ANTIVIRAL ACTIVITY OF SELECTED MEDICINAL PLANT EXTRACTS AS COMPARED TO ACYCLOVIR AGAINST HERPES SIMPLEX VIRUS

BY

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A thesis submitted in partial fulfilment for the award of the Degree of Masters of Science (Infectious Disease Diagnosis) in the School of Pure and Applied Sciences, Department of Biochemistry and Biotechnology of Kenyatta University

September, 2006
DECLARATION

This thesis is my original work and has not been presented for a degree in any other university or any other award.

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We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

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DEDICATION

To God for His gift of life and strength. To my beloved wife Immaculate and children; Julie, Petronilla and Paschal who endured my absence from home during the long study period, to my parents for their invaluable role in my education and to all relatives and friends who missed my social interaction during the study period. 
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## ACRONYMS AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>HSV:</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;:</td>
<td>Tissue culture infective dose that completely destroys 50% of cell culture replicates</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;:</td>
<td>Inhibitory concentration that reduces infectivity of virus by 50%</td>
</tr>
<tr>
<td>AIDS:</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>HIV:</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>UNLS:</td>
<td>US national library service</td>
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<tr>
<td>VZV:</td>
<td>Vericella zoster virus</td>
</tr>
<tr>
<td>DNA:</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>RNA:</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>CNS:</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>BVDU:</td>
<td>Bromovinyl deoxy uridine</td>
</tr>
<tr>
<td>DHPA:</td>
<td>(S) – 9 –(2, 3 – dihydroxypropyl) adenine</td>
</tr>
<tr>
<td>TK:</td>
<td>Thymidine kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>ACV</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>VTM</td>
<td>Virus transport media</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>REA</td>
<td>Restriction endonuclease analysis</td>
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<tr>
<td>HPF</td>
<td>High power field</td>
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<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
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<tr>
<td>DMF</td>
<td>Dimethyl formamide</td>
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<tr>
<td>VC</td>
<td>Virus control</td>
</tr>
<tr>
<td>CC</td>
<td>Cell control</td>
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<td>DC</td>
<td>Drug control</td>
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Herpes simplex virus (HSV) is among the most common opportunistic infections in immunocompromised individuals. The available drugs, mainly the nucleoside analogs such as acyclovir are losing efficacy due to development of resistance by some HSV strains. Alternative drugs are too expensive for many resource poor patients to access and therefore, there is need for a drug which will control HSV infections at affordable cost. The concept of using a locally available plant virucide seem to be an obvious alternative. For such purpose, the objective of this study was to determine the in vitro antiviral activity and therapeutic efficacy of selected medicinal plants: Latex of Plumeria alba, leaves from Melia azedarach, Vinca rosea and Warbugia ugandensis. Acyclovir was used as a reference drug for antiviral activity and for comparison with antiviral effects of the medicinal plants. The activity and efficacy were based on reduction of HSV – 1 titer in infected Vero cells and prevention of symptoms and death of infected 6 weeks old male mice. Leave extracts from Melia azedarach, Warbugia ugandensis and Vinca rosea did not reduce virus infectivity at concentration tolerated by the Vero cells. Hexane, dichloromethane and ethylacetate extracts from latex of Plumeria alba reduced virus infectivity by 0.25, 0.75 and 1.5 \( \log_{10} \) of reference virusTCID\(_{50} \) respectively. Whole latex reduced infectivity by 0.25 and 1.25 \( \log_{10} \) of reference virusTCID\(_{50} \) at 5% and 10% (v/v) of latex in maintenance media respectively. Although whole latex did not significantly prevent mortality of infected mice, prevention of skin lesions was significant at \( P < 0.05 \) compared to untreated infected mice. Acyclovir cream was superior in efficacy compared to the latex of Plumeria when used in traditional form. Thus all the mice treated with acyclovir cream survived. Prevention of skin lesions with acyclovir was however similar to latex. The cream containing 5% latex in petroleum jelly did not reduce virus infectivity in our in vivo assay using the animal model. Maximum amount of latex with zero toxicity to Vero cells was observed at 2.5% (v/v) of latex in maintenance media, but no virus inhibition was detected at same concentration. In vivo toxicity was assessed on skin tissues of mice after mice treatment with whole latex and processing for histology. Fibrocyte cell counts in the dermal layer of treated mice were increased by 32%, giving significant statistical difference of \( P < 0.05 \) compared to untreated ones. There were cell debris and thick network of tissue fibers in tissues of treated mice compared to untreated ones. The findings show that latex of Plumeria alba can be exploited for its antiviral properties for the treatement of HSV but there is need to isolate the active ingredient for further antiviral testing and toxicity studies.
CHAPTER ONE

1. INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1 Background information

Herpes Simplex Virus (HSV) infection is an emerging health problem especially among individuals with immune compromising diseases such as Acquired Immunodeficiency Syndrome (AIDS) and organ transplant recipients (Kurokawa et al., 2001). In the absence of effective therapy, the infection causes life-threatening complications (UNLS, 2004). Nucleoside analogs such as acyclovir (ACV) are the most common anti-herpes drugs, but some HSV strains are known to be resistant (Crumpacker et al., 1982). Improved analogs such as penciclovir, valacyclovir and vidarabine are effective but are relatively expensive (De Clerq, 2001). The spread of drug resistance combined with other shortcomings of the available anti-HSV emphasizes the importance of the development of new cost-effective drugs against HSV.
In Kenya, according to traditional medical practitioners, lesions attributed to HSV and Varicella Zoster Virus (VZV) heals on topical application of latex from *Plumeria alba*. The practitioners also report the effectiveness of a cream from the latex, developed by suspending 5% of latex in petroleum jelly. In addition, ethanol extracts from *Melia azedarach*, and *Vinca rosea* have been reported to possess activity against HSV while water extracts from *Warbugia ugandensis* is traditionally used against measles infection (Hudson, 1990; Olilo et al., 2002).

Against the above background, these plants could be a good source for the development of new and safe anti-herpes drugs. However, their virucidal or virustatic activity, toxicity and the active ingredients need to be investigated. The purpose of this study therefore was to determine whether latex of *Plumeria alba* as used traditionally is active against HSV and to identify the active fractions. The study also investigated toxicity. Besides *Plumeria alba*, the study sought to determine whether extracts obtained by 1:1 mixture of methanol and dichloromethane from leaves of *Melia azedarach*, *Vinca rosea*, and *Warbugia ugandensis* could give anti HSV activity.

### 1.1.2 Justification

It was envisaged that this study would enhance understanding of efficacy and safety of ethno-medical materials, in the management of HSV. It would also
Complement efforts in the search for new and effective drugs. Additionally it provides stimulus to the utilization and development of indigenous knowledge in health-care.

1.1.3 Hypothesis

Selected medicinal plant extracts have better antiviral activity and therapeutic efficacy than acyclovir against herpes simplex virus infections.

1.1.4 Objectives

General objective

The aim of this study was to determine therapeutic efficacy and safety of latex from Plumeria alba and extracts from Melia azedarach, Vinca rosea, and Warbugia ugandensis.

Specific objectives

- To determine in vitro antiviral activity of the solvent extracts from leaves of Melia azedarach, Vinca rosea, Warbugia ugandensis and Plumeria latex.
• To determine \textit{in vivo} therapeutic efficacy of whole latex and latex cream of \textit{Plumeria alba}.

• To determine \textit{in vitro} and \textit{in vivo} the toxicity of \textit{Plumeria alba} latex.
1.2 LITERATURE REVIEW

1.2.1 Classification and structure of Herpes Simplex Virus

The Herpes viruses are in the family *Herpesviridae* and include Varicella Zoster Virus (VZV), Epstein-Barr Virus (EBV), Human Cytomegalovirus (HCMV), Herpes Simplex Virus (HSV) and Human herpes viruses 6,7 and 8 (Reinke et al., 1999). The virion of the family *Herpesviridae* consists of a single large double stranded DNA, which forms a central electron dense core, an icosahedral capsid surrounded by an amorphous layer of protein, the tegument. The tegument is surrounded by an envelop, a lipid bilayer which has on its outer surface at least ten distinct glycosylated and several nonglycosylated viral proteins and polyamines (Richman et al., 1999; Wright and Archard, 1992). The viral DNA genome has molecular weight of approximately 100 million Kilo Daltons and consists of approximately 152-kilo bases.

Broadly, there are two types of HSV; Type 1 and 2. HSV-1 has a G+C content of 68 moles percent while HSV-2 has 69 moles percent (Richman et al., 1997). Each DNA of HSV-1 and HSV-2 encode at least 81 different polypeptides and consists of two stretches of unique sequences, $U_L$ and $U_S$, each flanked by relatively large inverted repeat DNA sequence. The $U_L$ and $U_S$ can invert relative to each other to yield four populations of DNA molecule differing solely in relative orientation of
these DNA sequences. HSV type 1 and 2 differ with respect to nucleotide sequence, restriction endonuclease cleavage site and apparent sizes of many viral proteins they encode.

Herpes simplex and Herpes zoster viruses are classified in the sub-family *Alphaherpesvirinae*, which is characterized by relative rapid replication cycle *in vitro*, ability to establish latent infection in sensory ganglia of the host and variable host range (Reinke *et al.*, 1999). HSV -1 and 2 vary in their clinical manifestations but are characterized by painful vesicular or ulcerative lesions at various mucocutaneous sites (Lennette *et al.*, 1995). Reactivation of the virus from neural latency typically produces recurrent lesions at or near the site of infection (Lennette *et al.*, 1995).

Immunocompromised patients and infected neonates suffer severe complications due to extensive local disease, visceral involvement, general dissemination and or encephalitis (Richman *et al.*, 1997). Most immunocompetent individuals experience sub-clinical infections with either HSV-1 or HSV-2, mainly following reactivation of latent virus and subsequent mucocutaneous replication (Lennette *et al.*, 1995). Infection may some times occur without symptoms and this usually lead to asymptomatic virus shedding, which is significant for oral HSV-1 transmission among children, sexually transmitted HSV in adults and mother to child transmission during birth (Baker and Amstay, 1984). Acyclovir resistant
strains are much less virulent in immunocompetent individuals but cause persistent symptoms in the immunocompromised (Lennette et al., 1995).

1.2.2 Epidemiology of HSV

HSV infections occur worldwide without seasonal variations (Lennette et al., 1995). Ninety percent of oral HSV is due to HSV – 1 and 90% genital herpes is due to HSV – 2 (Emmert, 2000). Many studies however indicate that infection episodes of HSV viruses are usually asymptomatic. Sixty three percent (63%) of a subset of adults enrolled in the Framingham Heart study was sero-positive but only 47% reported cold sore episodes. Only 20 – 30% of people with HSV – 2 antibodies gave history of genital herpes lesions (Lenette et al., 1995). Animal vectors for HSV have not been described; therefore, humans remain the sole reservoir for transmission to other humans. Over one third of the world’s population are estimated to have recurrent episodes of HSV infection and therefore capable of transmitting it during productive infection (Lennette et al., 1995)

1.2.2.1 Transmission of HSV

The Virus is transmitted from the infected to a susceptible individual during close personal contact. Transmission majorly occurs during a symptomatic shedding of
the virus by the contact source. Prevalence of unrecognized HSV-1 and 2 is high and is the main reservoir of infection (Richman et al., 1997). Transmission of HSV-1 occurs through contact with active ulcerative lesions or with oral secretions of asymptomatically excreting patients. Asymptomatic salivary excretion was reported in 2 – 9% of adults and 5 – 8% of children in USA, with highest infection rate among children in day care centers (Richman et al., 1997). Transmission risk to HSV – 2 is greater from exposure to lesions than asymptomatic shedding (Mertz et al., 1992). Transmission of male to female is more likely than female to male (Mertz et al., 1992). Transmission of HSV to the neonates usually occurs during delivery from an asymptomatic mother. Mothers who acquired genital herpes shortly before delivery are at much more risk of transmitting the virus to their neonates (25 – 25%) than those with asymptomatic HSV – 2 reactivations (Brown et al., 1991). Transmission can also occur from non-genital sites and none-maternal sources but are few. Primary infection occurring early in pregnancy also present risk to the child (Richman et al., 1997).

Geographical location, socio-economic status and age are the most determinant of frequency of HSV-1 (Richman et al., 1997; Lenette et al., 1995; Whitely, 1985). Infections occur early among children in developing countries due to lower socio-economic conditions and standard of hygiene. In Japan, France, Sweden, and Caucasian Americans, children below age 15 had prevalence less than 70%
while in Rwanda, Zaire, Senegal, Jamaica and Haiti, seroprevalence was above 95% (Whitely, 1985).

Because infection with HSV – 2 is usually acquired through sexual contact, antibodies are rarely detected before puberty (Richman et al., 1997). The prevalence of genital herpes infection in USA was estimated to approach 20 million persons in 1985 (Szklo, 1985). By 1989, over 50 million cases were estimated to exist in either the active or dormant stage (Magder et al., 1989). Due to earlier sexual maturity and easier acquisition, many researchers have found higher prevalence in females than males. Emonyi et al (1998) reported in Uganda, ratio prevalence as 1:1.8 of male to female. The highest prevalence of HSV – 2 was found in female prostitutes in USA and was virtually identical in Tokyo (Richman et al., 1997). Increased neonatal herpes have been reported to coincide with increased genital herpes (Prober et al., 1992).

1.2.3 Clinical spectrum of HSV

The pathogenesis begins with the virus coming into contact with mucosal surface or abraded skin. With virus replication at the site of infection, either an intact virion or capsid is transported retrograde by neurons to dorsal ganglia, where after another round of replication, latency is established. After latency is established, proper stimulus causes reactivation followed by ante grade transport of infectious
virus particles to the epithelium surface along peripheral sensory nerves. Virus becomes evident at mucocutaneous sites, appearing as skin vesicles or mucosal ulcers.

Histopathologic characteristics of HSV infection reflect viral mediated cellular death and associated inflammatory response. Viral infection induces ballooning of cells with condensed chromatin within the nuclei of cells, followed by degeneration of the cellular nuclei, generally within parabolas and intermediate cells of the epithelium. Cells lose intact plasma membrane and form multinucleated giant cells. With cell death, a clear fluid containing large quantities of virus appear between the epidermis and dermal layer. The vesicular fluid contains cell debris, inflammatory cells and multinucleated giant cells. Within the skin, there is an intense inflammatory response, more so with primary infection than recurrent. On healing, the vesicular fluid is absorbed and scarring is uncommon. When mucous membrane is involved, the vesicles are replaced by shallow ulcers (Richman et al., 1997). Herpes simplex – 1 generally occur in oropharyngeal area. The trigeminal ganglion become colonized and harbors the latent virus. Herpes simplex – 2 is generally acquired by genital contact with consequent seeding of the sacral ganglia (Richman et al., 1997)

Human herpes viruses produce a wide spectrum of diseases. Besides genital and oral infections, other conditions include herpetic whitlow, herpetic eye infections,
visceral HSV and other cutaneous infections. Immuno suppressed patients with
deficient cell mediated immunity and neonates have more severe infections and
more frequent recurrences than normal adult patients (Baker and Amstay, 1984).

1.2.4 HSV in immuno-compromised individuals

Patients compromised by either immunotherapy or underlying disease, such as
malnutrition are at risk for severe HSV infection. Severity of HSV in renal,
hepatic, bone marrow and cardiac transplant appear to be directly related to the
degree of immuno-suppressive therapy employed (Rand et al., 1977). The patient
may develop progressive disease involving respiratory tract, oesophagus or even
gastrointestinal tract. Reactivation of HSV infection in these patients can occur at
multiple sites and healing occurs over an average of 6 weeks (Richman et al.,
1997)

Since the first report of HIV/AIDS, the severity of HSV clinical disease in
severely immunocompromised host has been recognized as more frequent and
severe (Richman et al., 1997). Because of persistent and high level viral
replications, resistance to HSV therapy develops (Richman et al., 1997).
Treatment therefore requires higher doses of antiviral medication and
susceptibility testing. Association of HIV with genital herpes was noted as early
as 1981 (Carrasco et al., 2002). Suppressive therapy of HSV significantly increased survival of HIV patients (Carrasco et al., 2002). In HIV infected individuals, genital ulcer lesion may not only be the source HSV but a focus of HIV shedding due to the CD4 cells infiltrating the herpetic lesions (Carrasco et al., 2002).

1.2.5 Diagnosis of HSV infections

Although the vesicular lesions on skin and mucous membrane are usually diagnostic of HSV infection, clinical manifestations involving visceral organs and central nervous system require laboratory confirmation. The three approaches to laboratory diagnosis of HSV infections include the following:

1.2.5.1 Confirmation and typing of HSV isolates

The usual method for confirming HSV infection is isolation of the virus in a cell culture. Most infected specimens can be identified by discernible cytopathic effect (CPE) within 48 – 96 hours after inoculation (Lenette et al., 1995). Virus isolation permits additional testing of the isolate for sensitivity to antiviral drugs or analysis by restriction enzyme testing. Specimens which includes CSF, stool, urine, skin scrapings, throat, nasopharynx and conjunctivae swabs should be transferred in appropriate virus transport media (VTM) to virology laboratory in ice. Inoculation
is made onto cell cultures such as human foreskin fibroblasts and vero cells that are susceptible to CPE characteristic of HSV replication (Richman et al., 1997). While HSV CPE is a usually characteristic method of diagnosis, other viruses such as enterovirus, varicella or cytomegalovirus or toxicity of specimen such as urine or semen can be confused with HSV (Lenette et al., 1995). Herpes simplex typing by type specific monoclonal antibodies is currently the simplest method.

Type 1 and type 2, HSV typing can be accomplished simultaneously by the same technique. Other confirmatory methods are DNA detection, fluorescence antibody test, enzyme immuno assay or restriction endonuclease analysis (Lenette et al., 1995).

1.2.5.2 Serological assessment of HSV

Antibody detection of HSV infection is of little clinical value as therapeutic decision is hardly made solely on results of serologic studies (Richman et al. 1997). The use of ELISA antibody assay allows only definition of past infection or seroconversion but cannot distinguish HSV- 1 from HSV –2. Other commonly used tests for measurement of HSV antibodies are complement fixation tests, passive heamagglutination, neutralization, and immunofluorescence (Richman et al., 1997).
1.2.5.3 Polymerase Chain Reaction (PCR)

Though not cost effective diagnostic method, PCR is the method of choice for HSV infection for CNS (Richman et al., 1997). A primer from an HSV DNA sequence that is common to both HSV-1 and 2 is used to identify the infection agent in CSF. The method has a sensitivity of 95% and specificity that is approaching 100% (Aurelius et al., 1991; Troendle et al., 1993).

1.2.5.4 Restriction Endonuclease Analysis (REA)

This technique provides definitive sub typing of HSV isolates. The REA has been used to study HSV DNA sequence. The studies have revealed DNA differences among strains of HSV 1 and 2. No two epidemiological unrelated isolates of HSV have been found to be identical, suggesting that there may be a large number of HSV strains infecting man (Baker and Amstey, 1984)

1.2.6 HSV chemotherapy

Most of the antivirals against HSV are nucleoside analogs (compounds which mimic the purine and pyrimidine bases of DNA). Acyclovir (zovirax), DHPA (Ganciclovir) and BromovinylDeoxy Uridine (BVDU) are guanine nucleoside
substitute while vidarabine (Ara-A, vira-A) is an adenine nucleoside cogen
(Wright and Archard, 1992).

Acyclovir has become the standard of therapy of HSV infection and is the most commonly prescribed (Richman et al., 1997). The drug inhibits viral replication by chain termination after incorporation into the viral DNA (Lenette et al., 1995). The compound, (9, [2-hydroxyethoxymethyl] guanine, zovirax) is targeted at the viral DNA polymerase (De Clercq, 2001). The specificity is determined by specific viral-encoded thymidine kinase (TK), which ensures and confines the specific phosphorylation of the nucleoside analog to the virus infected cells. The enzyme phosphorylates acyclovir to monophosphate form and is further phosphorylated by cellular kinase(s) to the triphosphate form, which then interacts as a competitive inhibitor/alternate substrate with viral DNA polymerase. If incorporated into the nascent viral chain, the acyclovir obligatorily leads to chain termination (De Clercq, 2001).

1.2.6.1 Treatment indication of acyclovir

Acyclovir is widely used in the treatment of several manifestations of HSV and VZV infections. Indications include primary and recurrent herpes genitalis, herpes
labialis herpetic keratitis, herpetic encephalitis, herpes zoster and often-severe life threatening mucocutaneous HSV and VZV infections in immuno-compromised patients (AIDS patients, cancer patients and organ transplant recipients receiving immunosuppressive agents) (De Clercq, 2001). The derivative of ACV, valaciclovir came from the search for amino acid esters of acyclovir that would have increased oral bioavailability (De Clerq, 2001).

1.2.6.2 Application of acyclovir

The intravenous formulation is recommended for treatment of HSV encephalitis and VZV infection in immunocompromised subjects. Treatment of neonatal HSV infection is also recommended due to its superior toxicity profile (Richman, et al., 1997). For serious mucocutaneous, visceral or central nervous system disease due to HSV or VZV, parenteral acyclovir is the agent of choice unless resistance is suspected. For subjects with normal renal functions, a dose of 5mg/kg body weight 8hourly is appropriate in mucocutaneous disease. A dose of 10mg/kg body weight 8 hourly is recommended for VZV or invasive HSV disease. High VZV dose is indicated due to generally lower susceptibility of isolates. Intravenous and oral acyclovir decreases the period of virus shedding and speed of healing in patients with primary genital HSV infection but topical ointments application only demonstrate marginal efficacy (Richman et al., 1997). For recurrent genital HSV infection, oral acyclovir for 5 days reduces the duration of pain and virus
shedding, but topical ACV is clinically ineffective. Due to short period of recurrent oralabial HSV, the role of topical or oral acyclovir has been difficult to demonstrate (Spruance et al., 1984 and Spruance et al., 1982). Oral acyclovir has shown some benefits for treatment of herpes zoster in adults and varicella in children (Richman et al., 1997).

1.2.6.3 Acyclovir prophylaxis

Oral acyclovir was effective for the suppression of recurrent genital herpes, reducing recurrences by approximately 90% (Richman et al., 1997). Sub clinical shedding of HSV-2 from the genital tract in women can be suppressed by twice daily oral acyclovir. Successful prophylaxis of herpetic whitlow and recurrent erythematous multiform associated with recurrent HSV was also reported (Richman et al., 1997).

1.2.6.4 Acyclovir resistance

Although herpetic infections have been successfully treated with nucleoside analogs such as acyclovir, resistant viruses appear especially in the immunosuppressed patients such as organ transplant recipient and patients with HIV /AIDS (Kurokawa et al., 2001). The mechanism of resistance may be due to lack of viral thymidine kinase, which is not encoded by mutant strains, or mutation of
DNA polymerase gene (DeClerq, 2001; Lenette, et al., 1995). Being the most cost effective drug with very high body tolerance, its resistance justifies the search for new alternatives. Various reports and observations about the plants selected for the study below, identifies them as possible sources of effective anti HSV remedy.

### 1.2.7 Study plants

#### 1.2.7.1 Melia azedarach

*Melia azedarach* belongs to the family *Meliaceae*. The common name is Chinaberry and the Luo in Kenya call it “dwele”. It is a deciduous, wide spreading tree, which grows up to 50 feet tall. The plant grows in variety of soils and is cold – hardy and drought resistant. Chinaberry is a native of Asia where it is revered for its beauty and is used for its medicinal properties. Today, it is widely distributed from the mediteranian region to the tropics up to an altitude of 1800m (Ascher, 1995). Leaves, bark and seeds are poisonous to farm animals (Varamey, 2001; Kokwaro 1993). The plant was selected for study to determine whether extraction targeting broad range of extracts could improve reported moderate activity of ethanolic extract on HSV – 1. (Hudson, 1990)
1.2.7.2 Warburgia ugandensis

*Warburgia ugandensis* belongs to the family *Canellaceae*. Local vernacular names are; “Apacha” (Luyhia), “Muthiga” (Kikuyu), “Olologon” (Maasai), “Soget” (Kipsigis), “Sogo-Maita” (Luo) (Kokwaro, 1993). The tree grows unto 40m tall but often quite short (about 10m) with blackish rough pole and rounded compact crown of shiny dark green leaves. Flowers are greenish, fruits greenish purple and egg shaped (Gachathi and Campbell, 1989). The bark is chewed for toothache, the juice is swallowed for stomachache, constipation, cough, fever, muscle pain weak joints and general body pain, (Kokwaro 1993) water infusion is used as bath medicine against rashes caused by measles virus in Uganda (Olila *et al.*, 2002). It was selected because of its medicinal use on skin as bath medicine and the fact that it’s indicated for virus infection.

1.2.7.3 Vinca rosa

The plant is a common weed throughout the tropics. It is the source of vinca alkaloids, vincristine and vinblastine, used in cancer chemotherapy (Schmidt, 1994). The compounds have the property of being tubulin inhibitor, which is speculated to explain their antiviral property (Hudson, 1990). Vincristine was
found to be effective in reducing the production of infectious Rauscher and Friend leukemia virus in the spleen of mice in the order of 2.6-to 4 log₁₀ (Hudson, 1990).

When the two Vinca alkaloids vincristine and vinblastine were evaluated in the rabbit cornea infected with HSV - 1, they were significantly protective at concentrations of 1000 and 250μg/ml respectively (Hudson 1990). The crude extract of vinca rosea failed to reveal any mortality in mice following oral administration even at 10g/ml body weight within 24 hours of administration. The LD₅₀ value in mice by intraperitoneal route of administration based on 24 hours and 7 days mortality data were 3162.28 (1542.58 – 6482.67) and 954.99(667.83 – 1365.64) mg/kg respectively (Sharma et al., 1990).

1.2.7.4 Plumeria alba

Members of the genus Plumeria are typically small to medium size plants and contain latex, a white thick sticky liquid that has been used for treatment of warts (Schmidt, 1984). Latex of Plumeria alba is popular in Kenya for traditional treatment of herpes lesions caused by Vericella zoster and Herpes Simplex Virus on the skin. According to the author’s communication with ethno-medical practitioners, the medicine is applied on the lesions once a day and is reported to be effective and safe.
CHAPTER TWO

2. MATERIALS AND METHODS

2.1 Plant collection

Based on existing literature and ethno-medical information, plants were collected at Kenyatta University. A botanist assisted with the identification and collection of plant materials. The specimen of each plant was deposited at the department of complementary medicine. Each plant part was handled according to the texture of the material and investigation required as described below.

2.1.1 Leaves

Leaves of *Vinca rosea*, *Warbugia ugandensis*, and *Melia azedarach* were collected in the morning at between 8 and 10 am and delivered to drying room within 30 minutes of collection. The leaves were dried at room temperature for two weeks and the dry leaves ground in electric grinder to form powder.
2.1.2 Plumeria latex

*Plumeria alba* Latex was collected in plastic bags after making a cut on a branch and letting the liquid flow freely in the bags. The collected material was divided into two portions. One portion weighing 100g was mixed with 40 grams of silica gel (mesh size 70-230). The mixture formed a solid paste, which was dried and ground in pestle and mortar to form homogenous fine mixture this extract was designated ‘ground mixture’. The other portion was kept in a freezer at -20°C to be used in its whole form for bioassay and was designated ‘whole latex’.

2.2 Preparation of plant extracts for *in vitro* and *in vivo* bioassays

2.2.1 Leaves extracts

The extraction procedure for plant leaves was according to a method described by Houghton and Raman (1998), but modified to obtain a broad range of chemical components from single extract of each plant. Plant leaves were extracted with dichloromethane and methanol (1:1). Twenty grams of powdered plant material was put in 100ml conical flask and 60ml mixture of the solvents added to immerse the material. The material remained soaked in the solvent for 48 hours before the extract was filtered through cotton wool and the filtrate dried in a hood. The dried material was kept at -20°C in a freezer until required for bioassay.
Leave extract stock at 100mg/ml was prepared by dissolving 0.01g of extract in 100μl of dimethyl sulphoxide (DMSO). The stock was sterilized by filtration with a membrane filter of 0.45μm pore size before test concentration of 2, 1, 1.5 and 0.5mg/ml were prepared using maintenance media as diluent.

2.2.2 Ground mixture

The procedure for latex extraction as described by Houghton and Raman (1998) was adopted. The ‘ground mixture’ was sequentially extracted in a glass chromatography column using hexane, dichloromethane, ethyl acetate and methanol. Silica gel (mesh size 70-230) was packed in column to which 250ml of hexane was passed through. The ‘ground mixture’ of silica gel and latex weighing 87.31 grams was loaded into the column before extraction with 250ml volume of each solvent in sequence. Each extract was concentrated in a rota vapour and the solvent recycled until no more extract was obtained. The extracts were dried in a hood for 48 hours and kept in a freezer at -20°C until required for bioassay. Extracts from hexane, dichloromethane and ethyl acetate were dissolved in dimethyl formamide (DMF) to make stock of 100 mg/ml. The same stock concentration for methanol extract was prepared in DMSO. Test concentrations of 2, 1.5, 1 and 0.5mg/ml were prepared following the same procedure for leaves.
The stocks were filter sterilized using 0.45μm pore size filter before test concentrations were made in sterile maintenance media.

### 2.2.3 Whole *Plumeria* latex

The latex material previously kept in the freezer was thawed at room temperature before gum material was separated from the liquid portion to facilitate filter sterilization. The liquid was sterilized by filtration using filter of pore size 0.45μm. Test concentrations at 1, 2.5, 5, 10 and 20% of latex fluid in maintenance media were prepared in sterile media and used in *in vitro* bioassay. For *in vivo* assay, whole latex without removing the gum was used as described later in the *in vivo* procedure. The latex was collected freshly from the tree on the day the *in vivo* treatment began.

### 2.2.4 Latex cream

The cream is a product developed from *Plumeria* latex and is reported by traditional healers to have longer shelf life compared to whole latex. The cream contains 5% latex in petroleum jelly. The cream was used as is described later, in the *in vivo* assay.
2.3 Acyclovir

Acyclovir (Zovirax from Wellcome company LTD) was purchased and used in in vitro experiment as reference drug. One tablet of acyclovir containing 200mg of acyclovir was ground and dissolved in 20ml phosphate buffered saline to make 10mg/ml stock. The stock was sterilized with membrane filter of 0.45µm pore size before test concentrations of 20, 10, 5 and 2.5µg/ml were prepared in sterile maintenance media.

2.4 Cell and Virus Cultures

2.4.1 Cell cultures

Cells were grown and maintained following routine standard procedure at KEMRI and as recommended by WHO (1997). Vero cell lines were grown in 75cm² culture flasks at 36°C in Eagle’s minimal essential medium prepared in Earl’s balanced salt solution containing 10% inactivated calf serum, 10U/ml penicillin and 10µg/ml streptomycin. Cell monolayer cultures were maintained in the cultivation flasks at 36°C in the same chemically defined medium containing 2% calf serum. For experiments, seeding density was obtained from the flask by trypsinisation and seeded in 96-micro titer well plates (manufactured by Nunc)
company) at $1 \times 10^4$ well. The cells were grown for 24 hours at 36° C in 5% CO$_2$ before being used for *in vitro* experiments.

### 2.4.2 Virus cultures

HSV-1 7401H was obtained from KEMRI. One vial was removed from -80° C cold storage and immediately thawed at 37° C in a water bath. The thawed virus was inoculated in monolayer Vero cells, previously grown and maintained in 75 cm$^2$ tissue culture flask. The infected cells were incubated at temperature of 36° C in 5% CO$_2$ for 24 hours. Virus was harvested after cultivation by 3 times repeated freezing of culture at -80° C and thawing at 37° C. The culture medium was centrifuged at 3000rpm for 10 minutes and the recovered virus in the supernatant kept in a freezer at -80°C until required for titration and bioassay.

For titration, the stored virus was removed from freezer and immediately thawed in 37° C water bath. The stock was diluted to make 1:10$^1$, 1:10$^2$, 1:10$^3$, 1:10$^4$, 1:10$^5$, 1:10$^6$, 1:10$^7$ and 1: 10$^8$ dilutions in maintenance media. Growth media, in 96-well plate was removed after 24 hours of cell growth and replaced with 200µl volume of maintenance media to wash out metabolic waste of the previously growing cells. The maintenance media was removed after one hour and replaced with 200µl volumes of each virus dilution; each dilution was inoculated in four
wells. The infected cells were incubated at 36°C in 5% CO₂ for 3 days. Virus cytopathic effect (CPE) was examined using x10 power objective of inverted microscope and recorded in a titration sheet. Virus infectivity titer was calculated by Karba formula as described in the manual for the virological investigation of polio (WHO, 1997). The titer was expressed in tissue culture infective dose, which destroys 50% of cell culture, replicates (TCID₅₀). An infective TCID₅₀ dose of 10⁶/0.2 ml was obtained and used as 'control virus' in the experiments, where its titer reduction was attributed to antiviral activity of substance under investigation.

2.5 In vitro bioassays

2.5.1 Antiviral activity of diluent solvents

In order to establish the potential effect of diluent solvents. Percentage volumes of DMSO and DMF were prepared in maintenance media and used to determine effects of the solvents on virus replication, so as to eliminate any antiviral activity of solvents. This was achieved by subtracting activity due to solvents from extract solution. The percentage volumes were prepared to correspond with the solvent concentrations, when test extract were made from stock. Thus, 2%, 1.5%, 1% and 0.5% of DMSO and DMF were prepared to represent percentage concentrations
of the solvents when test extracts were prepared at 2mg/ml, 1.5mg/ml, 1mg/ml, and 0.5mg/ml from extract stocks.

2.6 Antiviral activity of plant extracts and acyclovir

*In vitro* assay was performed as described by Christon *et al* (1988). The TCID\textsubscript{50} of control virus was determined in groups of cell cultures maintained in the presence of leave extracts from *M. azedarach, V. rosea, W. ugandensis*, fractions of *Plumeria* latex and whole *Plumeria* latex. Control virus (TCID\textsubscript{50} titer of \(10^6/0.2\) ml) was diluted with various concentrations of each extract and acyclovir to achieve dilutions of \(1:10^1, 1:10^2, 1:10^3, 1:10^4, 1:10^5, 1:10^6\). Each virus dilution was inoculated in four wells of 96 microtiter plate and the plates incubated for 3 days in 5% CO\textsubscript{2} at 36°C. Control virus diluted in maintenance media without test substance was included in the experiment as virus control (VC). Fresh maintenance media was added to two wells in each plate to serve as cell control (CC). Extracts and acyclovir controls were included in each plate by putting 200\(\mu\)l of each test concentration in two wells and cytopathic effect examined to determine the effect of the substances on the cells. After 3 days of incubation, tissue culture infective dose (TCID\textsubscript{50}) was calculated on the basis of virus-specific cytopathic effect observed in each group of infected cells. Karba formular (WHO, 1997) was used to calculate TCID\textsubscript{50} as shown:
\[ \text{Log}_{10} \text{TCID}_{50} = L - d (S - 0.5), \]

Where:

- \( L \) = Log of lowest dilution used in the test;
- \( d \) = difference between log dilution steps
- \( S \) = sum of proportion of positive tests (i.e. cultures showing CPE)

Activities of the compounds were obtained by subtracting the log of experimental titer from the log of reference control titer as shown below:

\[ (\text{log}_{10} \text{ TCID}_{50} \text{ of reference control titer} - \text{log}_{10} \text{ TCID}_{50} \text{ of experimental titer}). \]

### 2.6 In Vivo antiviral activity of whole *Plumeria* latex

*In vivo* efficacy was done in pathogen free 6-weeks old male Swiss mice obtained from KEMRI. The mice weighed between 16 to 18 grams. The mice were randomly divided into six groups, each group comprising of 5 mice. Each group was put in a separate cage and fed with mice cubes from Unga limited and water *ad libitum*. The groups were labeled as group I, II, III, IV, V and VI. Where groups I – IV were experimental while groups V and VI were controls.

For viral inoculations, the animals were shaved on the right mid flank, using an electric shaver. Veet hair remover cream was applied on the shaved area and allowed to act on the remaining hair for 5 minutes before being washed with warm water. After 48 hours, the animals were inoculated with HSV-1. The shaved
right mid flank of mice was scratched with a bunch of gauge 26 hypodermic needles to facilitate virus infection in the skin. Groups I, II, III and IV, were inoculated with 50µl of control virus according to method of Tolo et al. (2002) and Kurokawa et al. (2001). Group V and VI were not infected and named latex control and negative control group respectively. Group I, II, and III received treatment with *Plumeria* cream, *Plumeria* latex and acyclovir cream respectively. Group IV received no treatment and was named positive control. Treatment of mice began 24 hours after infection, at the site of inoculation, once daily topically, for 7 days. Comparison of responses to infection in various treatments was done

2.7 Toxicity assays of whole *Plumeria* latex

This was carried out in two stages, the *in vitro* and *in vivo* assays.

2.7.1 *In vitro* toxicity assay

*In vitro* toxicity of whole *Plumeria* latex was tested by examining its effect on viability of Vero cells. The method was adopted from routine methods of determining viable counts of cells for subculture (Rovozzo, 1973), but modified by including latex in maintenance media. Vero cells were seeded at 1x10⁴ cells/well and grown for 24 hours. Growth medium was replaced with maintenance medium and left in the wells for one hour. The media was then
replaced with the solution of latex in maintenance media and the plate incubated in 5% CO₂ at 36°C for 3 days. The latex solution was removed and cells washed twice with phosphate buffered saline. Twenty micro liters of 0.25% trypsin was put in the wells and left to act on the cells for 3 minutes before 20 μl of 0.04% trypan blue solution was added and mixed with the cells. The cells were counted in four corner squares of hemocytometer and the count expressed in count/ml as shown in the formula, \( \text{Count / ml} = \frac{N}{4} \times 10^4 \times 2 \), where N is the cell counted in four corner squares of hemocytometer chamber, divided by 4 to obtain count per square and 2 is the dilution factor. Therapeutic index of latex was calculated by the method used by Horvath (1984). The index is obtained by calculating the ratio of maximum non-toxic concentration of latex to maximum concentration of latex that did not inhibit virus replication \( \log_{10} \text{maximum none toxic concentration} - \log_{10} \text{maximum none inhibitory concentration} \)

2.7.2 In Vivo toxicity assay

In vivo cytotoxicity was evaluated in 6-weeks old male mice. Ten mice were randomly divided into two groups comprising of 5 mice each, thus group VII and VIII. The mice were shaved on the right middle flank and scratched with a bunch of gauge 26 hypodermic needles. Group VII mice were the treatment group to which Plumeria latex was administered on the scratched area of the skin once a day for seven days. Group VIII mice were the control and were not treated. After
7 days, all the mice in both group VII and VIII were sacrificed and the shaved portions of skin removed for histology. The histological method of tissue processing and examination was as described by Leeson and, Leeson, 1970. The skin tissues were fixed in 10% formal saline for 3 days before the tissues were dehydrated with ethanol, cleared with xylene and infiltrated with paraffin wax in a 24-hour tissue processor machine. The infiltrated tissue was blocked with paraffin wax to facilitate sectioning for staining and microscopy. The blocks were cut in a microtome (Rotary HM 340) and the thin sections were floated in water bath at 37°C to enable them to stretch out before being fished out by attaching onto labeled clean slides. The sections attached on the slides were cleared free of paraffin wax by immersion in two changes of xylene and in descending percentages of ethanol at 100%, 90% 70% 50% and finally in water.

The sections were stained with hematoxylin and Eosin staining procedure (Leeson and, Leeson, 1970). Hematoxylin and Eosin stains cell nucleus dark blue, and both cytoplasm and connective tissue pink – red. Tissue changes due to toxic effects include increased proliferation of fibroblasts of the connective tissue, thickening of tissue fibers and infiltration of inflammatory cells (Kulonen and Pikkarainen, 1973). The tissues were examined for presence of inflammatory cells (neutrophils, basophils and eosinophils). The comparative appearances of connective tissue fibers of treated and untreated mice were examined and the number of fibroblast per high power objective determined. The fibroblasts were
counted in 5 fields using x 40 power objective and the average count per field calculated for each tissue. Mean count per group of mice was then calculated from the count per field of each tissue in the group.

2.8 Data analysis

Data analysis for drug efficacy was done by student t-test to compare the differences in mean days at which initial symptoms were observed and mean days at which symptoms progressed to zosteriform. Mean days at which death occurred was used to calculate mean survival time in the groups affected. Responses to various treatments were compared to infected but untreated mice (positive control group) to determine therapeutic efficacy. The student t-test was also used to determine statistical difference in mean fibroblast counts, to evaluate toxicity by mitotic response of mice to treatments with *Plumeria* latex. A ‘p’ value less than 0.05 was considered statistically significant.
CHAPTER THREE

3 RESULTS

3.1 Antiviral activity of diluent solvents

Baseline titer results of control virus in solvents are presented in table 1. The results showed that dimethyl sulfoxide did not have antiviral effect as the control virus titer remained at TCID$_{50}$ of $1 \times 10^6/0.2$ ml. Dimethyl formamide on the other hand showed antiviral effect and it reduced control virus up to 1.5 log$_{10}$ at a concentration of 2%.
Table 1: Effect of diluent solvents on virus replication

<table>
<thead>
<tr>
<th>% Of solvent in maintenance media</th>
<th>Virus yield in solvents TCID&lt;sub&gt;50&lt;/sub&gt;/0.2ml</th>
<th>Titer reduction in logs&lt;sub&gt;10&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMF</td>
<td>DMSO</td>
</tr>
<tr>
<td>0.5</td>
<td>1x10&lt;sup&gt;5.25&lt;/sup&gt;</td>
<td>1x10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>1%</td>
<td>1x10&lt;sup&gt;4.75&lt;/sup&gt;</td>
<td>1x10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.5%</td>
<td>1x10&lt;sup&gt;4.75&lt;/sup&gt;</td>
<td>1x10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>2%</td>
<td>1x10&lt;sup&gt;4.5&lt;/sup&gt;</td>
<td>1x10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

DMF-Dimethyl formamide, DMSO-Dimethyl sulfoxide

3.2 In vitro antiviral activity of plant extracts and whole Plumeria latex

Table 2 shows in vitro activities of plant extracts against HSV-1. The results are presented as log<sub>10</sub> reduction of reference virus titer. All leaf extracts had no antiviral activity but caused cell lysis at concentrations above 0.5mg/ml. Plumeria latex fractions at 1mg/ml showed differing antiviral activities. Ethyl acetate fraction gave the highest titer reduction of 1.5, followed by dichloromethane with 0.75 and hexane, 0.25. All the three fractions caused cell lysis at 1.5 mg/ml. Methanol fraction showed no activity below 1.5 but caused
cell lysis at 2mg/ml. Table 3 represents *in vitro* antiviral activity of whole *Plumeria latex*. The highest detectable antiviral activity of whole latex of 1.25 was lower compared to highest detectable activity of its fractions.
Table 2: *In vitro* antiviral activity of solvent extracts against HSV-1

<table>
<thead>
<tr>
<th>Medicinal Plant</th>
<th>Part used</th>
<th>Extraction solvent</th>
<th>TITER REDUCTION <em>(TCID&lt;sub&gt;50&lt;/sub&gt;/0.2ml)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5mg/ml</td>
</tr>
<tr>
<td><em>Vinca rosea</em></td>
<td>Leaves</td>
<td>DCM/MEOH</td>
<td>0</td>
</tr>
<tr>
<td><em>Melia azedarach</em></td>
<td>Leaves</td>
<td>DCM/MEOH</td>
<td>0</td>
</tr>
<tr>
<td><em>Warbugia ugandensis</em></td>
<td>Leaves</td>
<td>DCM/MEOH</td>
<td>0</td>
</tr>
<tr>
<td><em>Plumeria alba</em></td>
<td>Latex</td>
<td>HXN</td>
<td>0</td>
</tr>
<tr>
<td><em>Plumeria alba</em></td>
<td>Latex</td>
<td>DCM</td>
<td>0</td>
</tr>
<tr>
<td><em>Plumeria alba</em></td>
<td>Latex</td>
<td>EthAT</td>
<td>0</td>
</tr>
<tr>
<td><em>Plumeria alba</em></td>
<td>Latex</td>
<td>MEOH</td>
<td>0</td>
</tr>
</tbody>
</table>

*(TCID<sub>50</sub>/0.2ml) = (log<sub>10</sub> TCID<sub>50</sub> of reference control titer – log<sub>10</sub> TCID<sub>50</sub> of Experimental titer)*

HXN – hexane, DCM – Dichloromethane, EthAT – Ethyl acetate,

MEOH – Methanol, DCM/MEOH – Dichloromethane/Methanol mixture

CL – Cell lysis, NT – Not tested, 0 – No titer reduction obtained
### Table 3: In vitro antiviral activity of whole *Plumeria* latex

<table>
<thead>
<tr>
<th>Percentage of latex in maintenance media</th>
<th>0</th>
<th>1</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus yield (TCID&lt;sub&gt;50&lt;/sub&gt;/0.2ml)</td>
<td>1x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1x10&lt;sup&gt;5.75&lt;/sup&gt;</td>
<td>1x10&lt;sup&gt;4.75&lt;/sup&gt;</td>
<td>CL</td>
</tr>
<tr>
<td>Titer Reduction</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.25</td>
<td>1.25</td>
<td>CL</td>
</tr>
</tbody>
</table>

<sup>1</sup>(TCID<sub>50</sub>/0.2ml) = (log<sub>10</sub> TCID<sub>50</sub> of reference control titer − log<sub>10</sub> TCID<sub>50</sub> of experimental titer)  
CL = Cells lysis

### 3.3 In Vitro antiviral Activity of Acyclovir

Antiviral activity of acyclovir at 0 to 20μg/ml was determined from control virus titer (TCID<sub>50</sub> =1x10<sup>6</sup> /0.2 ml.). The results are presented in table 4. Acyclovir reduced virus titer by 4.75, 4.5, 3.75 and 1.5 logs for drug concentrations, 20, 10, 5, and 2.5μg/ml respectively.
Table 4: *In vitro* antiviral activity of acyclovir against HSV-1

<table>
<thead>
<tr>
<th>Acyclovir concentration µg/ml</th>
<th>20</th>
<th>10</th>
<th>5</th>
<th>2.5</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus yield (TCID&lt;sub&gt;50&lt;/sub&gt;/0.2ml)</td>
<td>1x10&lt;sup&gt;−1.25&lt;/sup&gt;</td>
<td>1x10&lt;sup&gt;−1.5&lt;/sup&gt;</td>
<td>1x10&lt;sup&gt;−2.25&lt;/sup&gt;</td>
<td>1x10&lt;sup&gt;−4.5&lt;/sup&gt;</td>
<td>1x10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Titer reduction&lt;sup&gt;+&lt;/sup&gt;(TCID&lt;sub&gt;50&lt;/sub&gt;/0.2ml)</td>
<td>4.75</td>
<td>4.5</td>
<td>3.75</td>
<td>1.5</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>+</sup>(TCID<sub>50</sub>/0.2ml) = (log<sub>10</sub> TCID<sub>50</sub> of reference control titer − log<sub>10</sub> TCID<sub>50</sub> of experimental titer).

### 3.4 *In vivo* antiviral activities of plant extract and acyclovir

Response of mice to different treatments with *Plumeria alba* cream, Plumeria *alba* latex and 5% commercial acyclovir cream are shown in tables 5 – 8. The responses were monitored among the six experimental groups as described in materials and methods section. *Plumeria* cream treatment results are presented in table 5. The results show that onset of symptoms began on day 4 post-infection. The transition from ulcer to severe zosteriform stage was so rapid that only 1 mouse was noticed with zosteriform symptom on day 6. The first death of mice occurred on day 6 and by day 8, all mice had died.
Table 5: Response of infected mice to *Plumeria* cream

<table>
<thead>
<tr>
<th>Day</th>
<th>Number of Mice and Conditions observed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Symptom</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

As shown in table 6, whole *Plumeria* latex prevented infection manifestation in 3 out of 5 mice by day 7. Of the affected mice, one mouse had a vesicle that lasted for less than 24 hours and one died. By day 14, four out five mice had died. Three died without skin lesions. In the group treated with whole *Plumeria* latex without infection, there were no symptoms or death observed.
Table 6: Response of infected mice to whole *Plumeria* latex

<table>
<thead>
<tr>
<th>Day</th>
<th>Number of mice and conditions observed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No symptom</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>14</td>
<td>1</td>
</tr>
</tbody>
</table>

Results of response of infected mice to treatment with acyclovir are presented in table 7. Though one mouse developed vesicles on day seven after infection, which lasted for only 24 hours, all the five mice survived for the entire period of the experiment. Compared with the treatment groups, the first symptom in the positive control (group IV) developed symptoms in day 4 and all died by day 9.
(Table 8). In the negative control (group VI), all the mice survived the entire period of experiment without any symptom.

Table 7: Response of infected mice to Acyclovir cream

<table>
<thead>
<tr>
<th>Day</th>
<th>Number of mice and conditions observed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No symptom</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
</tr>
</tbody>
</table>
Table 8: Response of infected and untreated mice (Positive Control)

<table>
<thead>
<tr>
<th>Day</th>
<th>No symptom</th>
<th>Vesicle</th>
<th>Ulcer</th>
<th>Zosteriform</th>
<th>Severe zosteriform</th>
<th>Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

3.5 A comparison of responses to treatments

Table 9 represent results of the response comparison of in vivo treated groups. Mice treated with latex cream did not delay onset of disease, disease progression or death. Whole Latex and acyclovir significantly delayed symptoms when each group’s mean day of disease onset was compared with infected but untreated mice (p<.05). Only 1 infected mouse in each group treated with whole latex and acyclovir developed vesicles on day 7. Mice treated with whole latex and positive control group however did not show significant difference in survival time or
mortalities, p= .337. However, severe forms of the disease, zosteriform or severe zosteriform (score 6/8) were not observed in infected mice treated with whole latex. Acyclovir prevented death in all mice receiving the treatment and also prevented severe forms of the disease. All mice in positive control group developed preliminary symptoms by day 5.2 and all were dead by the 9th day. Groups of uninfected mice without treatment (Negative control) and the group treated with whole latex without infection (latex control) survived and showed no symptoms. This showed that the deaths and disease observed were as a result of infections and not due to treatments or any other unexplained cause.
Table 9: A comparison of responses to treatments

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Treatment</th>
<th>Mean time (Days ±SD)</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Score 2/4</td>
<td>Score 6/8</td>
</tr>
<tr>
<td>I</td>
<td>Plumeria cream</td>
<td>4.2±0.45</td>
<td>6</td>
</tr>
<tr>
<td>II</td>
<td>Plumeria latex</td>
<td>7a</td>
<td>NO</td>
</tr>
<tr>
<td>III</td>
<td>Acyclovir cream</td>
<td>7a</td>
<td>NO</td>
</tr>
<tr>
<td>IV</td>
<td>Positive control</td>
<td>4.7±0.5</td>
<td>6.2±0.45</td>
</tr>
<tr>
<td>V</td>
<td>Latex</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>VI</td>
<td>Negative control</td>
<td>NO</td>
<td>NO</td>
</tr>
</tbody>
</table>

NO - indicates that no symptom was observed. NC - not calculated because all mice survived. Latex^c (latex control). *Only one mouse developed the symptom.

Score 2/4 (2 or 4) indicates mean time at which preliminary symptoms, vesicle or ulcer were initially observed. Score 6/8 (6 or 8) indicates mean time at which advanced symptoms, zosteriform or severe zosteriform were initially observed.
3.5 Toxicity assays

In vitro toxicity of whole *Plumeria* latex is presented in table 10. The maximum non-toxic concentration of *Plumeria* latex was 2.5% as no reduction in viability of cells was observed at this concentration. More than 2.5% concentration dose gave reduction in cell viability, proportional to the increased concentration.

### Table 10: Effect of *Plumeria* latex on Vero cells

<table>
<thead>
<tr>
<th>Percentage of latex in maintenance media</th>
<th>50</th>
<th>25</th>
<th>10</th>
<th>5</th>
<th>2.5</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>%Reduction of viable cell count</td>
<td>100</td>
<td>83</td>
<td>22</td>
<td>11.6</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 11 shows *in vivo* toxicity effect of whole *Plumeria* latex. Mean fibroblast counts in tissues of treated group of mice was 145/HPF and the untreated group was 110/HPF, indicating 32% more counts in treated mice. The mean counts of treated mice were statistically different from untreated group at p< 0.05. Connective tissue fibers of treated mice were much thicker compared to untreated group showing that the plant material affected the natural structure of skin tissue, suggesting increased cell death. No inflammatory cells particularly neutrophils,
Eosinophils or basophils were observed in all tissues, indicating that *Plumeria* latex does not elicit immunological reaction from the mammalian subjects.

**Table 11: Counts of fibroblast in the dermal layer of skin tissue of mice treated with whole *Plumeria* latex and the untreated mice**

<table>
<thead>
<tr>
<th>Fibroblast counts</th>
<th>Latex treated mice</th>
<th>None-treated mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group VII</td>
<td>Group VIII</td>
</tr>
<tr>
<td>162</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>136</td>
<td>113</td>
<td></td>
</tr>
<tr>
<td>152</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>134</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>141</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>Mean = 145</td>
<td>Mean = 110</td>
<td></td>
</tr>
<tr>
<td>SD = 11.79</td>
<td>SD = 4.28</td>
<td></td>
</tr>
</tbody>
</table>
3.6 Photographic presentations of antiviral activity and toxicity on various experimental settings

The photographic illustrations show observations made on Vero cells (photograph 1 and 2), experimental animals (photograph 3 to 9) and histological preparations of skin tissues (photograph 10). Photograph 1 shows non-infected Vero cells in cell control well, notice the cylindrical appearance (typical of fibroblastic cell morphology) and evenly spread monolayer of cells as opposed to photograph 2 where cells are rounded and are clumping together due to cytopathic effects of HSV-1. Photograph 3 shows mouse infected with HSV-1 on day two of experiment. The mouse is a representative of the group infected but not treated (positive control). Inoculation scar is visible but herpetic lesions have not developed.

In photograph 4, a positive control mouse has developed ulcer lesions (Score 2/4) on day five of experiment. In photograph 5, Symptoms have developed to severe zosteriform on day seven (Score 6/8). Photograph 6 is a representative mouse on day seven of latex treatment, notice latex particles around the healing site of inoculation. The site has no herpetic lesions as opposed to photograph 5. Photograph 7 is a mouse in the group treated with acyclovir on day seven of experiment, the inoculation scar has healed and there are no herpetic lesions. The
mouse resemble the one in photograph 8 which shows a representative of mice in the group that was not infected but scratched (Negative control). There is no scar or lesion as opposed positive to control mouse on day 7. Photograph 9 illustrates the lesion after death of infected mouse, the mouse was previously receiving *Plumeria* cream and was the first death recorded on day six of experiment. Photograph 10 shows histological preparation of skin tissues of mice for *in vivo* toxicity experiments. The photograph shows two tissues for comparison. Photograph (a) is a tissue of mouse that was not treated with *Plumeria* latex. Notice the difference with photograph (b) below it. Remarkable changes are thick connective tissue and pink debris, characteristic of protein materials ([Leeson and Leeson, 1970](#)). The debris was attributed to dead cells due to effect of *Plumeria* latex.
Cytopathic effects of HSV on Vero cells seen on the 3rd day, after inoculation. The changes seen are rounding up, enlargement and grouping of cells.

Photograph 1: Cytopathic effect of HSV-1 on Vero cells.

Cells in cell control well. The uninfected cells are cylindrical and evenly distributed.

Photograph 2: Monolayer of Vero cells without cytopathic effect
Inoculation site. No infection lesions were observable on day 2

Photograph 3: Positive control mouse before onset of symptoms on day 2

Photograph 4: Positive control mouse with ulcer lesion on day 5
Photograph 5: Positive control mouse with severe zosteriform on day 7

Severe zosteriform on day 7 of experiment

Photograph 6: Infected mouse treated with Plumeria latex on day 7

Inoculation scar of mouse treated with latex of Plumeria alba. The scar is surrounded with particles of latex. No infection lesion is observable
Photograph 7: Infected mouse treated with 5% commercial acyclovir cream on day 7. No inoculation scar or lesion was observable
Photograph 8: Uninfected and untreated mouse (Negative control) on day 7.

No scar or lesion was observable
Photograph 9: A dead mouse that was receiving *Plumeria* cream but died on day 6
Photograph 10: Tissue of untreated mouse (a) and Tissue of treated mouse (b). No inflammatory cells were seen in all tissues but tissue fibers of treated mouse appear thick with deposits of cell debris.
CHAPTER FOUR

4.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 DISCUSSION

The medicinal plants used in the study were selected on the basis of reported activity in various literature sources and information from traditional applications (Hudson 1990; Kokwaro, 1993; Olilo et al., 2002; Schmidt, 1984). Leaves of *M. azedarac*, *V. rosea* and *W. ugandensis* were extracted with an equal mixture of dichloromethane and methanol to obtain broad range of compounds that could be separated on polarity basis and tested individually, if initial screening results gave antiviral activity. Measurement of *in vitro* antiviral activity was achieved by monitoring reduction in log TCID$_{50}$ of reference control virus. The method measures infectious virions only and is widely accepted, since the aim of antiviral assay is to determine whether a compound/extract reduces infectivity of viruses without causing cell toxicity (Hudson 1990). The method was used to determine effects of guanisidine on Adenovirus and several compounds on HSV and Rubellavirus (Horvath, 1984; Christon et al., 1988).
To obtain the antiviral activity of plant extracts on the titer of reference control virus, effects of diluent solvents on virus replication were first determined and eliminated by subtracting the antiviral value if the solvent caused titer reduction.

Results of the solvent assays showed that Dimethylsulphoxide (DMSO) was the best solvent, as it did not reduce the replication of control virus. The TCID$_{50}$ titer of the control virus remained at 6log$_{10}$ for 2% DMSO, the largest percentage when test concentrations were made from stock. The solvent was used by Horvath et al (1984) up to a final concentration of 5% in maintenance media. The result implied that, any observed reduction of control virus titer by the extracts solution could directly be attributed to the effect of the extract under antiviral test.

Dimethylformamide (DMF), as opposed to DMSO, showed activity against HSV, reducing the titer of control virus by 0.75, 1.25, 1.25 and 1.5 log$_{10}$ at 0.5, 1, 1.5, and 2% respectively. Effect of the solvent on titer reduction was therefore subtracted from the total effect to obtained activity due to extract under assay (Hudson, 1990). Results of in vitro experiments showed no antiviral activity of leave extracts from V. rosea, M. azederach and W. ugandensis at 0.5mg/ml but caused cell lysis at 1mg/ml. The finding obtained with the leaves of M. azedarach was different from those obtained in a screening programme in Argentina where extracts obtained with ethanol was active at 50μg/ml against HSV-1 (Hudson 1990). The lack of activity could be attributed to variation in the amount of active
components in plants of two geographical locations or diluting effect of many inactive compounds due to broad-spectrum extraction with dichloromethane/methanol mixture.

Findings on *W. ugandensis* are similar to that by Olilo *et al* (2002) where no activity was obtained with trials of water and ethanol extracts on measles virus. This plant was selected on the basis of its use as bath medicine for skin ailments (Olilo *et al.*, 2002). Flower of *V. rosea* is known to contain the *Vinca* alkaloids, *vincristine* and *vinblastine* reported to protect rabbit from herpetic keratitis (Tokamaru and Avitabule, 1971). Results with the leaves indicate that the alkaloids either were too little in the extract or as in the case of *M. azederach* were diluted by inactive compounds.

The main plant of the study was *Plumeria alba*. The latex of this plant is used widely for treatment of skin lesion caused by *Herpes Simplex Virus* (HSV) and *Vericella Zoster Virus* (VZV). The plant latex is applied on the lesions once daily for seven days. The lesions are reported to heal within 2 weeks with less pain during the course of disease and after recovery. Treatment with *Plumeria* latex is reported to prevent development of scars that are usually associated with healing of infected skin after herpes zoster and the disease is reported to recur less
frequently. Latex cream was developed to prolong the shelf life of the medicine for up to 6 months. The ingredients of the cream are 5% latex in petroleum jelly.

Four solvent fractions of the latex, obtained by sequential polarity based extractions in hexane, dichloromethane, ethyl acetate and methanol extracts were tested separately to determine the most potent fraction. At 1mg/ml, the solvents fractions of *Plumeria* latex gave varied results against HSV-1. Hexane reduced virus yield by 0.5, dichloromethane 0.75 and ethyl acetate 1.5 log units. Antiviral activity was absent in methanol extract. This indicates that, compounds responsible for activity were concentrated in ethyl acetate fraction. Since activity was absent in methanol but distributed between hexane and ethyl acetate, the responsible compound(s) are therefore of moderate polarity and can be identified from ethyl acetate fraction after further chromatographic separations.

*Plumeria* latex is however used traditionally in its whole form without solvent fractionation. It was therefore evaluated for antiviral activity in this form, except that the gum was removed to facilitate filter sterilization. Percentage volumes of the fluid obtained in maintenance media gave reduction activity at 1.25 and 0.25 log units for 10% and 5% respectively. There was no antiviral activity at 2.5% and below. This finding seem to support the use of latex cream made of 5% w/w in petroleum jelly *in vivo* on human hosts, as observed in ethno medical practice.
Results of acyclovir activity indicated that it is superior to both *Plumeria* fraction and its whole form. The highest titer reduction activity of latex fraction was 1.5 logs at 1mg/ml. The same titer reduction was obtained by only 2.5μg/ml of acyclovir showing superior potency. The nucleoside analog however, was a pure compound as opposed to plant extracts that were a mixture of many compounds.

An *in vitro* experiment, however, even if accurately done is merely a preliminary screening procedure, whose results require to be confirmed *in vivo* (Hudson 1990). Its utility is limited when the ultimate aim of a compound is to control infections *in vivo*. This is due to the fact that, several conditions vary between *in vitro* and *in vivo* state. Variables of *in vitro* state that differ with *in vivo* experiments include; dissimilar physiological situation of *in vivo* cells and variety of cell types with different individual responses as opposed to single cell lines *in vitro* (Hudson, 1990). Cell culture experiments have thus been considered a mere preliminary screening activity and some workers have actually bypassed it completely and instigated *in vivo* screening. The argument held is that the only real test of a compound’s potential is to evaluate it in an animal model of infection (Horvarth, 1984; Hudson, 1990).

For *in vivo* experiment, latex was obtained fresh from the plant on the day treatment began and kept at 4°C for the seven-day period of administration.
Freshness of latex was required to conform with direction of use as advised by ethno-medical practitioners. The cream was prepared on the eve of experiment and kept as for the latex. The materials were topically applied as is done in traditional setting. Results are presented in mean days at which symptoms were initially observed or survival time in days as shown in Table 5 to 9. Preliminary symptoms of the infected mice are indicated as score 2/4 (vesicle /ulcer) and severe symptoms as score 6/8 (zosteriform/severe zosteriform). The scores were grouped as 2/4, or 6/8 for ease of recording and comparison, since progression intervals between 2 and 4; 6 and 8 were rapid and also difficult to differentiate. Efficacy was compared on the basis of delay of disease onset, survival period, prevention of lesion progression and death. The differences in mean days at which symptom or death were observed was analyzed by student t-test.

Results obtained indicated that infected mice treated with latex prevented development of symptoms in 4 mice out of 5. The only symptom, the vesicles observed in one mouse and recorded on the seventh day after infection healed within 24 hours. Although 4 out of the 5 mice died, 3 died without symptoms. The onset of vesicle on day 7 was significantly different compared with day 4.7, mean day of disease onset in infected but untreated group (positive control) \( p < 0.05 \).

Mean survival time of mice treated with *Plumeria* latex was increased, though not statistically significant compared to positive control group, \( p < 0.337 \). Mice treated with *Plumeria* cream and positive control showed no significant difference in
disease onset, progression or death. No symptoms or deaths were observed in latex drug control and negative control mice, suggesting that mice deaths could be attributed to virus effects but not latex toxicity or any other different cause.

All the mice treated with reference drug, the acyclovir survived up to the end of experiment but like latex, one mouse developed vesicles on day 7 which healed within 24 hours. The observations were recorded for 28 days but after 14 days of experiment, no further changes in observations were noted. In the group of mice treated with Plumeria cream and the positive control group, observations ended on day 8 and 9 respectively, when all the mice had died.

Although there was no significant different effect of latex on mice survival compared to positive control group (p<.337), it is significant that only one mouse developed vesicles that disappeared by 24 hours. Further more, no severe symptoms were observed. This indicates that the antiviral property in the latex material prevented replication of the virus on the superficial layer of skin tissues but death occurred due to poor penetration of the material. The virus thus continued to replicate in deeper layers of the skin resulting to dissemination of high viral load. Dissemination of virus results in fatalities because vital internal organs such as the brain and the liver are usually involved (Lenette, 1995). Acyclovir on the other hand prevented death of all mice and significantly delayed
onset of symptoms in the only one mouse affected. This was statistically different at p< 0.05 compared with untreated mice (positive control). It also prevented progression of lesion scores.

The findings with latex cream on mice model are different from the observations in traditional practice, where human hosts realize healing effect within 14 days. This can be explained in terms of immunity of human hosts. The 6-week-old mice had no previous encounter with HSV as compared to practical clinical situations of many patients, whose infections are usually secondary. The mice therefore faced a bigger challenge and were more likely to succumb to the infection.

*In vitro* toxicity studies of *Plumeria* latex gave results, which indicated that the latex is toxic to cell monolayers except from percentage volumes below 2.5%. A potent and selective antiviral compound at zero toxicity should have viral inhibition higher than 6log₁₀ (more than 99.9999% inhibition) with therapeutic index higher than 3log₁₀ (Hudson, 1990, Horvath, 1984). According to Horvath (1984), therapeutic index is based on the ratio of maximum concentration of a compound that show no toxic effect compared to maximum concentration of compound that has no detectable virus inhibition. The maximum percentage concentration of latex giving zero toxicity was 2.5% but at this concentration, no
virus inhibition was observed. This gave therapeutic index of zero (log 0.25 - log 0.25). Although compounds with therapeutic index of zero or negative value have the likelihood of inducing resistance by the infecting agents (Horvath, 1984), in vitro therapeutic index, may not necessarily apply in vivo where its use is intended (Hudson 1990). It however offers a useful guide to the potency of a compound. Compounds that inhibit virus replication at concentrations above none toxic levels, e.g. 5-ido-dUrd, are usually applied only in topical treatment of local virus infection (De Clercq, 1979; De Clercq et al., 1981). At the antiviral level that showed toxicity, the latex is correctly administered traditionally as a topical treatment.

Toxicity effect of latex on the skin of mice was investigated by histological examination of stained skin tissue of mice previously treated with latex following the same regime as for therapeutic application. Toxicity evaluation by this approach was designed to closely resemble therapeutic use. Toxicity assessment was based on determining whether the mice responded to latex by immunological reaction, increased mitosis of fibroblastic cells in the dermal layer of the skin or altered appearances of connective tissue fibre. Immunological response was evaluated by examining the tissues for the presence of neutrophils, eosinophils or basophils, while counts of fibroblast was used to determine mitotic activity of latex. The general appearance of connective tissue matrix was used to assess none
specific toxicity. No immunological indicator cells were noted, showing that latex does not activate immune system of mice. This finding could probably be different if latex penetrated into deep layers of skin tissue where contact with sensitive immune system is close. The finding of fibroblast count showed that latex caused increased mitosis of fibroblasts in dermis layer by 32%. This increase was statistically significant when t-test was used to compare means of treated and untreated mice group $p < 0.05$. The increase can be attributed to cytotoxicity and death of superficial cells, thereby activating cellular mitosis. This interpretation is supported by the fact that tissue debris was more in tissues of treated mice compared to untreated ones.

4.2 CONCLUSIONS

- Leave extracts of *Melia azedarach*, *Vinca rosea* and *Warburgia ugandensis* failed to give any antiviral response, indicating low efficacy as compared to acyclovir.

- The maximum titer reduction shown by latex fraction was $1.5 \log_{10}$ at 1mg/ml., giving lower activity as compared to acyclovir, which reduced infectivity of HSV by $4.75 \log_{10}$ at 0.02mg/ml.
The active principles in *Pumeria* latex are of moderate polarity since the antiviral activity was concentrated in ethyl acetate fraction.

On *in vivo* efficacy, therapeutic findings obtained, when each drug was used as indicated by traditional method or manufacturer, showed that acyclovir had better efficacy than latex or latex cream, since no mice died in the group treated with the nucleoside analog as opposed to the plant products.

Toxicity results indicate that latex of *Plumeria alba* is toxic to cell lines at antiviral concentrations *in vitro* and also stimulates mitotic activity *in vivo* due to its cytotoxic activity on epidermal cells.

The present study therefore disapproves the stated hypothesis but may be different if active principles of the medicinal plants were isolated and tested in pure form as for acyclovir. The low activity of the plant extracts against HSV may have been because inactive compounds present in crude plant materials diluted the available active principles.

The traditional therapeutic use can still be helpful as an alternative to conventional drugs when cost and benefit factors are taken into consideration.
4.3 **RECOMMENDATIONS**

- The active components in *Plumeria* latex need to be identified and evaluated to determine their antiviral potency and selectivity.

- *In vitro* activity need to be tested in variety of other cells since different cell lines are known to respond differently to actions of drugs and infections.

- The medicinal efficacy should also be evaluated on other animal to obtain broader data on animal models.
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