzyme link immunosorbent assay	NNRTI	Non-nucleotide reverse transcriptase inhibitor
EMEM	Eagle's minimum essential medium	Nacl	Sodium chloride
FBS	Fetal bovine serum	NFDM	Nonfat dry milk
FCS	Fetal calf serum	PBS	Phosphate buffer saline
FITC	Fluorescein isothiocyanate	PAGE	Polyacrylamide gel

			electrophoresis
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	PCR	Polymerase Chain Reaction
gB	Glycoprotein B	SI	Selectivity index (CC_{50}/EC_{50}
gC	Glycoprotein C	PFU	Plaque forming unit
gD	Glycoprotein D	PVDF	Polyvinyl difluoride
gH	Glycoprotein H	RPMI	Roswell park memorial institute medium
gL	Glycoprotein L	RNA	Ribo Nucleic Acid
gM	Glycoprotein M	RTPCR	Reverse transcriptase polymerase chain reaction
HCl	Hydrochloric acid	qRTPCR	Real-Time Quantitative Reverse Transcription polymerase chain reaction
HIV	Human Immunodeficiency Virus	SD	Standard deviation
HPTLC	High Performance Thin layer Chromatography	SDS	Sodium dodecyl sulfate
ICH	International conference on harmonization	TNF	Tumor necrosis factor
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	TOF-MS-ES+	Time-of-flight mass spectrometry
OD	Optical density	TT	Tukey's test
p.i.	Post-infection	TPCK Trypsin	L-1- Tosylamide-2-phenylethyl chloromethyl ketone trypsin
RP-HPLC	Reverse phase High Performance Liquid Chromatography	TK	Thymidine kinase
TRIF	TIR-domain-containing adapter-inducing interferon-β	TCM	Traditional Chinese medicine

DEDICATED TO

TO ALL THOSE KNOWN AND UNKNOWN SCIENTISTS WHO SPEND THEIR LIVES IN THE FOUR WALLS OF LABORATORY FOR THE BETTERMENT OF HUMANITY

Chapter 1

Plants with anti-HSV (Herpes Simplex Virus) potential

1.1. Virus and their Uniqueness

The word "*Virus*" in Latin means "poison" or 'lethal substance'. A virus is an ultramicroscopic metabolically inert 'acellular' infectious particle that can reproduce only inside its specific living host cell. Viruses are capable of infecting almost all types of living organisms, from microbes (Archea, Bacteria) to plants, and animals to human being, and are the most copious biological entity almost ubiquitous on earth.

Virus particles are equipped with necessary tools to aid their invasion into living host cells to multiply by manipulating the host cell machinery. The infectious viral particle or *virion*, is usually made up of nucleoprotein materials containing either DNA or RNA, that carry the genetic information's; and a surrounding protein covering or coat called as capsid to surround and protects the genetic material. However, some viruses contain an additional external lipid envelope to cover the protein coat when the virions are outside the host cell. Viruses are obligate intracellular parasites that utilize the host cell machinery to propagate and cause ailments as benign as a common wart, as irritating as a cold, or as deadly as the bloody African fever (Chattopadhyay *et al*., 1999). Despite the lack of cellular architecture viruses showed parasitic nature and thus, can be termed as the '*acellular parasites of cellular hosts*' (Chattopadhyay et al., 1999; 2009). From the evolutionary point of view viruses have several invasion strategies and each virion has its own unique configuration of surface molecules that enable them to enter into the host cells by precisely fitting their surface molecules with the molecules of target cell (Wagner & Hewlett, 1999; Chattopadhyay & Bhattacharya, 2008). For example, the viruses that cause Lassa fever, Ebola fever and AIDS spread easily, kills swiftly/quickly and have no cure or vaccine. Each strain of virus is unique in its surface antigenic structure, its receptors on host cells and its life cycle, and thus, viruses are *host* and *route* specific. As a consequence of genetic variation, variety in the mode of transmission, efficient replication and the ability to persist within the host, viruses have adapted to all forms of life and occupied numerous ecological niches, resulting in widespread diseases from microbes to plants, and livestock to humans (Chattopadhyay et al., 1999; Chattopadhyay & Naik, 2007). Moreover, viral genomes are highly error prone and thus, can mutate rapidly with very high rate (Shum et al., 2013), that results in the continuous change of its capsid or membrane proteins, surrounding the viral genome, to saves space and confuses the host. In addition, larger viruses\ contain an outer lipid membrane, embedded with glycoproteins, to recognize the weak points on the target cells through

which the virus particle can enter. In HIV infection, this weak point is the K+ channel of T4-lymphocytes, which is associated with a chemokine receptor (Dragic et al., 1996). Additionally, some viruses possess certain specific enzymes to establish infection within the host, e.g., the Influenza virus posse's the enzyme neuraminidases or lysozyme on their surface to hydrolyze the protective structures on host's plasma membrane. Once the virus particle has entered the plasma membrane of the target cell, it fuses its lipid membrane with a lysosome. Then, the proteases in the latter hydrolyze the protein capsid around the viral genome and the nucleic acids somehow escape into the cytoplasm. The viral nucleic acid then reaches the host chromosomes via nuclear pore. The viral genome essentially carry at least three different genes: *pol* (polymerase), *gag* (a gene-regulating protein), and *env* (envelope). In RNA virus, the *pol* gene delivers the reverse transcriptase, while other viruses may provide it during a previous infection of the host cell, and synthesizes a DNA-strand complementary to the viral RNA. The viral DNA is then incorporated into the cellular genome. On the other hand, the DNA viruses insert their genomes directly into the chromosomes of the host cell with the help of host's enzymes, and the viral DNA incorporated into host chromosome (DNA) as *proviral DNA*.

The integrated viral genome can enter into either, *lysogenic* or *lytic* cycle. In the lysogenic state, the viral genes may remain silent for a prolonged period, but upon adequate provocation, perhaps a second infection or an irradiation, it becomes active, i.e., it enters the lytic state. In the lytic stage, the viral genome takes command over the metabolic machinery of the host cell, using the host cell enzymes and the substrates to provide energy and building blocks for new virus particles. These processes deprive the host cell to utilize its essential substrates, making the infected cell starves that lead to the early death, while scores of new virus particles leave the dying cell. Alternatively, the infected cell becomes ''immortalized'' and provides a source of new viral particles during its extended life time.

1.2. Herpes Simplex Virus

Infections caused by Herpes Simplex Virus are of major concern owing to its silent epidemic nature, recurrent potential with periodic reactivation and frequent development of drug-resistance with variable manifestations. HSV causes primary infection as herpes labialis, herpes genitalis, corneal scarring with loss of sight (Jin et al., 2003), and travels

to the nerve ganglion to establish lifelong latency (Whitley and Roizman, 2001), followed by reactivation to cause recurrent infection at the primary site.

Primary infection with HSV-1 occurs predominantly in children. It is often asymptomatic. However, HSV-1 infection can result in a wide spectrum of clinical presentation, ranging from asymptomatic infection, to ulcerative and vesicular lesions of the skin, and to systemic infection of large organs. Furthermore, symptoms differ between primary and recurrent lesions. When symptoms do occur, vesicular lesions are usually intraoral, affecting the gingival and buccal mucosa (gingivostomatitis and pharyngitis). The vesicles formed then ulcerate, exposing infectious particles to the environment and therefore other individuals. Over the following 1-2 weeks, any symptomatic peripheral lesion in the mucosa usually resolves. During primary infection, some viral particles enter the endings of peripheral sensory nerves (predominantly c-type nerve fibres) and the virus travels retrogradely to the dorsal root ganglion (DRG) of the innervating dermatome. In oral infection the HSV is transported to the trigeminal ganglion, where a latent infection occurs, which is a common ability of all herpes viruses. (Jones, 2003).

Periodically, the virus reactivates from latency. However the factors responsible for this reactivation are still unclear, but include febrile illnesses, psychological stress, and systemic immune-compromised states. After reactivation, viral particles travel down the same peripheral nerves and re-infect the innervated area of skin. The most common sites for reactivation include the lips and adjoining skin (herpes labialis). Clinically, secondary infection is characterized by an early symptom of pain, burning, tingling, or itching, followed by macular lesion within 24 hours, which later become vesicular and then pustular and most often cleared within 2 weeks.

In the majority of cases, local peripheral HSV infections are not life threatening, although the morbidity can be both physically and psychologically debilitating. This is particularly in complicated HSV-1 infection, such as herpes keratitis (the second most common cause of non-traumatic corneal blindness), herpetic whitlow and eczema herpeticum, which may result from auto-inoculation from the oral lesion. HSV can also rarely result in life-threatening encephalitis. The HSV-1 infection is not restricted to the oral region. An increasing number of genital herpes infections, particularly in women under 25 years age, are now caused by HSV-1 (Malkin, 2004), and shedding genital herpes during child

birth increases the risk of neonatal infection. This has a high mortality rate and incidence of neurologic shriek in survivors (Whitley, 2004).

1.3. Biology of HSV-1

The name Herpes is derived from the Greek word '*herpein*' which means to creep', which refers to the chronic, latent or recurrent infections that occur or spread as skin lesions. Scientists remained bewildered for many years regarding the epidemiology of HSV infections until it was discovered in 1950 by Bumet and Buddingh showing the latent nature of HSV after primary infection and reactivate after later incitement. The other members of the herpes virus family also showed similar results which confirmed that latency is a shared property amongst them.

Conventionally, the viruses of *Herpesviridae* family share a common virion structure. The viral particle consists of a double-stranded DNA core surrounded by an icosahedral capsid consisting of 162 capsomeres enclosed by protein unstructured matrix called tegument, which, in turn, is surrounded by a bilayered lipid envelope with embedded branched glycoproteins. Referring to these morphological characteristics, various viruses infecting different hosts are classified as herpes viruses.

There are at least 25 viruses in the family of *Herpesviridae*, which is classified into three subfamilies: alpha (α), beta (β) and gamma (γ); and there are eight herpes virus types that frequently infect human beings (Table 1.1).

Table 1.1. Classification and disease profile of Herpes viruses

Type	Synonym	Subfamily	Pathophysiology
HHV-1	Herpes simplex virus-1 (HSV-1)	*Alphaherpesvirinae* (α-herpes virus)	Oral and/or genital herpes; usually orofacial
HHV-2	Herpes simplex virus-2 (HSV-2)	*Alphaherpesvirinae* (α-herpes virus)	Oral and/or genital herpes; usually genital
HHV-3	Varicella zoster virus (VZV)	*Alphaherpesvirinae* (α-herpes virus)	Chickenpox and Shingles
HHV-4	Epstein-Barr virus (EBV) Lymphocryptovirus (LCV)	*Gammaherpesvirinae (*γ-herpes virus)	Infectious mononucleosis, Burkitt's lymphoma, CNS lymphoma (in AIDS patients), Post-transplant lymphoproliferative syndrome (PTLD),

			Nasopharyngeal carcinoma.
HHV-5	Cytomegalovirus (CMV)	*Betaherpesvirinae* (β- herpes virus)	Infectious mononucleosis-like syndrome, retinitis etc.
HHV-6, 7	Roseolovirus	*Betaherpesvirinae* (β- herpes virus)	Roseola infantum or *exanthema subitum*
HHV-8	Kaposi's sarcoma-associated herpesvirus (KSHV) , a rhadino-virus	*Gammaherpesvirinae* (γ- herpes virus)	Kaposi's sarcoma, primary effusion lymphoma, some multicentric Castleman's disease.

1.3.1. Virus structure and replication

HSV belongs to a group of viruses, the genomes of which comprises a single large double-stranded DNA molecule (Roizman, 1996). The herpes simplex virion is divided into four segments: (1) a dense core containing viral DNA; (2) an icosahedral capsid; (3) a formless, unusual layer of proteins, known as tegument, which encompasses the capsid; and (4) an envelope. The structure of HSV-1 virion is presented in Fig. 1.1. The center contains the linear double-stranded DNA enveloped as a toroid. A little part of the viral DNA seems to be circular. Viral DNA contains at least 152 kbp. The variability in size is due to the variation in the number of reiterations of specific terminal and internal sequences (Whitley et al, 1997).

The capsid comprises of 162 capsomeres and is encircled by the firmly adhering tegument. There are three types of capsids which can be isolated from infected cells: A-capsids (procapsids) lack both scaffold proteins and viral DNA; B-capsids does not hold for the viral DNA yet contain the protein framework for it; C-capsids contain the viral genome (Gibson and Roizman, 1972; Sheaffer et al, 2001). Capsids comprises of four chief proteins: the significant capsid protein UL19 (VP5), VP26 accessory protein (UL35), and furthermore UL18 (VP23) and UL38 (VP19C) proteins, whose roles are not yet well studied. Six duplicates of the real capsid protein, VP5, frame the hexons, and five duplicates shape the pentons. Six duplicates of VP26 possess the external surfaces of the hexons shaped by VP5. A solitary particle of VP19C and two duplicates of VP23 shape a triplex that ties encompassing capsomeres to frame associations between them. In the focal point of each capsomere, there is a channel joining the virion external surface and center. The channels in hexamers are 4 nm in diameter, and in pentamers

they are marginally smaller, while in B-capsids these channels are totally shut. The capsid contains UL6 protein, which shapes the entry on the vertex of one of the 12 capsid, through which the viral genome is apparently stuffed into the capsid (Brown and Newcomb, 2011), and VP24 (UL26) protease, breaking the scaffold during DNA packaging.

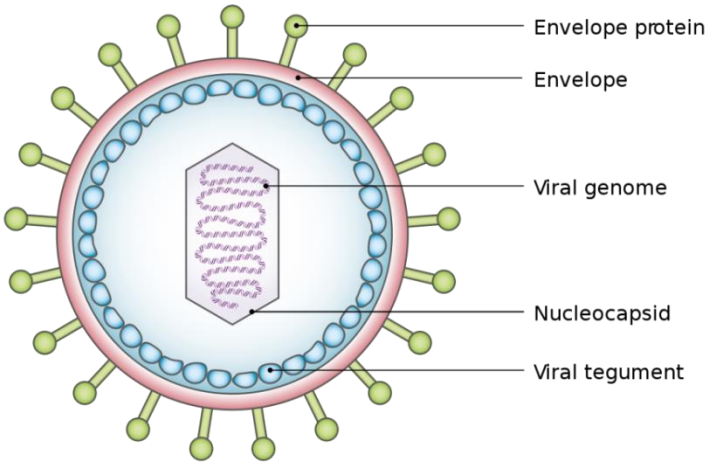


Fig. 1.1. HSV-1 virion structure

(Adopted from https://en.wikipedia.org/wiki/Viral_tegument)

The tegument consisting of 26 proteins, participates in the transport of capsid to the nucleus and other organelles (UL36, UL37, ICP0) (Radtke et al, 2010). Some of the proteins (VP1-2, UL36) also plays a pivotal role in, entry of viral DNA into the nucleus (Jovasevic et al, 2008), activation of early genes transcription (VP16, UL48) (Ace et al, 1989), clampdown of cellular protein synthesis, and degradation of mRNA (VHS, UL41) (Barzilai et al, 2006). The RNA-binding proteins US11, UL47, and UL49 of the tegument apparently bind to viral and cellular transcripts wrapped in the virion.

The virion outer envelope comprises of a bilayer lipid and 11 glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK), membrane gL, and gM (Chowdhury et al, 2013). It also contains two unglycosylated membrane proteins (UL20 and US9). The bilayer lipid is a result of virus exit of host cell by exocytosis which is formed from host cell membrane during the process.

1.3.2. Life cycle of HSV

The life cycle of HSV is classified into six steps: (i) attachment of virion and host cell entry; (ii) transport of the viral particle or virion to the nucleus; (iii) viral genes expression;

(iv) replication; (v) assembly of virion and (vi) exit or egress of the new virion from the infected cell (Fig. 1.2).

1.3.2.1. Virion attachment and entry

The viral particle or virion attach to the cell surface by viral glycoproteins gB and gC, which act together with the cell surface glycosaminoglycans, specifically with heparan sulfate (Herold et al, 1994). The viral entry into the host cell involves the union of the viral outer envelope with the plasma membrane which is facilitated by the interaction of four glycoproteins, gD, gB, and the heterodimer gH/gL (Gianni et al, 2009; Avitabile et al, 2009). Glycoprotein gD can bind to three types of receptors: nectin-1 and nectin-2, herpes virus entry mediator (HVEM), and 3-O-sulfated heparan sulfate (3-O-S-HS). (Baldwin et al, 2013). It also activates membrane fusion by interacting with the gB and gH/gL complex. (Satoh et al, 2008).

1.3.2.2. Transport of virion to the nucleus

After the completion of envelope fusion the virion along with the tegument and capsid are transported to the nucleus. The tegument and the capsid remains attached with each other and gets transported to the nucleus by the cytoskeleton proteins of the infected cell (Zaichick *et al*., 2013). After reaching the nucleus the capsid, injects the linear viral DNA into the nucleus, through the capsid portal, formed by the creation of a twelve units of the capsid protein pUL6 (Trus *et al*., 2004).

1.3.2.3. Viral gene expression in host cell nucleus

Viral transcription, replication and the assembly of progeny capsids (Fig. 1.2) take place within the host cell nucleus. Once the viral DNA is injected into the host cell nucleus, the virion DNA releases its multifunctional tegument protein VP16 along with virus shut off protein VHS that stops the host cell protein synthesis and subsequently degradation of cellular mRNA. As a result the host cell protein synthesis and cellular replication stops and replication machinery goes under the control of the virus. Vital cellular processes such as transcription (Jenkins and Spencer, 2001), linking of the cellular RNA, protein biosynthesis (Hardy and Sandri-Goldin, 1994), and cellular response to infection are also hindered. All these steps add fuel to the viral replication and transcription. The host cell RNA-polymerase II in turn synthesizes the viral mRNA with the contribution of viral

factors in all steps in infection. The cascade of HSV genetic events are sequential and divided into three stages as immediate early (IE), early (E), and late (L) gene synthesis.

For the transcription of IE genes, the presence of the tegument protein VP16 is crucial (Mackem and Roizman, 1982). VP16 protein released from tegument interacts with the transcription factor and trigger the release of host cell factor – 1 (HCF1) and then together with HCFC1 protein forms a complex to activate transcription of IE or α genes.

An interesting characteristic of VP16 is its ability to regulate the methylation and demethylation of histone H3 that binds with non-nucleosomal viral DNA at the IE, E and L gene promoters during infection. During infection, VP16 is responsible to trigger a cascade of viral gene expression by activating the viral α gene promoters and removal of histone H3. (Herrera and Triezenberg, 2004). After that five genes (ICP0, ICP4, ICP22, ICP27 and US1.5) belonging to the group of IE genes, activate the transcription of E genes. The main function of the IE gene-encoded proteins is the activation of E gene expression. Proteins and enzymes encoded by the E genes are involved in viral genome replication (e.g. HSV DNA polymerase, UL30), regulation of nucleotide metabolism (e.g. thymidine kinase, UL23), suppression of IE genes, and activation of L genes. The level of expression of DNA polymerase reaches its maximum only 4 h after infection (Yager and Marcy, 1990). After initiation, viral DNA synthesis shifts from a replication mechanism to a rolling-circle mechanism (Skaliter and Lehman, 1994), which concatemeric molecules that are cleaved during the process of nucleocapsid assembly.

1.3.2.4. Viral DNA replication

The commencement of DNA replication in HSV is the unwinding of the double helix by by UL9 and additionally ICP8 (UL29) proteins in the AT-rich regions of the oriL or oriS origins of replication. The latter are available in one duplicate in UL of the genome, and in two duplicates in US of the genome. ICP8 ties ssDNA fragments, and UL9 binds particularly to oriS and loosens up it. At that point helicase– primase complex made out of UL5, UL8, and UL52 proteins is stacked. Its helicase movement effectively catalyzes unwinding of dsDNA just if single-stranded shade of more prominent than six nucleotides is accessible. The leading and lagging DNA strands are integrated by viral DNA polymerase (UL30) complexed with processivity factor UL42 (Zuccola et al, 2000).

Notwithstanding the viral proteins, a couple of cell proteins including DNA ligase, topoisomerase II, and different segments of the DNA repair and homologous recombination frameworks seemed to take an interest in the replication (Weller and Coen, 2012). Some viral proteins like thymidine kinase (UL23), ribonucleotide reductase (UL39, UL40), deoxyuridine triphosphatase (UL50), uracil N-glycosylase (UL2), and soluble nuclease (UL12) partake in nucleotide digestion. These proteins are basic for viral DNA synthesis and repair in light of the fact that the production of the relating host cell enzymes is stifled.

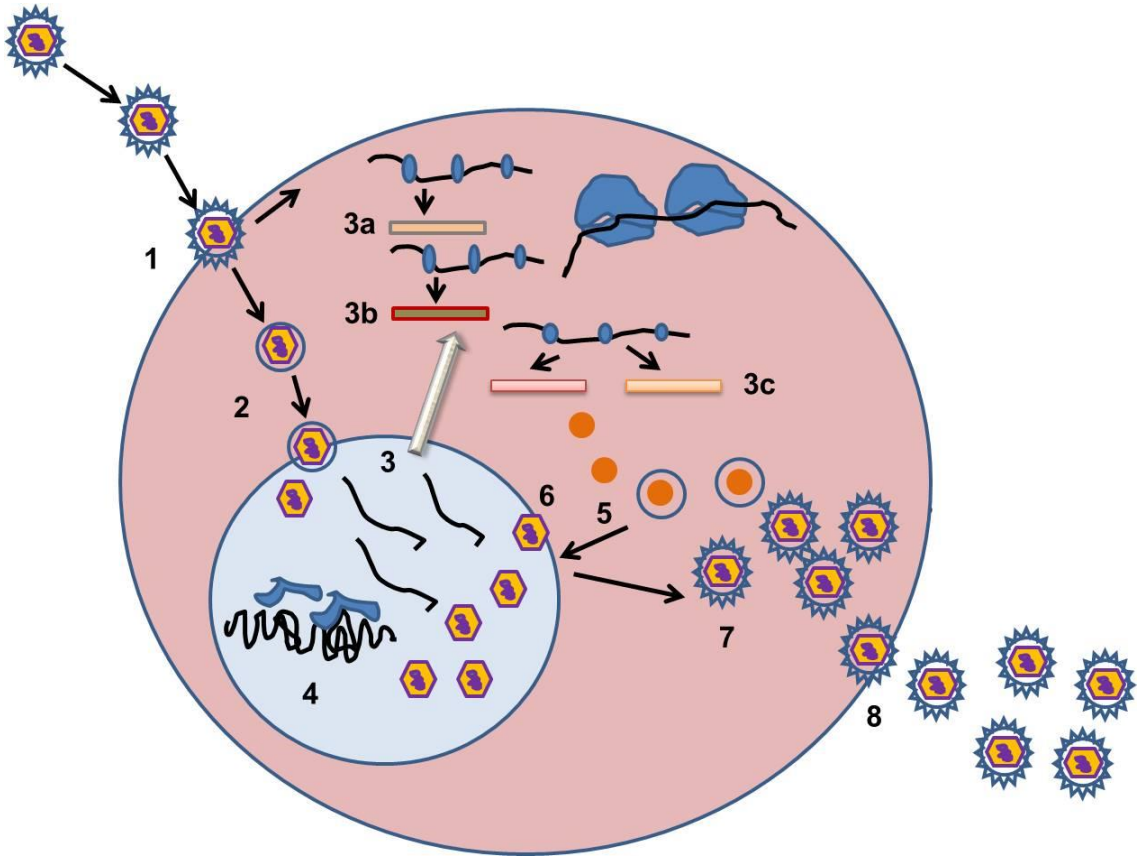


Fig. 1.2. HSV-1 life cycle: 1) virion attachment and entry into the cell; 2) transport to the nucleus; 3) viral gene expression: immediate early (a), early (b), and late (c); 4) viral DNA replication; 5) nucleocapsid assembly; 6) capsid maturation; 7) primary envelope formation; 8) egress.

1.3.2.5. Assembly, maturation, primary envelop formation and egress

After the inception viral DNA replication, the late genes expression, particularly encoding capsid proteins, encourages the gathering of progeny virions. The capsid assembly and viral genome packing happen in the nucleus (Fig. 1.2). This follows the exit or egress of nucleocapsid from the nucleus via nuclear pore or by budding through the nuclear membrane. The capsid is transported from the nucleus to the cytoplasm with the help of UL36 and UL37 proteins, (Sandbaumhuter et al, 2013). There the development of virion and external envelop groundwork take place. The exit of the virion from the host cell by exocytosis leads to envelope development (Fig. 1.2). And in addition amid virus entry into the cell, the exit of virions is related with microtubule-based transport and with the UL37 interaction with molecular motor dystonin. (Pasdeloup et al, 2012).

1.3.2.6. HSV Latency – a unique evolutionary survival mechanism

An interesting property of Herpes viruses is their ability to establish a latent infection. After primary infection, HSV-1 either replicates productively in epithelial cells to proceed for lytic cycle or enters sensory neuron axons and moves to the neuronal cell nucleus. There, the viral DNA remains circular and does not possess any lytic gene expression known as true latency; however, latency associated transcripts (LATs) are expressed and then spliced to give some mRNAs. However the exact reason and mechanism of latency is still not known fully. By repressing the lytic cycle the viral genome continues to replicate within the neuronal nucleus until some trigger cause the virus to migrate back from the neuron to the previously infected region. Thus, low level of viral replication and shedding takes place in some infected people due to partial latency. Recent views on the functions of LATs are conflicting, but their suggested major function is generation of miRNAs and siRNAs that downregulate ICP0 and other lytic gene expression (Webre et al, 2012).

1.4. Global estimate of HSV-1

According to WHO latest reports the estimated worldwide prevalence of HSV-1 infection among 0–49 year olds in 2012 was 67% averaged across all ages,. In 2012 it was estimated that there were 3709 million individuals aged 0–49 years with prevalent HSV-1 infection globally. However the new evaluations highlight that HSV-1 is also an important source of genital herpes. It has been figured out that 140 million people aged 15–49

years were having prevalent genital HSV-1 infection worldwide in 2012. Among them based on statistical significance it was assumed that 50% of incident infections in this age group were genital, and up to 239 million people of 85% of new HSV-1 infections were genital. The total figure of new infections was estimated to be 118 million. It was an intimidating figure as two-thirds of HSV-1 infections occurred in those aged 0–5 years in Eastern Mediterranean, half in Western Pacific, and one-third in Europe. Compared to that, around half of new HSV-1 infections in the Americas occurred in those aged 15–49 years.

In developed countries less number of people are getting infected with HSV-1 as children because of obvious reasons like better hygiene and living conditions. But they are at risk of contracting it genitally through oral sex after they become sexually active.

Estimates for HSV-1 prevalence by region among people aged 0-49 in 2012:

- Americas: 178 million women (49%), 142 million men (39%)
- Africa: 350 million women (87%), 355 million men (87%)
- Eastern Mediterranean: 188 million women (75%), 202 million men (75%)
- Europe: 207 million women (69%), 187 million men (61%)
- South-East Asia: 432 million women (59%), 458 million men (58%)
- Western Pacific: 488 million women (74%), 521 million men (73%)

Estimates of new HSV-1 infections among people aged 0-49 in 2012:

- Americas: 6 million women, 5 million men
- Africa: 17 million women, 18 million men
- Eastern Mediterranean: 6 million women, 7 million men
- Europe: 5 million women, 5 million men
- South-East Asia: 13 million women, 14 million men
- Western Pacific: 11 million women, 12 million men

In summary, these WHO estimates indicate an enormous burden of HSV-1 globally, with regional variation in the age at which HSV-1 is acquired (Looker et al, 2012).

1.5. HSV diseases

1.5.1. Gingivostomatitis and orolabial HSV infection.

Primary HSV infections are mostly asymptomatic. The most common manifestations of primary HSV-1 infection are acute herpetic gingivostomatitis and acute herpetic pharyngotonsillitis (Fig.1.3a). Neonatals and small childrens of age group 6 months to 5 years are the main victims of herpetic gingivostomatitis. It can also affect people of any age group. The mode of transmission is through infected saliva from another child or adults (Ajar and Chauvin, 2002). Usually, mucosal signs are preceded by fever, anorexia, and apathy. Although a self-limiting disease, this oral infection can cause significant mouth discomfort, burning pain around the blisters, and difficulty with eating and drinking (Huff et al, 1981)

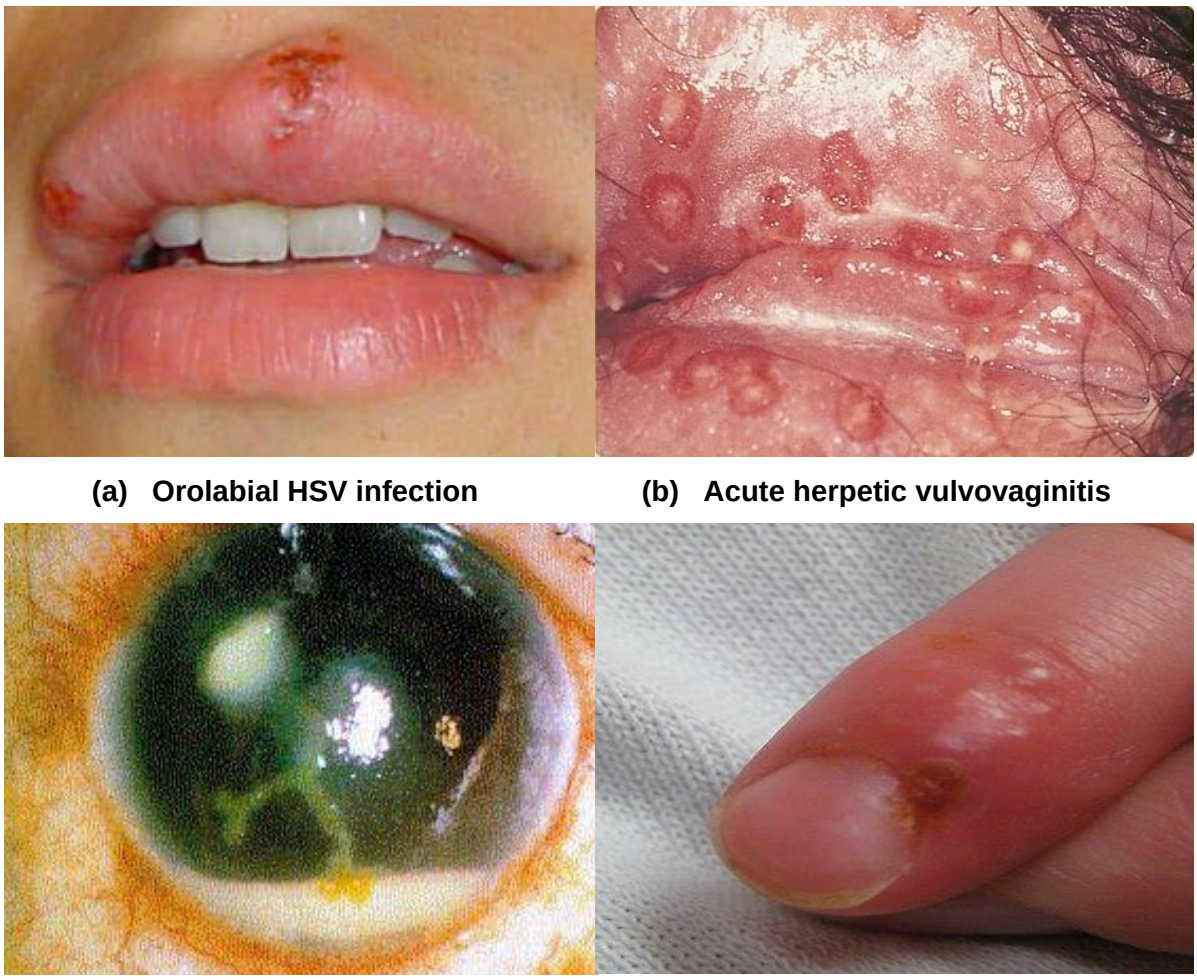
1.5.2. Primary genital herpes (or herpes progenitalis)

1.5.2.1. Acute herpetic vulvovaginitis

Primary genital herpes infections such as acute herpetic lesions of the female genitals are characteristic of infections caused by HSV-2 but it can also be caused by HSV-1. The primary lesions consist of small grouped vesicles localized on genitalia (Fig.1.3b). The symptoms are generally increased vaginal discharge along with severe discomfort during urination, and also include burning, fever, and malaise. Adding more to that the vaginal mucosa is inflamed and edematous (Staikov et al, 2015). As the amount of HSV-1 genital infections is on the rise (Tuokko et al., 2014), it is a matter of dreaded concern that the systemic infections of the newborn are more likely because of HSV-1 than HSV-2 (Brown et al., 2007; Välimaa et al., 2013).

1.5.2.2. Acute balanitis and urethritis

In case of acute balanitis and urethritis, herpetic lesions generally appear on the glans penis, prepuce, and the shaft. Vesicles and erosions on the scrotum, thighs, and buttocks are rarely observed. Herpetic urethritis occurs in 30-40% of male homosexuals and its major symptoms are mucoid discharge and dysuria. Men having sex with men with herpetic proctitis are more vulnerable to develop lesions in the perianal area and rectum. (Staikov et al, 2015).



(a) Orolabial HSV infection **(b) Acute herpetic vulvovaginitis**

(c) HSV keratitis **(d) Herpetic whitlow**

Fig. 1.3. Diseases caused by HSV: (a) Orolabial HSV infection; (b) Primary genital HSV infection (acute herpetic vulvovaginitis); (c) Ocular HSV infection (HSV keratitis); (d) Herpetic whitlow

1.5.3. Ocular HSV infections

HSV keratitis is clinically diagnosed, based on distinctive features of the corneal lesion (Fig.1.3c). Several symptoms that can be observed clinically are pain in the eye, blurred vision, photophobia, tearing and redness (Wilhelmus, 1987). In addition to local damage to the eye, HSV infection can result in changes in the trigeminal fibres connected to the area (Rousseau et al., 2015). The infection can be persistent and recurrent. Even with the current medication available in the developed countries, HSV is the leading cause of blindness due to infectious origin (Roizman et al., 2013),

1.5.4. HSV infection with neurologic manifestations

Herpes simplex encephalitis is a life-threatening disease primarily caused by HSV-1 (Steiner, 2011; Whitley and Roizman, 2001). In encephalitis, lytic viral infection and immune responses both can lead to dreadful consequences. In case of enteric nervous system, the virus might spread and cause damage to the digestive tract resulting in stoppage of peristaltic movement, which is being considered as lethal following CNS infection (Khoury-Hanold et al., 2016). Encephalitis is usually caused by HSV-1 whereas HSV-2 is the more likely causative agent in HSV meningitis (Bradshaw and Venkatesan, 2016; Välimaa et al., 2013; Whitley and Roizman, 2001; Whitley et al., 1998).

Children with previous infections, such as measles, rubella, varicella, or mumps are susceptible to acute disseminated encephalomyelitis, a secondary infection commonly occurring in teenagers (Ito et al, 2000). HSV-1, when present in the brain of patients, who are the carrier of type 4 allele of the apolipoprotein E gene, may develop Alzheimer disease (Itzhaki, 2014). Another complication of HSV infection which is more common in children is post-infectious optic neuritis. It is usually bilateral in nature. The major clinical manifestations of optic neuritis include periocular pain, sub-acute unilateral loss of vision, and impaired color vision (Voss et al, 2011). Although less common, HSV infection also involves peripheral nervous system, having symptoms of neuropathic pain, sensory deficit, motor deficit, and in some cases poliradiculoneuropathy also (Guillain-Barré syndrome) (Steiner, 2011).

1.5.5. Herpetic facial paralysis.

Facial paralysis caused by HSV is known as idiopathic facial palsy or Bell's palsy which mainly occurs due to reactivation of HSV-1 from the geniculate ganglion. Shedding of HSV-1 in saliva was traceable by polymerase chain reaction during the acute phase of the disease (Furuta et al, 1998). Inflammation also plays a major role in pathogenesis of this ailment.

1.5.6. HSV gladiatorum

Herpes gladiatorum is a clinical manifestation of HSV infection that affects individuals participating in contact sports such as rugby or wrestling (Belongia et al, 1991). In case of this disease the trigeminal, cervical, or lumbosacral dermatomes are usually involved. HSV-1 is inoculated usually through scratched skin resulting in lesions appearing in 1–2

weeks. Recurrences are frequent and troublesome that may involve the face, neck, and ears.

1.5.7. Herpetic whitlow

Herpetic whitlow, caused by HSV-1 or HSV-2, is a painful infection predominantly witnessed in health care professionals. Herpes whitlow is found to occur near finger or toe nail and is a medical problem of grave concern (Fig.1.3d) where primary and recurrent herpetic whitlows often lead to painful neuritis in the affected areas. The disorder may last more than 3 weeks and patients could benefit from episodic or suppressive acyclovir (Lewis, 2004).

1.5.8. Herpes simplex infection with gastroenterologic manifestation

A rare complication of HSV infections is herpatitis, which often leads to acute liver failure and is often clinically unsuspected. The only way to combat this disease is liver transplantation or it may lead to death. The major clinical manifestations include symptoms like fever and coagulopathy, without any skin related problems.

Recently patients having gastric erosions with nonappearance of peptic ulcer were diagnosed with a focally enhanced inflammation associated with HSV seropositivity as the main characteristic of the infection. Recently a few cases of HSV gastritis and colitis and also cases of HSV duodenitis in patients with Crohn's disease have been reported (Lee et al, 2013).

1.5.9. Neonatal HSV infections

Neonatal herpes is a severe and fatal form of HSV infection that usually occurs in neonates during delivery from an ongoing reactivation of the mother's genital herpes and especially in primary infection (Brown et al., 2003). The major problem with this infection is that even with medication it can become systemic and fatal. Some other neonatal HSV infections are congenital intrauterine infection which is associated with skin vesicles or scarring, eye lesions (chorioretinitis, microphthalmia, and cataract), certain neurological manifestations such as intracranial calcifications, microcephaly, seizures, encephalomalacia, growth retardation, and psychomotor development (Straface et al, 2012).

1.6. Management of HSV infection

Management of viral infection is a major concern in public health care. The strategies to control viral infections includes: (i) public health measures that minimize the risk of infection; (ii) anti-infective drugs for infected individuals, and (iii) vaccination to the exposed or potentially vulnerable individuals. However, to provide these benefits to the people of underdeveloped and developing world is a challenge as infection can and will occur despite the best effort of the mankind. The effective public health measures can able to combat water and air borne infections; but public health measure is never totally effective to the entire population, particularly in developing and underdeveloped world. Hence, vaccination to the vulnerable and exposed individuals is the second strategy. However, till date there is no effective vaccine for all the infections and developing suitable vaccine for most of the viral infections is not yet possible due to number of factors including the ability of the viruses to mutate rapidly, as well as emerging and re-emerging viral infections. Thus, treatment with suitable drugs to the infected population is the only way to control and manage the viral diseases, though the development of suitable drug is a huge challenge for new emerging and re-emerging viral infections.

There is no fully effective antiviral drug and drug development protocol, which can produce a drug that kill a pathogenic virus without damaging the host cell. Hence, scientists are looking into the features of an ideal antiviral drug. Ideally an antiviral drug must (i) effectively inhibit some essential viral processes or specific steps of viral life cycle; (ii) prevent the development of drug-resistant viral strains; (iii) have broad spectrum of activity; and (iv) minimum or no effect on host cell or system.

Till date there are only 40 antiviral drugs approved by the Food and Drug Administration (FDA), USA, and most of these antivirals are nucleoside or nucleotide analogues. The anti-HSV drugs among them are listed in Table 1.2. Incorporation of these nucleoside analogues into replicating viral genome either prevents its replication or results in incomplete viral replication and reproduction. One such widely used nucleoside analogue is Acyclovir, the most effective and frequently prescribed gold-standard antiviral drugs discovered so far since 1970's.

Acyclovir (ACV), a nucleoside analogue, is a selective drug against HSV (Männistö and Tuominen, 2012), which after entering into the infected cell undergoes metabolic break

down in three steps: first it converted to acyclovir monophosphate by the viral enzyme thymidine kinase (TK). Then cellular kinases enzymes of host cell add another phosphate in two sequential steps to form the active drug acyclovir-triphosphate. The acyclovir triphosphate competes with 2-deoxyguanosine triphosphate (dGTP), a substrate for viral DNA polymerase and finally leads to the chain terminator. In actual infection, the HSV releases its naked capsid that delivers DNA to the nucleus of the host cell; and the active drug acyclovir triphosphate exerts its action on the viral DNA in the nucleus.

There are few derivatives of ACV, ie. valacyclovir, famciclovir, ganciclovir, effective against other herpes viruses as well. Prophylactic usage of nucleoside analogues on patients with a risk of contracting the virus has yielded favorable results, but unfortunately not full protection (Anderson et al., 2016). ACV is a well-tolerated drug, but there is a very low risk that ACV can result in side effects in patients with underlying renal disease; a state called Cotard's syndrome (Lindén and Helldén, 2013). In addition, prophylactic usage of ACV against HSV cannot entirely prevent viral reactivation (Johnston et al., 2012) and prophylactic usage can promote the emergence of ACV resistant strains (Duan et al., 2009; van Velzen et al., 2013). As HSV TK is a non-essential gene for HSV, the virus can replicate without a functional thymidine kinase (Coen and Schaffer, 1980). A virus negative for TK is, however, attenuated, and can manifest in reduction of spread with replication and reactivation capabilities as well as temperature sensitivity (Shimada et al., 2007). Rarely, HSV DNA polymerase mutation can also lead to ACV resistance in addition to TK (Burrel et al., 2013; Chibo et al., 2004; Suzutani et al., 2003). As these strains are often attenuated, their detection might be underestimated. ACV resistant HSV usually emerges in immunocompromised patients (Frobert et al., 2014; Stránská et al., 2005). When an ACV resistant disease emerges, the drug options are few. Foscarnet is available (Männistö and Tuominen, 2012), but is not well tolerated, and over half of the treated patients develop HSV resistance (Danve-Szatanek et al., 2004). Moreover, TK can remain (somewhat) functional but at the same time develop drug resistance (Darby et al., 1981). Even with all the available antivirals, HSV remains a significant medical burden and there is a need for treatment modalities with differing mechanisms of action.

Table 1.2. List of FDA approved anti-HSV drug and their mode of action.

Name of Drug	Mode of action
Acyclovir	Acyclovir-triphosphate serves as a competitive substrate for viral DNA polymerase, and its incorporation into the DNA chain results in termination of viral replication.
Brivudin	Competitive inhibitor of viral DNA polymerase
Famciclovir	Act as a substrate for viral DNA polymerase, and thereby inhibit DNA synthesis.
Foscarnet	Inhibits pyrophosphate binding on viral DNA polymerases.
Ganciclovir	Competitively incorporate during viral DNA synthesis, leading to the DNA chain termination.
Penciclovir	Act as a competitive inhibitor of deoxyguanosine triphosphate, to inhibit DNA polymerase activity.
Vidarabine	Inhibits viral DNA polymerase.

1.7. Role of medicinal plants in herpes virus infection

Medicinal plants offer a potential alternative for drug discovery because of their wide use in folk medicine and promising therapeutic potential (Chattopadhyay et al., 2015). A wide variety of phytochemicals including alkaloids, flavonoids, terpenoids, polyphenolics, coumarins, lignans etc were reported to be useful for therapeutic applications (Tombacz *et al*., 2014; Bag *et al.* 2014; Bag *et al.,* 2013; Mukherjee *et al.,* 2013; Chattopadhyay & Bhattacharya, 2008; Naithani *et al*., 2008; Chattopadhyay *et al*., 2006; Khan *et al.,* 2005) due to their interferences on viral entry or other steps like replication, maturation, assembly along with their antioxidative potential (Tombacz *et al*., 2014; Naithani *et al*., 2008; Christopher & Wong, 2006).

Due to amazing structural diversity and broad spectrum of bioactivity phytochemicals are increasingly explored as a source of complementary antivirals. Several reports on the identification/discovery of new drugs from traditional medicines indicated its use as a potential source for antiviral drug development (Chattopadhyay, 2006; Naithani *et al*., 2008; Bag *et al.,* 2013; Mukherjee *et al.,* 2013; Bag *et al.* 2014; Chattopadhyay *et al*., 2015; Ojha *et al*., 2015). In that respect, the additional chemical diversity of natural sources (microbes, marine flora and fauna, plants) can also be explored. Research on the antiviral potential of natural products is mainly focused on plants, particularly the ethnomedicinal plants (Kinghorn, 2001; Vlietinck & Vanden Berghe, 1991), as

demonstrated by the increasing number of literature on antiviral potential of plant based phytochemicals (Chattopadhyay *et al*., 2009; Chattopadhyay & Khan, 2008; Mukhtar *et al*., 2008; Chattopadhyay & Bhattacharya, 2008; Chattopadhyay & Naik, 2007; Chattopadhyay, 2006; Khan *et al*., 2005; Cos *et al*., 2004).

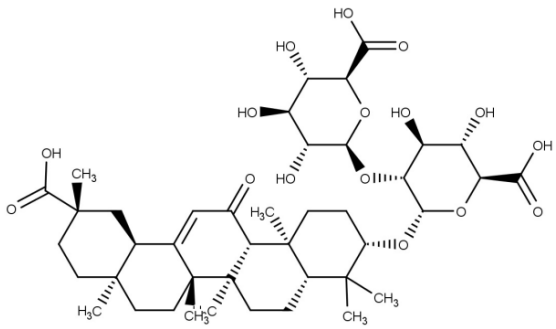
1.8. Anti-HSV potential of plant extracts and related compounds

1.8.1. *Glycyrrhiza glabra*

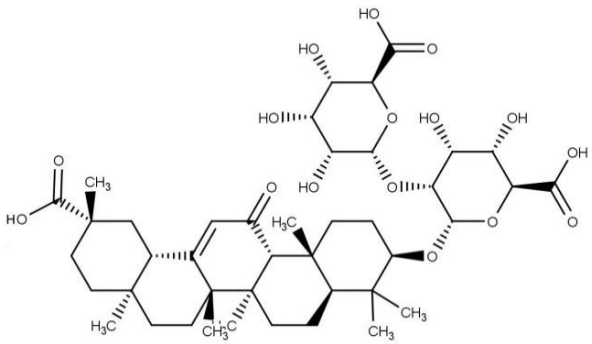
The pretreatment of *Glycyrrhiza glabra* extract on HSV-1 treated Vero cells showed significant anti-viral activity against HSV-1. Furthermore, incubation of HSV-1 virus with methanol extract for two hours previous to viral infection and also pre-treatment of Vero cells with extract for two hours led to a significant decrease in TCID50. In addition, virus incubation with aqueous extract presented remarkable anti HSV-1 activity (Ghannad, 2014)**.** It has been reported that glycyrrhizin significantly interrupted cellular adhesion in HSV (Huang et al, 2012). In this study rat cerebral capillary vessel endothelial cells (CCECs) and polymorphonuclear leukocytes (PMN) were isolated and the intercellular adhesion between these cells was examined by micropipette aspiration technique. Treatment of glycyrrhizin significantly reduced adhesion force and stress between CCEC and PMN. Based on the outcomes it was concluded that, glycyrrhizin lowered the inflammatory responses in HSV infection by inhibition of adhesion between CCEC and PMN. (Huang W et al, 2012).

In another report the effect of glycyrrhizin (1), on herpetic encephalitis introduced on mice by HSV-1 inoculation was studied (Sekizawa et al, 2001). Glycyrrhizin was administered intraperitonealy in mice suffering from herpetic encephalitis and it was observed that the survival rate of mice was increased in average about 2.5 times and the replication of HSV-1 in the brain was reduced to 45.6% (Sekizawa et al, 2001).

The triterpene glycyrrhizic acid (2), one of the major compound of *Glycyrrhiza glabra*, is known for its anti-inflammatory and antimicrobial activity. It was found that glycyrrhizic acid happened to be a strong inducer of Beclin 1, which resulted in inhibition of HSV-1 replication (Laconi et al, 2014). Therefore, glycyrrhizic acid exhibited its anti-HSV-1 activity by establishing a resistant state to HSV-1 replication (Laconi et al, 2014).



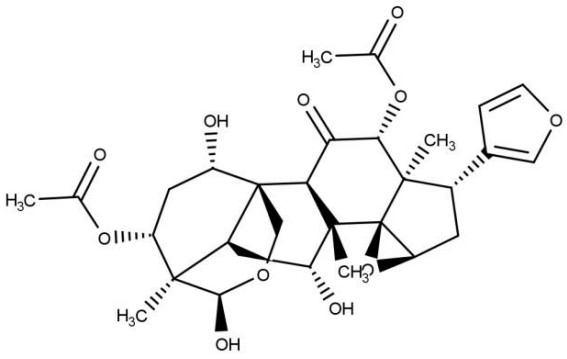
Glycyrrhizin (1)



Glycyrrhizic acid (2)

1.8.2. *Melia azedarach*

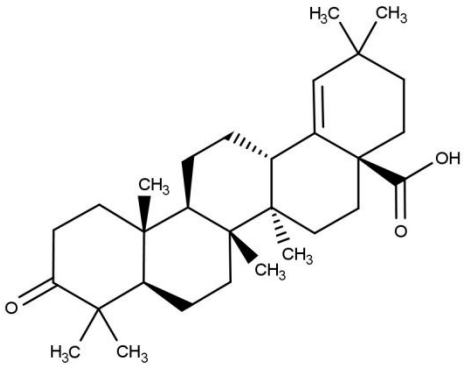
Melia azedarach (chinaberry) root bark and fruit has been used for traditional Chinese remedies against several infectious diseases. From the leaves of this tree a limonoid compound was isolated which was found to inhibit HSV *in vitro* (Alche et al, 2003). Also a protein called Meliacin isolated from the leaves was broadly studied which led to the discovery that it interfered with HSV DNA synthesis, maturation and envelope formation (Alche et al, 2002). This protein also inhibited formation of herpetic keratitis in mouse model when applied topically (Pifarre et al, 2002). A compound 28-deacetylsendanin (3) present in the fruit, was found to inhibit HSV replication and reduce production of HSV thymidine kinase (Kim et al, 1999). Another study reported that 1-cinnamoyl-3,11-dihydoximeliacarpin isolated from chinaberry inhibited replication of HSV and also exhibited profound immunomodulatory activity (Barquero et al, 2006). An interesting study reported that an aqueous extract of *Melia toosendan* (a close relative of *Melia azedarach*) prevented viral attachment *in vitro* (Hsiang et al, 2001).



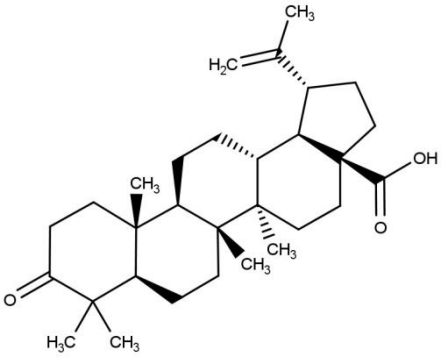
28-deacetylsendanin (3)

1.8.3. *Rhus javanica*

Rhus javanica, as a medicinal herb, exhibited oral therapeutic activity against HSV *in vivo*. Two major anti-HSV compounds; moronic acid (4) and betulonic acid (5) were isolated from the ethyl acetate extract and subjected to anti-HSV activity, both *in vitro* and *in vivo*. Moronic acid was found to be a major anti-HSV compound as quantified in the ethyl acetate extract. The EC_{50} of moronic acid and betulonic acid against wild-type HSV-1 were found to be 3.9 and 2.6 µg/ml, respectively as determined by plaque reduction assay. However, the selectivity index of moronic acid (16.3) was greater than betulonic acid (6.2). Susceptibility of wild-type HSV-1 to moronic acid was similar to that of acyclovir-phosphonoacetic acid-resistant HSV-1, thymidine kinase-deficient HSV-1, and wild-type HSV-2. Oral administration of moronic acid to HSV-1 infected mice significantly slowed down the progress of skin lesions and/or prolonged the mean survival times of infected mice without toxicity compared to the control group. Moronic acid was found to be more effective against the virus yields in the brain than those in the skin. Thus, moronic acid was considered as a major anti-HSV compound from *Rhus javanica* while the mode of action was different from that of ACV (Kurokawa, 1999).



Moronic acid (4)



Betulonic acid (5)

1.8.4. *Echinacea*

A complete, holistic treatment of HSV infected patient requires the drug that directly interferes with HSV and also supports the immune system. One such plant species is *Echinacea* spp. that acts to strengthen the immune system. Besides being a

macrophage stimulator, various extracts of species of *Echinacea* was shown to exhibit *in vitro* anti-HSV activity, with the presence of alkenes and alkylamides being most associated with inhibition (Binns et al, 2002). In another study it was found that *Echinacea purpurea* polysaccharide reduces the latency rate in HSV-1 Infections. The treatment with *Echinacea purpurea* as an immune-stimulator, before infection, enhances the efficient immune responses leading to reduced viral multiplication in the eye. Subsequently, it also significantly affected and diminished the establishment of a latent infection (Ghaemi et al, 2009).

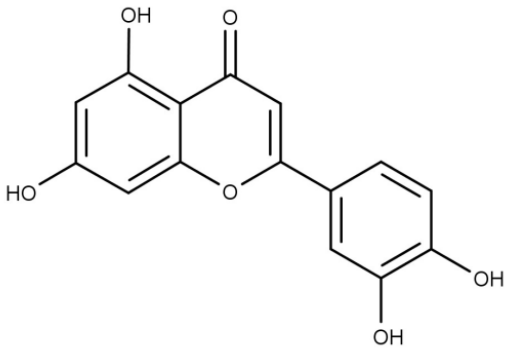
A uniform preparation of the *Echinacea* (Echinaforce, an ethanol extract of roots of *E. purpurea*) was assessed for HSV-1 infection in human bronchial epithelial cells (BEAS-2B), and in two other human cell lines. The *Echinacea* preparation exhibited effective virucidal activity by modulating the HSV-1 induced secretion of IL-6 and IL-8 (CXCL8), additional to several other chemokine. (Sharma, 2009). A single centre, prospective, double blind, placebo-controlled cross-over trial was conducted to assess whether *Echinaforce* could prevent or decrease the frequency and severity of genital herpes recurrences, where over a one-year period, 50 patients took part in the study receiving 6 months placebo and 6 months *Echinaforce* each. However no statistically significant benefit could be detected in this study (Vonau et al, 2001).

1.8.5. *Terminalia chebula*

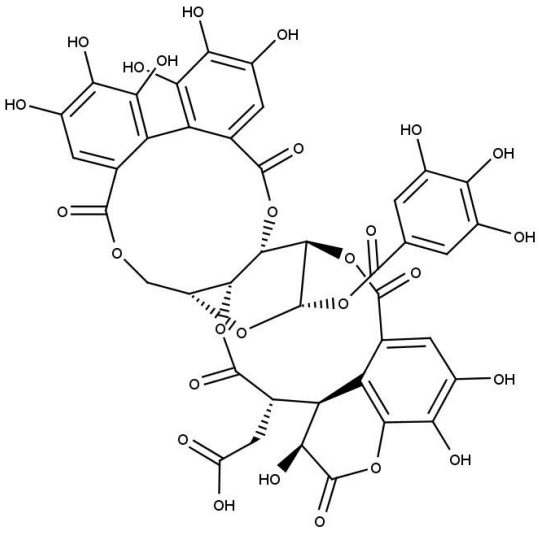
T. chebula fruits were famous home-based therapy for cough and cold. The healing activity against herpes simplex virus was established in different studies. The study performed with *T. chebula* extracts along with acyclovir against HSV-1 displayed their anti-HSV-1 activity profoundly (Kurokawa et al, 1995).

T. chebula is rich in tannins that are polyphenolic secondary metabolites found in higher plants. Hydrolyzable tannins possess structures generally consisting of gallic or ellagic acid esters conjugated to a sugar moiety. These polyphenols having high affinity for proteins and polysaccharides are thought to be the major bioactive compounds found in the leaves and the fruit of *T. chebula*. Two hydrolysable tannins chebulagic acid (7) and punicalagin (6) were isolated from dried fruits of *T. chebula* that showed good potential in inhibiting HSV-1 infection by targeting viral glycoprotein-glycosaminoglycan interactions to inhibit HSV-1 entry and cell-to-cell spread at EC_{99} doses of 60 µM and 40 µM

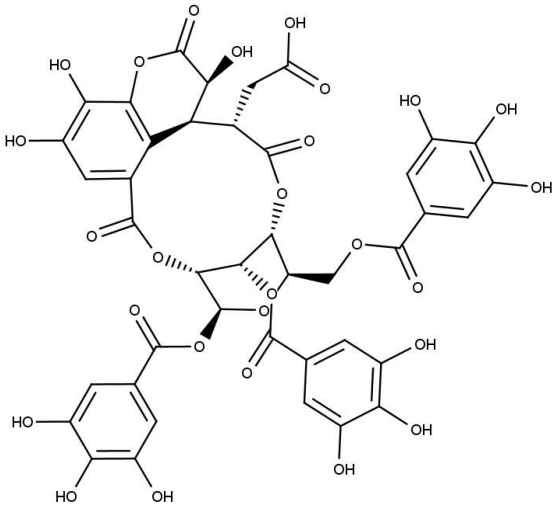
respectively (Lin et al, 2011). It has been reported recently that *T. chebula* fruit extract along with the isolated compounds chebulagic acid and chebulinic acid (8) inhibited HSV-2 by effectively preventing the attachment and penetration of the HSV-2 to Vero cells along with significant inhibitory activity against virus replication (Kesharwani et al, 2017).



Punicalagin (6)



Chebulagic acid (7)



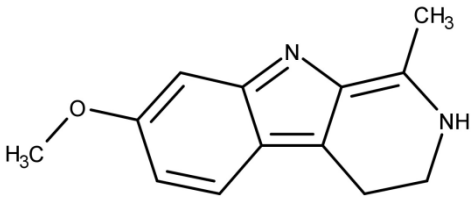
Chebulinic acid (8)

1.8.6. *Ophiorrhiza nicobarica*

Ophiorrhiza nicobarica a traditional herb used by the Shompen and Nicobarese tribes of the Nicobar Islands, India, against skin ailments (Dagar and Dagar, 1991), having antimicrobial and anti-inflammatory activities (Chattopadhyay et al., 2006, 2007). Both the herb and its isolated compound 7-methoxy-1-methyl-4,9-dihydro-3H-pyrido[3,4-b]

indole, also known as harmaline (9), exhibited strong antiviral activity against wild-type and clinical isolates of HSV-1 and HSV-2. Further it was found that harmaline did not interfere in viral entry but the recruitment of lysine-specific demethylase-1 (LSD1) and the binding of immediate-early (IE) complex on ICP0 promoter leading to the suppression of viral of immediate-early (IE) gene synthesis and thereby the reduced expression of ICP4 and ICP27. Moreover, harmaline at its virucidal concentration was nontoxic and reduced virus yields in cutaneously infected Balb/C mice (Bag et al, 2014).

In another study it was reported that both *O. nicobarica* extract and isolated harmaline was strongly effective against wild-type and clinical isolates of HSV-2. Harmaline possessed significant anti-HSV-2 activity in a dose-dependent manner and was effective at 2-4 h post-infection. Further it was observed that at 5 µg/ml harmaline suppressed viral IE gene expression and interfered with the recruitment of LSD-1 by HCF-1. The *in vivo* studies revealed that harmaline at its virucidal concentration was nontoxic and reduced virus yield in the brain of HSV-2 infected mice in a concentration dependent manner, compared to vaginal tissues (Bag et al, 2013).



Harmaline (9)

1.8.7. Other plant extracts with anti-HSV activity

During the last four decades many screening programs were conducted globally to evaluate the *in vitro* and *in vivo* antiviral activity of many plant extracts and herbal products. Many of them revealed strong anti-herpes virus activity, while isolated molecules from some plant extracts could be used as potential lead for development of anti-herpes virus drugs (Yarnell & Abascal, 2005; Khan et al., 2005; Chattopadhyay et al., 2006; Chattopadhyay & Khan, 2008). The *in vitro* and sometimes *in vivo* anti-herpes virus activities of many plant extracts are reported like *Pongamia pinnata* inn, *Beta vulgaris*, *Callisia grasilis*, *Annona* sp, *Polygonium punctatum*, *Lithraea molleiodes*, *Sebastiania braseiliensis*, *S. klotzschiana* (EC_{50}=39-169 µg/ml), *Eupatorium articulum* (EC_{50}=125-250 µg/ml), *Melaleuca leucadendron* fruits, *Nephelium lappaceum* pericarp

(199µg/ml), *Barleria lupulina* Lind, *Clinacanthus nutans* (Burm.f) Lindua, *Nepeta nepetella*, *N. coerulea*, *N. tuberosa* (EC_{50}=150-500 µg/mL), *Byrsonima verbascifolia* (EC_{50}=2.5 µg/ml), *Holoptelia integrifolia*, *Myrica rubra*, *Thea* (*Camellia*) *sinensis*, *Pterocarya stenoptera, Buxus hildebrandtii*, *Cissus hamaderohensis*, *Cleome socotrana*, *Dracaena cinnabari*, *Exacum affine*, *Jatropha unicostata* and *Kalanchoe farinacea* (EC_{50}= 0.7-12.5 µg/mL) mainly against HSV-1 and HSV-2. (Khan et al., 2005; Chattopadhyay & Bhattacharya, 2008; Chattopadhyay & Khan, 2008). The British Columbian ethnomedicines *Cardamine angulata*, *Conocephalum conicum*, *Polypodium glycyrrhiza* showed anti-HSV-1 activity (McCutcheon et al., 1995). Significant anti-HSV activity was also found with *Byrsonima verbascifolia* extract, a folk remedy for skin infections (Rao et al., 1969). The aqueous extracts of *Sanguisorba minor magnolii*, *Dittrichia viscosa* and *Nepeta nepetella* of Iberian Peninsula was found to inhibit HSV-1 at 50-125 µg/ml (Glatthaar-Saalmuller et al., 2001).

The ethnomedicinal plants from many South Asian regions were found to possess very promising anti-HSV activity. The Nepalese ethnomedicine *Nerium indicum* was found to inhibit HSV at low concentrations (Alche et al., 2000) while the Chinese folk medicine *Rheum officinale* and *Paeonia suffruticosa* inhibited HSV entry (Hsiang et al., 2001). Similarly, *Senecio ambavilla*, an ethnomedicinal plant of La Reunion Island exhibited HSV-1 inhibitory activity and anti-poliovirus activities (Rajbhandari et al., 2001). Some of the folk medicinal plant extracts of Thailand such as *Aglaia odorata*, *Moringa oleifera* and *Ventilago denticulate* were reported to inhibit thymidine kinase-deficient and phosphonoacetate-resistant HSV-1. They also delayed the growth of skin lesions at 750 mg/kg/per dose, increased the mean survival times and effectively reduced the mortality of infected mice similar to acyclovir (Fortin et al., 2002). Interestingly *Ceratostigma willmattianum* extract, an ethnomedicinal plant from China inhibited the viral adsorption, replication and transcription of HSV-1 (EC_{50} 29.46 mg/L) and HSV-2 (EC_{50} 9.2 mg/L) (Chen et al., 2004). *Senna petersiana* extract, a folk remedy for sexually transmitted diseases, showed strong anti-HSV activity (Dong et al., 2004).

The aqueous extract of *Carissa edulis* (Forssk.) Vahl (Apocynaceae) root from Kenya, significantly (100%) inhibited plaque formation in Vero E6 cells infected with 100PFU of wild type HSV (7401H of HSV-1 and Ito-1262 strains of HSV-2) or resistant HSV strains (TK(-) 7401H and AP(r) 7401H of HSV-1) at 50 µg/ml *in vitro* with minimal cell

cytotoxicity (CC_{50} 480 µg/ml). The oral dose (250 mg/kg) of the extract significantly (50%) hindered the onset of HSV infections in a murine model using Balb/C mice, which was cutaneously infected with wild type or resistant strains of HSV. It also augmented the mean survival time from 28 to 35% and reduced the mortality rate (70-90%) of infected mice (Tshikalange et al., 2005). A list of potential plant extracts having anti-HSV activities is provided in Table 1.3. The aqueous extract of leaves and stems of *Phyllanthus orbicularis* (Euphorbiaceae) exhibited selective antiviral indexes of 12.3 and 26 against bovine HSV-1 and HSV-2, respectively. It was also responsible for impairing the replication of both HSV-1 and HSV-2 in a concentration-dependent manner, partly due to a direct interaction with virus particles or inhibiting their entry into the cell (Alvarez et al, 2009).

A number of studies were carried out with propolis - the resin collected by bees from plants and reported the HSV inhibitory activity. For example, aqueous extract of propolis inhibited HSV-1 by 50% in Vero cells and 80–85% inhibition corneal HSV-1 infection in rabbits. Another report revealed that Brazilian hydroalcoholic propolis extract was effective for the treatment of vaginal lesions caused by HSV-2. In addition to that Schnitzler et al. have proposed that the HSV inhibitory potential of propolis is because of the presence of several phytochemicals including polyphenols, flavonoids and phenylcarboxylic acids (Schnitzler et al, 2010).

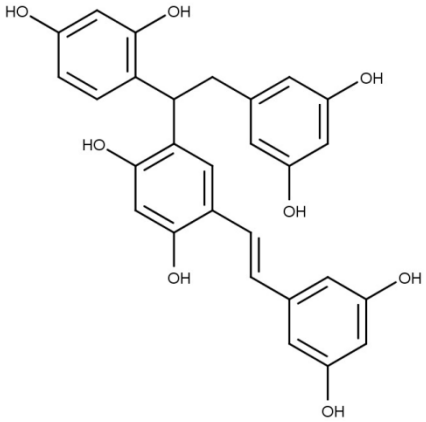
1.9. Isolated compounds having anti-HSV activity

1.9.1. Flavonoids

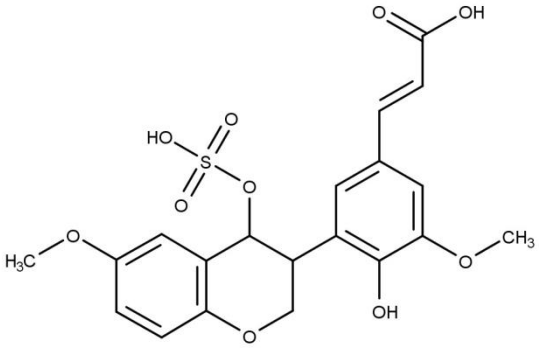
Flavonoids, such as flavones, flavonols, flavanones, isoflavones, anthocyanidins, chalcones and flavanonols, are reported to have broad antiviral activity (Ojha et al., 2015; Jeong & Kim, 2015; de Sousa et al., 2015) and are abundant in plant seeds, citrus fruits, olive oil, tea and red wine.

Flavonoid-based polymer has displayed substantial activity against HSV-1 and HSV-2 (Nowakowska, 2007). Chalcones are considered as precursors of flavonoids and isoflavonoids which are abundant in edible plants with diverse array of pharmacological activities. Dihydrochalcones derived from *Millettia leucantha* (Leguminosae) showed anti-herpes simplex virus (HSV) activity (Phrutivorapongkul et al, 2003). Likhitwitayawuid

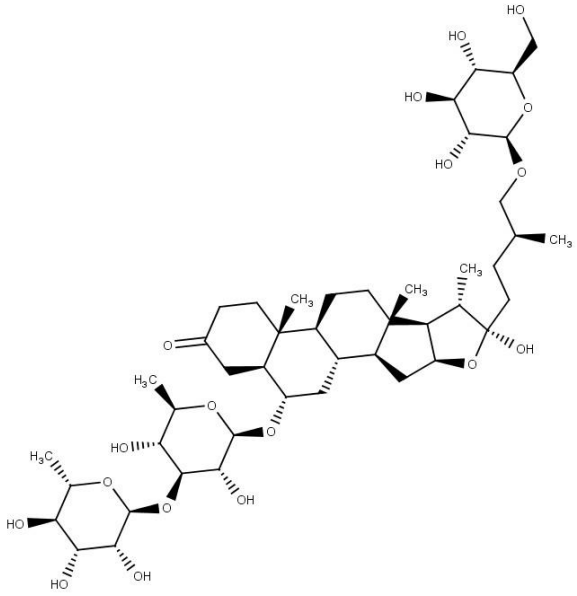
et al. described the isolation and anti-HSV activities of a series of phenolic compounds identified from the heartwood of *Artocarpus gomezianus*, including the new antiherpetic flavone artogomezianone (10) (Likhitwitayawuid et al, 2006).



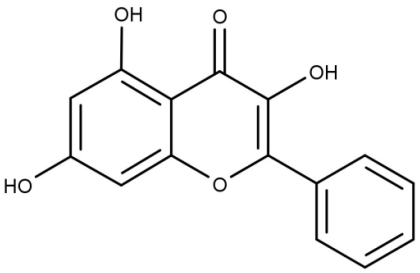
Artogomezianone (10)



Torvanol A (11)



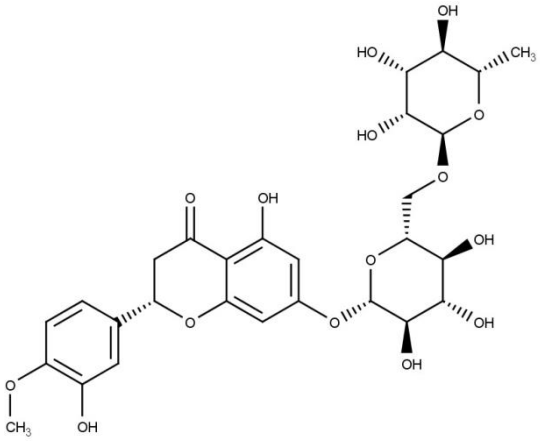
Torvoside H (12)



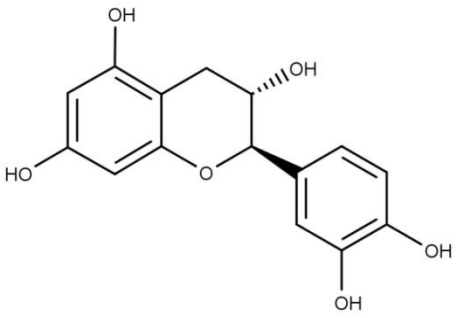
Galangin (13)

The C-4 sulfated isoflavone torvanol A (11) and the steroidal glycoside torvoside H (12), isolated from *Solanum torvum* fruits, had strong anti-HSV-1 activity (Arthan et al, 2002). While antibacterial galangin (3,5,7-trihydroxy-flavone) (13) isolated from *Helichrysum aureonitens* had significant activity against HSV-1 (Meyer et al, 1997). Similarly the isoquercitrin of *Waldsteinia fragarioides* have anti-HSV activity. Kaul et al. (Kaul et al,

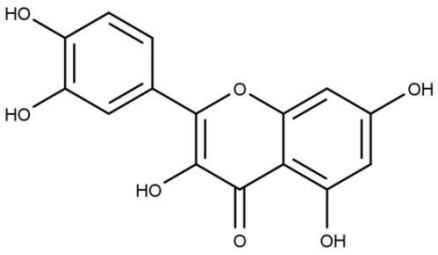
1985) reported that hesperidin (14), catechin (15) and quercetin (16) of orange and grape inhibit replication of HSV-1. Interestingly oxyresveratrol (17) of *Millettia erythrocalyx* and *Artocarpus lakoocha* inhibit HSV (Likhitwitayawuid et al, 2005). The amentoflavone (18) and robustaflavone (19) isolated from *Rhus succedanea* and *Garcinia multiflora* inhibited HSV-1. The bioflavonoids cinnamoylbenzaldehyde and lawinal (20) of *Desmos* spp. (Wu et al, 2003), mulberroside C (21) and leachianone G of *Morus alba* root inhibit HSV-1 (Du et al, 2003). Morin (22), a flavonoid group, isolated from *Maclura cochinchinensis* have powerful anti-HSV-2 activity, as the free hydroxyl groups are responsible for antiviral activity (Bunyapraphatsara et al, 2000).



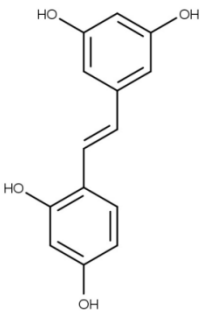
Hesperidin (14)



Catechin (15)



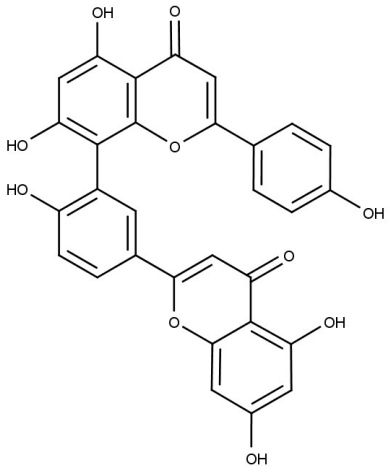
Quercetin (16)



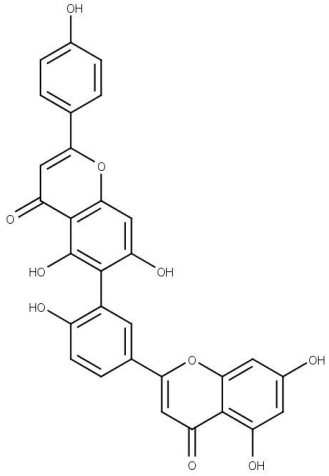
Oxyresveratrol (17)

Recently a bioflavanoid luteolin (23), isolated from *Pedilanthus tithymaloides* L. showed promising antiviral activity against the wild type and clinically isolated strains of HSV-1 and HSV-2 by abating HSV induced p65 nuclear translocation thereby blocking HSV induced NF-κB activation, with significant down-regulation of viral ICP0 and ICP27 leading to the inhibition of HSV replication along with the inhibition of IκB-α degradation (Ojha et al, 2015). Various combinations of flavones and flavonols have been shown to

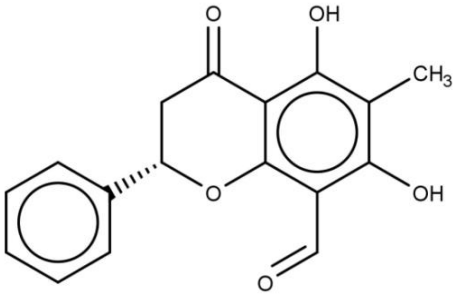
exhibit synergism. Kaempferol (24) and luteolin show synergistic effect against HSV. Synergism has also been reported between flavonoids and other antiviral agents. Quercetin is reported to potentiate the effects of 5-ethyl-2-dioxyuridine and acyclovir against HSV (Cushnie & Lamb 2005). Moreover, it has been reported that flavonols are more active than flavones against HSV-1 and the anti-HSV activity was noticed in order of galangin, kaempferol, and quercetin (Cushnie & Lamb, 2005). A list of isolated compounds having significant anti-HSV activity is provided in table 1.4.



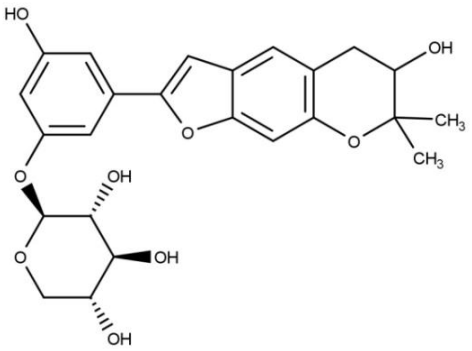
Amentoflavone (18)



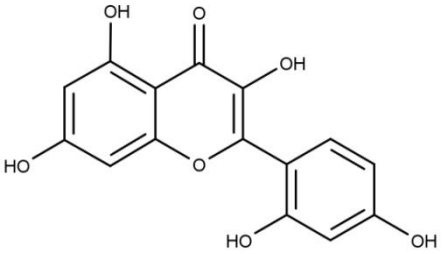
Robustaflavone (19)

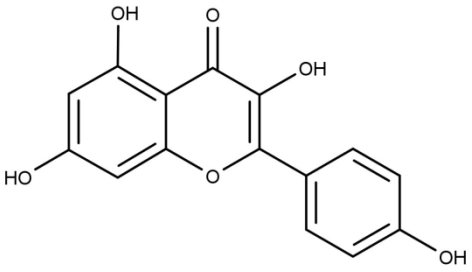


Lawinal (20)

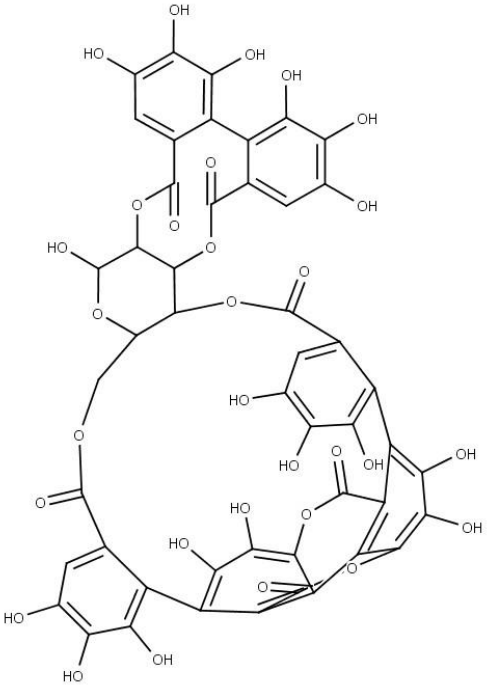


Mulberroside C (21)





Morin (22) **Kaemferol (24)**



Luteolin (23)

1.9.2. Phenolics and polyphenols

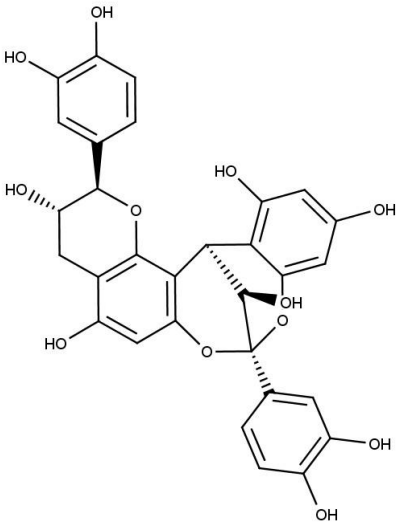
The simplest bioactive phytochemicals with a single substituted phenolic ring belongs to a wide group of phenylpropane that are in the highest oxidation state and have wide range of antiviral activities (Chattopadhyay and Naik, 2007).

The polyphenol rich extract of *Agrimonia pilosa* and *Punica granatum* of southern Mainland China, showed anti-HSV-1 activity (Hegde et al, 2003). The prodelphinidin-di-O-gallate isolated from *Myrica rubra* bark demonstrated in vitro anti-HSV-2 activities by inhibiting viral attachment and penetration, reducing viral infectivity and affecting the late stage of infection cycle (Cheng et al, 2003). Polyphenols and proanthocyanidins isolated from *Hamamelis virginiana* bark had remarkable anti-HSV-1 activity (Erdelmeier et al, 1996) while proanthocyanidin A1 (24) isolated from *Vaccinium vitis-idaea* block HSV-2 attachment and penetration to the host cell (Cheng et al, 2005), but oligomeric procyanidins of *Crataegus sinaica* significantly inhibit HSV-1 (Shahat et al, 2002).

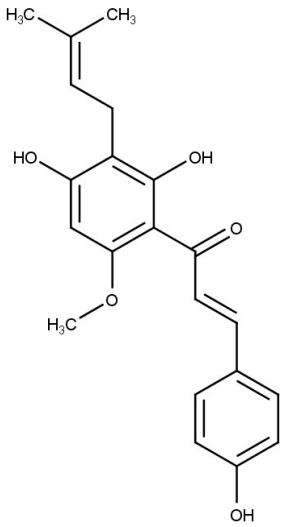
The aqueous extract of *Plantago major*, a popular ethnomedicine used in Ayurveda, traditional Chinese medicine and Chakma Talika Chikitsa, for treating several ailments

showed anti-herpes activity against HSV-1 and HSV-2 due to caffeic acid and its derivatives. A xanthohumol (25)-enriched *Humulus lupulus* (hop) extract having moderate activity against HSV-2 and HSV-1 might serve as a lead for synthesizing more active anti-HSV agent (Buckwold et al, 2004).

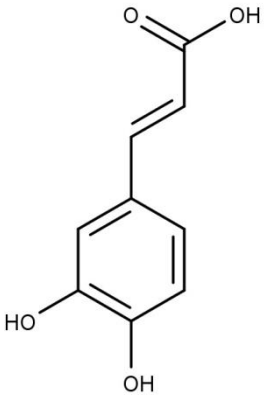
Caffeic acid (26) isolated from *Plantago major* possess significant antiviral activities against both HSV-1 and HSV-2 where its mode of action was found to be at the multiplication stages, post infection of both HSV-1 and HSV-2 (Chiang et al., 2002). Curcumin (27) from *Curcuma longa* was found to exhibit anti-HSV-1 activity by inhibiting P300/CBP histone acetyltransferase of HSV-1 (Kutluay et al, 2008). Recently Ojha et al reported a promising anti-HSV activity of chlorogenic acid (28) from *Odina wodier* which prevents attachment of the virus to the host cell (Ojha et al, 2013).



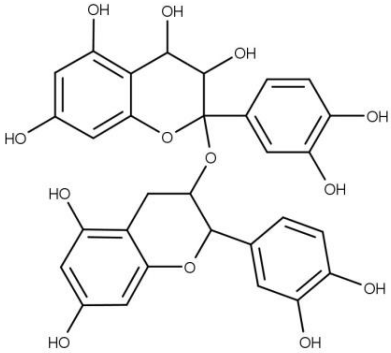
Proanthocyanidin A1 (24)



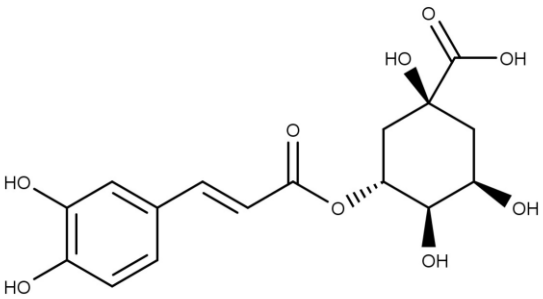
Xanthohumol (25)



Caffeic acid (26)



Curcumin (27)



Chlorogenic acid (28)

Table 1.3. Plant extracts and Isolated compounds having anti-HSV activity

Plant species	Family	Active constituent	Mechanism of HSV inhibition	Reference
Achyranthes aspera	Amaranthaceae	Oleanolic acid	Active against HSV-1 and HSV-1 by inhibition of the early stage of multiplication	Mukherjee et al, 2013
Actinodaphne hookeri	Lauraceae	Actinophnine	Inhibits HSV-1 replication.	Montanha et al., 1995
Artocarpus lakoocha	Moraceae	Oxyresveratrol	Inhibits early and late phase of viral replication with pretreatment in one-step growth assay of HSV-1 and HSV-2. It also inhibited late protein synthesis.	Chuanasa et al, 2008
Azadirachta indica	Meliaceae	Sulfonoquinovosyldiacylglyceride	Significantly down regulate the production of HSV induced pro-inflammatory cytokines such as TNF-α, IL-1β, IL-12 and IL-6.	Bharitkar et al, 2014
Camellia sinensis	Theaceae	Epigallocatechin gallate (EGG), Palmitoyl-EGCG (p-EGCG)	Binds to gB and gD glycoproteins resulting in inhibition of HSV	Isaacs et al, 2008
Cassia javanica	Fabaceae	Ent-epiafzelechin-(4α-8)-epiafzelechin (EEE)	Prevents HSV-2 penetration and interfered with replication at the late stage of viral life cycle.	Cheng et al, 2006

Coptidis rhizoma	Ranunculaceae	Berberine	Prevents virus penetration and inhibits immediate early genes ICP0 and gD. It also activates the NfκB signaling pathway to inhibit the viral replication.	Chin et al, 2010; Song et al, 2014
Crataegus sinaica	Rosaceae	procyanidin C-1	Reduces HSV-1 titer due to extracellular antiviral activity.	Shahat et al, 2002
Curcuma longa	Zingiberaceae	Curcumin	Inhibits P300/CBP histone acetyl transferase of HSV-1.	Kutluay et al, 2008
Digitalis lanata	Plantaginaceae	Glucoevatromonoside	Inhibition of HSV proteins synthesis (ICP27, UL42, gB, gD), the blockage of virus release and the reduction of viral cell-to-cell spread.	Bertol et al, 2011
Euphorbia jolkini Bioss	Euphorbiaceae	Putranjivain A	Inhibits viral attachment and penetration, and also interfered with the late stage of HSV replication.	Cheng et al, 2004
Euphorbia segetalis	Euphorbiaceae	Lupenone	Exhibited strong viral plaque inhibitory effect against HSV-1 and HSV-2.	Madureira et al., 2003
Ficus benjamina	Moraceae	Quercetin 3-O-rutinoside, Kaempferol 3-O-robinobioside	These three flavones were highly effective against HSV-1 reaching a selectivity index (SI) of 266, 100 and 666.	Yarmolinsky et al, 2012
Glycyrrhiza glabra	Fabaceae	Glycyrrhizin, glycyrrrhizic acid	Inhibits HSV-1, through induction of CD4+ T cells. Glycyrrhizic acid inhibits HSV1 replication.	Utsunomiya et al, 1995; Laconi et al, 2014; Huang et al,

				2012
Houttuynia cordata	Saururaceae	Quercetin, Isoquercitrin	Blocks viral binding and penetration and also suppress HSV replication	Hung et al, 2015
Humulus lupulus	Cannabaceae	Xanthohumol	Inhibits HSV-1 and HSV-2 infection with an SI of 1.9 to 5.3 respectively.	Buckwold et al, 2004
Hypericum connatum	Guttiferae	Hyperbrasilol B, Amentoflavone and Luteoforol	Inhibits cytopathic effect (CPE) and reduce viral titer of HSV-1 DNA viral strains KOS and VR733.	Fritz et al, 2007
Ilex asprella	Aquifoliaceae	Asprellanoside A, Oblonganoside H	Asprellanoside A and oblonganoside H exhibited anti-HSV-1 activity with TIC values of 0.14 and 0.18 mM, respectively.	Zhou et al, 2012
Limonium sinense	Plumbaginaceae	Samarangenin B	Inhibits expression of HSV-1 α gene, ICP0 and ICP4 genes.	Kuo et al, 2002
Maclura cochinchinensis (Lour.) Corner	Moraceae	Morin	Exhibited anti-HSV-2 activity at an EC_{50} value of 53.5 µg/ml, due to presence of free hydroxyl groups.	Bunyapraphatsara et al, 2000
Mallotus peltatus	Euphorbiaceae	Ursolic acid	Inhibits HSV-1 and HSV-2 by inhibiting the early stage of multiplication	Bag et al, 2012
Melaleuca alternifolia	Myrtaceae	Isoborneol	Inactivate HSV-1 replication by inhibiting glycosylation of viral glycoprotein gB.	Armaka et al, 1999
Melia azedarach L.	Meliaceae	1-cinnamoyl-3, 11-dihydroxymeliacarpin (CDM), 28-Deacetylsendanin (28-DAS),	Modulates the NF-κB signaling pathway by reduced activation in HSV-1-infected conjunctival cells.	Barquero et al, 2006; Alche et al., 2002; Kim et al, 1999

		Meliacine		
Morus alba	Moraceae	Leachianone G, Mulberoside	Interferes with virus adsorption into host cells.	Du et al, 2003
Myrica rubra	Myricaceae	prodelphinidin B-2 3,3-di-O-gallate (PB233-OG)	Inhibits HSV-2 attachment to host cells	Cheng et al, 2003
Odina wodier	Anacardiaceae	Chlorogenic acid	Prevents attachment of HSV-1 on the host cell surface	Ojha et al, 2013
Ophiorrhiza nicobarica	Rubiaceae	Harmaline	Inhibits HSV-1 infection by interfering with the viral immediate early transcriptional events.	Bag et al, 2013; Bag et al, 2014
Pedilanthus tithymaloides	Euphorbiaceae	Luteolin	Inhibits HSV-2 infection by modulation of NFκB signaling cascade.	Ojha et al,2015
Peganum harmala	Zygophyllaceae	Harmaline & Harmine	Inhibits HSV-1 infection by interfering with the viral immediate early transcriptional events.	Rashan, 1990
Phyllanthus urinaria	Phyllanthaceae	Excoecarianin	Inhibits HSV-2 by inactivation of viral particles.	Cheng et al, 2011
Plantago major	Plantaginaceae	Caffeic acid	Inhibits HSV-1 multiplication mainly before the completion of viral DNA replication, but not thereafter.	Chiang et al., 2002; Ikeda et al, 2011
Podophyllum peltatum L.	Berberidaceae	Podophyllotoxin, α-pelatin, β-pelatin	Inhibits replication of HSV-1.	McKee et al, 1997;Bedows & Hatfield, 1982
Prunella vulgaris	Lamiaceae	Lignin–carbohydrate	Blocks HSV-1 binding and inhibits penetration	Zhang et al, 2007

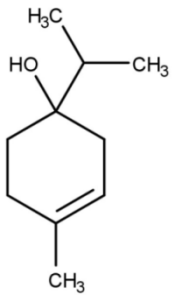
		complex (PPS-2b)	into host cells.	
Pterocarya stenoptera	Juglandaceae	Pterocarnin A	Inhibits HSV-2 entry (attachment and penetration) into host cells.	Cheng et al, 2004
Rheum tanguticum	Polygonaceae	Emodin	Inhibits the nuclease activity of HSV-1 UL12 alkaline nuclease and induces the accumulation of viral nucleocapsids in the nucleus in a dose-dependent manner.	Hsiang and Ho, 2008; Xiong et al, 2011
Rhus javanica	Anacardiaceae	Moronic acid Betulonic acid	Significantly retarded the development of skin lesions and/or prolonged the mean survival times of infected mice without toxicity compared with acyclovir. Moronic acid suppressed virus yields in the brain more efficiently than those in the skin.	Kurokawa et al, 1999
Scoparia dulcis L.	Plantaginaceae	Scopadulcic acid B	Inhibits HSV-1 replication.	Hayashi et al, 1988
Solanum torvum	Solanaceae	Torvanol, torvoside	Inhibits HSV-1activity with IC_{50} values of 9.6 and 23.2 µg/ml.	Arthan et al, 2002
Syzygium aromaticum Merr. et Perr.	Myrtaceae	Eugeniin	Inhibits viral DNA and late viral protein synthesis, but not cellular protein synthesis at its inhibitory concentrations.	Takeshi & Tanaka, 1981; Kurokawa et al, 1998
Tanacetum vulgare	Asteraceae	Parthenolide	Inhibits HSV-1 infection by altering the expression of p65, p38, c-Jun N-terminal kinase (JNK) proteins and also viral proteins	Alvarez et al, 2011

			including gB. gD and ICP0.	
Tanacetum vulgare	Compositae	Spiroketalenol	Inhibits HSV-1 and HSV-2 glycoprotein accumulation in host cells.	Alvarez et al, 2015
Terminalia arjuna	Combretaceae	Casuarinin (1)	Inhibits HSV-2 entry (attachment and penetration).	Cheng et al, 2002
Terminalia chebula	Combretaceae	Chebulagic acid and punicalagin	Target viral glycoprotein-glycosaminoglycan interactions to inhibit HSV-1 entry and cell-to-cell spread.	Lin et al, 2011
Teucrium polium	Lamiaceae	Rosmarinic acid	Inhibits concentration-dependent HSV-1 plaque formation.	Ansari *et al*., 2014
Trichosanthes kirilowii	Cucurbitaceae	Trichosanthin	Inhibits HSV-1 by modulating p38 MAPK protein and Bcl-2 gene	Ye et al, 2016
Tripterygium wilfordii	Celastraceae	Triptofordin C-2	Inhibits immediate early (IE) genes of HSV-1	Hayashi et al, 1996
Undaria pinnatifida	Asteraceae	Galactofucan	Inhibits HSV binding and entry into the host cell.	Thompson and Dragar, 2004

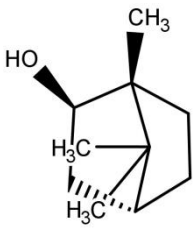
1.9.3. Terpenoids

Monoterpene compounds such as thymol, gamma-terpinene, 1,8-cineole, alpha-pinene, p-cymene, alpha-terpinene, citral, terpinen-4-ol (29) and alphaterpineol were purified from extracts of tea tree, thyme and eucalyptus. These compounds have demonstrated in-vitro antiviral activity against HSV-1 by 80% inhibition of virus (Astani et al, 2010). Isoborneol (30) is a monoterpene, found in a wide range of essential oils, exhibited *in vitro* virucidal effect against HSV-1, whereas at the concentration of 0.06%, it showed total inhibition of viral replication. Its mechanism of action is related to inhibition of glycosylation of viral polypeptides (Armaka et al, 1999). From *Tripterygium wilfordii* 13 sesquiterpenes were isolated and tested in vitro against HSV-1. Only Triptofordin C-2 (31) inhibited viral protein synthesis of infected cells when added at early steps of HSV-1 replication and demonstrated inhibition of translation of the transcripts of the immediate

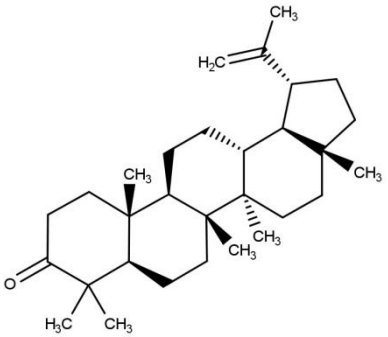
early (IE) genes with selectivity index (>10) (Hayashi et al, 1996). Putranjivain A (32), a diterpene isolated using the acetone–aqueous extraction from all parts of *Euphorbia jolkini*, demonstrated antiviral effect against HSV-2 in Vero cells with an EC_{50} value of 6.3 μM. It inhibited viral attachment and penetration, and also interfered with the late stage of HSV replication (Cheng et al, 2004). *Euphorbia segetalis* exhibit strong viral plaque inhibitory effect against HSV-1 and HSV-2 due to tetracyclic triterpene lupenone (33) (Madureira et al, 2003).



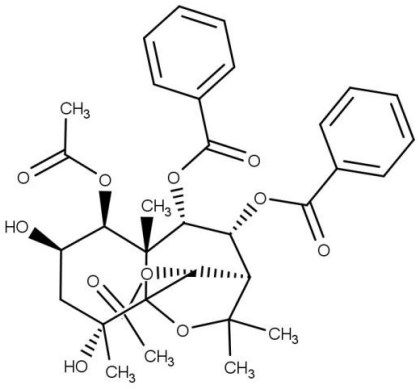
Terpinen-4-ol (29)



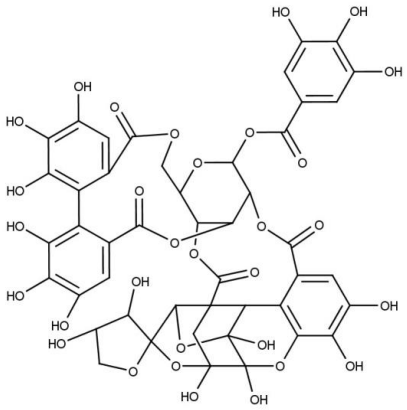
Isoborneol (30)



Lupenone (33)



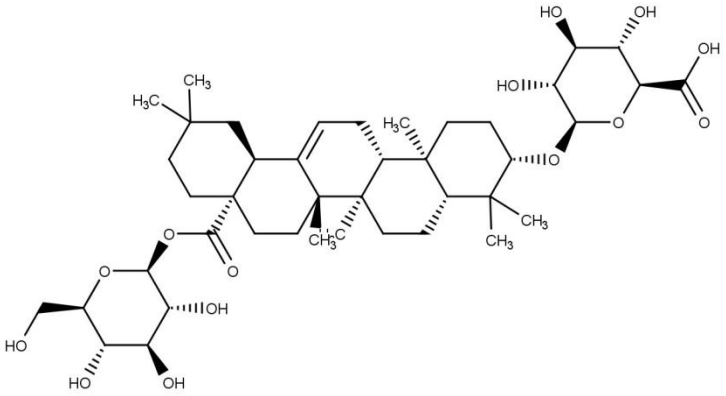
Triptofordin C-2 (31)



Putranjivain A (32)

Tetranortriterpenoid 1-cinnamoyl-3,11-dihydroxymeliacarpin was obtained from *Melia azedarach* and has displayed in-vitro anti-herpetic activity by impeding NF-κB activation in HSV-1 infected conjunctival cells (Bueno et al, 2009). Notoginsenoside ST-4i, a saponin isolated from the Chinese herb *Panax notoginseng*, demonstrated remarkable *in vitro* inhibitory activities against HSV-1 and HSV-2 with EC_{50} values of 16.4 and 19.44 μm, respectively. It inhibits penetration of HSV *in vitro* in the host cells (Pei et al, 2011).

Chikusetsusaponin IVa (34) isolated from *Alternanthera philoxeroides* (Mart.) induced inhibitory effect *in vitro* on both HSV-1 and HSV-2 along with exerting anti-HSV-2 activity *in vivo* in a mouse model. The anti-HSV-2 activity was due to direct inactivation of virus particles and to the inhibition of release of progeny viruses from infected cells (Rattanathongkom et al, 2009). The inhibitory activity of glycyrrhizin (GR) was examined on infected mice with HSV-1, and the results indicated that GR eliminated the increased susceptibility of thermally injured mice to HSV infectivity during the induction of CD4+ contrasuppressor T cells (Utsunomiya et al, 1995).

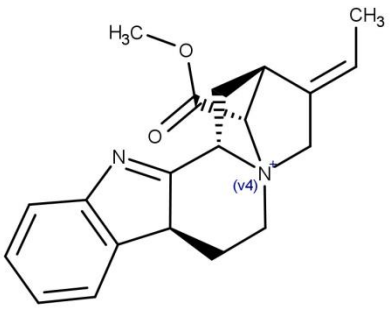


Chikusetsusaponin IVa (34)

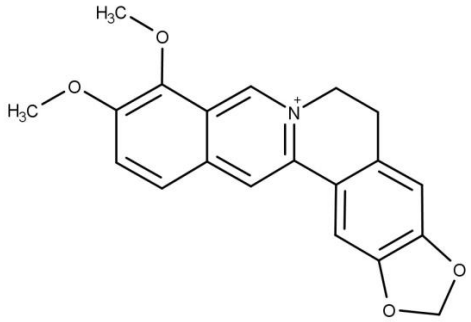
1.9.4. Alkaloids

Zhang et al. have recently isolated a novel alkaloid, 17-norexcelsinidine (35) from the twigs and leaves of *Alstonia scholaris*. This substance demonstrated significant *in vitro* inhibitory activity against HSV with EC_{50} of 1.09 µg/ml (Zhang et al, 2014). Berberine (36) is an alkaloid extracted from Chinese herb *Coptidis rhizoma* and demonstrated *in vitro* antiherpetic activity with EC_{50} of 24.4 and 26.8 µM, against HSV-1 and HSV-2 respectively. Moreover, it prevented the penetration of virus into the host cell and inhibits the immediate early genes ICP0 and gD. It also activated the NfκB signaling pathway to inhibit the viral replication (Chin et al, 2010; Song et al, 2014). Another alkaloid FK-3000 obtained from the methanol extract of *Stephania cepharantha* showed *in vivo* anti-HSV-1 activity. It effectively reduced skin lesions and prolonged the period of survival time in mice (Nawawi et al, 2001). Capsaicin, and its cis isomer (Civamide) form of the genus Capsicum, reduced recurrent infection of HSV-2 in guinea pigs (Bourne et al, 1999). Ren et al. reported that a crude alkaloid extract from the root of a Chinese herb *Tripterygium*

hypoglaucum showed *in vitro* antiviral activity against HSV-1 infection with an EC_{50} of 6.5 µg/ml (Ren et al, 2010).



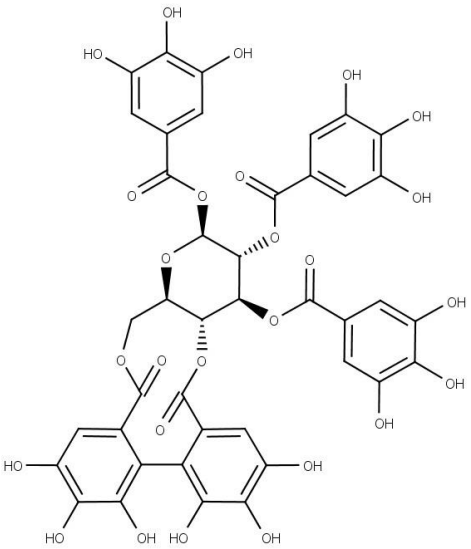
17-norexcelsinidine (35)



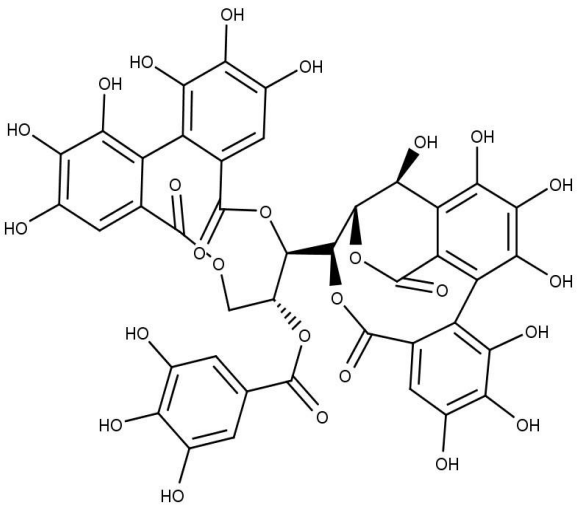
Berberine (36)

1.9.5. Tannins

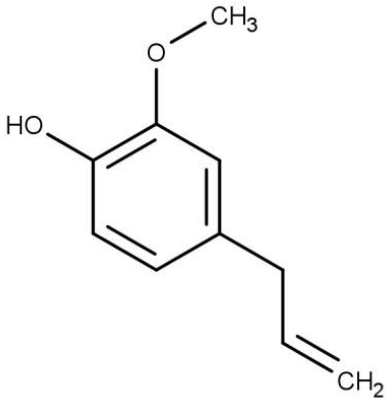
Tannins are grouped into hydrolysable and condensed tannins. Hydrolysable tannins are based on gallic acid, while the condensed tannins proanthocyanidins are derived from flavonoid monomers. The phenolic samaragenin B of *Limonium sinensi* suppress HSV-1 replication by regulating macromolecular synthesis (Kuo et al, 2002); while eugeniin (37) and eugenol (38) from *Geum japonicum* and *Syzygium aromaticum* block viral DNA polymerase and thereby inhibit acyclovir-resistant and thymidine kinase-deficient HSV-1 and wild-type HSV-2 (Kurokawa et al, 1998).



Eugeniin (37)



Casuarinin (39)



Eugenol (38)

Hydrolyzable tannin casuarinin (39) from *Terminalia arjuna* bark is virucidal and inhibit HSV-2 attachment and penetration and also disturbs the late events of virus replication (Cheng et al, 2002). The crude extract of *Cocos nucifera* L. and one of the fractions rich in catechin also showed inhibitory activity against acyclovir-resistant HSV-1 (Esquenazi et al., 2002).

1.9.6. Polysaccharides

Recently Thompson and Dragar reported that galactofucan, a sulfated polysaccharide from aqueous extract of seaweed *Undaria pinnatida* exhibits anti-HSV activity at noncytotoxic dose by inhibiting viral binding and entry into the host cell against clinical strains of HSV-1 and HSV-2, with EC_{50} of 32 and 0.5 µg/ml respectively, demonstrating its significant activity against HSV-2 (Thompson and Dragar, 2004). Stevian, the heterogeneous anionic polysaccharide with different ionic charges, extracted from *Stevia rebaudiana* and *Achyrocline flaccida* inhibit the replication of four serotypes of human rotavirus and HSV-1 by blocking the virus attachment to cell surface (Takahashi et al, 2001); while the acidic polysaccharides of *Cedrela tubiflora* inhibit HSV-2 and VSV replication (Craig et al, 2001). The anionic polysaccharide of Japanese medicine *Prunella vulgaris* had specific anti-HSV activity by competing for cell surface receptor, unlike other anionic carbohydrates (Xu et al, 1999).

Four neutral polysaccharides isolated from the aerial part of *Basella rubra* L. (BRN-1, BRN-2, BRN-3 and BRN-4) showed that only BRN-3 demonstrated antiviral effect on HSV-2 in Vero cells by inhibition of virus adsorption to host cells (Dong et al, 2012).

The discovery of novel anti-viral drugs deserves not only great efforts but sophisticated infrastructure and coordination of diverse discipline. Interestingly, it was found that traditional medicines, like Ayurvedic, Traditional Chinese medicine (TCM), Chakma medicines etc are good sources for potential antiviral drugs. The pharmaceutical industry is increasingly targeting medicinal plants with the aim of identifying lead compounds, focusing particularly on suitable alternative or complementary antiviral agents. A wide variety of active phytochemicals, including the flavonoids, terpenoids, organosulfur compounds, limonoids, lignans, sulfides, polyphenolics, coumarins, saponins, chlorophyllins, furyl compounds, alkaloids, polyines, thiophenes, proteins and peptides are found to have therapeutic applications against genetically and functionally diverse viruses (Khan *et al*., 2005; Chattopadhyay & Naik, 2007; Mukhter *et al*., 2008; Chattopadhyay & Bhattacharya, 2008; Chattopadhyay *et al*., 2009; Bag *et al*., 2014; Chattopadhyay *et al*., 2015). While different approaches are used to discover potential 'lead' molecules as 'anti-viral substance' from natural sources, including extracts and pure compounds of herbal medicines, our aim is to search for crude extract or isolated phytochemicals from medicinal plants with antiviral activities.

Chapter 2

Scope, objective and plan of work

2.1 Scope and rationale of the present study

2.2 Objective of the work

2.3 Framework of the study

2.1. Scope and rational of the present study

Globally, viral diseases caused a considerable number of morbidity and mortality, and are one of the major threats to public health. Among them, infections caused by Herpes simplex virus type 1 (HSV-1) is of major concern owing to its silent infection nature, epidemic potential, ability to produce recurrent infection with periodic reactivation, and frequent development of drug-resistance with variable manifestations. Herpes simplex virus 1 (HSV-1) infection is common worldwide, with an estimated 140 million people (67%) of the world population reportedly HSV-1 seropositive. The prevalence of HSV-1 varies with age, race, geographic location, and socioeconomic status. HSV-1 is transmitted via oral secretions and typically infects the squamous surfaces of the lips and mouth. Although HSV-1 infection is principally associated with oral and facial diseases, it can affect any other organs such is the case with some genital herpes. Recently it has been observed that 85% of the new HSV-1 infections were genital in nature which is a matter of serious concern among researchers. Most diseases caused by HSV-1 are self-limiting; however, they can be life-threatening in immune-compromised patients or neonates. Besides, HSV-1 can establish lifelong latent infection in sensory neurons and recurrent lesions at or near the point of entry into the body. An individual with latent HSV infection can remain asymptomatic for an extended period and currently there is no drug or vaccine available to eradicate the virus or its latency. Although HSV infection is successfully treated by FDA approved antiviral drugs including acyclovir, valacyclovir, famciclovir and penciclovir, but none can eliminate the virus from the host, nor prevent or stop latency or recurrent infection, except to kill the growing viruses. Moreover, widespread use of these nucleoside analogues generates drug-resistant strains which may cause more severe infections, especially in neonates and immune-compromised patients. Furthermore, the development of effective and safe HSV vaccines is not yet possible because of high costs, strain differences and complexity, which emphasizes the need for new effective compound against HSV. One of the best strategies to develop non-nucleoside antiviral lead is the screening of plants based on ethno-medicinal usage and subsequent detailed investigation, that may be effective against wild type and clinical strains and less likely produce drug resistant mutants.

Natural products and synthetically modified natural product derivatives are preferred due to potential and wide spectrum of biological activities, higher safety margin and lesser

costs than the synthetic drugs. Based on suitable models and approaches, uses of plants as HSV-1 inhibitory activity should be explored further. The therapeutic potential of plants depends on particular type of bioactive compounds present in the specific species. Standardization of plant materials with analytical techniques such as high-performance liquid chromatography (HPLC) is an essential tool to get the optimal concentrations of the known active constituents present, and in preserving their activities. The plants from traditional and other resources need to be evaluated based on utilization to find effective leads from natural resources useful in the treatment of HSV-1 infection.

Natural products, which provided many novel drug leads, are known to be an important source of anti-HSV-1 agents. Traditional medicines have been used to prevent or treat HSV-1 infections for a long time. Accordingly, a large number of natural products, including pure compounds and standard extracts, isolated from traditional herbs or other plants, have been examined for their antiviral effects on HSV-1. As an important source of novel anti-HSV-1 agents, natural products deserve further screening, studying and evaluation.

Based on literature review, it was found that some plants from Indian system of medicine showed specific efficacy against wound healing and skin related ailments. Therefore, these works were encouraged to evaluate the HSV-1 inhibitory potential of selected medicinal plants being used in our traditional system of medicine. The underlying mechanism of HSV-1 infection of most bio-active extract/fraction and its principal constituent were further explored by time-of-addition assay, Reverse transcriptase PCR (RT-PCR), immune-flurecsence assay and western blot analysis, to identify potential leads against HSV-1 infection.

2.2. Objective of the work

The present study was an attempt to investigate two selected Indian medicinal plant species *Dillenia indica* and *Boswellia serrata* for their phytochemical and biological potential related to HSV-1 inhibition and subsequent isolation and identification of potential lead molecule from these plants which could significantly inhibit HSV-1 infection. The work was divided into several major sections that includes collection and authentication of plant materials; screening of Indian medicinal plants for their anti-HSV

activity based on traditional uses and selection of potential candidates among them for further exploration; extraction and bio-assay guided isolation followed by standardization using active marker components through HPLC and HPTLC method; detailed evaluation of anti-HSV activity; and assessment of mechanism of inhibition of potential lead compounds.

Prominent objectives of the work were based on the following aspects:

- Selection of plants on the basis of ethno-medicinal uses.
- Collection and authentication of the selected plant materials.
- Extraction of the plant materials by using methanol as solvent depending on the literature review and condition of the raw material.
- Screening of anti-HSV-1 potential of selected plant extracts and selection of potential candidates for detailed investigation against HSV-1 infection.
- Evaluation of antiviral activity through bio-activity guided fractionation.
- Analysis of bio-active extracts and fractions through a reverse phase high performance liquid chromatography (RP-HPLC) and high performance thin layer chromatography (HPTLC) techniques using specific marker compounds and particular solvent system.
- Determination of the mode of inhibition of the bioactive extracts/fractions and active constituent against HSV-1 infection by time-of –addition study, immunofluorescence assay and semi-quantitative RT-PCR analysis.
- Investigation of host immune response and subsequent signaling pathway by semi-quantitative RT-PCR and western blot analysis.

Based on ethno-medicinal perspective it was decided to evaluate the anti-HSV-1 activity of two selected Indian medicinal plants and to isolate and identify potential leads from these plants against HSV-1 infection.

2.2. Framework of the study

The work was mainly designed to evaluate the Indian medicinal plants and their active constituent against HSV-1 infection. The plan of the work is represented in the schematic diagram (Figure 2.1).

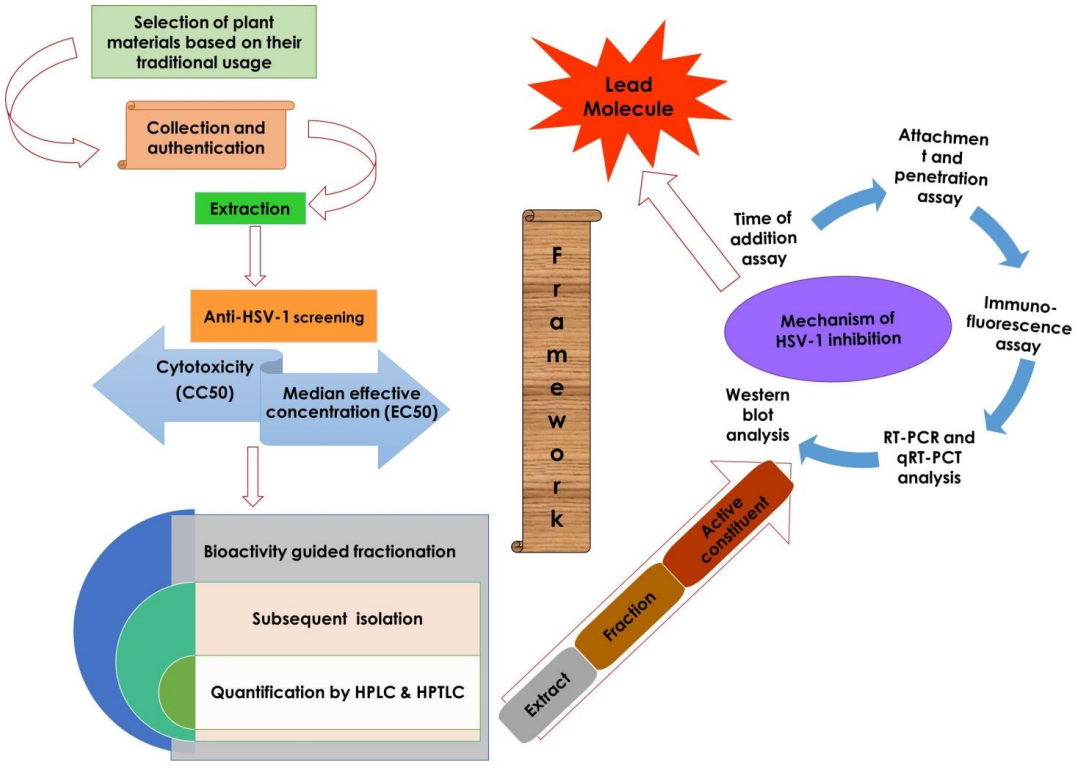


Figure 2.1 Work plan of the study

Chapter 3

Screening of Indian medicinal plants against HSV-1 infection

3.1. Rational for selection of medicinal plants for evaluation of anti-HSV-1 activity

The modern medicine has gradually developed over the years by observational and scientific efforts from traditional medicines, and even today the ancient wisdom of ethno-medicines is an important source of drug development. Ethno-botanical literature of diverse culture described the usage of folk-remedies including the plant extracts, infusions and powders for the management of diverse diseases including the diseases of viral origin (Butler, 2008). There is an increasing need for new compounds with antiviral activity as the treatment of viral infections with the available antivirals is often unsatisfactory with the problem of resistance, viral latency and conflicting efficacy in recurrent infections (Chattopadhyay and Naik, 2007). Interestingly, traditional medicines, like Ayurvedic, Traditional Chinese Medicine (TCM), Chakma medicines, etc., were believed to be a good source for potential drug development. A wide variety of active phytochemicals including the alkaloids, coumarins, flavonoids, lignans, limonoids, organosulfur, peptides, polyphenolics, saponins, terpenoids etc are reported to have therapeutic applications against genetically and functionally diverse viruses, due to their broad range of bioactivities like antioxidant, free radical scavenging, inhibition of viral entry, reproduction, DNA or RNA synthesis, or assembly (Chattopadhyay et al, 2008). Ethnopharmacology provides an alternative approach for the drug discovery, as many plants used in traditional medicines have significant pharmacological activities (Chattopadhyay et al., 2015). A detailed investigation on the efficacy and safety of selected plants used in local health care system may help in the development of invaluable herbal lead.

Thus, we have selected eight ethnomedicinal plants, based on their use in diverse ailment by different communities of India, to evaluate their antiviral efficacy against *in vitro* HSV-1 infection. The selected plants were traditionally used against skin, intestinal and sexual ailments by different tribal communities of India (Table 3.1). Moreover, literature review showed that these selected plants contain diverse bioactive phytochemicals like polyphenolics, flavonoids, terpenoids, arabinogalactan in *Dillenia indica* (Yazan and Armania, 2014); terpenoids in *Boswellia serrata* (Abdel-Tawab et al., 2011), which can contribute to the treatment of viral infections including HSV-1. Hence, the present study was undertaken to evaluate the antiviral potential of eight selected

plants against HSV-1infection, based on their traditionally usage for skin ailments and related conditions by local and tribal populations in India.

Table 3.1. Profiles of selected plants with their traditional use

Plant name and Voucher No	Local name	Family	Parts used (Dosage, Frequency & duration)	Traditional uses
Aegle marmelos L. (Corr.) SNPS-JU/2014/2103	Bel	Rutaceae	Leaf paste/juice: 5-10 leaves (paste or juice), topically applied or taken orally twice for a week. Leaves (2-5) sallow fried in cow ghee, taken 2-3 times a day for dry cough of children. Fresh Fruits: burnt in cow dung fire, mixed with molasses help to prevent acidity. Ripe fruits: Twice daily for constipation.	Wound healing, urination, body odor and ear trouble Leaf paste topically used against wound, boils, dry cough, and abscess by local and tribal communities: *Sabar*, *Lodha*, *Munda*, *Santal* of West Bengal; *Bodo* and *Kacharies* of Asssam. Other uses include astringent, eye problem, diarrhea, dysentery; and root Juice for fever (Saikia et al., 2006; Kala, 2006).
Butea monosperma (Lam.) Kuntze SNPS-JU/2014/1459	Palash	Fabaceae	Flowers: soaked overnight in a glass of water, filtered and taken in empty stomach. Leaf decoction: One tablespoon for 3 days after fifth day of menstruation for conception. Seed powder mixed with goat milk as aphrodisiac and	Flowers are used in skin diseases, as contraceptive, high blood pressure, gout, diarrhoea, astringent, leprosy, thirst, and burning sensation; leaf decoction and seed powder for conception by *Santal*, *kandha*, *ganda* and *sabara*

			contraceptive.	tribes of Bengal and Orissa (Patil et al., 2006)
Dillenia indica Linn. SNPS-JU/2012/2102	Chalta	Dilleniaceae	Fruits & bark paste applied 2-3 times daily for 5-7 days to burst abscess, and relieve pain. Leaf paste used 3-4 times a day for 5 days in edema. Oil is applied twice daily for 10 days to reduce skin diseases.	Fruits are used to rupture abscess, relieve pain, stimulate appetite, prevent dandruff, constipation, dysentery, stomachache and fever by *Lepcha* tribes of Dzongu valley, North Sikkim, and *Apatami, Mongpa, Singpho, Tangsa* tribes of Arunachal Pradesh, India. Oil is applied topically in skin diseases by *Garo* tribes (Gandhi and Mehta, 2013; Khongsai et al., 2011).
Enhydra fluctuan Lour. SNPS-JU/2014/2101	Helencha	Asteraceae	Leaves (4-5): boiled in water (2 cups or 100 ml) and used in empty stomach for moths as blood purifier. Leaf juice (10 ml) mixed with *Centella asiatica* and cucumber juice (2-5 ml) and taken twice for 3 weeks.	Leaf juice is externally applied in skin diseases, blood purification and to reduce prickly heat by *Jaintia* and *Garo* tribes of Meghalaya (Jaiswal, 2010). It is also used in hypertension and excess bile secretion.
Morus alba L. SNPS-JU/2013/1950	Tunt	Moraceae	Leaf paste of *M. alba*, *Eupatorium odoratum, Euphorbia hirta*,	Leaf used as antibacterial, diaphoretic, hypoglycemic (Singh et al., 2013) and

			Ficus benghalensis used twice for 7 days to heal wound	treating cuts and wounds by *Santal*, *Sabar* tribes of West Bengal and *Kuruma* tribes of Kerala, India (Thomas et al., 2014).
Boswellia serrata L. SNPS-JU/2014/2015	Shallaki	Burseraceae	Paste of dried resin applied for arthritis and skin related ailments.	Oleo-gum resin of *Boswellia serrata* is used for the treatment of skin diseases, syphilis, polyuria, gonorrhea, diabetes, anti-obesity, arthritis (Abdel-Tawab et al., 2011).
Piper beetle L. SNPS-JU/2011/1052	Paan	Piperaceae	Leaf decoction: 5-10 ml daily for 7 days to treat cough. Leaf paste applied 3-times daily to cuts, boils and scabies. Warmed leaves (2-5): stop nose bleed. Leaf (2-4) heated in Ghee and used daily for 5-7 days to reduce dry cough.	Leaf is used by various tribal communities of Eastern and North Eastern India including *Santal*, *Sabar*, *Munda*, *Lodha*, etc and other communities of India for dry cough, inflammation, cuts, boils and infection (Rai et al., 2011).
Stereospermum suaveolens Roxb. SNPS-JU/2014/1463	Atkapali, Parul	Bignoniaceae	Root, Leaf, Bark paste: Used twice daily for 7-10 days; consume 2-3 small tablets (5 gm paste) twice daily for 15 days.	Used for skin ailments, piles, inflammation, malaria, gonorrhea, and bronchitis (Muchandi and Chandrashekhar, 2011) by local and tribals communities like *Santal*, *Munda* of West Bengal; *Bodo* and *Miris* of Assam.

3.2. Screening of antiviral activity of selected ethnomedicinal plants against HSV-1

The main criterion for selection of the eight Indian medicinal plants is their effectiveness against skin related ailments as per their ethnomedicinal perspective. In the current study the methanol extracts of the selected plant materials were screened with the aim of identifying the most effective plant extracts against HSV-1 infection in Vero cells.

3.2.1. Reagents and chemicals

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

3.2.2. Collection and authentication of plant materials

The plant samples, selected on the basis of their traditional use in literature, were collected from different locations of Eastern and North Eastern India. The plant materials were authenticated by Dr. S. Rajan, Field botanist, The Medicinal Plant Collection Unit, Ooty, Tamilnadu, Govt. of India. The voucher specimen numbers of the plants were preserved at the School of Natural Product Studies, Jadavpur University, Kolkata for further reference.

3.2.3. Extraction of the plant material

The air dried and powdered plant materials were extracted with methanol (95%) for 72 hrs at room temperature. The extracts were then filtered and evaporated under vacuum at 40-45 ºC using an Eyela Vacuum Evaporator (Eyela, Japan) to get the crude dried extract. All the extracts were stored at 4 ºC until further use; and when required the extracts were dissolved in 0.1% dimethyl sulfoxide (DMSO) and diluted with DMEM, to obtain stock solution of 1 mg/ml.

3.2.4. Viruses and the cell line

African green monkey kidney cells (Vero cells, ATCC, Manassas, VA, USA) was grown and maintained in Eagle's minimum essential medium (EMEM), supplemented with 5-10% fetal bovine serum (FBS, Invitrogen, USA). The standard strains of HSV-1F (ATCC 733), purchased from the ATCC, USA, along with the clinical isolate VU-09 (HSV-1) was used. After plaque purification, the viruses were grown in the above media and the virus

stocks were stored at -80 ºC for future use, and whenever required the virus stocks were grown on Vero cells to determine the titer(s) by plaque reduction assay for further study.

3.2.5. Determination of cytotoxicity by MTT assay

To monitor the cellular toxicity of the test extracts on Vero cell the MTT assay was used. Vero cells (1.0×10^5 cells/ml) grown as monolayers were cultured onto 96 well plates at 37 ºC in 5% CO_2 for 6 hrs. Then different concentrations of the test extracts were added to each culture wells at a final volume of 100 µl, in triplicate, using DMSO (0.1%) and Acyclovir (ACV) as a negative and positive control, respectively. The extract treated cells were incubated at 37 ºC with 5% CO_2 for 72 hrs and then the MTT reagent (10 µl) was added to each well. After 4 hrs incubation at 37 ºC in 5% CO_2, the formazan was solubilized by adding diluted HCl (0.04 N) in isopropanol, and the absorbance was measured at 570 nm with a reference wavelength of 690 nm by an ELISA reader. The 50% cytotoxic concentration (CC_{50}) was calculated as:
[(sample absorbance-cell free sample blank)/mean media control absorbance)]/100% (Bag et al., 2014).

3.2.6. Determination of antiviral activity by CPE reduction and MTT assay

The initial inhibitory potential of test extracts on HSV-induced cell was determined by CPE reduction (Chattopadhyay et al, 2015), followed by MTT assay. Briefly, Vero cells (1.0×10^5 cells/ml) seeded on 96-well plates were grown for 24 hours and then infected with the virus (MOI: 1.0). The virus infected cells were then exposed to the increasing concentrations of the test extracts, with suitable control. After 72 hrs of incubation at 37 °C in 5% CO_2 the MTT assay was carried out as described previously (Bag et al., 2014).

3.2.7. Determination of antiviral activity by Plaque reduction assay (PRA)

To further confirm the antiviral activity of the test extracts PRA was used with ACV and DMSO (0.1%) as control. Serial dilutions of the test agents and ACV in EMEM was added to the virus infected Vero cell monolayers (MOI: 1.0) and overlaid with overlay medium containing test agents, to enable the virus to spread via cell-to-cell route to form plaques. The plaques developed after 3 days of incubation were fixed with 4% paraformaldehyde and stained with methylene blue (0.03%) in 70% methanol. The

effective concentration of the test extracts that inhibited the number of viral plaques by 50% (EC_{50}) was interpolated from the dose-response curves (Lin et al., 2011).

3.3. Results

3.3.1. Assessment of cytotoxicity and anti-HSV activity

The cytotoxicity (CC_{50}) and median effective concentrations (EC_{50}) of the test extracts, presented in Table 3.2, revealed that four, out of eight extracts tested, have significant activity against all the HSV-1 isolates tested, at a concentration that are non-cytotoxic to the Vero cells.

Table 3.2. Assessment of Cytotoxicity and antiviral activity of methanol extracts of eight selected plant extracts against HSV-1

Plant species and parts used	CC_{50}[a]	HSV-1F (at MOI: 1.0)		VU-09 (HSV-1 isolate) at MOI of 1.0	
		EC_{50} [b]	SI[c]	EC_{50} [b]	SI[c]
Aegle marmelos (leaves)	32.4 ± 5.2	ND	-	ND	-
Butea monosperma (leaves)	329.2 ± 7.4	107.5 ± 3.9	3.06	111.35 ± 6.8	2.95
Dillenia indica	427.4 ± 8.4	56.19 ± 5.8	7.6	61.24 ± 7.2	6.0
Enhydra fluctuans (leaves)	> 1000	525 ± 5.32	>1.90	522 ± 5.32	>1.91
Morus alba (leaves)	604.2 ± 3.7	ND	-	ND	-
Boswellia serrata	56.6 ± 3.4	5.2 ± 1.8	10.88	6.2 ± 2.1	9.12
Piper beetle (leaves)	792.8 ± 5.6	ND	-	ND	-
Stereospermum suaveolens (leaves)	664.6 ± 3.29	ND	-	ND	-
Acyclovir	130 ± 3.8	2.1 ± 0.5	61.9	2.2 ± 0.4	59.09

[a] 50% cytotoxic concentration for Vero cells in µg/ml.

[b] Concentration of test agent (µg/ml) producing 50% reduction of virus induced plaques.

[c] Selectivity index (SI)=CC_{50}/EC_{50}.).

ND, Not detectable.

The methanol extracts of *Dillenia indica* fruits and *Boswellia serrata* oleo-gum resins exhibited significant antiviral activity towards HSV-1F with EC_{50} of 56.19 and 5.2 µg/ml; while VU-09 isolate was inhibited at EC_{50} of 61.2, and 6.2 µg/ml, respectively. However, the extracts of *Enhydra fluctuans* and *Butea monosperma* exhibited anti-HSV activity but at lower concentrations, while the other four extracts failed to produce significant inhibitory activity against the tested isolates of HSV-1. The results of CPE inhibition assay, followed by MTT and plaque reduction assay were found to be comparable, and the extracts of those four plants exhibited anti-viral activity in those widely used assay system. Based on the selectivity index (SI), the preferential antiviral activity (EC_{50}) of the test extracts in relation to its cytotoxicity (CC_{50}) revealed that *D. indica* and *B.serrata* were most effective among all the extracts and thus, subjected to further evaluation of their anti-HSV activity.

3.4. Discussion

The development of new antiviral agents from traditional medicine, capable of inhibiting herpes virus infection, represents an attractive strategy, particularly for the patients suffering from recurrent and chronic infections as well as in immune-compromised individuals and neonates, who often generate ACV-resistant HSV strains. In our continued quest for identifying new leads from ethnomedicinal plants of traditional use we have evaluated nine plant extracts selected on the basis of their traditional use in skin infections and related ailments. The cytotoxicity study revealed that the crude methanol extracts of the test plants had different CC_{50} due to the variable concentration of bioactive compound(s) present within those plant extracts. While the antiviral activity, studied by CPE reduction, MTT assay and PRA showed that four extracts, namely *Butea monosperma, Dillenia indica*, *Enhydra fluctuans*, and *Boswellia serrata* had significant antiviral activity, as they effectively inhibit HSV-1 infection in Vero cells without reducing cell viability at 50% effective concentration (EC_{50}). Interestingly all these four extracts also inhibited the growth of VU-09, and HSV-1 strain isolated from human patients. Most importantly two of these plant extracts (*D. indica* and *B. serrata*) showed significant inhibitory activity against HSV-1 infected vero cells at EC_{50} of 56.19 µg/ml and 5.2 µg/ml with SI of 7.6 and 10.88 respectively, indicating that these plants extracts need to be studied further with other members of herpes virus family.

The results of this study provide evidence that the ethnopharmacology can be a guide for the screening of biologically active plant materials even for antiviral screening. Here, we have used 100 % inactivation to define the antiviral activity of an extract, and found that some extracts had partial antiviral activity, probably due to their varied chemical nature. Out of eight plant extracts tested, *D. indica* and *B. serrata* exhibited potent anti-HSV activity whereas extracts of *B. monosperma* and *E. fluctuans* revealed insignificant inhibitory activity. The other four extracts namely *Aegle marmelos, Morus alba, Piper beetle* and *Stereospermum suaveolens* showed no anti-HSV at all. One of the reasons for search of antivirals from ethnomedicinal plants is the wide acceptability of traditional medicines for their long-standing use and their relatively low toxicity profile, as the widely used antiviral drug acyclovir, a nucleoside analogue that targets the thymidine kinase of DNA polymerase of herpes viruses (Kleymann, 2003) neither able to eliminate the virus from the host nor able to prevent recurrent infections. Moreover, the extensive and long-term use of acyclovir and related analogues yielded frequent drug-resistant strains due to mutations in viral thymidine kinase and/or DNA polymerase by altering the substrate sensitivity (Piret and Boivin, 2011). Moreover, the efficacy of therapeutic vaccines against primary and recurrent HSV infection has failed (Coleman and Shukla, 2013) and thus, search for natural alternative is the top priority to control and prevent HSV infections and its transmission. Our results indicated that the elucidation of active constituents and the possible mode of action of these two plants extract (*D. indica* and *B. serrata*) showing promising inhibitory potential against HSV-1, may provide useful leads for the development of effective antiviral agents.

3.5. Conclusion

The outcome of our study elucidate that four out of eight extracts possess some degree of anti-HSV-1 activity. Among these four, extracts of *D. indica* and *B. serrata* were significantly higher in their SI values. On this basis these two plant extracts were chosen for further investigation of anti-HSV-1 activity. Thus *D. indica* and *B. serrata* were targeted as the potential sources of finding leads for antiviral efficacy against HSV-1 which is described in detail in subsequent chapters.

3.6. Publication

Screening of ethnomedicinal plants of diverse culture for antiviral potentials. Indian Journal of Traditional Knowledge (CSIR-NISCAIR, India) 2016, 15 (3), 474-481.

Chapter 4

Evaluation of anti-HSV activity of *Dillenia indica* Linn fruit extract and identification of potential lead

4.1. *Dillenia indica* Roxb. – A profile

4.1.1. Botanical taxonomy

Scientific classification			**Vernacular names**		
Kingdom	:	Plantae	English	:	Elephant apple
Division	:	Phanerogamae	Sanskrit	:	Bhavya
Class	:	Dicotyledonae	Hindi	:	Girnar
Order	:	Dilleniales	Bengali	:	Chalta
Family	:	Dilleniaceae	Tamil	:	Uva
Genus	:	Dillenia	Telugu	:	Peddakalinga
Species	:	Indica	Marathi	:	Karambal

4.1.2. Plant description

Dillenia indica L. (Family-Dilleniaceae), also known as elephant apple tree are native to the forests of India, and the Indo-Malaysian region up to Tropical Australia. The fruits are sour in taste and are well known by the Indian communities as a flavoring agent for curries, and preparation of jam and jelly (The wealth of India 1952).

Figure 4.1a. *Dillenia indica* L. plant

Figure 4.1b. Fruit of *Dillenia indica*

It is a medium-sized evergreen tree reaches up to 15m in height, trunk is straight but not much high and branches are spreading and forming round-shady head. Bark is reddish brown in colour and exfoliating where young branchlets are brown pubescent, glabrescent and contains leaf scars. Leaves are 15-36 cm long, fascicled at the ends of the branches, oblong-lanceolate, acuminate, sharply serrate with the nerves close into

the serratures, not forking at the margins. Its branches are used to make good firewood. The flowers are large, 15-20 cm in diameter, with five white petals and numerous yellow stamens. Fruits are aggregate and globose, 5–12 cm in diameter, indehiscent with persistent sepals, fleshy and slightly swollen. The fruits are greenish yellow in colour and edible. The fruit is an aggregate of 15 carpels, each carpel contain five seeds embedded in an edible fibrous pulp. Seeds are imbedded in glutinous pulp, compressed, with hairy margins (Yazan and Armania, 2014; Gandhi and Mehta 2013).

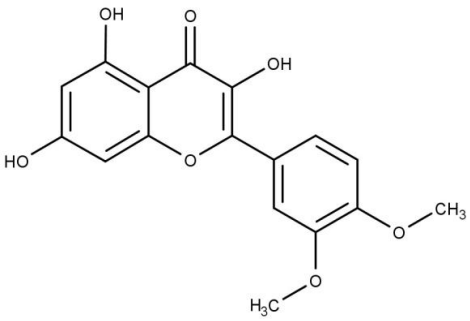
4.2 Ethno-pharmacological uses of *D. indica*

D.indica has been reported for its traditional usage in several countries for various ailments. The fruit juice of *D. indica* is used as a cooling beverage to treat fever and to relieve fatigue (Shome et al., 1980). Although mainly a source of food for elephant and monkey, the ripped fruits are taken orally to increase appetite and overcome weakness (Poonam & Singh, 2009), as laxative (Prasad et al., 2008) and medication for abdominal pain (Kritikar & Basu, 2003). The fruit of the plant is used by the native communities in Mizoram, India, as a remedy for jaundice (Rai & Lalramnghinglove, 2010). The fruit is also used as a treatment for diabetes mellitus among the Khamptis traditional healers (‘‘Chau yau’’) in Arunachal Himalaya, Northeast of India (Tag et al., 2012). In contrast, the rural community of the Dhemaji district of Assam, Northeast India, use the leaves of the plant, locally known as ‘‘Outenga’’, for the same purposes (treatment of diabetes) (Sood et al., 2005; Tarak et al., 2011). The mucilaginous substance from the fruit is traditionally used as a cosmetic product to reduce dandruff among Assamese people in the North East of India (Saikia et al., 2006). The ethnic communities of Dibru-Saikhowa Biosphere Reserve of Northeast India were reported to use the decoction of the floral body of *D. indica* as a treatment for dysentery (Purkayastha et al., 2005). Furthermore, the leaves juice (mixed with sugar) and decoction of the leaves of *D. indica* are taken orally to treat fever and cough, and constipation, among the Taungya community in Terai Arc Landscape, India (Poonam & Singh, 2009). The juices from the leaves, bark, and fruits are mixed together and taken orally (2–5 times daily) as a treatment for cancer and diarrhea among the native people of Mizoram, India (Sharma et al., 2001). In South Vietnam, the leaves of *D. indica*, locally known as ‘‘So ba’’ are traditionally used to treat intestinal diseases (Dung & Loi, 1991) and malaria-like symptoms (Nguyen-Pouplin et

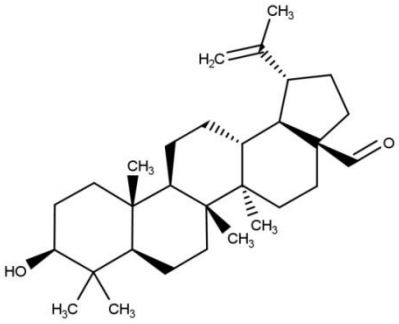
al., 2007). Among the traditionally used medicinal plants of Bajali Subdivision, Barpeta District, Assam the fruit juice of *D.indica* is used to prevent different kinds of skin diseases. Fruit juice is also used to increase the quantity of semen (Kalita et al, 2015). The seed oil of *D.indica* is widely used for garnish in indigenous ayurvedic medicine for nervousness and the mucilage is applied on wounds of burns (Janick & Paull, 2008).

4.3. Phytochemical profile of *D. indica*

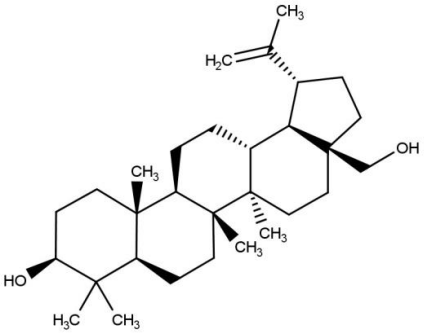
D. indica is a rich source of triterpenoids, flavonoids, tannins and various other phytoconstituents. Stem bark of *D. indica* contains 10% tannin, dillenetin (1), betulinaldehyde (2), betulin (3), betulinic acid (4), flavonoids like rhamnetin, isorhamnetin (5), lupeol (6), myricetin (7), naringenin (8), quercetin (9) and kaempferol glucoside (Shah, 1978; Khanum & Khan, 2007; Khare, 2007). The ethanol extract of stem bark afforded two flavonoids viz., kaempferol glucoside and quercetin derivative as well as a triterpenoids (Srivastava & Pande, 1981). It has also been reported that methanolic extract of stem yielded four compounds lupeol, betulinaldehyde, betulinic acid and stigmasterol (10) using column chromatographic separation (Parvin et al., 2009).



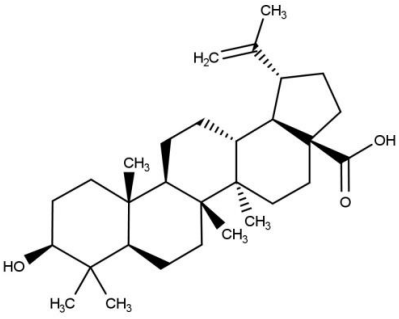
Dillenetin (1)



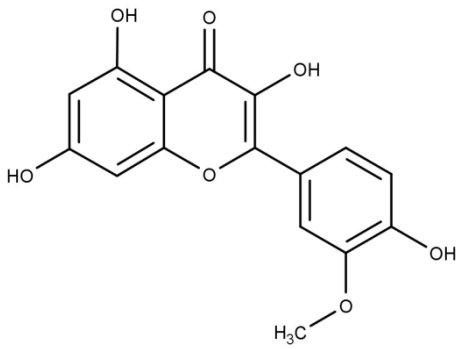
Betulinaldehyde (2)



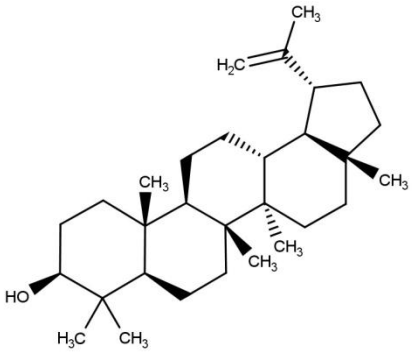
Betulin (3)



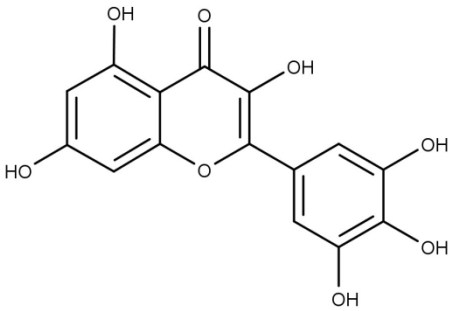
Betulinic acid (4)



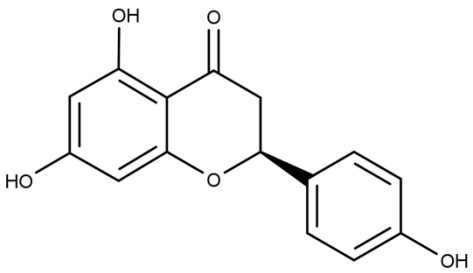
Isorhamnetin (5)



Lupeol (6)

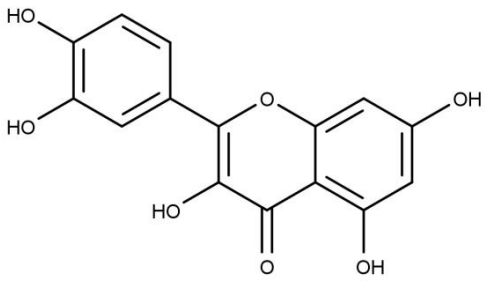


Myricetin (7)

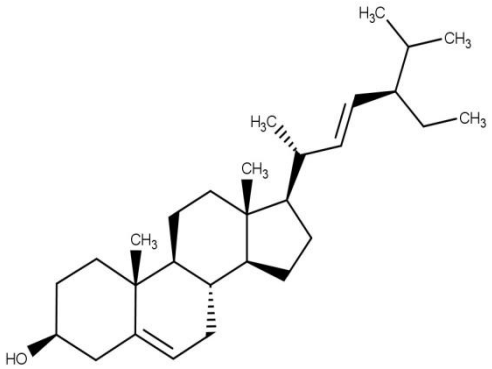


Naringenin (8)

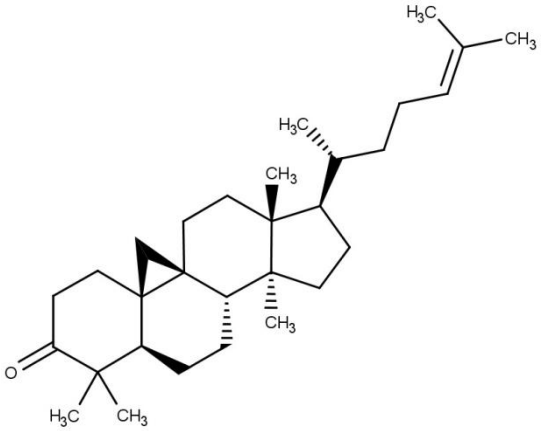
Leaves of *D. indica* were found to contain cycloartenone (11), n-hentriacontanol, sitosterol, betulin, and betulinic acid (Mukherjee, 1981). Methanolic extract of leaves after fractionation with n-hexane and chloroform also yielded compounds like sitosterol (12), stigmasterol as well as dillenetin (Md. Muhit et al., 2010). Further phytochemical studies has been performed on acid hydrolyzed extracts of dried leaves which showed presence of kaempferol (13) (Bate-smith & Harborne, 1975). The presence of betulinic acid in *D. indica* extracts has also been reported previously (Kumar et al., 2010). Flavonoids such as gallic acid (14) and dihydrokaempferol were also identified in ethanol extract of *D. indica*. In addition based on spectroscopy data and characteristic reaction including positive Liebermann-Burchard and Zimmerman's test, a new hydroxylactone was isolated and identified as 3β-hydroxylupane-13β-28-lactone (Banerji et al., 1975). Fruit of *D. indica* contain about 34% of total phenolics in methanolic extract (Md. Abdille et al., 2005) and polysaccharide like arabinogalactan. The presence of fixed oil, colouring matter, sterols, glycosides, saponins, proteins, free amino acids, sugars, free acids and tannins in the seeds has also been reported (Uppalapati & Rao, 1980).



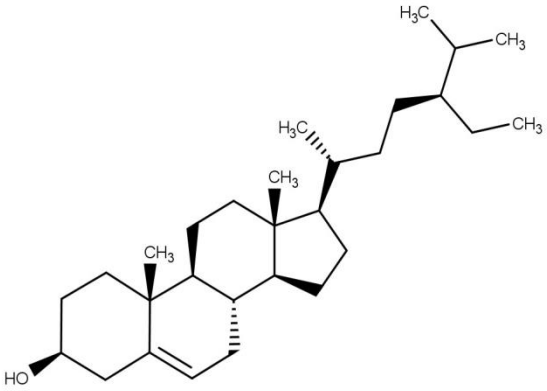
Quercetin (9)



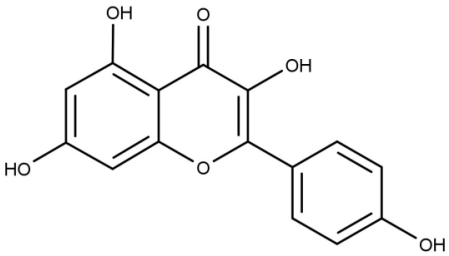
Stigmasterol (10)



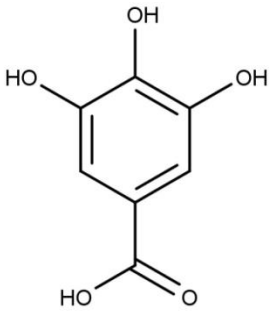
Cycloartenone (11)



Sitosterol (12)



Kaemferol (13)



Gallic acid (14)

D. indica is one of the major sources of Betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid). It is highly distributed in the bark, timber, pericarp and fruit of the plant. In recent years some significant biological properties of betulinic acid have drawn the attention of the researchers. It has been reported to have many important biological and pharmacological activities, including antimelanoma, anticancer, anti-inflammatory and

anti-HIV (Kumar et al., 2010). Betulin is also a pentacyclic triterpenoid having structural resemblance to betulinic acid (Yazan and Armania, 2014).

4.4. Pharmacological activities of *D.indica*

A lot of pharmacological investigations of *D.indica* have been carried out which are summarized as follows:

Pharmacological activity	Preclinical/Clinical/Mechanistic studies	References
Anticancer activity	• Betulinic acid as the major compound is responsible for the anti-leukemic properties of *D. indica* towards human leukemic cell lines (U937, HL60, and K562), which was likely due to induction of apoptosis.	Kumar et al., 2010
Anti-diabetic activity	• Petroleum ether extract and ethyl acetate fraction of *D. indica* leaves showed prominent anti-diabetic effect in alloxan-induced diabetic Wistar rats and Type-1 and Type-2 diabetic models in streptozotocin (STZ) induced Wistar rats.	Kumar et al., 2011a, 2011b
Anti-oxidant activity	• The methanol extract of *D. indica* fruit exhibited the significant anti-oxidant activities as per b-carotene-linoleate model system, 2,2-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging assay, and phosphomolybdenum method.	Abdille et al., 2005
Anti-microbial activity	• *D. indica* leaves extract exhibited antimicrobial properties against Gram positive (*B. cereus*, *Bacillus megaterium*, *B. subtilis*, *Staphylococcus aureus*, and *Sarcina lutea*) and Gram negative bacteria (*E. coli*, *P. aeruginosa*, *Salmonella paratyphi*, *Salmonella typhi*, *Shigella boydii*, *Shigella dysenteriae*, *Vibrio mimicus*, and *Vibrio parahemolyticus*).	Apu et al., 2010

Anti-inflammatory activity	• The stem barks extract of *D. indica* showed significant anti-inflammatory activity in the carrageenan-induced edema and acetic acid-induced capillary permeability models.	Yeshwante et al., 2009
Anti-malarial activity	• *D. indica* leaves extract showed significant effect against *Plasmodium falciparum.*	Nguyen-Pouplin et al., 2007
Anti-diarrhoeal activity	• The methanol extract of *D. indica* roots significantly reduced the charcoal induced Gastro Intestinal (GI) motility in mice and decreased the movement of GI tract.	Bose et al., 2010
Hepatoprotective activity	• The n-hexane extract of *D. indica* seeds showed hepatoprotective activity in wistar rats by reducing the activity of bilirubin, serum enzymes, urea and lipid peroxidation creatinine.	Reddy et al., 2010
Wound healing activity	• A glycolic extract from the fruits of *D. indica* showed significant wound healing activity lonely or in mixture with micro current stimulation to skin wounds surgically produced on the back of Wistar rats.	Migliato et al., 2011

Methanol extract of *D. indica* exhibited potent anticancer activity in leukemic cell lines (U937, HL60 and K562). Further, successive solvent extraction (based on polarity) reveals that the ethyl acetate fraction is the most cytotoxic due to high concentration of betulinic acid presence. Structure elucidation of betulinic acid as the major compound in the ethyl acetate fraction showed that it was likely responsible for the anti-leukemic properties of the fruits of *D. indica* (Kumar et al, 2010). Further research concerning the synthesis of colloidal silver nanoparticles of fruit extract is intended to improve pharmacological action of *D. indica* (Singh et al, 2013).

Anti-diabetic and anti-hyperlipidemic activity of *D. indica* leave was also evaluated in the *in vivo* animal model. It has been found that daily oral administration of 250 and 500 mg/kg extract significantly reduced the blood glucose level compared to the untreated group. It has also shown to reduce the cholesterol and triglyceride level in diabetic rats

after oral administration of the extract for 21 days (Kumar et al, 2011a). Subsequently further studies were conducted by the same group of researchers on the anti-diabetic and cholesterol-lowering properties of the bioactive fraction of *D. indica*. It was shown that single dose (400 mg/kg) of ethyl acetate fraction reduced the Type-1 and Type-2 diabetic effect in Wistar rats.

Beside the anti-diabetic effect it also reduced serum cholesterol and triglyceride level in diabetic rats treated with the ethyl acetate fraction (Kumar et al, 2011b). The fruit extracts of *D.indica* exhibited potent anti-oxidant activity due to its phenol content (Abdille et al., 2005). Apu et al., 2010 reported the antimicrobial properties of leaves extract of *D. indica* against Gram positive and Gram negative bacteria. Nguyen-Pouplin et al., reported the anti-malarial properties of leaves extract of *D. indica* against *Plasmodium falciparum* (Yazan and Armania, 2014).

4.5. Lead finding from *Dillenia indica* extract against HSV-1

4.5.1. Collection and authentication of plant materials

The fruits of *Dillenia indica* Linn. (family, Dilleniaceae) was purchased from the local market at Kolkata. Plant material was authenticated by Dr. S. Rajan, Field botanist, The Medicinal Plant collection unit, Ooty, Tamilnadu, Govt. of India. A voucher specimen number (SNPS-JU/2012/2102) of the plant has been preserved in the School of Natural Product Studies, Jadavpur University, Kolkata for further reference (Figure 4.2).

Figure 4.2. Voucher specimen of *D. indica*

4.5.2. Reagents and chemicals

Betulinic acid (B8936-5MG) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent were purchased from Sigma Chemical Co. (St Louis, MO, USA). And Dulbecco modified Eagle medium (DMEM), phosphate buffer saline (PBS), fetal bovine serum (FBS), Penicillin and streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). Primers for semi-quantitative PCR were obtained from IDT, California, USA.

4.5.3. Extraction and fractionation of *D.indica*

The collected, dried fruits were made into small pieces by cutter mill to become pulverized. 500 g of the powdered sample was extracted with methanol for three days at room temperature consecutively. The process was repeated for three times. The collected solvent extract was filtered and evaporated under vacuum at 45-50 ^{o}C through the rotary evaporator (Eyela N-1001S-W, USA). The yield of the crude methanol extract of *D. indica* (ME) was 179 g. After words ME was suspended in water and partitioned successively with ethyl acetate and n-butanol. Each fraction was evaporated under vacuum in order to obtain 41 g ethyl acetate fraction (EFA), 36.8 g n-butanol fraction (NBF) and 68 g aqueous fraction (AF), respectively. Each sample was kept in borosilicate glass vials at 4 ^{o}C prior to further study. The yields of the fractions were calculated by taking ME as initial amount.

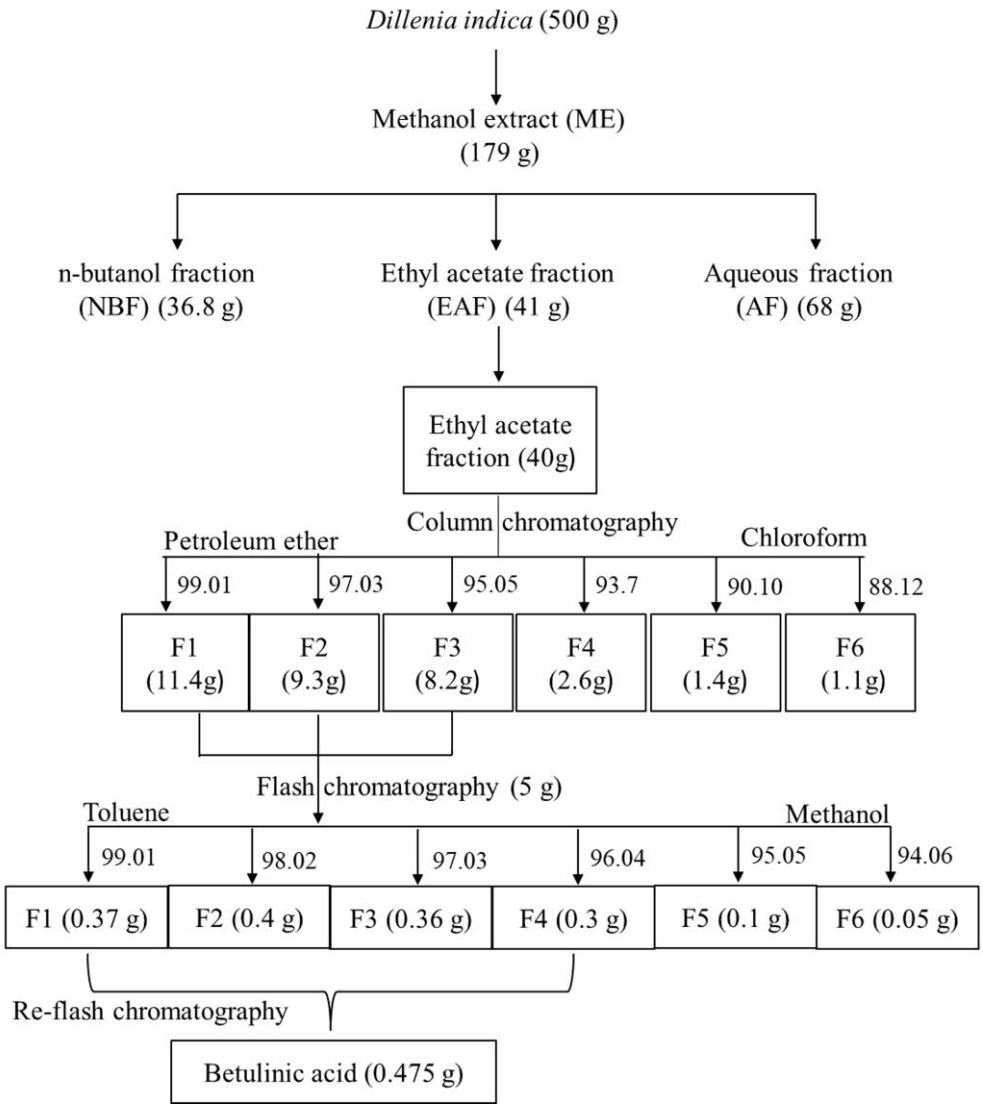
Extraction yield:

ME (Methanol extract of *D. indica*) : Yield 35.8 % w/w
AF (Aqueous fraction of *D. indica*) : Yield 34.98 % w/w
EAF (Ethyl acetate fraction of *D. indica*) : Yield 22.90 % w/w
NBF (n-butanol fraction of *D. indica*) : Yield 20.55 % w/w

4.5.4. Bioactivity guided isolation of active compound

The MTT assay for determination of anti-HSV-1 activity was performed with every fraction of the extract. The isolation of active compound was executed with the most active EAF according to Kumar et al., (2010) with modifications. Briefly the fractionation of EAF (40 g) was carried out on silica gel column using solvents of increasing polarity i.e., petroleum ether (PE) to chloroform, eluted with the mixtures of PE: chloroform gradient 99:1, 97:3, 95:5, 93:7, 90:10, 88:12 (v/v) to yield six Fractions (F_{1-6}). Among these, the bioactive fraction F1, F2 and F3 were combined together and subjected to flash chromatography (Biotage-Isolera one, Sweden) using a silica gel Cartridge (Biotage® SNAP cartridge KP-Sil, 30g) with a step gradient of toluene-methanol solvent system. The chromatographic separation, visualized at 210 nm, yielded six (F'_{1-6}) sub-fractions, out of which F'1 (0.37 g), F'2 (0.4 g), F'3 (0.36 g) and F'4 (0.3 g) were identified as betulinic acid (BT).

Thus, the sub-fractions 1-4 were combined and subjected to repeated flash chromatography on silica gel Cartridge (Cartridge KP-Sil 10g), until a white color crystal was obtained by re-crystallization with methanol. The white solid crystal was characterized and identified as BT using molecular ion peak from time-of-flight mass spectrometry (TOF-MS-ES+). Scehme-I represented the bioassay-guided isolation of BT from *D.indica*.



Scheme-I: A schematic representation of the extraction and fractionation of *Dillenia indica* fruit. [Yield of the fraction in (g)]

4.5.5. Characterization and quantification of betulinic acid by HPLC

RP-HPLC method was developed to identify and quantify the percentage of purity of isolated BT. Separation was achieved by Waters Spherisorb column (C18; 250×4.6 mm, 5µm particle size) of a Water HPLC system (Milford, MA, USA) consisting of 600 controller pumps, dual-wavelength UV-Vis detector, equipped with an in-line degasser AF 2489 and a Rheodyne 7725i injector (USA) with 20 µl loop. The quantitative estimation was performed with Empower-2 software program. The mobile phase was optimized by acetonitrile and Milli-Q water (88:12) with phosphoric acid at pH 3.0. A standard calibration curve was prepared by diluting standard BT (purity ≥ 98 % purchased from Sigma-Aldrich) to different concentration (1-500 µg/ml) range. The detection was carried out at 210 nm. Additionally, BT was identified with the help of molecular ion peak from time-of-flight mass spectrometry (TOF-MS-ES+).

For preparation of standard and sample solutions a primary stock solution (1 mg/ml) containing BT was prepared by dissolving in methanol and subsequently diluted to 1-500 µg/ml for optimization of mobile phase. Extract and fractions of *D. indica* were dissolved in the same way to get 1 mg/ml of sample solution. Standard and sample solutions were filtered through 0.45 µm syringe filter prior to injection into the HPLC column. Amount of BT present in the fractions were calculated after triplicate injection (n=3) by comparing and interpolating the extract peak area (response) with respect to the standard marker.

4.5.6. Standardization of *D.indica* by HPTLC method

4.5.6.1. Equipments

A Camag HPTLC system equipped with a sample applicator Linomat V, twin trough plate development chamber, TLC Scanner III with photo documentation device and Wincats integration software 4.02 (Switzerland) was used for this experiment. Fine Chem. Ltd (Mumbai, India). TLC aluminum plates pre-coated with silica gel 60 F_{254} (10 × 10 cm, 0.2 mm thick) used were obtained from E. Merck Ltd (Mumbai, India).

4.5.6.2. Chromatographic conditions

Chromatography was performed on 10 × 10 cm aluminium backed silica gel 60 F_{254} HPTLC plates. Samples were applied to the plates, as 5 mm bands, by Camag Linomat V automated spray-on band applicator equipped with 100 µL syringe (Hamilton, USA).

Standard betulinic acid was dissolved in HPLC grade methanol to produce a 1 mg/ml solution. The concentrated extract of the plant was reconstituted in HPLC grade methanol to produce a 10 mg/ml solution. The solutions were filtered through a 0.45 µ Whatman's syringe filter. This solution was aspirated through a 100 µl Syringe and thus applied band-wise with the CAMAG Linomat V. Following that the plates were developed in a CAMAG twin trough chamber using the solvent system Petroleum ether: ethyl acetate: toluene (7:2:1, v/v/v). After development the plates were then dried under warm air flow.

Post-chromatographic derivatization was done by spraying anisaldehyde-sulphuric acid reagent To prepare the reagent, 0.5 ml of anisaldehyde is dissolved in 50 ml of acetic acid and 1ml of concentrated sulfuric acid is added. The plates were subjected to spraying by anisaldehyde-sulphuric acid reagent and then heated at 50^{o}C for 10 min. Densitometric scanning was performed at 580 nm with a Camag TLC scanner III equipped with Wincats software, using deuterium light source; the slit dimension was 6.00 × 0.45 mm. After the development, bands in the extracts were identified by matching their R_f values with those obtained for standard.

4.5.7. 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 isolates (VU 09 and VU07), isolated from clinical specimens were obtained from ICMR Virus Unit repository and the quality control wild type strain of HSV-1F (ATCC-733) 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 370 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 ^{o}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.

4.5.8. 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 1.0×10^5 cells/ml. After incubation at 37 ^oC in 5% CO_2 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 ^oC with 5% CO_2 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 at 37 ^oC, the formazan was solubilized by adding diluted HCl (0.04 N) 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 by the formula :

percentage (%) of cell viability = [(sample absorbance - cell free sample blank)/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 (Bag et al., 2014; Ojha et al., 2013).

4.5.9. Antiviral study using MTT assay

The antiviral activity of extract, fractions and isolated compound *of D. indica* against HSV-1 was evaluated by MTT assay, a sensitive and accurate method for rapid screening of antiviral agents. Vero cells were seeded onto 96 well plates with a concentration of 1.0×10^5 cells/ml. After incubation at 37 ^oC in 5% CO_2 for 6 h, the virus at (multiplicity of infection; MOI: 1) was added and incubated for 1 h. Different concentrations of test drugs were added to culture wells in triplicate at a final volume was 100 µl in each well. The maximum concentration of DMSO (0.1%) serves as a negative control and acyclovir as a positive control for HSV assay. After incubation at 37 ^oC with 5% CO_2 for 3 days, the MTT test was carried out as described above. Viral inhibition rate was calculated as:

percentage (%) of viral inhibition = [(Atv - Acv) / (Acd - Acv)] / 100%

where Atv 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 (EC_{50}) was defined as the concentration which achieved 50% inhibition of virus-induced cytopathic effects (Chattopadhyay et al., 2009).

4.5.10. Plaque reduction assay (PRA)

Vero cells monolayers seeded in 6-well plates (2 x 10^6 cells per well) were treated with serial dilutions of the test extract, fractions and isolated compounds betulinic acid of *D. indica* for 1h at 37 °C and then separately challenged with HSV-1 (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^{o}C in 5% CO_2, the supernatant was removed, and the wells were fixed with methanol and stained with Giemsa (Sigma). Viral inhibition (%) was calculated as follows:

percentage (%) of viral inhibition = [1 - (number of plaques) in test/(number of plaques) in control] X 100%,

where “(number of plaques) in test” indicates the plaque counts from virus infection with the test extract or isolated compound BT treatment, and “(number of plaques) in control” indicates the number of plaques derived from virus-infected cells with DMSO treatment (Bag et al., 2013; Bag et al., 2014). The 50% effective concentration (EC_{50}) 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).

4.5.11. Dose-response assay

A study was conducted to analyze the dose-dependent effect of the test agents on the HSV infected Vero cell. Vero cells monolayer at 1.0 × 10^5 cells/ml was grown onto 96 well plates. After incubation at 37 ^{o}C in 5% CO_2 for 6 h, HSV-1 (at 1.0 MOI) was added and incubated for 1 h for viral adsorption. Different concentrations (1, 2.5, 5, 10, 25, 50 and 100 µg/ml) of the test extract, fractions and isolated BT 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 ^{o}C in 5% CO_2, the MTT assay was carried out as described earlier (Mukherjee et al., 2013).

4.5.12. 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 the most active ethyl acetate fraction (EAF) and isolated compound betulinic acid (BT). Following three different approaches Vero cells were treated with EAF (58.5 µg/ml) or BT (5.5 µg/ml), at their EC_{99} concentrations for 1 h (short term) and 24 h (long term) as pre-infection. The cells were then infected with the virus for 1 h followed by PRA. For co-infection cells were treated simultaneously with virus and the agents, incubated (1 h at 37°C), washed and subjected to PRA. For post-infection, the virus-infected cell was washed with PBS and overlaid with the overlay media containing the test agents. Further, Vero cells pre-treated with EAF or BT for 1 h was infected with the virus in presence of test agents followed by the addition of overlay medium containing EAF or BT (throughout). The viral plaques developed after 72 h post-infection were stained and counted (Lin et al., 2011)

4.5.13. Viral inactivation assay

HSV-1 (10^4 PFU/ml) mixed with EAF (58.5 µg/ml) or BT (5.5 µg/ml) was incubated at 37 °C for 1 h. The virus-drug mixture was immediately diluted 50-fold (final inoculums 50 PFU/well) with DMEM containing 2% FBS and added to Vero cells seeded in 6-well plates. After adsorption for 1 h at 37°C, the cells were washed with PBS, and covered with the overlay medium, incubated at 37 °C for 72 h at 5% CO_2, and subjected to PRA (Lin et al., 2011).

4.5.14. Viral attachment assay by Plaque reduction and Immunofluorescence

To study the effect of test agents on viral attachment we used modified PRA, with indirect Immunofluorescence assay (IFA). Vero cells grown in 6-well plates were prechilled at 4°C for 1 h and incubated with HSV-1 (100 PFU) for 3 h at 4 °C in presence of EAF (58.5 µg/ml), BT (5.5 µg/ml), heparin (100 µg/ml) and DMSO (0.1%). The cells were washed twice with PBS, and covered with the overlay medium, incubated at 37°C for 72 h at 5% CO2 and subjected to PRA (Lin et al., 2011).

To confirm the above results IFA was performed. The treated prechilled cells described above, were washed twice with PBS, fixed with prechilled 4% paraformaldehyde in PBS

on ice for 1 h, and then blocked with 5% bovine serum albumin (BSA) in 0.1% PBS-triton X100 solution at 4°C. The cells were permeabilized with 0.1% triton X100 in PBS, and incubated overnight with FITC-labelled anti-HSV-1 mouse monoclonal antibodies (Dako Cytomation, Denmark). After another wash, secondary rabbit polyclonal antibodies (Dako Cytomation, Denmark) and DAPI were added, to observe under Axiovert 40 CFL inverted epifluorescence microscope (Carl Zeiss, Germany) (Bag et al., 2012).

4.5.15. Viral Penetration Assay

The inhibition of viral penetration was performed by a standard method with minor modifications. Vero cell monolayers chilled at 4°C for 1 h was incubated with HSV-1 (100 PFU/well) for 3 h at 4°C, and then in presence of EAF (58.5µg/ml), BT (5.5µg/ml), heparin (100 µg/ml) or DMSO (0.1%) for 20 min at 37°C to facilitate penetration. After inactivating the extracellular virus by citrate buffer (pH 3.0) for 1 min, the cells were washed with PBS, incubated for 72 h with overlay media at 37°C, and stained to count viral plaques (Lin et al., 2011).

4.5.16. Reverse transcriptase PCR (RT-PCR) and Quantitative real time PCR (qRT-PCR)

Vero cells infected with HSV-1 (MOI =1.0) was incubated for 1 h and treated with citrate buffer (pH 3.0) to inactivate extracellular viral particles. The infected cells were added with DMEM containing EAF (58.5µg/ml), BT (5.5µg/ml), or DMSO (0.1%) and incubated at 37 ºC in 5% CO_2. The total cellular RNA, isolated at 4, 8 and 12 h post-infection by TRIzol (Invitrogen, USA) was used to produce cDNA with high-capacity cDNA reverse transcription kit (SuperScript Vilo^{TM} First stand cDNA synthesis kit, Invitrogen, USA). Standard PCR amplification was carried out for the resultant cDNA, using primers against HSV-1 immediate-early (ICP27), early (TK), and late (gD) genes, with GAPDH gene as control. For comparison, Vero cells were co-incubated at 37°C with HSV-1 in presence of EAF (58.5 µg/ml), BT (5.5 µg/ml), or DMSO (0.1%) at zero time (co-addition) for 1 h. The cells were washed with PBS and overlaid with DMEM. Likewise, total cellular RNA was harvested from the samples at 4, 8, and 12 h post-infection, and subjected to RT-PCR (Lin et al., 2011). Sequences of the PCR primers have been listed in Table 4.1.

Moreover, the quantitative real-time PCR was conducted with 12 h post-infected cDNA samples, in triplicate, using SYBR green Supermix with primers at a concentration of 0.5

µM, using untranscribed sample and water as negative controls in a 96-well plate using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, USA). The data for each sample were calculated as the percent difference in the CT value with the housekeeping gene GAPDH (∆CT= CT GAPDH - CT gene of interest), and then as the fold expression relative to mock-infected controls.

Table 4.1. Primer used in Reverse Transcriptase PCR (RT-PCR) assay Real-Time PCR (qPCR) assay

Gene	Primer Sequence
ICP 27	Forward primer, 5'- GCCGCGACGACCTGGAATCG -3'
	Reverse primer, 5'- TGTGGGGCGCTGGTTGAGGAT -3'
TK	Forward primer, 5'-GGCATGCCTTATGCCGTGACCGAC -3'
	Reverse primer, 5'- CCAGGTCGCATATCGTCGGTATGG-3'
gD	Forward primer, 5'- ATGGGAGGCAACTGTGCTATC -3'
	Reverse primer, 5'- CTCGGTGCTCCAGGATAAAC -3'
GAPDH	Forward primer, 5'-AAGGTCGGAGTCAACGGATT-3'
	Reverse primer, 5'-CTGGAAGATGGTGATGATGGGATT-3'

4.5.17. Statistical Analysis

Results were expressed as mean ± standard deviation (n = 3). The one-way Analysis of variance (ANOVA) test was performed to determine the significant mean difference between samples followed by multiple comparisons Tukey's test (TT) in Graph-Pad Prism version 5 (Graph Pad Software, USA). This test was used in post-hoc analysis of ANOVA to find out means that are significantly different from each other. A value of $P < 0.05$ was considered to be statistically significant as compared to the respective control.

4.6. Results

4.6.1. Identification and Quantification of BT

The isolated compound was obtained from the most active fraction EAF. It is a white crystalline solid (0.47 g) with m.p. 276-278 ^{o}C. The white solid crystal was characterized and identified using molecular ion peak by comparing its time-of-flight mass spectrometry (TOF-MS-ES+) data (Figure 4.5). The molecular weight of the compound was 456.7 g/mol. Its molecular peak was obtained at m/z 456 $[M]^{+}$. Its m/z values of

fragmentation were found to be 572, 457, 412, 394 and 275. The fragmentation resembled that of betulinic acid (BT) which was confirmed by previously reported mass-ES+ data in the literature (Borrás-Linares et al., 2014; Budzikiewicz et al., 1963). Thus the isolated compound from the active EAF fraction was identified as BT.

The percentage of purity of BT was calculated from the RP-HPLC method. The calibration curve of BT was constructed by plotting mean peak area against concentrations. Standard and isolated BT showed a separate and distinct peak at a retention time of 9.220 and 9.286 at 210 nm (Figure 4.3 and Figure 4.4). Linearity curve was plotted in concentration ranges 1-500 µg/ml and tested by the least square regression analysis. The standard calibration curve of BT was linear with r^2 = 0.995 in five concentrations range. The percentage purity of the isolated BT was calculated 97.14 % w/w.

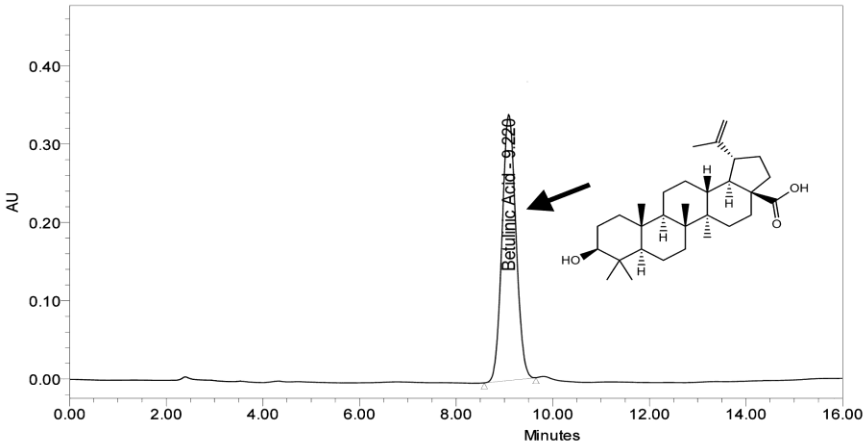


Figure 4.3. RP-HPLC chromatogram of standard betulinic acid

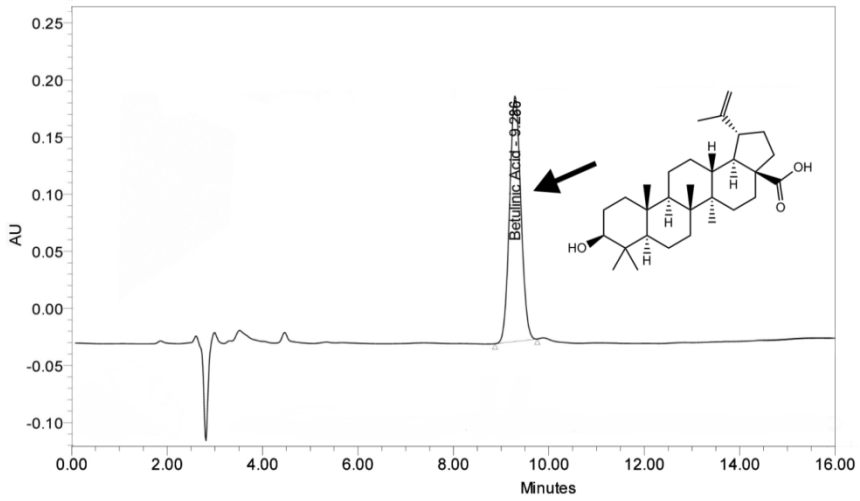


Figure 4.4. RP-HPLC chromatogram of isolated betulinic acid from *D.indica*

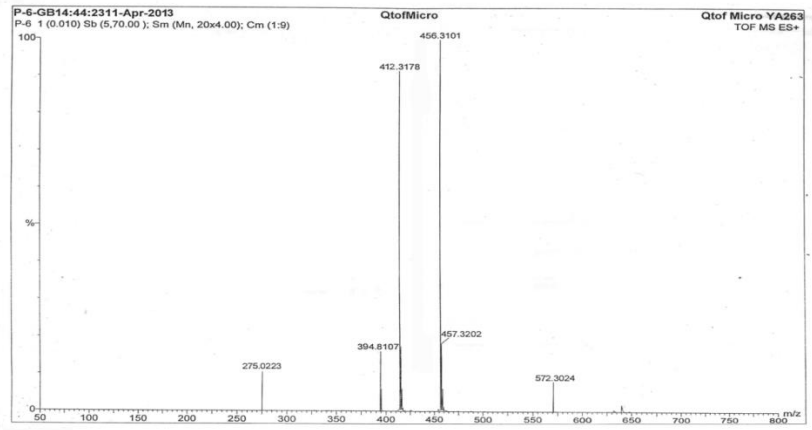


Figure 4.5. TOF-MS-ES+ spectra of betulinic acid

The percentage content of the active compound BT in all the extract and fractions (except aqueous fraction) was determined by RP-HPLC method as they showed inhibitory activity against HSV-1. The content of BT in EAF calculated from the calibration curve was found to be 9.47 ± 4.71 % (w/w); while the content of BT in ME and NBF was 2.34±1.02 % and 0.49±0.09 % (w/w), (Figure 4.6, Figure 4.7 and Figure 4.8) respectively. The HPLC method was validated in accordance with International Conference on Harmonization guidelines (ICH) Q2R1 guidelines. The method validation studies revealed that the RP-HPLC method was simple, accurate, rapid and reproducible.

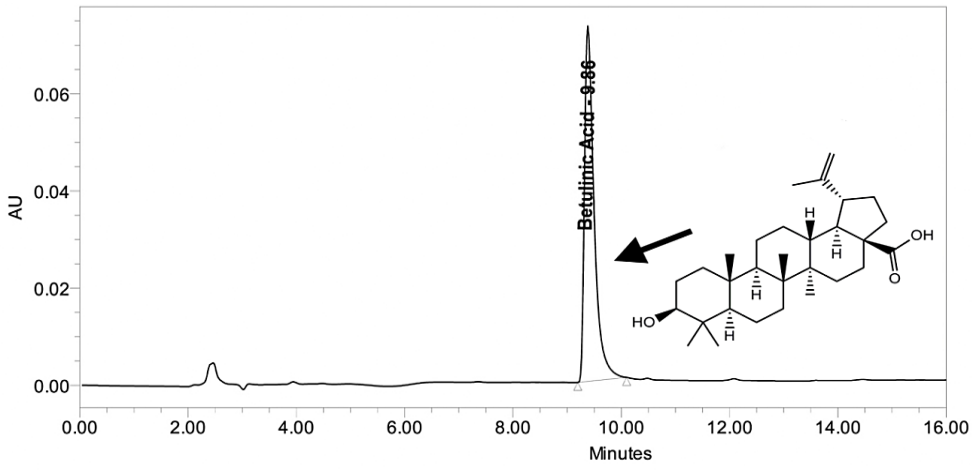


Figure 4.6. RP-HPLC chromatogram of methanol extract of D.indica (ME)

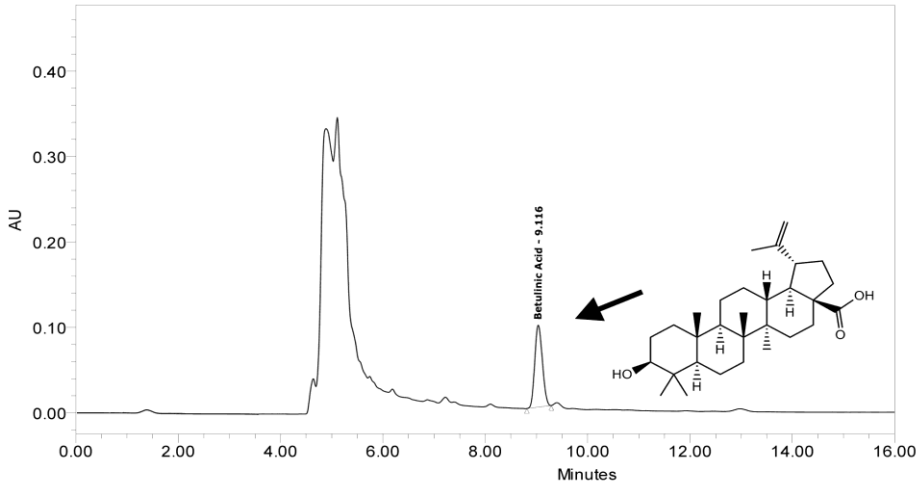


Figure 4.7. RP-HPLC chromatogram of ethyl acetate fraction of *D.indica* (EAF)

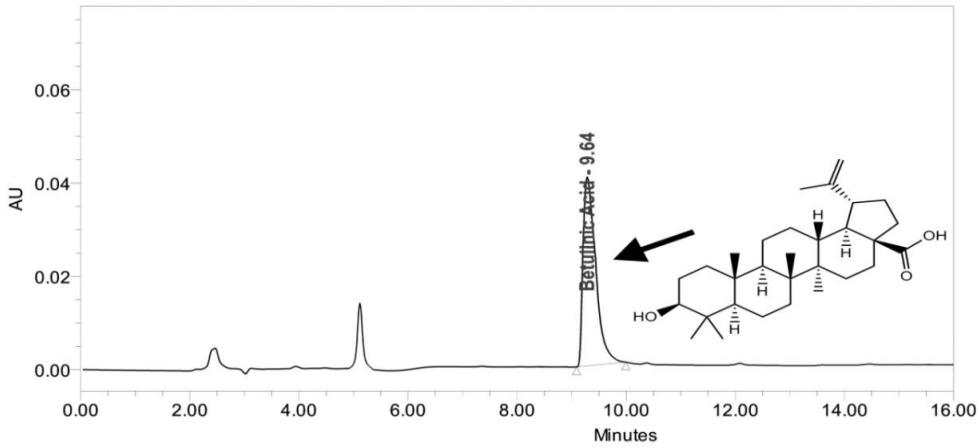


Figure 4.8. RP-HPLC chromatogram of n-butanol fraction of *D.indica* (NBF)

4.6.2. HPTLC standardization of *D. indica*

As the ethyl acetate fraction was found to be most active against HSV-1 the EAF was also subjected to HPTLC standardization. Standard betulinic acid showed single peak in HPTLC chromatogram (Figure 4.9 A) and ethyl acetate fraction of *D. indica* showed nine peaks (Figure 4.9 B). Among the peaks one peak from the extract matched with standard betulinic acid (R_f 0.54). The percentage of betulinic acid in *D. indica* was found to be 9.43 ± 2.71 % (w/w).

(A) **(B)**

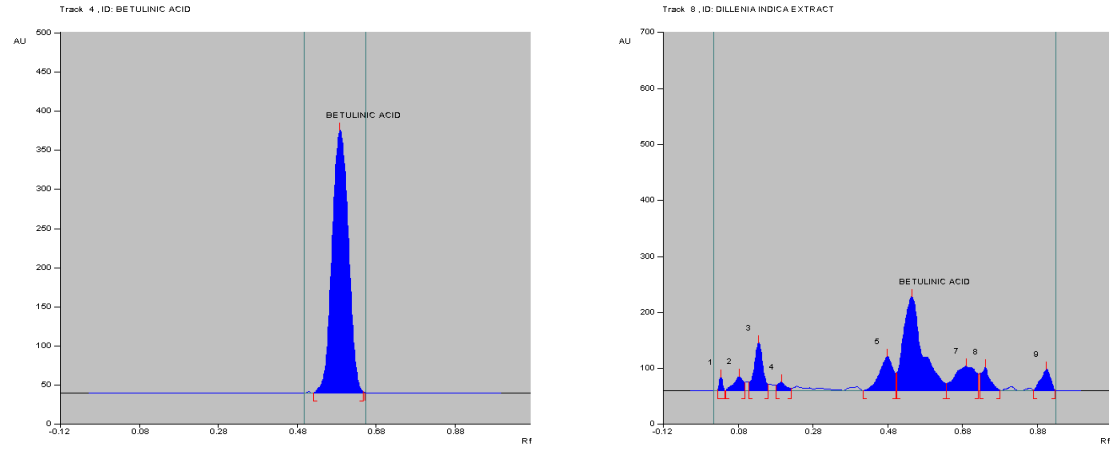


Figure 4.9. (A) HPTLC chromatogram of betulinic acid; (B) HPTLC chromatogram of *Dillenia indica*

The mobile phase resolved betulinic acid efficiently from the extract. Calibration curve was plotted based on the application of different concentrations of betulinic acid ranging from 2–10 µg, the correlation coefficient (r) value of 0.97697 was indicative of good linear dependence of peak area on concentration. The calibration curve was represented by the linear equation Y = 62.128X + 43.389, where Y represented the response as area and X represented the concentration (Figure 4.10). The HPTLC method was validated in accordance with International Conference on Harmonization guidelines (ICH) Q2R1 guidelines. The method validation studies revealed that the HPTLC method was simple, accurate, rapid and reproducible.

(A) **(B)**

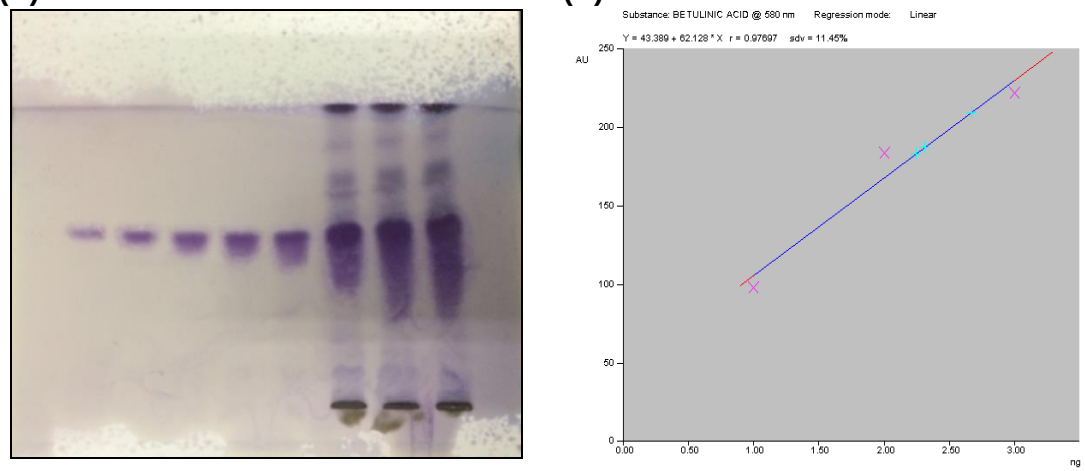


Figure 4.10. (A) Photodocumation of TLC plate at 580 nm; (B) Calibration curve of betulinic acid

4.6.2. 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 (ME), fractions (EAF and NBF) and isolated betulinic acid (BT) exhibited a cytotoxic effect (CC_{50}) on Vero cells at concentrations much higher than their EC_{50} while the aqueous fraction (AF) had no activity against HSV-1. Results presented in Table 4.2 revealed that the CC_{50} of ME, EAF, NBF and AF were found to be 427.4, 309.6, 310.7 and 173.3 µg/ml, respectively; while it was 54.8 µg/ml for BT. The cell morphology at cytotoxic and non-cytotoxic concentration of the most active fraction EAF and BT is presented in Figure. 4.11.

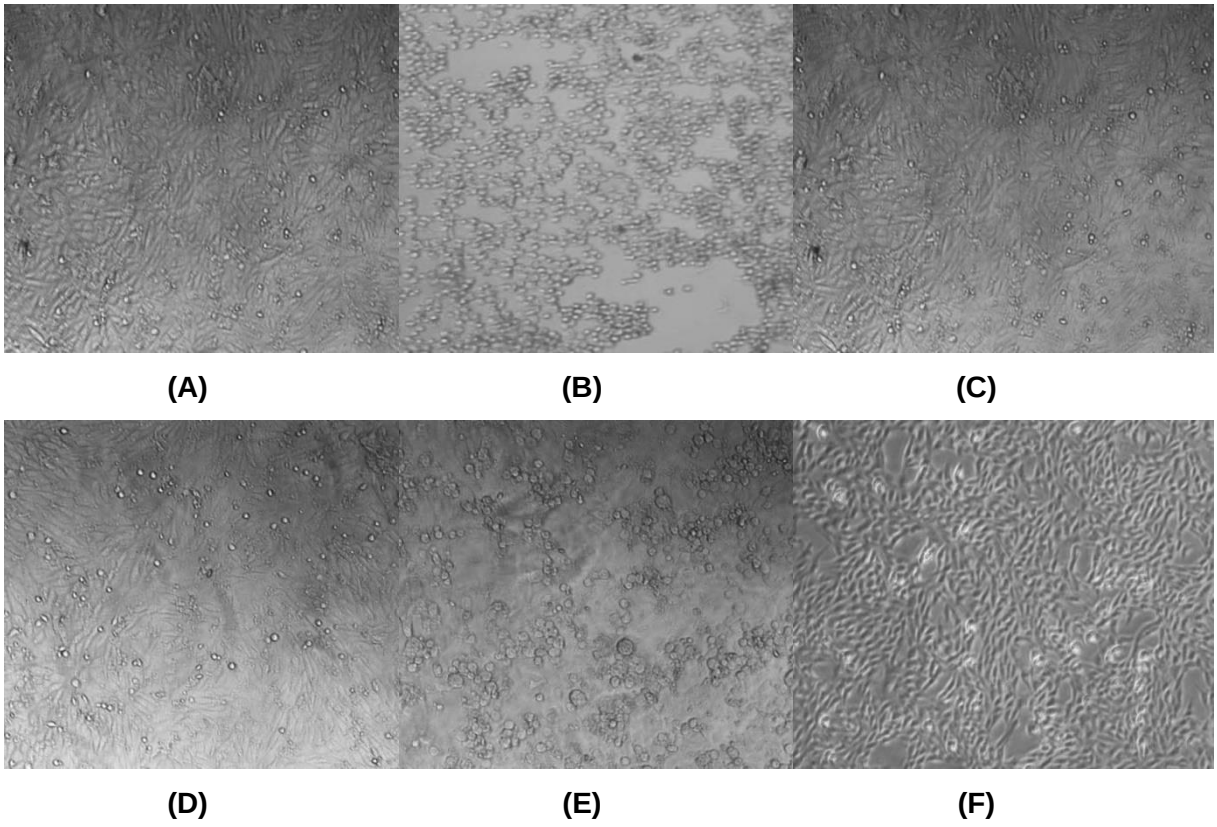
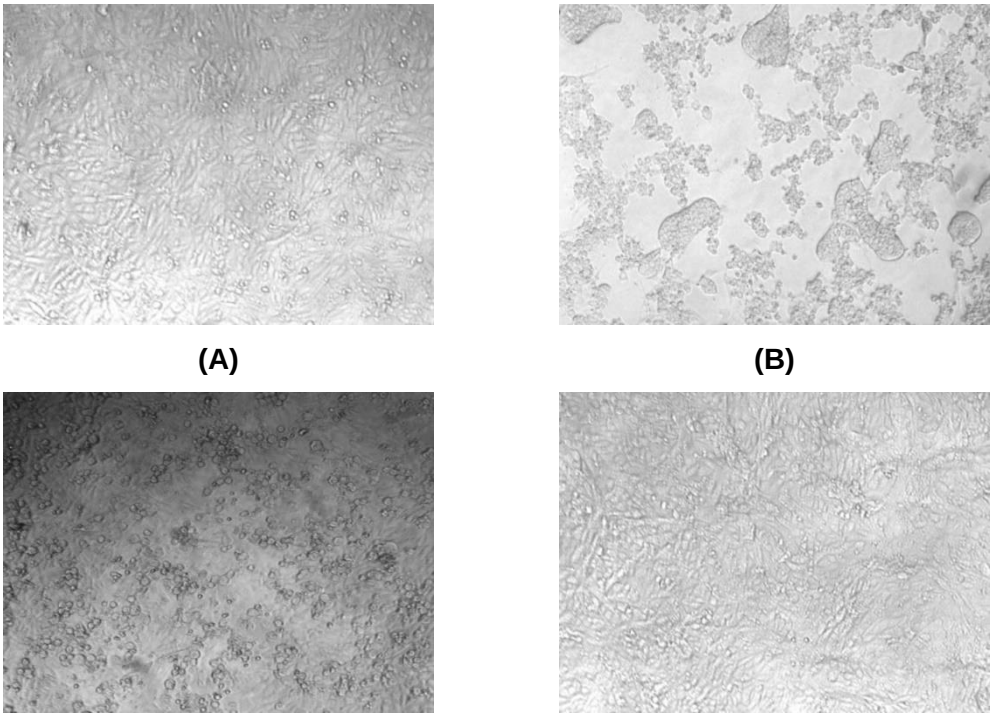


Figure 4.11. **(A) Cell control; (B) cytotoxic concentration; (C) non-cytotoxic concentration of EAF (100 µg/ml); (D) Cell control; (E) cytotoxic concentration; (F) non-cytotoxic concentration of BT (15 µg/ml).**

4.6.3. Inhibition of HSV infection

The antiviral activity of the test extract, fractions and betulinic acid was determined against HSV-1F and two clinical isolates (VU 09 and VU 07) 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 agents at EC_{50} of 56.19-63.5 (ME), 30.3-37.6 (EAF), 71.42-82.4 (NBF) and 3.2-3.9 (BT) µg/ml respectively, inhibited all the strains tested, which was far below their CC_{50} concentration (Table 4.2), but AF had no anti-HSV activity. Based on higher selectivity index (SI) values which measure the preferential antiviral activity (EC_{50}) of a drug in relation to its cytotoxicity (CC_{50}), EAF (8.23-10.2) and BT (14.05-15.22) were chosen for further analyses. Subsequently, nearly complete (EC_{99}) inhibition of viral growth was achieved at 58.5 µg/ml of EAF and 5.5 µg/ml of BT against HSV-1F. The virus infected cell morphology at EC_{50} and EC_{99} concentration of the EAF and BT are presented in Figure. 4.12.



(A) **(B)**

(C) **(D)**

(E)

(F)

Figure 4.12. (A) Cell control; (B) Virus control; (C) EC_{50} concentration of EAF, (D) EC_{99} concentration of EAF; (E) EC_{50} concentration of BT, (F) EC_{99} concentration of BT.

Table 4.2. Assessment of cytotoxicity and anti-HSV activity of *D. indica* and its constituent by MTT and plaque reduction assay

Test Drug	CC_{50}[a]	HSV-1F		HSV-1 (VU-09)		HSV-1 (VU 07)	
		EC_{50}[b]	SI[c]	EC_{50}[b]	SI[c]	EC_{50}[b]	SI[c]
Methanol extract (ME)	427.4 ± 4.4	56.19 ± 5.8	7.6	61.2 ± 5.2	6.0	63.5 ± 4.2	5.81
Ethyl acetate fraction (EAF)	309.6 ± 3.5	30.3 ± 2.9	10.2	37.6 ± 4.8	8.23	35.8 ± 5.6	8.64
n-Butanol fraction (NBF)	310.7 ± 6.2	71.42 ± 5.2	4.35	82.4 ± 3.9	3.77	77.9 ± 3.6	3.98
Aqueous fraction (AF)	173.3 ± 5.4	-	-	-	-	-	-
Betulinic acid (BT)	54.8 ± 3.3	3.6 ± 0.8	15.22	3.2 ± 1.2	17.12	3.9 ± 0.8	14.05
Acyclovir	130 ± 3.8	2.1 ± 0.5	61.9	2.2 ± 0.4	59.09	>30.00	-

[a]50% cytotoxic concentration for Vero cells in μg ml^{-1}.

[b]Concentration of test agent (μg ml^{-1}) producing 50% reduction of virus induced plaques.

[c]Selectivity index (SI) = $CC_{50}\,EC_{50}^{-1}$; Clinical isolates: VU 09 and VU 07.

4.6.4. Dose-dependent effect of EAF and BT

To determine the effect of different doses of the ethyl acetate fraction and betulinic acid on HSV post-infection, we exposed the virus (HSV-1F) infected Vero cells to EAF and BT at two fold concentrations (1, 2.5, 5, 10, 25, 50 and 100 µg/ml) for 3 days. The results presented in Figure. 4.13 showed that the EAF at 58.5 µg/ml and BT at 5.5 µg/ml exhibited nearly complete (EC_{99}) inhibition of HSV-1 indicating a high correlation between drug concentration and its inhibition rate.

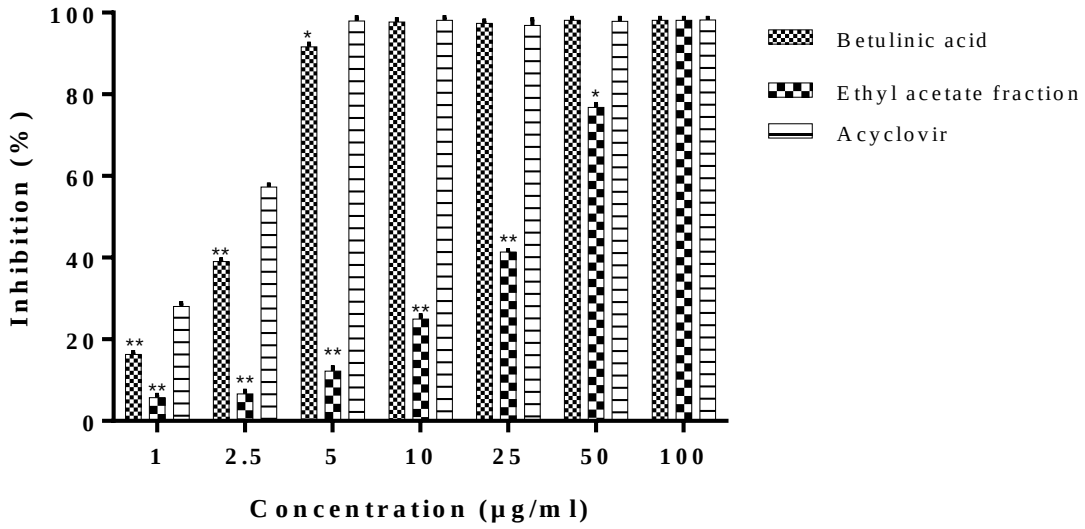


Figure 4.13. Dose dependent anti-HSV-1 effect of ethyl acetate fraction, betulinic acid and acyclovir on HSV-1. Each bar represents the Mean ± SD of three independent experiments (, P<0.001; *, P<0.05 compare with control).**

4.6.5. Effect of EAF and BT on time-of-addition assay

To test the time course analysis with EAF and BT, in order to investigate the possible mechanism of action of anti-HSV activity, EAF and BT were added at different time points of the virus life cycle (pre-entry, entry, and post-entry), and efficacy was determined by PRA. Results demonstrated that both long and short term pretreatment of Vero cells with EAF or BT were unable to protect the Vero cells against HSV-1 infection. Pre-incubation of HSV-1 with the test drugs caused a significant suppression of HSV multiplication. However, the fraction and the compound were found to be effective in preventing plaque formation when added during virus adsorption and in early stages of

replication after entry (Figure 4.14). The data also indicated that HSV-1 infection was severely impaired when EAF or BT were present at the time of infection. Hence, results suggest that the antiviral activity of EAF and BT is not due to their direct effects on Vero cells but on the viral particles to inhibit its entry into host cells.

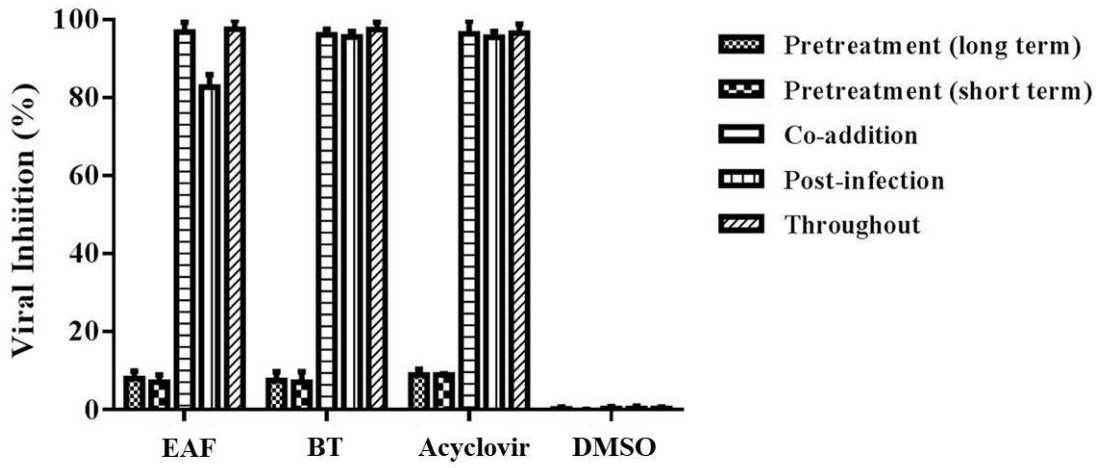


Figure 4.14. Time-of-addition assay of EAF and BT on HSV-1 plaque formation. Vero cells seeded in 6-well plates were treated with EAF/BT at pre-, co- and post-infection using DMSO (0.1%) and Acyclovir as the control. After 72 h of incubation, viral plaques were stained and counted. The data represent the Mean ± SD of three independent experiments with each treatment performed in triplicate.

4.6.6. Effect of EAF and BA on viral inactivation, attachment and penetration assay

In order to evaluate the antiviral mechanism of EAF and BT, we investigated their effects on the virus particles themselves. EAF and BT were pre-incubated with virus particles and then diluted to a sub-therapeutic concentration prior to infection. It was observed that both EAF and BT could interact with virus particles, irreversibly, to prevent HSV infection (Figure 4.15 a). This suggests that EAF and BT can bind to virus particles and neutralize virus infectivity.

The ability of EAF and BT to affect viral attachment and penetration was assessed. The attachment assay was carried out at 4°C, which allows for virus binding but prevents entry. Heparin, a competitive HSV binding inhibitor, was included as a positive control in both experiments. The attachment assay indicated that the test agents irreversibly interact with the virion to prevent HSV-1 infection (Figure 4.15 b).

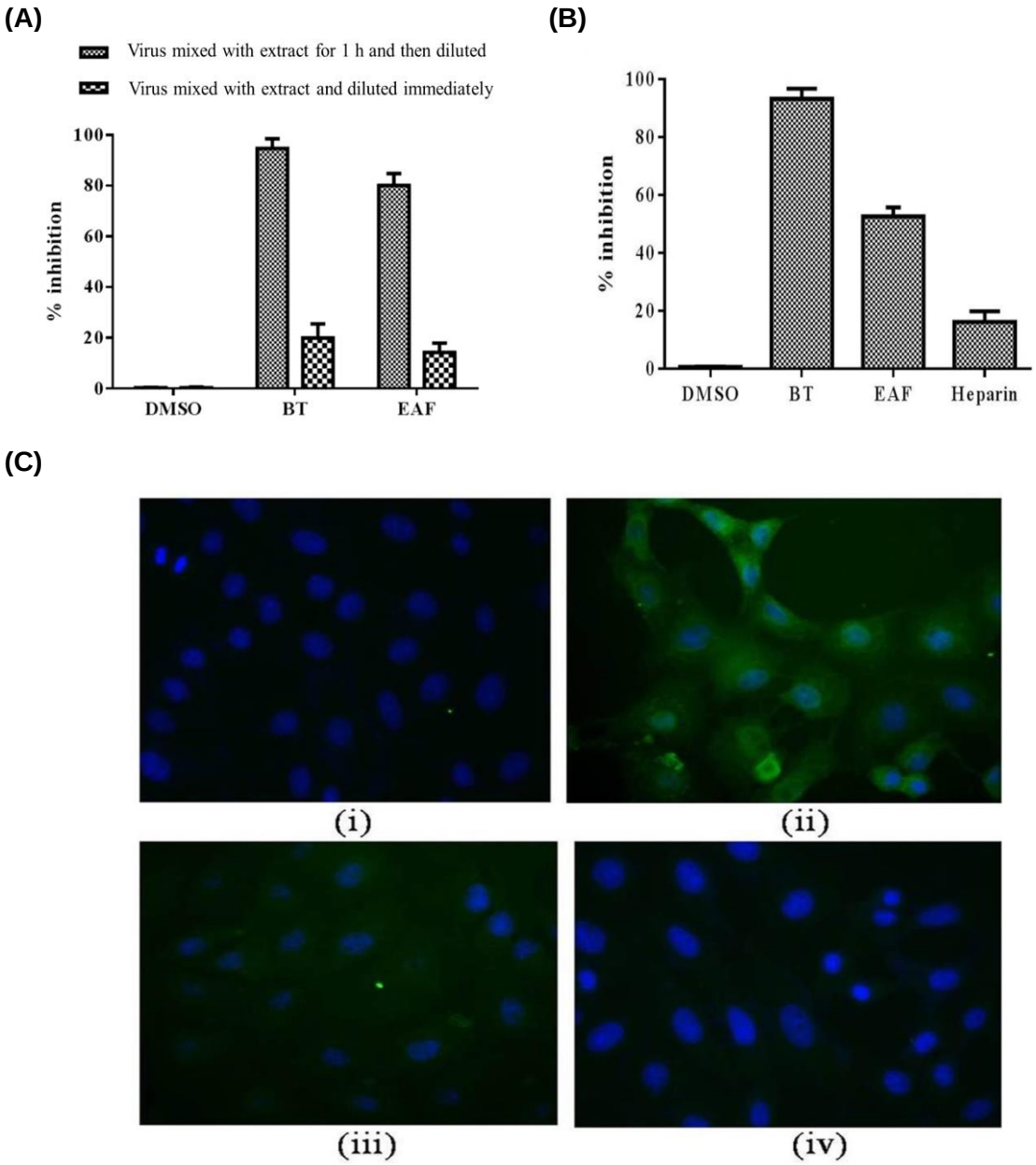


Figure 4.15. (A) Inhibition of HSV-1 entry in presence of EAF or BT by inactivating viral particles and thereby preventing virus binding and internalization into Vero cells. (B) Attachment assay by modified PRA and (C) Indirect Immunofluorescence assay; where (i) Cell control, (ii) Virus control, (iii) Co-infected Vero cells with EAF and (iv) Co-infected Vero cells with BT. The data shown are Mean ± SD of three independent experiments with each treatment performed in duplicate.

On the other hand the indirect IFA, to detect the bound virus particle on Vero cells, revealed that both EAF and BT inhibited HSV-1 attachment to the host cell surface

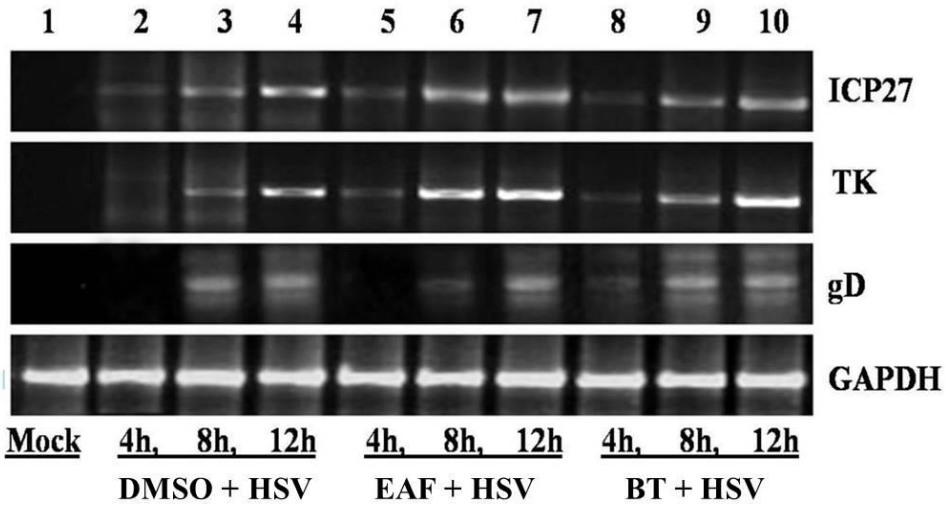
(Figure 4.15 c**)**. Together, these results indicated that EAF and BT might interact with the viral glycoprotein and/or cellular receptor during attachment.

To further assess the effects of EAF and BT on virus penetration step, HSV-1 particles were allowed to first bind to Vero cells at 4°C and were subsequently allowed to fuse with and penetrate the host cell membrane by shifting the temperature to 37°C in the presence or absence of the test agents. The viral penetration assay showed no significant activity during the viral penetration like heparin, which was effectual at blocking HSV-1 attachment, but incapable of preventing virus penetration.

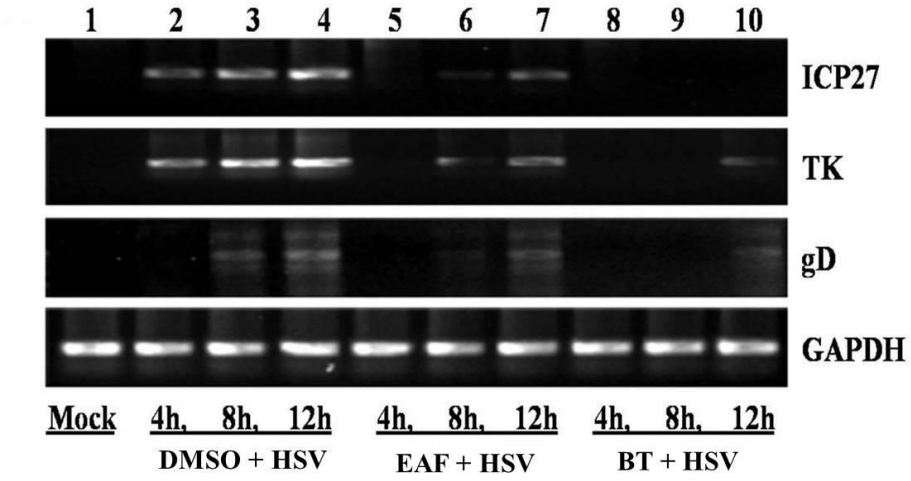
4.6.7. Effect of EAF and BT on HSV RNA expression

The time-of-addition assay suggests that the EAF and BT inhibited HSV-1 infection when the treatment was carried out during virus attachment and entry into the Vero cell. To address this possibility, we evaluated the effects of EAF and BT on HSV-1 mRNA expression after virus entry. The results established that EAF (58.5 µg/ml) and BT (5.5 µg/ml) have no effect on the viral mRNA expression after penetration, since the immediate-early (ICP27), early (TK gene), and late (gD) transcripts were unaffected by EAF or BT (Figure 4.16 a). On the other hand, both EAF and BT prevent the development of viral mRNA synthesis when added together with HSV-1 (Figure 4.16 b and Figure 4.16 c). These findings confirm that the test agents inhibited HSV attachment, but failed to inhibit transcription after penetration of the host cell.

(A)



(B)



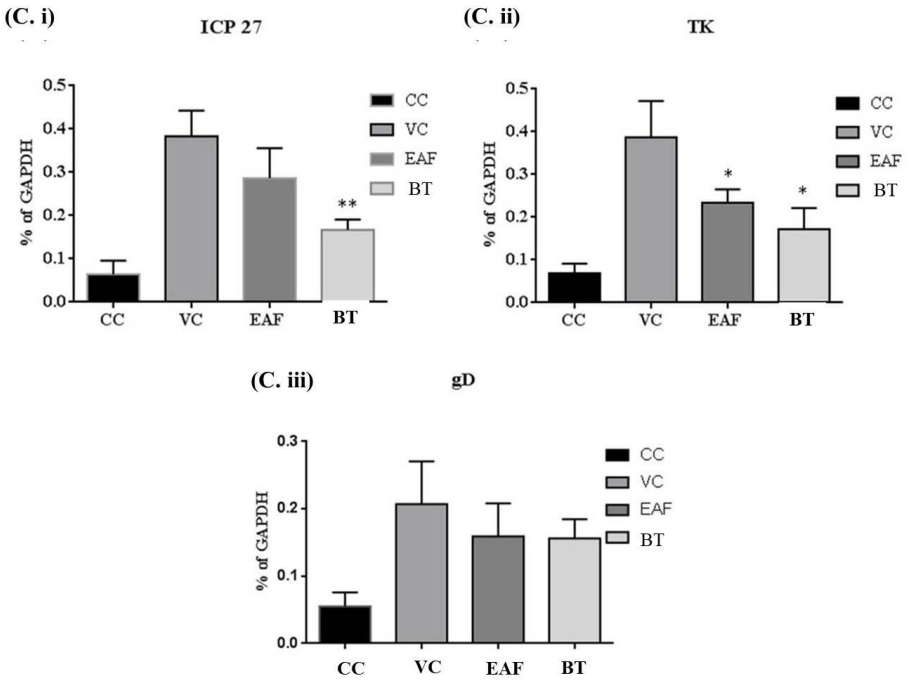


Figure 4.16. Effect of EAF and BT on HSV-1 transcription, following entry into the host cell. (A) Total cellular RNA isolated from the HSV-1 infected Vero cells, untreated or treated with EAF or BA for 4, 8 and 12 h p.i., were subjected to RNA isolation and cDNA synthesis, and then amplified by RT-PCR. (B) Co-incubated with HSV-1, EAF or BA for 1 h, washed and overlaid without tests agent, then performed RT-PCR analysis, as described in panel (A). (C) Co-incubated 12 h p.i., cDNA samples from panel (b) were used to analyze the expression of (i) ICP27, (ii) TK and (iii) gD genes by qRT-PCR.

4.7. Discussion

The development of new antiviral lead molecules, capable of inhibiting herpes virus infection, represents an attractive strategy, particularly in immunocompromised individuals who often generate ACV-resistant HSV strains. In search for new molecules from ethnomedicinal source with different mode of action, we have evaluated the detailed antiviral potential of the most active ethyl acetate fraction of *D. indica* fruit and one of its major active constituent betulinic acid against clinical and wild-type isolates of HSV-1. Our results revealed that the test agents effectively inhibited HSV-1 infection in Vero cells without reducing the cell viability.

The present study for the first time, demonstrated the anti-HSV activity of *D. indica* fruit. The extract ME and its fractions EAF and NBF possess significant antiviral activity among which EAF was found to be most active fraction against HSV-1 infection with a SI of 8.64 – 10.2. Further bioactivity guided fractionation by conventional column and successive flash chromatography from EAF, leads to the isolation of a triterpene BT. The RP-HPLC quantification of BT in various fractions showed that EAF contains the highest percentage of BT, which has potent *in vitro* anti-HSV activity against the tested isolates. The standardization of EAF by HPTLC analysis also displayed similar results.

The cytotoxicity study revealed that the crude ME, EAF, NBF and BT had different CC_{50} due to the variable concentration of bioactive compound(s), and the antiviral activity was far below the CC_{50} dose. Further, EAF and BT revealed dose-dependent antiviral activity, as the infection of Vero cell infected by HSV-1 was significantly prevented by the EAF or BT. Interestingly, EAF and BT were found to inhibit HSV-1 infection in a dose-dependent manner, with an EC_{50} of 30.3 and 3.6 µg/ml, respectively. However, nearly 99 % inhibition of HSV-1 was recorded at 58.5 µg/ml for EAF and 5.5 µg/ml for BT.

Then to know the time or steps of viral life cycle affected by the test agents we conducted the time-of-addition assay. Here, we observed that EAF and BT were effective against HSV-1 infection when the test agents were added during virus adsorption or attachment, and throughout multiple cycles of viral replication. Specifically, the viral infection was sternly subdued when the test agent(s) were concurrently added with the virus during infection. On the other hand, pretreatment of Vero cells with test agents exhibited no effect against the infection, indicated that neither EAF nor BT has

any direct action on host cell surface or entry factors. Thus, the antiviral effect of EAF and BT was attributed to their ability of interacting with viral particles directly.

For further determination of the possible mode of action of EAF and BT on viral life cycle, viral inactivation, attachment and penetration assay were carried out. The results showed that HSV-1 attachment was significantly inhibited only when BT and HSV virion were in contact with each other. Pretreatment of host cells with the EAF or BT, followed by removal of unabsorbed drug, indicated that masking cell surface receptors or entry factors by the extract or its phytoconstituents such as triterpenoids are unlikely, while viral binding assays revealed that both EAF and BT blocked viral attachment to the host cell. Thus, the EAF and BT may bind to one or more glycoproteins of the infectious virions and make them inert, leading to the impaired glycoprotein function and there by prevent attachment and entry of the virion to the host cell. Thus, the use of BT and/or EAF of *D. indica* fruit extract may help to improve the projection of anti-HSV therapy and reduced the risk of drug resistance by lowering its dose.

This was further confirmed by our subsequent data that EAF and BT directly inactivated HSV-1 particles, perhaps by binding with surface glycoproteins to make the virion impaired. Moreover it was also observed that both EAF and BT were unable to affect HSV-1 mRNA expression after virus penetration, since levels of immediate-early (ICP27), early (TK), and late (gD) viral gene transcripts were unaffected by the compounds (Figure 4.16 a). On the other hand, both EAF and BT suppressed HSV-1 mRNA synthesis when added together with the virus at the same time (Figure 4.16 b). These findings suggest that neither EAF nor BT inhibit HSV-1 transcription following penetration of the host cell. Thus, our results collectively demonstrated that EAF and BT of *D. indica* fruit exhibited potent antiviral activity against HSV-1 by interfering with the viral entry via attachment, fusion and penetration to host cell; and the isolated BT does not have any significant effect on intracellular replication of HSV.

4.8. Conclusion

The outcome of our study elucidated that betulinic acid from *D. indica* might be a potential candidate against HSV infections, due to its inhibitory ability and moderately high SI value. Furthermore, the compound is ubiquitous and the toxicity of this plant derived BT is comparatively lower, while the present anti-HSV drugs have high toxicity,

side effects and problem of drug resistance in long term use. Therefore betulinic acid can be considered as an effective lead against HSV-1 infection that merits a greater attention, and further investigation.

4.9. Publication

Dillenia indica fruit extract and its major constituent inhibits Herpes Simplex Virus entry in host cell. Indian Journal of Medical Research (Medknow Publications on behalf of the Indian Council of Medical Research, India). Accepted for publication on 12th April, 2018.

Chapter 5

Evaluation of anti-HSV activity of *Boswellia serrata* Roxb. oleo-gum resin extract and identification of potential lead

5.1. *Boswellia serrata* Roxb. – A profile

5.1.1. Botanical taxonomy

Scientific classification			**Vernacular names**		
Kingdom	:	Plantae	English	:	Indian frankincense, Indian oilbanum
Division	:	Magnoliophyta	Sanskrit	:	Sruva
Class	:	Magnoliopsida	Hindi	:	Shallaki
Order	:	Sapindales	Bengali	:	Gandhabiroja
Family	:	Burseraceae	Tamil	:	Kunkiliyam
Genus	:	*Boswellia*	Telugu	:	Guggilamu
Species	:	*Serrata*	Marathi	:	Dhupali

5.1.2. Plant description

Boswellia serrata Roxb. is a species characteristic of the tropical dry deciduous forests and occurs in very dry teak forests or in dry mixed deciduous forests in association with species such as *Terminalia* spp., *Anogeissus latifolia* and *Acacia leucophloea*. It is usually found on the slopes, ridges of hills and on flat terrains. It achieves a bigger size on fertile soils. It remains unaffected by drought and can resist fire outbreak better than any other species in its zone of existence. The tree is very hard and strong in nature and serves as a nurse tree for other species. In the westernmost state of India, Maharashtra, the occurrence of this tree is prevalent throughout the dry deciduous forests.

Figure 5.1a. *Boswellia serrata* Roxb. plant

Figure 5.1b. *Boswellia serrata* Roxb. oleo-gum resin

Boswellia serrata Roxb. is a slight-sized to big deciduous tree with a mild scattering crown and incredibly drooping branches. It typically has a brief bole that is 3-5 m in height. Once in a while it may grow taller, if grown in a completely stocked wooded area. Normally, it reaches a girth of 1.2-1.8 m and a height of 9-15 m. The bark is very thin, greyish- green, ashy or reddish in shade with a chlorophyll layer below the thin external layer. Leaves are exchange, exstipulate, imparipinnate, 20-45 cm in dimension, congested towards the tops of the branches. Leaflets possess a dimension of 17-31 cm; opposite, 2.5-8 cm x 0.8-1.5 cm, basal pairs often smallest, sessile, lanceolate or ovate-lanceolate, crenate, very variable in size (Alam et al, 2012).

5.2. Ethno-pharmacological uses of *B. serrata*

Boswellia serrata has been prescribed in several ancient medicinal texts such as Ayurveda and Unani system of medicine. The gum-resin of *Boswellia serrata* Roxb. is extensively used for treating osteoarthritis of the knee. It relieves pain and improves joint mobility and flexibility. It is also used in treating rheumatoid arthritis (Anonymous). The exuded oleo-gum resin is also useful in urinary disorders, goiter, gout, piles, cutaneous and nervous diseases (Simonsen et al, 2000; Chatterjee et al, 2003; Dymock et al, 2005). The bark is sweet, acrid, cooling and tonic. It is good for Pitta, asthma, dysentery, ulcers and hemorrhoids. It is useful in diarrhea, piles and skin diseases; mixed with butter applied as a poultice on bleeding or suppurating wounds (Prajapati and Kumar, 2005). It is also moderately effective in removal of scars. With duck fat it is useful in ringworm infection (Ghani, 2002). It is useful in healing the wet wounds and ulcers (Baiter, 2003). In China, *B. serrata* has been used as a skin remedy for bruises and infected sores (Michie and Cooper, 1991).

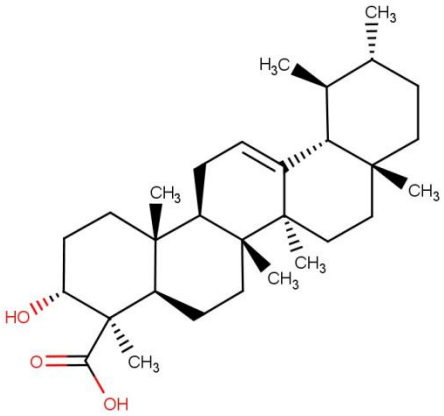
B. serrata is used with oil and camolian earth in hot inflammation of the breast in confined women (Sina, 1998). It functions as a memory enhancer when administered as infusion in fasting. It is applied with sodium nitrate to remove dandruff and drying the ulcer. *B. serrata* exudate is dissolved in alcohol to treat earache. It is also useful in red and chronic pterygium and also for treating cancer of the eye. *B. serrata* with honey is useful in phlegmatic cough. It is given along with other drugs in inflammation of organs and

pneumonia. It stops vomiting and even hematemesis. It facilitates digestion and is used for treatment of diseases of digestive tract such as dyspepsia. It is useful in haemoptysis and is used as external application to relieve pain and inflammation (Baiter, 2005; Sina, 1998; Ghani and Khazianul, 2002).

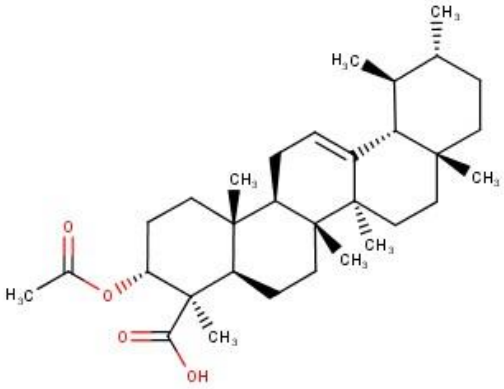
5.3. Phytochemical profile of *B. serrata*

It has been reported that *B. serrata* contain 60-85% resins (mixtures of terpenes), 6-30% gums (mixture of polysaccharides) and 5-9% essential oil (Rijkers et al., 2006). Resin portion consists of pentacyclic triterpenes called boswellic acids that are considered to be the main active constituents (Goyal et al., 2011). Gum portion comprises of pentose and hexose sugar along with some oxidizing and digestive enzymes. And the essential oil is the mixture of monoterpenes, diterpenes and sesquiterpenes.

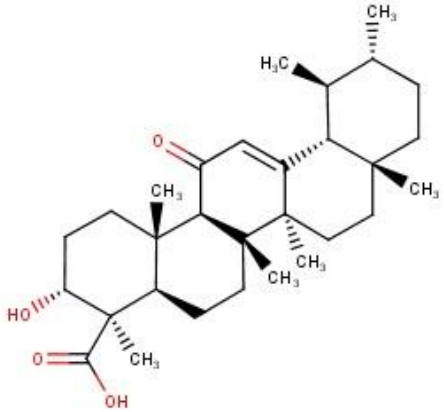
The main components of gum resin exudate are volatile oils (5–15%), pure resin (55–66%) and mucus (12–23%). The gum resin typically contains 30% boswellic acids (Basch et al., 2004). Boswellic acids with the molecular formulas of $C_{32}H_{52}O_4$ are pentacyclic triterpenes, which may exist in an α-configuration (geminal methyl groups at C-20) or a β-configuration (vicinal methyl groups at C-19/C-20). Furthermore, boswellic acids may contain an oxo-moiety at C-11 and an acetyl moiety at the C-3 OH group. Various pharmacological studies indicate that β-configurated derivatives exert stronger bioactivities as compared to the respective α-isomers (Poeckel and Werz., 2006). The four major boswellic acids (pentacyclic triterpenic acids) found in *B. serrata* namely: β-boswellic acid (BA) (1), acetyl-β-boswellic acid (2), 11- keto-β- boswellic (3) acid and 3-O-acetyl-11-keto-β boswellic acid (4) were found to be responsible for the inhibition of pro-inflammatory enzymes (Abdel-Tawab et al., 2011). Apart from these, other boswellic acids such as 3-alpha-acetoxy-11-keto-beta-boswellic acid (5), 3-O-acetyl-9,11-dehydro-beta-boswellic acid (6), α-boswellic acid (7) and 3-O-Acetyl-alpha-boswellic acid (8) also constitutes the gum resin of *B. serrata*.



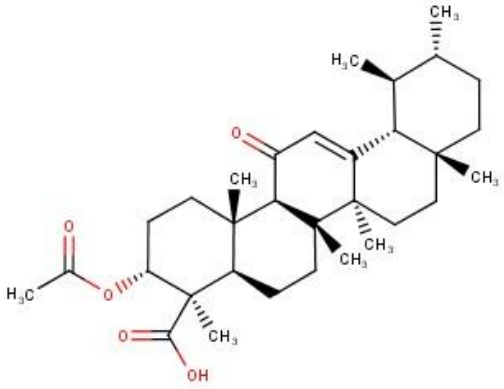
β-boswellic acid (1)



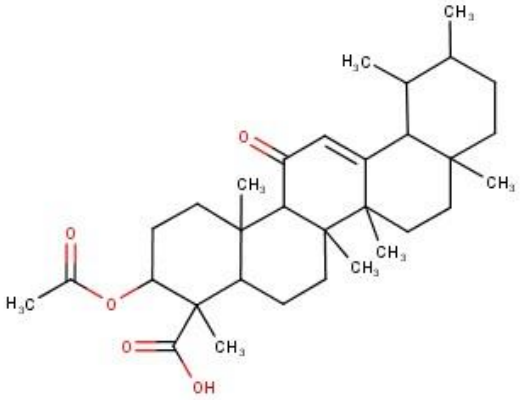
3-Acetyl-beta-boswellic acid (2)



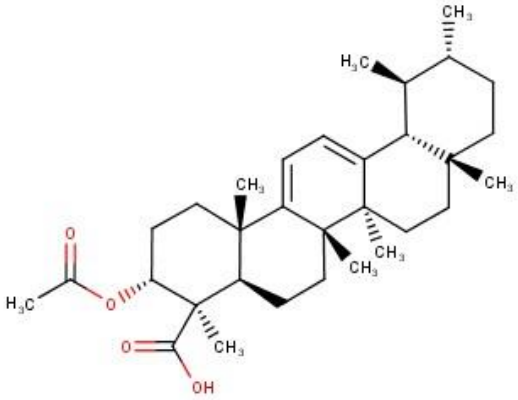
11-Keto-beta-boswellic acid (3)



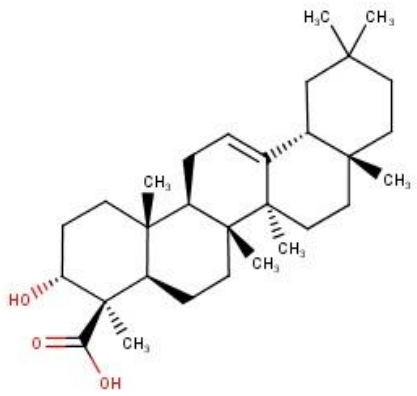
3-O-Acetyl-11-keto-beta-Boswellic Acid (4)



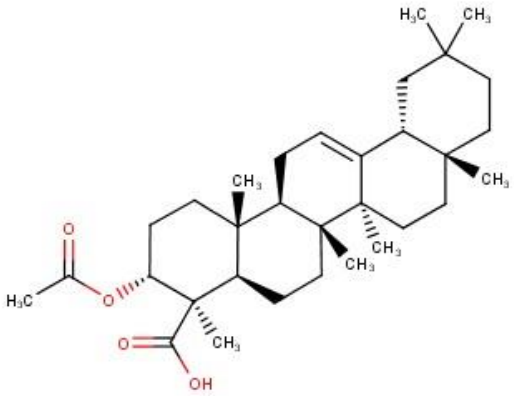
3 alpha-acetoxy-11-keto-beta-boswellic acid (5)



3-O-acetyl-9,11-dehydro-beta-boswellic acid (6)



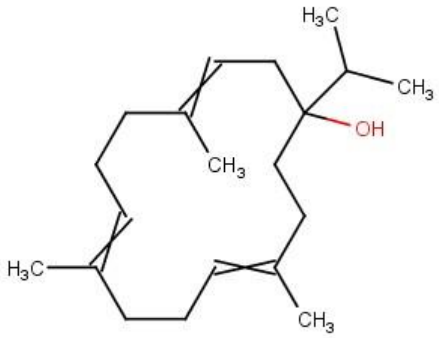
α-boswellic acid (7)



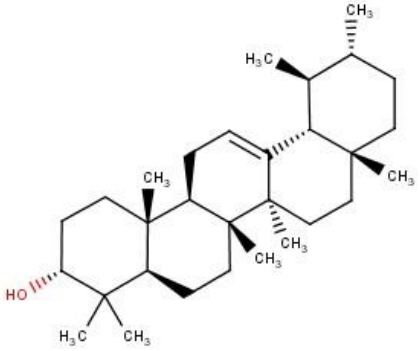
3-O-Acetyl-alpha-boswellic acid (8)

It has also been reported that the resinous part of *B. serrata* contains terpenes: monoterpenes (α-thujone); diterpenes (macrocyclic diterpenoids such as incensole, incensole oxide, iso-incensole oxide, a diterpene alcohol known as serratol (9); triterpenes (such as α-amyrin and 3-epi-β-amyrin (10)) and tetracyclic triterpenic acids (tirucall-8, 24-dien 21- oic acids) (Mannino et al., 2016; Zhang et al., 2013).

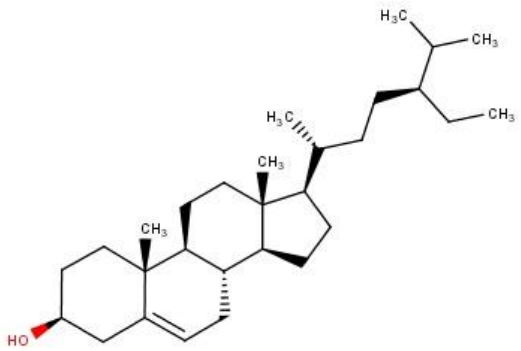
The bark is reported to contain: tannin, 9.1; pentosans, 18.3; lignin, 28.8; holocellulose, 48.7% and β-sitosterol (11). On steam distillation, the fresh leaves gave an essential oil having the following composition; Volatile oil: p-Cymene, 2.2; methylchavicol, 4.0; δ-limonene, 3.9; α-terpineol, 13.6; α-pinene, 2.5;bornyl acetate, 20.0; α-terpineolene, 1.9; α-phellandrene and δ-thujone.[37,38]. The seeds contain moisture, 9.0; crude protein, 8.0; pentosans, 29.3; and water sol mucilage, 1.2%.



Serratol (9)



3-epi-β-amyrin (10)



β-sitosterol (11)

5.4. Pharmacological activities of *B. serrata*

A lot of pharmacological investigations of *B. serrata* have been carried out which are summarized as follows:

Pharmacological activity	Preclinical/Clinical/Mechanistic studies	References
Anti-inflammatory activity	• Mixture of boswellic acids inhibited 25-46% paw edema in rats inhibited 25-46% paw edema in rats • Boswellic acid showed significant activity by inhibiting 35% of inflammation when tested in Papaya Latex Model. • *B. serrata* extract showed improvement of inflammation of periodontium on the plaque induced gingivitis in a double blinded randomized clinical trial • Acety-11-keto-β boswellic acid from *B. serrata* reduces chronic inflammation through the inhibition of NF-κB	Siddiqui., 2011; Gupta et al., 1992; Samani et al., 2011; Cuaz-Perolin et al. 2008
Anti-asthmatic Activity	• *B. serrata* improved disease condition in patients with chronic bronchial asthma showing improvement in physical symptom and sign of dyspnea, bronchi, number of attacks, increase in stimulation of mitogen activated protein kinase (MAPK) and mobilization of intracellular calcium ion.	Gupta et al., 1998

Skin diseases	• *B. serrata* extract dissolved or dispersed in a suitable carrier such as fatty alcohols, or fatty acids improves the stability of products used for topical application. • Acetyl-11-keto-β boswellic acid is reported to be an effective topical agent to soften facial lines and relax the skin	Eyre et al., 2003: Qurishi et al., 2010
Inflammatory Bowel Disease	• *B. serrata* extract and acetyl-11-keto-β-boswellic acid treatment in ileitis induced Sprague-Dawley rats, decreased leukocytes adherence (up to 98%), tissue injury scores and macroscopic and microscopic inflammation of the gut mucosa due to the specific, non-competitive inhibition of 5-lipoxygenase. • The gum resin of *B. serrata* was effective to induce the remission in about 80% of the patients with ulcer colitis grade II and III, when was applied 350 mg three times a day over period of six weeks.	Krieglstein et al., 2001; Gupta et al., 1997
Anticancer Activity	• *B. serrata* alcoholic extract inhibited tumor growth by inhibiting cell proliferation and cell growth due to the interference with biosynthesis of DNA, RNA and proteins, in mice with ehrlic ascites carcinoma and S-180 tumour. • Topical application of Boswellin with 12-Otetradecanoylphorbol-13-acetate to mice previously treated with dimethylbenz-anthracene caused 87- 99% inhibition in the number of tumor. • Boswellic acids induced apoptosis in protein synthesis dependent and not associated with free radical scavenging activity. • In glioblastoma patients, boswellic acids showed concentration dependent inhibition of glioma cell proliferation and anti-edema effect.	Tsukada et al., 1986; Huang et al., 1997; Xia et al., 2005; Boker et al., 1997

Anti-arthritic Effect	• *B. serrata* showed 34% and 84% inhibition of paw swelling with 50 and 100 mg/kg doses in adjuvant-induced poly-arthritic rats. • *B. serrata* extract treatment reduced knee pain, increased knee flexion and increased walking distance in patients of knee osteoarthritis. • In a comparative study of *B. serrata* with valdecoxib in patients of knee osteoarthritis, *B. serrata* showed a slower onset of action but the effect persisted even after stopping therapy while the action of valdecoxib faded rapidly after stopping the treatment.	Singh and Atal, 1984; Kimmatkar et al., 2003; Sontakke et al., 2007
Cerebral tumors	• A *B. serrata* extract (named as phytopharmacon H15) caused a reduction of the peritumoural brain edema of 22 to 48% patients with brain tumors. • Use of 3600mg/ day of *B. serrata* extract on patients with malignant glioma decreases the fluid around the tumor and signs of brain damage. • Patients receiving radiotherapy plus certain amount of *B. serrata* extract showed 75% reduction of cerebral edema.	Simmet and Ammon, 2001; Kirste et al., 2011
Hypoglycemic Activity	• Herbal formulation containing *B. serrata* gum-resin produced significant anti-diabetic activity on non-insulin dependent diabetes mellitus in streptozocin induced diabetic rat model.	Al-Awadi et al., 1991

It was reported that the boswellic acids mixture inhibited 25-46% paw edema in rats, substantiating its anti-inflammatory property (Siddiqui., 2011). The boswellic acid from *B. serrata* inhibited 35% of inflammation when examined in Papaya Latex Model (Gupta et al., 1992). It has been found that acety-11-keto-β boswellic acid from *B. serrata* attenuated chronic inflammation by inhibition of NF-κB signaling cascade (Cuaz-Perolin et al. 2008). Also it was observed that boswellic acids were found to be responsible for the inhibition of

leukotriene biosynthesis and thereby reducing and preventing the inflammation in chronic inflammatory diseases like asthma. In a clinical study patients with chronic bronchial asthma were treated with *B. serrata* preparation of 300 mg thrice daily for a period of six weeks. The results demonstrated that physical symptom and sign of dyspnea, bronchi and number of asthmatic attacks in 70% of the patients were improved and stimulation of mitogen activated protein kinase (MAPK) and mobilization of intracellular calcium ion was increased (Gupta et al., 1998).

In an animal study on leukocyte-endothelial cell interactions in inflammatory bowel disease *B. serrata* extract and acetyl-11-keto-β-boswellic acid, decreased leukocytes adherence (up to 98%), attenuated tissue injury scores, and significantly reduced macroscopic and microscopic inflammation of the gut mucosa in ileitis induced Sprague-Dawley rats. It was possibly due to the specific, non-competitive inhibitoion of 5-lipoxygenase, the key enzyme of leukotrienes by boswellic acids (Krieglstein et al., 2001). Interestingly it was observed that treatment of alcoholic extract of *B. serrata* in mice induced with ehrlic ascites carcinoma and S-180 tumour resulted in inhibition of tumor growth. This was because of inhibition of cell proliferation and cell growth due to interference in DNA, RNA and proteins biosynthesis (Tsukada et al., 1986).

B. serrata showed 34% and 49% inhibition of paw swelling when administered 50 and 100 mg/kg doses respectively as compared to controls in mycobacterium adjuvant-induced poly-arthritic in rats. It was also found that mixture of boswellic acid showed 45-67% anti-arthritic activity. The fraction was found to be effective in both adjuvant arthritis (35-59%) and established arthritis (54-84%) (Singh and Atal, 1984). A randomized double blind placebo controlled crossover study was conducted to assess the efficacy and safety of *B. serrata* extract in patients (n=30) suffering from knee osteoarthritis. Treatment of *B. serrata* extract to all the patients resulted in decrease in knee pain, increased knee flexion and increased walking distance (Kimmatkar et al., 2003). *B. serrata* was also compared with valdecoxib in 66 patients suffering from knee osteoarthritis in a randomized, prospective, open-label, comparative trial conducted for a period of six months. The effect was assessed with WOMAC scale at baseline and monthly interval upto one month after drug discontinuation. It was finally observed that *B. serrata* showed a slower onset of action but

the effect remained even after discontinuing *B. serrata treatment* as compared to the action of valdecoxib which showed speedy recovery but faded rapidly after stopping the treatment (Sontakke et al., 2007).

The impact of *B. serrata*, has also been studied in patients with brain tumors. An ethanolic extract from the gum resin of *B. serrata* (coined as phytopharmacon H15) caused a reduction of the peritumoural brain edema of 22 to 48% when administered for a period of seven days (Simmet and Ammon, 2001). Another study revealed that administration of 3600mg/ day of *B. serrata* extract (60% boswellic acids) on patients with malignant glioma, seven days before surgery, reduces the fluid around the tumor with average of 30% in 8 of the 12 patients. The signs of brain damage were also augmented during the treatment. Recently another interesting clinical study revealed that simultaneous treatment of *B. serrata* extract in patients having malignant cerebral tumors receiving radiotherapy showed that 75% reduction of cerebral edema was detected in 60% of the patients getting the extract (Kirste et al., 2011).

5.5. Lead finding from *Boswellia serrata* extract against HSV-1

5.5.1. Collection and authentication of plant materials

The oleo-gum-resin of *B. serrata* Roxb. (family, Burseraceae) was purchased from the local market at Kolkata. The resin was authenticated by Dr. S. Rajan, Field botanist, The Medicinal Plant collection unit, Ooty, Tamilnadu, Govt. of India. A voucher specimen number (SNPS-JU/2014/2105) of the plant has been preserved in the School of Natural Product Studies, Jadavpur University, Kolkata for further reference (Figure 5.2).

Figure 5.2. Voucher specimen of *B. serrata*

5.5.2. Reagents and chemicals

β-Boswellic acid (80342-5MG) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent were purchased from Sigma-Aldrich, Inc (St Louis, MO, USA). And Dulbecco modified Eagle medium (DMEM), phosphate buffer saline (PBS), fetal bovine serum (FBS), Penicillin and streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). Primers for semi-quantitative PCR were obtained from IDT, California, USA. Antibodies against IkB-α, NF-κB/p65, p38, p-p38, JKN1/2, p-JNK1/2, GAPDH and ECL kit were purchased from Abcam (Cambridge, MA, USA).

5.5.3. Extraction of plant materials

The dried oleo-gum-resin (250 gm) was extracted with methanol (95%) for 72h at room temperature and the process was repeated for three times. The collected solvent extract was filtered and evaporated under vacuum at 45-50 ^{o}C through the rotary evaporator (Eyela N-1001S-W, USA). The yield of the crude methanol extract of *B. serrata* (BSE) was found to be 21.3%. The extract was stored at 4ºC; and when required, dissolved in 0.1% DMSO and diluted with DMEM to obtain a stock solution of 1 mg/ml.

5.5.4. Liquid chromatography electrospray ionisation mass spectrometry of *B. serrata*

The LC-ESI-MS and LC-ESI-MS/MS analysis of BSE were performed on Waters tandem quadrupole detector triple quadrupole MS (USA), equipped with H-Class acquity UPLC (Waters, USA) system, and electrospray ionization (ESI) source. ACQUITY UPLC® BEH C18 1.7µm column was used in negative ion mode LC-ESI-MS experiments. 5 µl of sample injected through auto-sampler into UPLC flow. The mobile phase consisted of (A) 1:1 (v/v) acetonitrile: methanol and (B) 0.1 mM ammonium acetate in water. A linear gradient elution was performed at the flow rate of 0.250 ml/min as 95–40% B in 0–14 min; 40% B in 14–16 min; 20% B in 24–32 min; and 5% B in 35-40 min. Nitrogen served as the nebulizing and drying gas at flow rates of 30 and 650L/h, respectively. The ESI source potentials for every experiment were capillary voltage 3.5kV; cone potential at 30V. The source and desolvation temperatures were 120 and 350°C, respectively. The mass resolution was set

approximately 1000 and the mass analyzer was scanned between 150Th and 750Th in 0.5 s. Online tandem mass spectra of various ions were measured by precursor ion selection in MS1 followed by collision-induced dissociation and analysis of the daughter ions by MS2. Data acquisition and processing were carried out using Mass Lynx V4.1 software change note 714 software (Waters Corporation, Milford, MA01757).

5.5.5. Standardization of *B. serrata* extract by RP-HPLC method

5.5.5.1. HPLC instrument and chromatographic conditions

HPLC method was developed to quantify the major secondary metabolite present in the bioactive extract of *B. serrata*. Waters (Milford, MA, USA) HPLC system consisting of a 600 controller pump, dual UV-Vis detector with an in-line degasser AF 2489 and a rheodyne7725i injector (USA) having a 20µl loop was used for standardization of *B. serrata* extract (BSE) using standard β-boswellic acid (≥95% pure; Sigma Aldrich, St Louis, MO, USA) as active marker. Separation was achieved using Waters Spherisorb column (C18; 250×4.6mm, 5µm particle size), and quantitative estimation was performed with Empower 2 software. A mobile phase containing solvent A (acetonitrile) and B (0.1M acetic acid, pH 3.0 in water) (85:15) was optimized and run through C-18 HPLC column with security guard column system. Elution was carried out at 1.0 ml/min in an isocratic mode at 210 nm.

5.5.5.2. Standard and sample solution

A primary stock solution (1mg/ml) containing β-boswellic acid was prepared by dissolving in methanol and subsequently diluted to 10-80 µg/ml for optimization of mobile phase. Extract of *B. serrata* was dissolved in the same way to get 1 mg/ml of sample solution. Standard and sample solutions were filtered through 0.45 µm syringe filter prior to injection into the HPLC column. Amount of β-boswellic acid present in the extract was calculated after triplicate injection (n=3) by comparing and interpolating the extract peak area (response) with respect to the standard marker.

5.5.5.3. Calibration curve (linearity)

The linear calibration plot of β-boswellic acid was constructed by means of linear regression analysis between peak areas and concentrations after triplicate injection (n=3) of the prepared standard solutions (10-80 µg/ml) to distinguish the reproducibility of the detector response. Peak areas of β-boswellic acid obtained from the chromatogram were plotted against different concentrations of the standard. The amount of standard compound in the solution was obtained by linear regression analysis of peak areas in chromatograms within the linear range of the detector.

5.5.6. Standardization of *B. serrata* by HPTLC method

5.5.6.1. Equipments

A Camag HPTLC system equipped with a sample applicator Linomat V, twin trough plate development chamber, TLC Scanner III with photo documentation device and Wincats integration software 4.02 (Switzerland) was used for this experiment. Fine Chem. Ltd (Mumbai, India). TLC aluminum plates pre-coated with silica gel 60 F_{254} (10 × 10 cm, 0.2 mm thick) used were obtained from E. Merck Ltd (Mumbai, India).

5.5.6.2. Chromatographic conditions

Chromatography was performed on 10 × 10 cm aluminium backed silica gel 60 F_{254} HPTLC plates. Samples were applied to the plates, as 5 mm bands, by Camag Linomat V automated spray-on band applicator equipped with 100 µL syringe (Hamilton, USA).

Standard β-boswellic acid was dissolved in HPLC grade methanol to produce a 1 mg/ml solution. The concentrated ethyl acetate fraction of the plant was reconstituted in HPLC grade methanol to produce a 10 mg/ml solution. The solutions were filtered through a 0.45 µ Whatman's syringe filter. This solution was aspirated through a 100 µl Syringe and thus applied band-wise with the CAMAG Linomat V. Following that the plates were developed in a CAMAG twin trough chamber using the solvent system hexane: acetone (7:3, v/v). After development the plates were then dried under warm air flow. Densitometric scanning was performed at 254 nm with a Camag TLC scanner III equipped with Wincats software, using

deuterium light source; the slit dimension was 6.00 × 0.45 mm. After the development, bands in the extracts were identified by matching their R_f values with those obtained for standard.

5.5.7. Viruses and the cell line

African green monkey kidney cells (Vero cells, ATCC USA) was grown and maintained in DMEM, supplemented with 5-10% FBS and antibiotic-antimycotic mix (Penicillin G sodium 100 Units/ml, Streptomycin sulfate 100 µg/mL and Amphotericin B 100U; Gibco-BRL, Grand Island, NY). The DMEM with 2% FBS was served as maintenance medium. The standard strain of HSV-1F (ATCC-733, USA) and the clinical isolate of HSV-1 (VU-09) were used. After plaque purification, the viral stocks were propagated in Vero cells and stored at -80 ºC. Whenever required the virus stocks were grown in Vero cells to determine the titer(s) by plaque assay.

5.5.8. Preparation of mouse peritoneal macrophages

Male BALB/c mice (18-20 gm), acclimatized for 15 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). 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 ºC 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 ºC in 5% CO2 for 6 h, washed with PBS to remove the non-adherent cells, and further incubated for 24 h (Ojha et al., 2015).

5.5.9. Determination of cytotoxicity by MTT assay

Vero cell (1.0×10^5 cells/ml) monolayers cultured in 96 well plates for 6 h was treated with BSE or BA (0-1000 µg/ml) in each well at a final volume of 100 µl, in triplicate, using DMSO (0.1%) and acyclovir as control. The cells were incubated at 37 ºC with 5% CO_2 for 72 h and MTT (Sigma Aldrich, St Louis, MO, USA) assay was carried out following manufactures protocol. The absorbance was read at 570 nm with a reference wavelength of 690 nm by an ELISA reader to calculate the % of cell viability using the formula: cell viability (%) = At /As × 100%, where "At" and "As" refer to the absorbance of the test compounds and the solvent control (DMSO), respectively. The 50% cytotoxic concentration (CC_{50}) causing visible morphological changes in 50% of Vero cells with respect to cell control were determined (Bag et al., 2014; Ojha et al., 2013).

5.5.10. Determination of antiviral activity by MTT and Plaque reduction assay (PRA)

The antiviral activity of BSE and BA, tested by MTT assay, was confirmed by plaque reduction assay (PRA). Vero cell monolayers (100 pfu/well) infected with clinical isolate (VU 09) and wild type HSV-1F were treated with serial dilutions of the BSE and BA followed by addition of overlay medium (1% methylcellulose). The plaques developed after 72 h incubation were fixed with 4% paraformaldehyde and stained with crystal violet (0.03% in 70% methanol). The antiviral concentration of 50% effectiveness (EC_{50}) was defined as the concentration which achieved 50% inhibition of virus-induced cytopathic effects (Chattopadhyay et al., 2009).

5.5.11. Dose-response assay

A study was conducted to analyze the dose-dependent effect of the BSE and BA on the HSV infected Vero cell. Vero cells monolayer at 1.0×10^5 cells/ml was grown onto 96 well plates. After incubation at 37 ^oC in 5% CO_2 for 6 h, HSV-1 (at 1.0 MOI) was added and incubated for 1 h for viral adsorption. Different concentrations (1, 2.5, 5, 10, 25, 50 and 100 µg/ml) of BSE and BA 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 ^{o}C in 5% CO_2, the MTT assay was carried out as described earlier (Mukherjee et al., 2013).

5.5.12. Time-of-addition assay

Time-of-addition assay was conducted to investigate the possible steps of HSV-1 infection cycle affected by the BSE and BA, following four different approaches: Vero cells ($1.0x10^6$ cells/ml) cultured in six-well plates were treated with BSE or BA (10 or 30 µg/ml) for 1h (pre-infection) and then infected with HSV-1F (100 pfu/well) and washed with PBS. In co-infection, cells were treated simultaneously with the virus and BSE and BA, incubated for 1h at 37°C, washed and covered with overlay media. In post-infection the cells infected with the virus for 1h, treated with BSE or BA at intervals of 1, 2, 4, 6, 8, 10 and 12h and subjected to PRA (Lin et al., 2011).

5.5.13. 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 (MOI: 1) infected Vero cells were treated BSE (10 µg/ml) or BA (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 SDSPAGE 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).

5.5.14. Isolation of RNA and semi-quantitative RT-PCR

HSV-1 (1.0: MOI) infected Vero cells treated with BSE and BA (10 and 30 µg/ml) at 1h, 2h and 4h p.i, were subjected to RNA isolation to determine the expressions of viral genes. To

determine cytokines and iNOS expressions; drug treated Vero cells at 12h p.i. were subjected to RNA isolation using RNeasy Mini kit (Qiagen, Germany), as per the manufacturers' protocol and subsequent RT-PCR analysis. 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-RadMJ Mini, Hercules, CA, USA). The cDNA (10%) was subjected to standard PCR amplification using the primers of respective genes, using gapdh as internal standard. The sequences of the forward and reverse primer are listed in Table 5.1. The respective DNA and proteins bands were analyzed using a model GS-700 Imaging Densitometer and Molecular Analyst software (version 1.5; Bio-Rad Laboratories, CA, USA) (Ojha et al., 2015).

Table 5.1. **Primer used in Reverse Transcriptase (RT)-PCR assay**

Gene	**Primer Sequence**
ICP 27	Forward primer, 5'-CCTTTCTCCAGTGCTACCTG-3'
	Reverse primer, 5'-GCCAGAATGACAAACACGAAG-3'
DNA Pol	Forward primer, 5'-CAGAACTTCTACAACCCCCA-3'
	Reverse primer, 5'-TAGATGATGCGCATGGAGTA-3'
gD	Forward primer, 5'-ATGGGAGGCAACTGTGCTATC-3'
	Reverse primer, 5'-CTCGGTGCTCCAGGATAAAC -3'
IL-1β	Forward primer, 5'-TCATGGGATGATGATGATAACCTGCT -3'
	Reverse primer, 5'-CCCATACTTTAGGAAGACAGGGATTT -3'
IL-6	Forward primer, 5'-CCCCCAGGAGAAGATTCCAAAG -3'
	Reverse primer, 5'-TTCTGCCAGTGCCTCTTTGCT -3'
IL-12	Forward primer, 5'-AGTTTGGCCAGGGTCATCC -3'
	Reverse primer, 5'-CTGAAGTGCTGCGTTGATGG -3'
IL-10	Forward primer, 5'-GGACAACATACTGCTAACCGA -3'
	Reverse primer, 5'-AAAATCACTCTTCACCTGCTCC -3'
IL-4	Forward primer, 5'-GAACGAGGTCACAGGAGAAGG -3'
	Reverse primer, 5'-TGTGGACTTGGACTCATTCAT -3'

IFNγ	Forward primer, 5'-AACGCTACACACTGCATCTTGG -3'
	Reverse primer, 5'-GACTTCAAAGAGTCTGAGG -3'
iNOS	Forward primer, 5'-AAACCCCTTGTGCTGTTCTCA -3'
	Reverse primer, 5'- CTCTCCACTGCCCCAGTTTT-3'
TNFα	Forward primer, 5'- ACATTCGAGGCTCCAGTGAATTCGG-3'
	Reverse primer, 5'- GGCAGGTCTACTTTGGAGTCATTGC-3'
GAPDH	Forward primer, 5'-CAAGGCTGTGGGCAAGGTCA-3'
	Reverse primer, 5'-AGGTGGA AGAGTGGGAGTTGCTG-3'

5.5.15. Measurement of Nitric Oxide

Macrophages cultured in 24 well plates (1×10^6 cells/well) were treated with HSV-1 (at 1 MOI), and incubated for 1h. The activated cells were then treated with BSE (10 µg/ml) and BA (30 µg/ml) for 24 h. The concentration of nitric oxide (NO) in the supernatant was determined by Griess reagent (Sigma, USA) (Bhattacharjee et al., 2009).

5.5.16. Densitometry analysis

The respective RNA and proteins bands were analyzed using a model GS-700 Imaging Densitometer and Molecular Analyst software (version 1.5; Bio-Rad Laboratories, CA, USA) (Ojha et al., 2015).

5.5.17. Statistical analysis

Results were expressed as mean ± standard deviation (n = 3). The one-way Analysis of variance (ANOVA) test was performed to determine the significant mean difference between samples followed by multiple comparisons Tukey's test (TT) in Graph-Pad Prism version 5 (Graph Pad Software, USA). This test was used in post-hoc analysis of ANOVA to find out means that are significantly different from each other. A value of $P < 0.05$ was considered to be statistically significant as compared to the respective control.

5.6. Results

5.6.1. Characterization of *B. serrata* extract by LCMS/MS

LCMS/MS analysis for detection and identification of major compounds of *B. serrata* was carried out using their m/z values. All target compounds revealed in negative mass mode with their corresponding [M-H]− ions. The MS full scan spectra for BSE showed deprotonated precursor. Total three compounds were identified - β-boswellic acid, 11-Keto-β-boswellic acid, and 3-O-Acetyl-11-keto-β-boswellic acid. The molecular ion [M-H]− and fragmentation pattern of these compounds were found to be similar to the previous literatures (Sharma et al., 2016; Umar et al., 2014). Further, spectral similarity has been searched through 'The Metabolomics Innovation Centre' (TMIC) with reference spectra of β-boswellic acid (HMDB0002388) (Allen et al., 2015). The spectral similarity between the reference and the observed spectrum was very high. Table 5.2 summarizes the detail of identified compounds through LC-MS/MS. Total Ion Chromatogram (TIC) and MS/MS spectra are provided in Fig. 5.3 and 5.4.

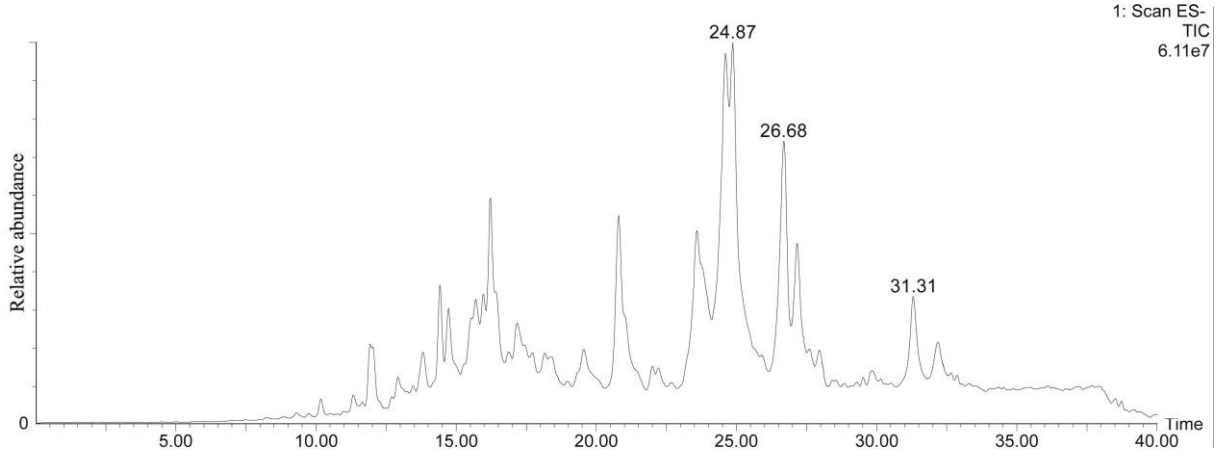


Figure 5.3. Total ion chromatogram (TIC) of methanol extract of *Boswellia serrata* in the negative ion mode.

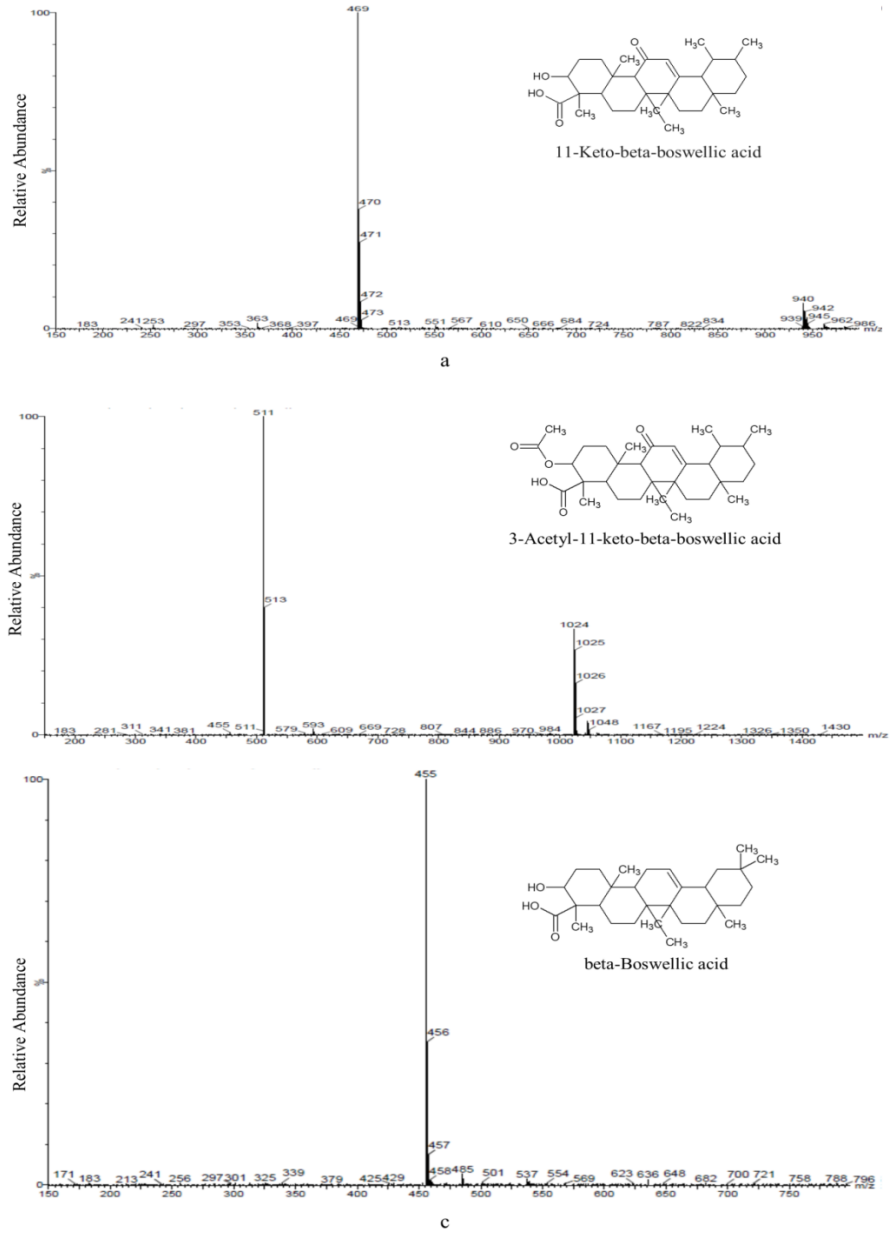


Figure 5.4. MS/MS spectra boswellic acids from BSE. a) 11-Keto-β-boswellic acid (KBA) (deprotonated precursor [M-H]− ions at m/z 469); b) 3-O-Acetyl-11-keto-β-boswellic acid (AKBA) (deprotonated precursor [M-H]− ions at m/z 511); c) β-Boswellic acid (deprotonated precursor [M-H]− ions at m/z 455).

Table 5.2. Boswellic acid and its derivatives identified in *Boswellia serrata* oleo-gum resin extract by LC-MS/MS

Retention Time	Molecular formulae	Exact mass	Deprotonated mass $[M-H]^-$	Proposed compounds
24.87	$C_{30}H_{46}O_4$	470.694	469	11-Keto-β-boswellic acid (KBA)
26.68	$C_{32}H_{48}O_5$	512.731	511	3-O-acetyl-11-keto-β-boswellic acid (AKBA)
31.31	$C_{30}H_{48}O_3$	456.711	455	β-Boswellic acid

5.6.2. Quantitative analysis by RP-HPLC

The calibration curve of β-boswellic acid was constructed by plotting mean peak area against concentrations. The HPLC chromatogram showed a separate distinct peak of BA at a retention time of 11.868 (Figure 5.6). Linearity curve was plotted in five concentration ranges 10-80 µg/ml and tested by the least square regression analysis. The standard calibration curve of β-boswellic acid was linear with r2 = 0.9967. From calibration curve, BA concentration in BSE was calculated and was found to be 1.06 ± 0.03 % (w/w) (Figure 5.5; Figure 5.6). The HPLC method was validated in accordance with International Conference on Harmonization (ICH) Q2R1 guidelines. The method validation studies revealed that the RP-HPLC method was simple, accurate, rapid and reproducible.

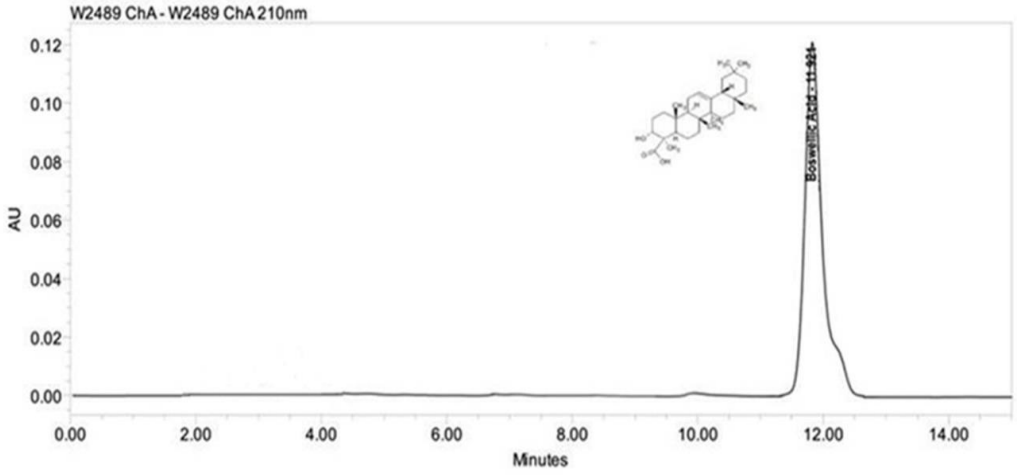


Figure 5.5. RP-HPLC chromatogram of standard β-boswellic acid

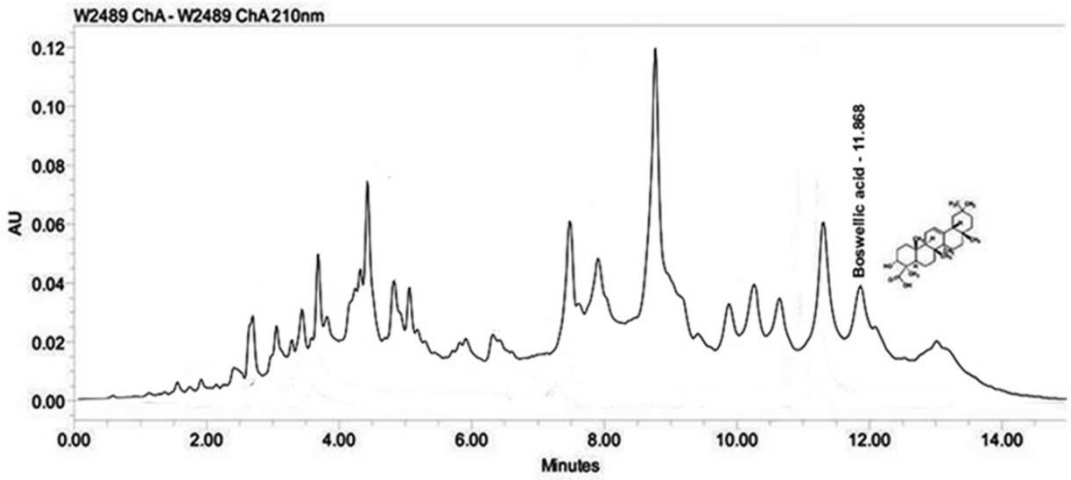


Figure 5.6. RP-HPLC chromatogram of *Boswellia serrata* extract

5.6.3. HPTLC standardization of *B. serrata*

Standard β-boswellic acid showed single peak in HPTLC chromatogram (Figure 5.7 A) and methanol extract of B. *serrata* showed eight peaks (Figure 5.7 B). Among the peaks one peak from the extract matched with standard betulinic acid (Rf 0.46). The percentage of betulinic acid in B. *serrata* was found to be 1.04 + 1.13 % (w/w).

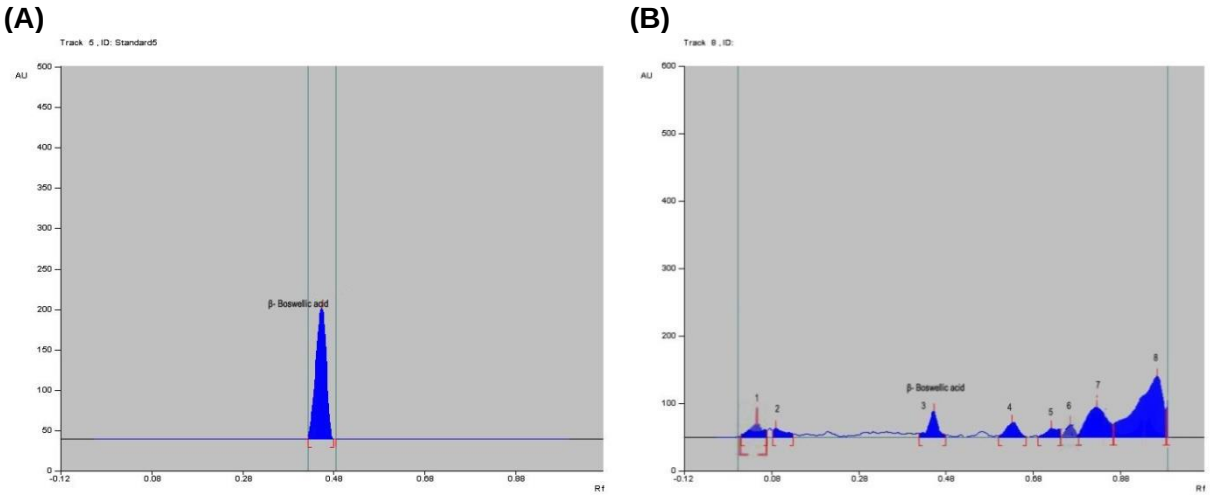


Figure 5.7. (A) HPTLC chromatogram of β-boswellic acid; (B) HPTLC chromatogram of *Boswellia serrata*

The mobile phase resolved β-boswellic acid efficiently from the extract. Calibration curve was plotted based on the application of different concentrations of β-boswellic acid ranging from 2–10 µg, the correlation coefficient (r) value of 0.97222 was indicative of good linear dependence of peak area on concentration. The calibration curve was represented by the linear equation Y = 6.496X + 6.674, where Y represented the response as area and X represented the concentration (Figure 5.8). The HPTLC method was validated in accordance with International Conference on Harmonization (ICH) Q2R1 guidelines. The method validation studies revealed that the HPTLC method was simple, accurate, rapid and reproducible.

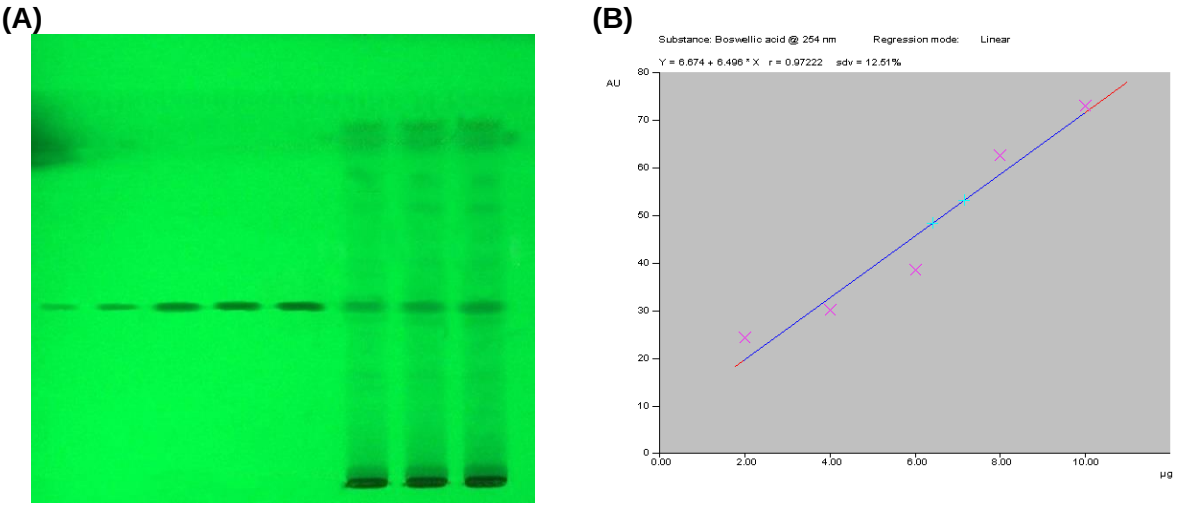


Figure 5.8. (A) Photodocumation of TLC plate at 254 nm; (B) Calibration curve of β-boswellic acid

5.6.4. Assessment of cytotoxicity and anti-HSV activity

The antiviral activity of *B. serrata* oleo-gum resin and β-boswellic acid was determined against HSV-1F, and clinical isolate of HSV-1 (100 PFU) using PRA with acyclovir (5-10 µg/ml) and DMSO (0.1%) as control. The results showed a dose-dependent inhibition of viral plaque formation by BSE and BA (data not shown). The PRA demonstrated that BSE at 5.2–6.2 µg/ml and BA at 12.1–14.63 µg/ml inhibited all the strains tested, far below their CC_{50} concentration (Table 5.3). Moreover, nearly-complete (EC_{99}) inhibition of viral growth was achieved at 10 µg/ml of BSE and 30 µg/ml of BA against HSV-1.

Table 5.3. Assessment of cytotoxicity and anti-HSV activity of *B. serrata* and β-Boswellic acid by MTT and plaque reduction assay

Test Drug	CC_{50}[a]	HSV-1F at MOI: 1		HSV-1 (VU-09)	
		EC_{50}[b]	SI[c]	EC_{50}[b]	SI[c]
Boswellia serrata	56.6 ± 3.4	5.2 ± 1.8	10.88	6.2 ± 2.1	9.12
β-Boswellic acid	105.5 ± 5.2	12.1 ± 2.8	8.71	14.63 ± 3.1	7.21
Acyclovir	130 ± 3.8	2.1 ± 0.5	61.9	2.2 ± 0.4	59.09

[a] 50% cytotoxic concentration for Vero cells in µg/ml.
[b] Concentration of test agent (µg/ml) producing 50% reduction of virus induced plaques.
[c] Selectivity index (SI) = CC_{50}/EC_{50}.
Clinical isolate: VU-09

5.6.5. Dose-dependent effect of EAF and BA

To determine the effect of different doses of the BSE and BA on HSV post-infection, we exposed the virus (HSV-1F) infected Vero cells to BSE and BA at two fold concentrations (1, 2.5, 5, 10, 25, 50 and 100 µg/ml) for 3 days. The results presented in Figure 5.9 showed that the BSE at 10 µg/ml and BA at 30 µg/ml exhibited nearly complete (EC_{99}) inhibition of HSV-1 indicating a high correlation between drug concentration and its inhibition rate.

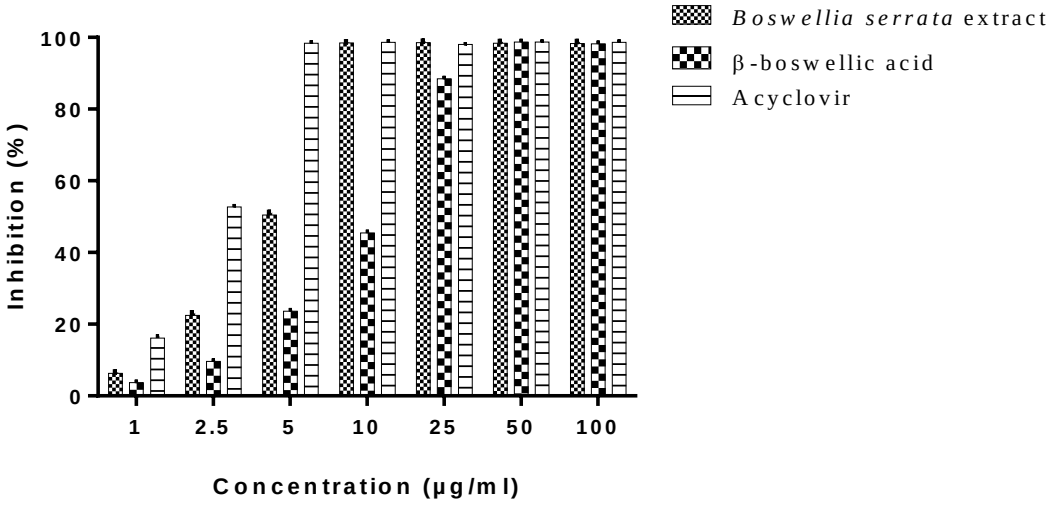


Figure 5.9. Dose dependent anti-HSV-1 effect of *B. serrata* extract, β-Boswellic acid and acyclovir on HSV-1. Each bar represents the Mean ± SD of three independent experiments (, P<0.001; *, P<0.05 compare with control).**

5.6.6. Time-of-addition assay

To investigate the possible stage of viral life cycle affected by BSE and BA we conducted the time-of-addition assay. Our results revealed that BSE and BA significantly inhibited HSV-1 within 1–4h post-infection, which signifies the effect at an early stage of virus infection (Figure 5.10).

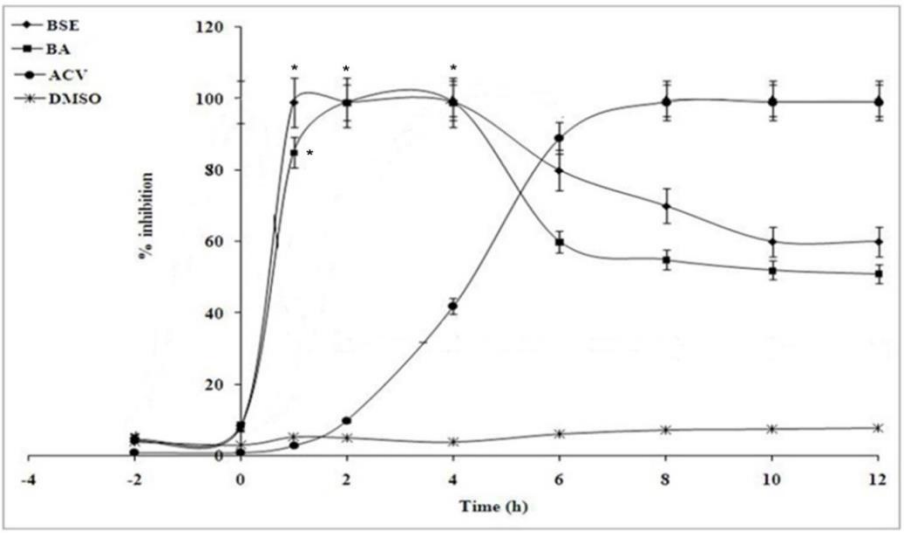


Figure 5.10. Time of Addition assay of BSE ((10 µg/ml)) and BA ((30 µg/ml)) on HSV-1 infected Vero cell, using DMSO and Acyclovir (ACV) as controls. Each curve represents the mean ± SD of three independent experiments (*, P<0.05, compared to control).

5.6.7. Effect of BSE and BA on viral RNA expression

To address the outcomes of time-of-addition assay, we evaluated the effects of BSE and BA on viral mRNA expression after virus entry. Vero cells infected with HSV-1 for 1h was inactivated by citrate buffer, washed, and exposed to the test agent's at1, 2, and 4h post-infection, and the total cellular RNA was isolated. Our results demonstrated that BSE and BA down-regulated the viral mRNA expression, as levels of immediate-early (ICP27), early (DNA-Pol), and late (gD) gene transcripts were failed to express at 1 and 2h post-infection in presence of BSE and BA, while at 4h post-infection the gene expression was minimal

(Figure 5.11). These findings clearly suggest that both BSE and BA inhibit the early stage of HSV transcription following penetration of the host cell.

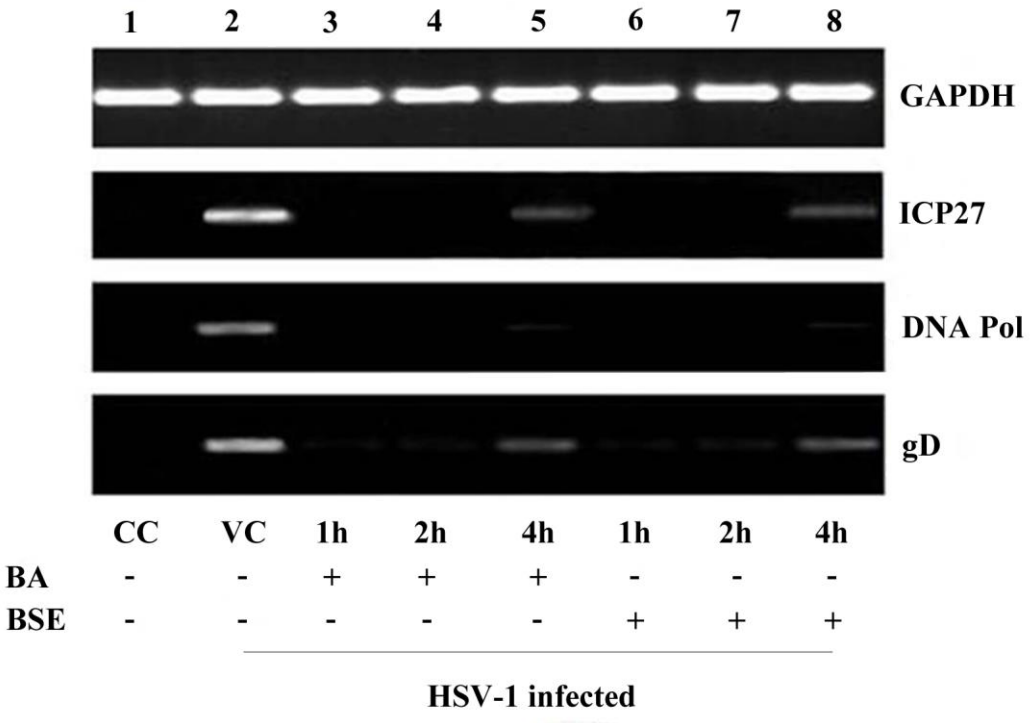


Figure 5.11. Effect of BSE (10 µg/ml) and BA (30 µg/ml) on HSV-1-induced ICP27, DNA polymerase and gD gene expression. Lane 1: cell control; lane 2: cell + HSV-1; Lane 3,4,5: cells + HSV-1 + BSE at 1h, 2h and 4h post-infection; Lane 6,7,8: cells + HSV-1 + BA at 1h, 2h and 4h post-infection. The data are expressed as Mean ± SD from triplicate experiments yielding similar results (*, P<0.05; **, P<0.01).

5.6.8. Effect of BSE and BA on NF-κB, p38, and JNK1/2 in HSV-infected macrophages

Our study on signaling pathway to ascertain the in vitro mechanism of BSE and BA, by which they control HSV infection in macrophages, revealed an expected result as it has been proved that BSE is an inhibitor of NFκB. For any system to produce an anti-inflammatory response it needs to reduce production of NFκB, thereby creating an obstacle in the canonical pathway of inflammation. The level of IκBα, an endogenous NFκB inhibitor

was examined by western blot analysis and it revealed that there was a steep upregulation of IκBα. This proves that HSV-1 induced the degradation of IκBα, was blocked by both BSE and BA at 12 h p.i. It was also observed that there is a down-regulation of both p-NFκB and NFκB within 30 min of treatment with BSE and or BA. The modulation of NFκB was also observed in both the cytoplasmic and nuclear extract. Inhibition of pro-inflammation via NFκB pathway influences the MAPK adaptor molecules, resulting in the inhibition of p38 phosphorylation, consequently down-regulating pp38. But the effect of BSE and BA was insignificant on JNK1/2 (Figure 5.12).

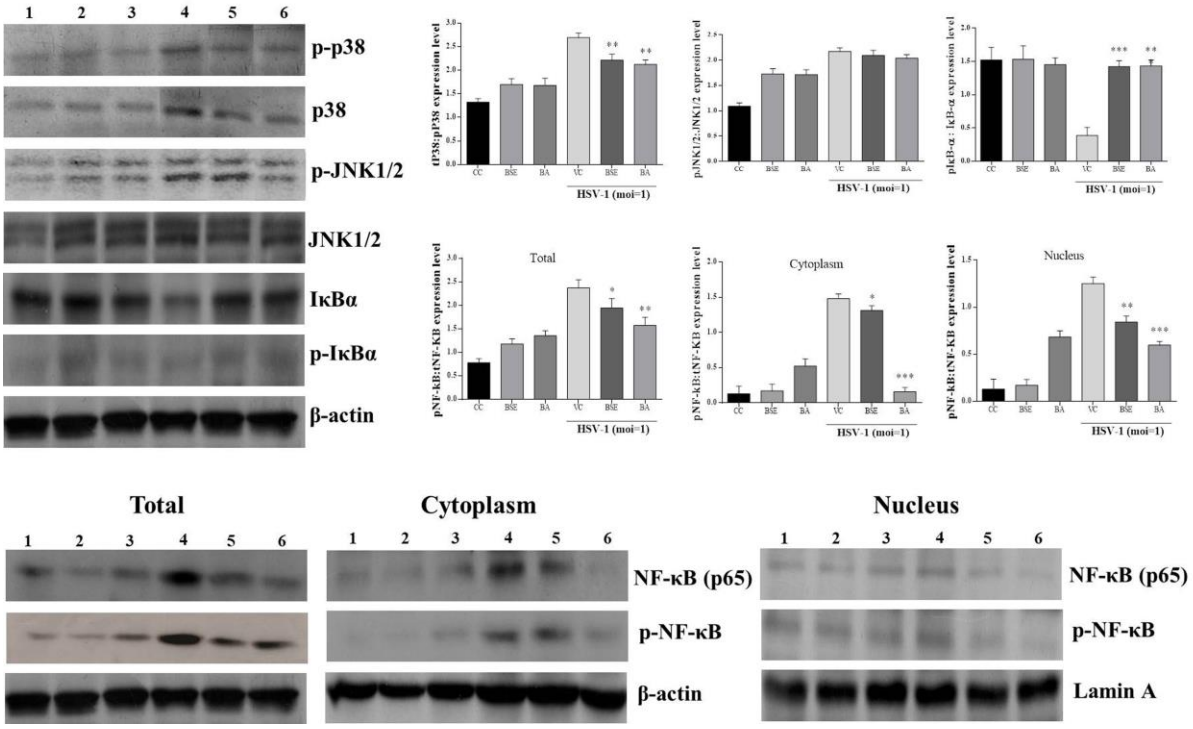


Figure 5.12. Western blot analysis of HSV-1-induced p38, JNK1/2, IκBα and NF-kB activation, using β-actin and Lamin A as internal control, on HSV-1-infected peritoneal macrophage(s) treated with *Boswellia serrata* extract (BSE) (10 µg/ml) or boswellic acid (BA) (30 µg/ml). The data are expressed as mean± SEM from triplicate experiments yielding similar results (*, p<0.05; **, p<0.01, *** p<0.001).

5.6.9. Effect of BSE and BA on inflammatory cytokines in HSV-1-infected macrophages

The HSV-1 infection into host cell triggers the release of proinflammatory cytokines associated with the activation of NFκB and MAP kinase. Therefore, the role of BSE and BA in modulating the expression of HSV-induced pro-inflammatory cytokines was assessed by reverse transcriptase PCR. As shown in Figure 5.13 both BSE and BA were effectively down-regulated the expression of TNF-α and IL-β with considerable inhibition of IL-6 as well, which are considered as the hallmark of pro-inflammatory and chemotactic dynamics. We further investigated the action of BSE and BA on the expressions of cytokines of Th-1 (IL-12, IFN-γ) and Th-2 (IL-10, IL-4). Interestingly, IL-12 and IFN-γ were found to be down-regulated by both BSE and BA, while the Th-2 cytokines were up-regulated (Figure 5.13).

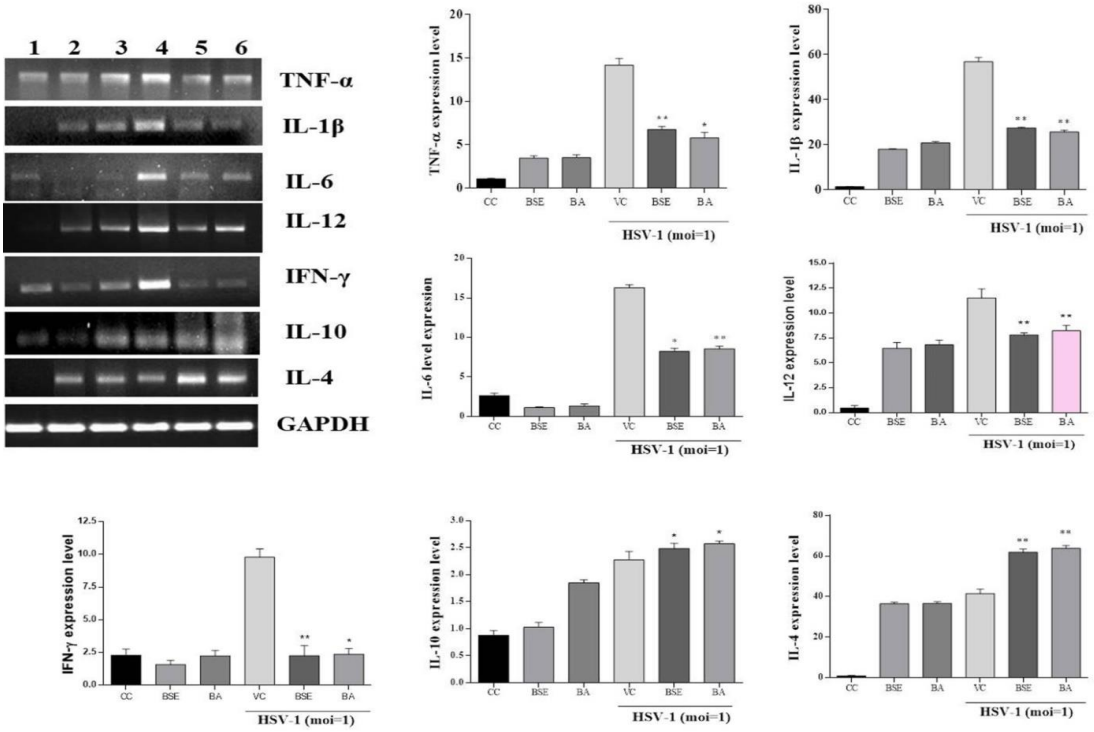


Figure 5.13. RT-PCR analysis of cytokines of BSE (10 µg/ml) and BA (30µg/ml) treated HSV-1 infected macrophages. Data are expressed as Mean ± SD from triplicate experiments, yielding similar results. Asterisks indicate statistically significant (*, P<0.05;, P<0.01) induction of cytokines' release, compared to the infected macrophages.**

5.6.10. Effect of BSE and BA on HSV-1 induced NO production

As HSV infection induces the production of pro-inflammatory mediator nitric oxide (NO), we have evaluated the effect of BSE and BA on NO and iNOS gene expression in mouse peritoneal macrophages. The results revealed that NO production in virus-infected and BSE and BA treated macrophages was significantly reduced, while iNOS expression was suppressed, compared to the untreated cells (Figure 5.14).

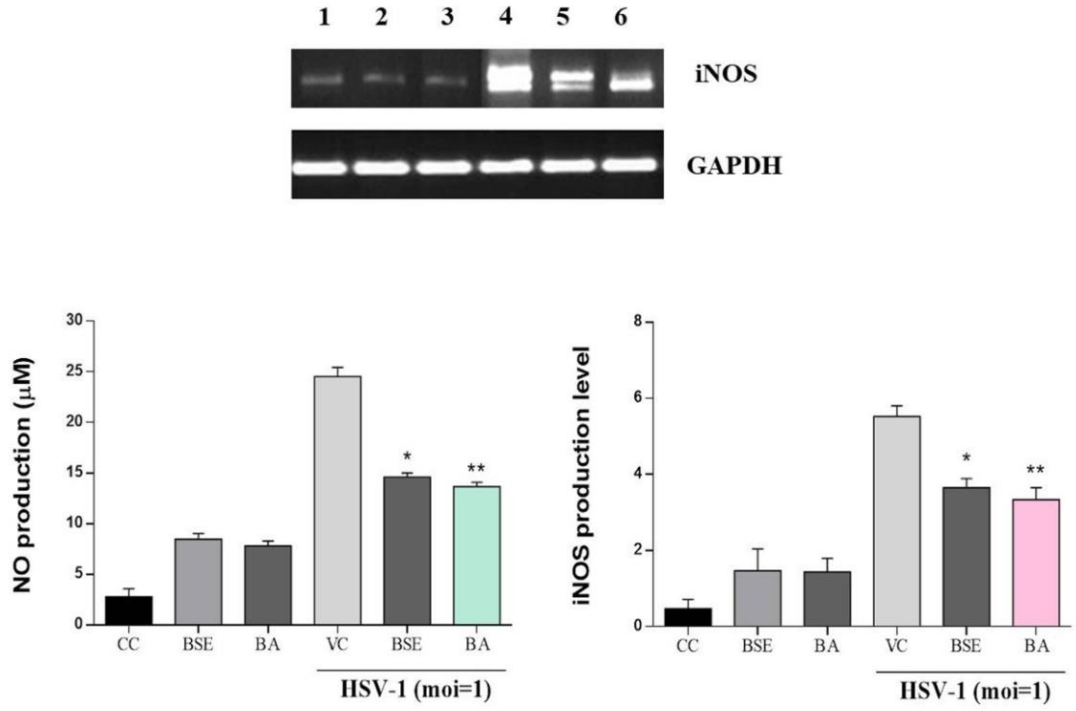


Figure 5.14. (A) Effect of BSE (10µg/ml) and BA (30µg/ml) on NO production in HSV-1 infected macrophages. The concentration of NO was determined by Griess reagent. Data are expressed as Mean ± SD from triplicate experiments, yielding similar results (m moles of nitrite). Asterisks indicate a statistically significant increase (*, P<0.05; **, P<0.01) in nitrite generation, compared to the infected macrophages. (B) RT–PCR analysis for iNOS2 mRNA expression of HSV-1-infected macrophages treated with the BSE or BA.

5.7. Discussion

The present study demonstrated a potent anti-HSV activity of oleo gum-resin extract of BSE (EC_{50} 5.2 - 6.9 µg/ml). The LC-ESI-MS/MS analysis of BSE leads to the identification of three major boswellic acids namely 11-Keto-β-boswellic acid (KBA), 3-O-acetyl-11-keto-β-boswellic acid (AKBA) and β-Boswellic acid. Although other β-configured derivatives of boswellic acids are present in the extract β-boswellic acid was opted as the choice of active marker because it is the first of its form. Also the anti-inflammatory potential of β-boswellic acid is less explored than the other β-configured boswellic acids.

The RP-HPLC analysis of the bioactive BSE revealed the presence of a pentacyclic triterpene β-boswellic acid as an active constituent with a content of 1.06 ± 0.03 % (w/w). In addition to that the HPTLC analysis was also used as a tool for standardization of BSE which revealed the content of BA as 1.04 + 1.13 % (w/w). Further, significant *in vitro* anti-HSV-1 activity of BA (CC_{50} 105.5; EC_{50} 12.1-14.63 µg/ml with SI of 8.71-7.21) indicated that the extract and its major constituent had different EC_{50}, far below their CC_{50} dose. Further, the BSE and BA revealed dose-dependent antiviral activity by preventing the HSV-infection with higher SI values. Interestingly, the SI of the BSE was much higher than its active constituent BA, probably due to the synergistic effect of other active compounds present in the bioactive gum-resin extract of *Boswellia serrata* that produce higher anti-HSV activity than its active marker. Significantly, both BSE and BA inhibit wild-type and the clinical isolates of HSV-1 in a dose-dependent manner (Table 5.3). However, complete inhibition of viral plaque formation was found at 10 µg/ml of BSE and 30 µg/ml of BA.

The time-of-addition assay between 0 and 12h post-infection revealed that BSE and BA possess a similar inhibitory effect by interfering with the early stage of viral replication, as the maximum inhibition was at 1, 2 and 4h post-infection. To verify these data we have isolated the viral m-RNA from the drug-treated and untreated cells at different time intervals and subjected to RT-PCR analysis for expressions of viral transcripts of ICP27, DNA-Pol and gD genes. Interestingly, these genes failed to express in the resultant c-DNA product of the drug-treated HSV-infected cells, which clearly signifies the anti-viral activity of both BSE and its compound BA.

The first line of response in an HSV-infected macrophage is inflammation, where the activation of canonical NFκB occurs mostly as an initiative to secrete host-protective cytokines to clear out the virus from the host. But due to evolutionary pressure the virus has developed various ways to bypass this innate signaling mechanism required for cytokine production and instead hijack the functionality of this transcription factor for its own replication and spread of infection. A recent report showed that the viral DNA polymerase processivity factor UL42 interacts with p65/RelA and p50/NF-κB1 to block NFκB translocation to the nucleus in response to stimuli, such as TNF-α from host response. While another study showed that ICP27 directly inhibits NFκB activation with decreased production of IFN-γ, IL12, IL-6, and TNF-α; and ICP27 is an early viral protein that inhibits the host cell apoptosis. Thus, if the HSV virion was inhibited in early phase of infection by inhibiting the host-induced Th-1 pro-inflammatory response, the chances of rid-of the virus will be more effective. Ours results suggest that BSE and BA are capable of effectively modulating host's immune mechanism during early stage of virus infection.

HSV-infection leads to a cascade of intracellular signaling events that results the secretion of cytokines and other inflammatory mediators that constitute the pro-inflammatory response. Effect of the ability of BSA and BA to down-regulate virus-induced TNF-α production showed that both agents inhibited TNF-α production. Similarly, to determine the inhibitory effect of BSE and BA on pro-inflammatory cytokine we have studied the expression of IL-1β and IL-6 *in vitro*. Here also we observed similar effect of BSE and BA on IL-1β and IL-6 expression (Figure 5.13) like TNF-α; thus, the efficacy of both agents to reduce IL-1β and IL-6 expression revealed its role in inhibiting downstream mediators of inflammation. Here Th1 cytokines were inhibited but cytokines associated with Th2 were enhanced. Further, the inhibitory effect of BSE and BA on IL-12 and IFN-γ indicated that these agents have important regulatory and modulatory activities in HSV-infections, especially during an enhanced Th1 response. Additionally, BSE and BA showed enhanced Th2 cytokines IL-10 and IL-4. As different disease manifestations are associated with prominence of one or the other Th1 and Th2 phenotypes, so the increase in Th2 cytokines

by BSE and BA can further aid in its anti-inflammatory action by negative regulation of Th1 cytokines and inflammatory cytokines TNF-α, IL-1-β and NO.

A significant decrease in NO production was observed with BSE or BA treatment, presumably from the suppression of iNOS gene induction, as evidenced by RT-PCR (Figure 5.14). Here, we observed that the expression of iNOS was down-regulated as compared to HSV-1 infected untreated cells, suggesting that the reduced iNOS would have resulted in the inhibition of NO, in HSV-induced macrophages. Therefore, the inhibition of these pro-inflammatory mediators showed to have tremendous therapeutic value.

Further, signaling studies using Western blot revealed that NFκB is down-regulated during the treatment with BSE and BA (Figure 5.12). The NFκB is by far the most significant molecule in HSV-1 infected host. Collectively, our results demonstrated that BSE and BA are able to suppress HSV-infection in the host cell as an effective therapeutic tool. HSV-infection is known to be influenced by cellular signaling pathways and transcription factors, including the activation of MAP kinase that promotes viral replication. Influenced by this observation, we have investigated the effect of BSE and BA upon MAPK pathway. The results revealed that the down-regulation of Th-1 stimulating pp38 exert inhibitory effect on phosphorylation of p38 by BSE and BA, but has insignificant effect on JNK. Thus, the inhibition of NFκB and p38 MAP kinase pathway by *B. serrata* and β-boswellic acid also inhibit the expression of pro-inflammatory genes, denoting that the BSE and BA successfully control the virus spread at the early stage of infection; which can be explored to develop a therapeutic lead for the management of HSV-infection.

5.8. Conclusion

The outcome of the study elucidate that gum-resin extract of *B. serrata* and its constituent β-boswellic acid exhibited potent antiviral activity against wild-type and clinical isolate of HSV-1 *in vitro* by inhibiting the early replication. Moreover, the BSE and BA significantly inhibited HSV-induced p38 and NFκB activation, resulting in the inhibition of HSV-1 replication. However, further studies are required to examine the antiviral efficacy of other major constituents of *B. serrata*. Thus considering all the experimental results β-boswellic acid can

be considered as an effective lead for therapeutic potential against HSV, due to its low cytotoxicity and significant anti-herpetic activity.

5.9. Publication

Boswellia serrata oleo-gum-resin and β-boswellic acid inhibits HSV-1 infection *in vitro* through modulation of NF-κB and p38 MAP kinase signaling. Phytomedicine (Elsevier Science, USA) 2018, 51, 94-103.

Chapter- 6

Summary and conclusion

Summary

HSV infection is globally prevalent in individuals of all age groups, and a matter of serious concern as 60-90% of global population are infected with this virus. Currently available antiviral drugs acyclovir and its related agents successfully managed the infection but neither able to eliminate the virus from the host not can prevent recurrent infections. Moreover, frequent development of HSV resistance to anti-herpetic drugs is considered a serious problem. Thus, identification of new anti-herpetic lead is the need of the hour.

The increasing use of traditional medicines demands scientifically validated evidence for the principles behind treatments and effectiveness of any drug. Several medicinal plants are reported to possess the ability of inhibiting and preventing different viruses and viral diseases including the HSV infection in human. Medicinal plants are traditionally used since ages and are still utilized by diverse indigenous and marginalized populations throughout the globe. Although substantial amount of evidence is on anti-HSV plants, extracts of different plant parts or isolated phytocompounds, but most of those plants are neither studied in detail nor are the bioactive anti-HSV molecules, reported so far, established for clinical use.

In this context, this study was designed to evaluate the HSV-1 inhibitory potential of selected Indian medicinal plants with an aim to find the potential leads from them. The study deals with the screening of eight medicinal plants, selected on the basis of their traditional usage against skin related ailments, against HSV-1 wild and clinical isolates. Preliminary screening results revealed that two plants, namely *Dillenia indica* and *Boswellia serrata*, were most effective against HSV-1 *in vitro*. Thus, this thesis highlighted the scientific evaluation of these two important medicinal plant extracts against HSV-1 infection along with their mode and molecular mechanism of anti-HSV action. Further, bioactivity guided fractionation and chromatographic evaluation were carried out for identification of antivirally active phytoconstituents. The results are useful for further assessment of anti-HSV-1 activity to explore lead molecules for the development of effective antiviral drugs.

Chapter 1 describes a brief introduction of Herpes Simplex Virus-1 (HSV-1) comprising of its structure, life cycle including replication and mechanism of infection, global

prevalence, epidemiology of various disease manifestations and treatment strategies using FDA approved drugs. The seroprevalence data displayed the global impact of HSV-1, suggesting immediate intervention for the proper management of HSV infection considering it a matter of utmost importance. In addition, the mechanisms of antiviral efficacy of the existing drugs, especially acyclovir, against HSV are also included. The various limitations of these antiviral drugs are described highlighting the importance of identification of alternative lead molecules from nature, particularly from plant sources. The Chapter also included the detailed study of various medicinal plants having HSV inhibitory potential, highlighting extensive literature survey on the major aspects of HSV inhibitors from medicinal plants including their extract and isolated constituents. The extent of efficacy of different types of phytoconstituents including the flavonoids, terpenoids, alkaloids, tannins, glycosides etc. against HSV are briefly described to identify potent and safe leads as HSV-1 inhibitors from vast source of medicinal plants.

Chapter 2 describes the scope and rationale of the study along with the specific objective of the work. A framework of the study was designed, based on the specific objectives, and the detailed of the experiments performed.

In Chapter 3 the screening of selected Indian medicinal plants against HSV-1 infection in Vero cells is described. Ethnopharmacological relevance is the basis for selection of any medicinal plant. Hence, we have conducted extensive literature survey of Indian medicinal plants among which eight were selected based on their traditional usage against skin related ailments and wounds. Usage against skin infection was given the priority as the HSV-1 infect the skin mucosa of human and silently spread from infected to uninfected individuals through cuts, wound, body fluid and sex. The plants thus selected includes *Aegle marmelos*, *Butea monosperma*, *Dillenia indica, Enhydra fluctuans, Morus alba, Boswellia serrate, Piper beetle* and *Stereospermum suaveolens*. These plants were collected and authenticated by a field botanist and were subjected to extraction with methanol as solvent. All the extracts were then assessed for determination of cytotoxicity (CC_{50}) by MTT assay. Depending on the CC_{50} values the doses for determining the median effective concentrations (EC_{50}) against HSV-1 infection were selected and subjected to antiviral screening by cytopathic effect reduction, MTT and plaque reduction assay. The results revealed that out of eight extracts tested, two have moderate and two were having significant antiviral activity

against the test isolates of HSV-1 at concentrations that are non-cytotoxic to the Vero cells. The methanol extracts of *Dillenia indica* and *Boswellia serrata* exhibited significant antiviral activity against HSV-1 with EC_{50} of 56.19 - 61.2 and 5.2 - 6.2 µg/ml respectively. In addition to that extracts of *Enhydra fluctuans* and *Butea monosperma* exhibited anti-HSV activity at lower concentrations, while the other four extracts failed to inhibit the tested isolates. The results of MTT and plaque reduction assay were found to be comparable. Based on the selectivity index (SI), the preferential antiviral activity (EC_{50}) of the test extracts in relation to its cytotoxicity (CC_{50}), revealed that *D. indica* (SI=7.6) and *B. serrata* (SI=10.88) were most effective among the extracts tested and thus, were further explored to find potential leads against HSV-1 infection.

Based on the study the following article has been published.

- Screening of ethnomedicinal plants of diverse culture for antiviral potentials. *Indian Journal of Traditional Knowledge* (CSIR-NISCAIR, India) 2016, 15 (3), 474-481.

The detailed study of the antiviral potential of *Dillenia indica* L. extract and its isolated constituent betulinic acid (BT) against HSV-1 infection is described in Chapter 4. The bioactivity guided fractionation of *D. indica* extract showed that the ethyl acetate fraction (EAF) is the most effective one against HSV-1 isolates tested. Column chromatography and successive flash chromatography of EAF revealed that one of the major active component BT possess potent anti-HSV-1 activity. The percentage purity of BT (97.14% w/w) was calculated by using RP-HPLC technique. Further standardization of various bioactive fractions of *D. indica* by BT using RP-HPLC method showed that the ethyl acetate fraction contained the highest amount of BT (9.47 ± 4.71 % (w/w)). Standardization of EAF by HPTLC method showed similar results. The EC_{50} of EAF and BT was found to be 30.3-37.6 µg/ml and 3.2-3.9 µg/ml respectively, against HSV-1 isolates, as determined by MTT and plaque reduction assay. Subsequently, nearly complete (EC_{99}) inhibition of viral growth was achieved at 58.5 µg/ml of EAF and 5.5 µg/ml of BT against HSV-1F. Both EAF and BT displayed very promising SI of 10.2 and 15.22, respectively.

In order to explore the mechanism of HSV-1 inhibition by EAF and BT the time of addition assay was conducted. The results showed that the antiviral activity of EAF and

BT was not due to their direct effects on Vero cells but on the viral particles by inhibiting its entry into the host cells. Further, viral inactivation assay revealed that pre-incubation of EAF and BT with virus particles resulted in HSV-1 inhibition suggesting that EAF and BA can bind to virus particles to neutralize virus infectivity. Similarly inhibition of HSV-1 attachment to the host cell surface by EAF and BA was confirmed by indirect immunofluorescence assay. While the viral penetration assay showed no significant activity of EAF and BT against HSV-1. To confirm this mode of action of test agents RT-PCR and qRT-PCR studies were carried out to know the effects of EAF and BT on HSV-1 mRNA expression after virus entry. The results established that EAF and BT had no effect on the viral mRNA expression after penetration, since the immediate-early (ICP27), early (TK gene), and late (gD) transcripts were unaffected by EAF or BT. On the other hand, both EAF and BT prevent the development of viral mRNA synthesis when added together with the virus. These findings confirm that the test agents inhibited HSV attachment, but not the transcription, after penetration, of the host cell.

Collectively our results clearly suggest that EAF and BT were effective against HSV-1 infection by inhibiting attachment of the virus to the host cells.

Based on the study the following article has been published.

- *Dillenia indica* fruit extract and its major constituent inhibits Herpes Simplex Virus entry in host cell. *Indian Journal of Medical Research* (Medknow Publications on behalf of the Indian Council of Medical Research, India). Accepted for publication on 12th April, 2018.

Chapter 5 deals with the possible mechanism of HSV-1 inhibition of β-boswellic acid (BA) from oleo-gum resin extract of *Boswellia serrata* Roxb. The gum resin of *B. serrata* is one of the popularly used traditional medicines against skin diseases. *B. serrata* is a rich source of triterpenoids boswellic acids and its derivatives. The *B. serrata* extract (BSE) was subjected to LCMS/MS analysis and three compounds were identified - β-boswellic acid, 11-Keto-β-boswellic acid, and 3-O-Acetyl-11-keto-β-boswellic acid. Although there are many boswellic acids in *B. serrata,* BA was chosen as the active marker against HSV-1 infection. The *B. serrata* extract (BSE) was standardized by using both HPLC and HPTLC. RP-HPLC analysis revealed that BA content in BSE was 1.06 ± 0.03% (w/w) whereas HPTLC analysis revealed 1.02 ± 0.01% (w/w) of BA in BSE. The

MTT and plaque reduction assay demonstrated that BSE at 5.2-6.2 µg/ml and BA at 12.1-14.63 µg/ml inhibited the HSV-1 isolates tested far below their CC_{50} concentrations. While nearly-complete (EC99) inhibition of viral growth was achieved at 10 µg/ml of BSE and 30 µg/ml of BA. Interestingly, the SI of BSE (10.88) was much higher than its active constituent BA (8.71), probably due to the synergistic activity of various compounds present in the bioactive extract of *B. serrata* that produce higher activity than its active marker. Time-of-addition assay revealed that both BSE and BA possess a similar inhibitory effect between 0 and 12 h post-infection by interfering with the early stage of viral replication, as the maximum inhibition was recorded at 1, 2 and 4 h post-infection. The effect of BSE and BA on viral mRNA expression after entry, by RT-PCR analysis, revealed that BSE and BA down-regulated the viral mRNA expression, as levels of immediate-early (ICP27), early (DNA-Pol), and late (gD) gene transcripts failed to express at 1 and 2 h p.i. in presence of test agents, while the gene expression was insignificant at 4 h p.i.. These findings clearly suggest that both BSE and BA inhibit the early stage of HSV-transcription following host cell penetration.

The role of BSE and BA in modulating HSV-induced pro-inflammatory cytokines' expression was assessed by RT-PCR on isolated mouse peritoneal macrophages, infected with HSV-1 followed by treatment of BSE and BA at their EC_{99} concentrations. Results indicated that BSE and BA effectively down-regulated the expression of TNF-α and IL-β with considerable inhibition of IL-6, indicating that both agents inhibit downstream mediators of inflammation. Further investigation showed that Th-1 cytokines (IL-12 and IFN-γ) were down-regulated by BSE and BA, while the Th-2 cytokines (IL-10 and IL-4) were up-regulated. A significant decrease in NO production was observed with BSE or BA treatment, presumably from the suppression of iNOS gene induction, as evidenced by RT-PCR.

Western blot analysis showed the down-regulation of NF-κB and p38 after BSE and BA treatment, but the effect was insignificant on JNK1/2. Thus, the inhibition of NF-κB and p38 MAP kinase pathway by BSE and BA also inhibited the expression of pro-inflammatory genes, thereby controlling the virus spread at an early stage of infection.

All these experimental outcomes suggested that BSE and BA were effective against HSV-1 infection, which functions through modulation of NF-κB and p38 MAP kinase signalling at early stage of infection.

Based on the study the following article has been published.

- *Boswellia serrata* oleo-gum-resin and β-boswellic acid inhibits HSV-1 infection *in vitro* through modulation of NF-κB and p38 MAP kinase signaling. Phytomedicine (Elsevier Science, USA) 2018, 51, 94-103.

Conclusion

There is a need for new remedies against herpes simplex virus (HSV), a widespread pathogen. Despite the fact that the available antivirals, especially acyclovir, can inhibit the virus number but failed to eliminate the virion from the host or prevent the recurrent infection, there are situations where the viral disease takes over the immune system of the host. In these cases, neither the immune system nor the drugs are capable of preventing damage caused by the infection. Such cases are common in immunocompromised patients and in HSV keratitis. Moreover, frequent development of acyclovir resistance leads to complicated situation which highlighted the need for alternate therapeutic molecule.

There are multiple approaches in antiviral drug development against HSV and a need for development of new treatment modalities. One of these approaches is development of potential leads from natural resources such as medicinal plants. From time immemorial medicinal plants have been an important source of remedy against infectious diseases such as HSV infection. So isolation, identification and characterization of potential leads against HSV could be an effective strategy for development of new anti-HSV drugs. A similar approach is followed here.

In relation to ethnomedicinal uses, extracts of eight selected Indian medicinal plants were screened against HSV-1 to determine antiviral efficacy. The prescreening results showed that two plants namely *Dillenia indica* and *Boswellia serrata* are significantly effective against HSV-1. Detailed study of these plant extracts lead to the isolation and identification of active constituents betulinic acid (from *D. indica*) and β-boswellic acid (from *B. serrata*), respectively. Both of these constituents inhibited HSV-1 infection in Vero cells following different mechanism of action. Betulinic acid was found to be effective against HSV-1 by inhibiting the virus attachment to the host cell, thereby

preventing its entry to the host cell. Whereas β-boswellic acid inhibited HSV-1 infection by modulating the host immune response against the virus at an early stage of infection.

Thus, these two compounds betulinic acid and β-boswellic acid could be considered as effective leads against HSV-1, which need to be explored for preclinical and successive clinical studies, for the development of antiviral drug.

Chapter 7

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