A HERBAL REMEDY
FOR
HERPES SIMPLEX VIRUS INFECTION

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the Degree of Master of Science (Infection Diagnosis) of

Kenyatta University

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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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I dedicate this work to my parents, REV. WALTER EDWIN TOLO and MRS. MARY NANZALA TOLO, whose tenderness and love made me what I am today.
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Abstract

Herpes Simplex Virus (HSV) infection has emerged as a major opportunistic infection particularly in immunosuppressed persons and therefore a serious disease in high Human Immunodeficiency Virus/Aquired Immunodeficiency Syndrome (HIV/AIDS) prevalence areas as seen in sub-Saharan Africa. Present drugs for HSV management, such as the nucleoside analogue acyclovir, are experiencing resistance from the re-emerging strains of HSV. In this study, aqueous extracts from 12 medicinal plants were investigated for anti-HSV activity. The plants were collected within Kenya from medicinal plants used widely for the management of various diseases using information obtained from literature search and ethnomedical use. Plaque reduction assay was used to determine the in vitro anti-HSV activity for the extracts by evaluating the ability of the extracts to inhibit the formation of plaques in Vero E6 cells infected with 100 Plaque Forming Unit (PFU) of HSV. A 50% inhibition at 50μg/ml extract concentration was registered as active. A murine model, in which BALB/c mice were cutaneously infected with HSV and treated with extract, was used for in vivo determinations of therapeutic efficacy for the extract showing the best antiviral activity in vitro. Acyclovir was used as a reference drug for both in vitro and in vivo investigations. In vitro and in vivo cytotoxicity of the extracts was determined by cell lysis and weight lose or death respectively. Of the 12 plants tested, three extracts from Carissa edulis (Forssk.) Vahl (Apocynaceae), Maytenus heterophylla (Eckl. & Zeyh.) Robson (Celastraceae) and Periploca
linearifolia Dill & A. Rich. (Asclepiadaceae) exhibited remarkable anti-HSV activity \textit{in vitro} for both the wild type (HSV-1, HSV-2) and the resistant strains (TK\(^{-}\) HSV-1, AP\(^{r}\) HSV-1) with an EC\(_{50}\) of 15-60\(\mu\)g/ml with no cell cytotoxicity (CC\(_{50}\) 300-480 \(\mu\)g/ml). \textit{Carrisa edulis} root-bark extract had the best activity \textit{in vitro}, with a therapeutic index of between 31.8 and 69.6 for both susceptible and resistant HSV strains. The therapeutic efficacy of \textit{C. edulis} extract was demonstrated in mice infected with wild type or resistant strains of HSV since an oral dose of 250mg/kg significantly delayed the onset of HSV infections by over 50% and increased the mean survival time by 28% to 35% relative to the infected untreated mice (\(p < 0.05\) Vs control by Student’s t-test). The mortality rate for mice treated with extract was also reduced by between 70% and 90% as opposed to the infected untreated mice which had 100% mortality (\(p < 0.05\) Vs control by repeated measure analysis of variance (ANOVA)). No acute toxicity was observed in mice at the oral dose of 250mg/kg. The anti-HSV activity seemed to arise from terpenoidal and phenolic compounds detected in these extracts.

These results show that the water extracts from \textit{C. edulis}, \textit{M. heterophylla} and \textit{P. linearifolia} have anti-HSV activity. \textit{C. edulis} root bark extract has both \textit{in vitro} and \textit{in vivo} activity and therefore can be exploited as an alternative herbal remedy for HSV infections.
# Table of Contents

Declaration .................................................. ii
Dedication .................................................... iii
Acknowledgement ........................................... iv
Abstract ...................................................... vi
Table of Contents .......................................... viii
List of Tables ................................................ xv
List of Plates ............................................... xvi
List of abbreviations .................................... xvii

**CHAPTER ONE** ............................................. 1

1.0 Introduction ........................................... 1
1.1 Justification ........................................... 5
1.2 The hypothesis ......................................... 5
1.3 Objectives of the study ............................... 6
1.3.0 Main objective ....................................... 6
1.3.1 Specific objectives .................................. 6

**CHAPTER TWO** ............................................. 7

2. Literature Review ....................................... 7
2.0 Herpes Simplex Virus ................................. 7
2.0.1 History ............................................... 7
2.0.2 Herpes Simplex Virus structure and composition 8
2.0.3 Classification of herpes simplex virus .......... 10
2.1 Herpes Simplex Virus Infection .................... 11
2.1.4.6 HSV Infections in Immunocompromised hosts 31
2.1.5 Diagnosis 32
2.1.5.0 Collection of specimens 32
2.1.5.1 Detection of virus 32
2.1.5.1.0 Cell culture 32
2.1.5.1.1 Other HSV detection techniques 33
2.1.6 Measurement of HSV 34
2.1.7 Chemotherapy of HSV infection 36
2.1.7.0 Interferon-α 36
2.1.7.1 Antiviral agents 37
2.1.7.1.0 Nucleoside analogs 37
2.1.7.1.0.1 Idoxuridine (5-iodo-2'-deoxyuridine) 37
2.1.7.1.0.2 Vidarabine 37
2.1.7.1.0.3 Acyclovir {9-[(2-hydroxyethoxy)methyl]guanine, Zovirax} 38
2.1.7.1.1 Non-nucleoside antivirals 39
2.1.7.1.1.0 Foscarnet (trisodium phosphonoformate) 39
2.1.7.1.1.1 Phosphonoacetic acid (PAA) 39
2.1.8 Use of anti-HSV vaccines 40
2.2 Anti-viral resistance 41
2.3 Medicinal plants and traditional medicine 43
2.3.0 Medicinal plants 43
2.3.1 Traditional medicine 44
CHAPTER THREE

3. Materials and methods

3.0 Medicinal plants

3.0.1 Collection of the medicinal plants

3.0.2 Initial processing of the medicinal plant parts

3.0.3 Preparation of the aqueous extracts

3.0.4 Preparation of stock solutions of the extracts

3.1 Acyclovir (ACV)

3.2 Viruses and cells

3.2.0 Cells

3.2.1 Viruses

3.2.2. Virus titration

3.3 In vitro Assays

3.3.0 Plaque reduction assay

3.3.1 EC$_{50}$ determination

3.3.2 Cell cytotoxicity assay (CC$_{50}$)

3.3.2.1 Trypan blue exclusion test
3.3.3. Virus yield reduction assay 62

3.4 In Vivo assays 63

3.4.0 Therapeutic efficacy of Carissa edulis root bark extract in male and female BALB/c mice cutaneously infected HSV 63

3.4.1 Determination of acute toxicity of the Carissa edulis root bark extract in uninfected mice 65

3.5 Phytochemical screening 66

3.5.0 Thin Layer Chromatography (TLC) of the total extracts 66

3.5.0.1 Determination for the presence of nitrogenous compounds 67

3.5.0.1.1 Alkaloids 67

3.5.0.2 Determination for the presence of phenolic compounds 68

3.5.0.2.1 General presence of phenolics 68

3.5.0.2.2 Presence of specific phenolics 68

3.5.0.2.2.1 Flavonoids 68

3.5.0.2.2.2 Anthraquinones 68

3.5.0.3 Determination for the presence of terpenoids 69

3.5.0.3.1 General presence of terpenoids 69

3.5.0.3.2 Presence of specific terpenoids 69

3.5.0.3.2.1 Cardiac glycosides 69

3.5.0.3.2.2 Steroids 70

3.5.0.3.2.3 Saponins 70

3.6 Data analysis 71
CHAPTER FOUR

4. Results 72

4.0 In vitro assays 72

4.0.1 Plaque reduction assay for all medicinal plants 72

4.0.2 Determination of EC₅₀ and CC₅₀ for the three medicinal plant extracts with high anti-HSV activity 73

4.0.3 Plaque assay results for Carissa edulis RB extract 74

4.0.4 Virus yield reduction assay 75

4.1 In Vivo assays 78

4.1.0 Progression of infection in untreated BALB/c mice following a cutaneous wild type 7401H HSV-1 infection 78

4.1.1 Therapeutic efficacy of Carissa edulis root bark extract in male BALB/c mice infected with wild type strain 7401H HSV-1 79

4.1.2 Therapeutic efficacy of Carissa edulis root bark extract in female BALB/c mice infected with wild type strain 7401H HSV-1 80

4.1.3 Therapeutic efficacy of Carissa edulis root bark extract in female BALB/c mice infected with acyclovir resistant strain (AP⁵) HSV-1 86

4.1.4 Therapeutic efficacy of Carissa edulis root bark extract in male BALB/c mice infected with acyclovir resistant strain (AP⁵) HSV-1 86

4.1.5 Therapeutic efficacy of Carissa edulis root bark extract in male BALB/c mice infected with thymidine kinase deficient (TK⁻) strain HSV-1 88

4.1.6 Therapeutic efficacy of Carissa edulis root bark extract in male BALB/c mice infected with wild type strain HSV-2 90
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1.7</td>
<td>Determination of acute toxicity of the <em>Carissa edulis</em> root bark extract in uninfected mice</td>
<td>90</td>
</tr>
<tr>
<td>4.2</td>
<td>Phytochemical Screening</td>
<td>93</td>
</tr>
<tr>
<td>4.2.0</td>
<td>Phytochemical screening for <em>Carissa edulis</em>, <em>Maytenus hetrophylla</em> and <em>Periploca linearifolia</em> RB extracts</td>
<td>93</td>
</tr>
</tbody>
</table>

**CHAPTER FIVE**

5. Discussion

5.0 Conclusion and Recommendations

References

Appendix
List of Tables

Table 1: *In vitro* anti-HSV activity of the medicinal plant extracts on wild type 7401H strain herpes simplex virus type 1 (HSV-1) 73

Table 2: EC$_{50}$ and CC$_{50}$ determination for the three medicinal plant extracts with high anti-HSV activity 74

Table 3: *In vitro* activity of *Carissa edulis* RB extract on the growth of HSV in Vero E6 cells 77

Table 4: *In vitro* activity of acyclovir (Zovirax) on the growth of HSV in Vero E6 cells 78

Table 5: Effect of *Carissa edulis* root bark extract on cutaneous wild type strain 7401H HSV-1 (1 x 10$^6$ PFU) infection in male BALB/c mice 83

Table 6: Effect of *Carissa edulis* root bark extract on cutaneous wild type strain 7401H HSV-1 (1 x 10$^6$ PFU) infection in female BALB/c mice 84

Table 7: Effect of *Carissa edulis* root bark extract on cutaneous acyclovir resistant (AP$^r$) strain HSV-1 (1 x 10$^6$ PFU) infection in female BALB/c mice 87

Table 8: Effect of *Carissa edulis* root bark extract on cutaneous infection with acyclovir resistant (AP$^r$) strain HSV-1 (1 x 10$^6$ PFU) in male BALB/c mice 88

Table 9: Effect of *Carissa edulis* root bark extract in male BALB/c mice cutaneously infected with thymidine kinase deficient (TK$^-$) strain HSV-1 (1 x 10$^6$ PFU) 89

Table 10: Effect of *Carissa edulis* root bark extract on cutaneous infection with wild type strain HSV-2 (1 x 10$^6$ PFU) in male BALB/c mice 91

Table 11: Toxicity of *Carissa edulis* root bark extract in uninfected BALB/c mice following treatment with 250mg/kg extract 92

Table 12: Phytochemistry of *Carissa edulis*, *Maytenus heterophylla* and *Periploca linearifolia* aqueous root bark extracts 94
List of Plates

Plate 1: Anti-HSV activity of *Carissa edulis* RB extract. Vero E6 cells were infected with 100 PFU HSV and treated with 20-50μg/ml extract. 76

Plate 2: Progression of HSV infection in untreated BALB/c mice following a cutaneous wild type strain 7401H HSV-1 (1 x 10^6 PFU) infection. The scoring of infection. 82

Plate 3: Therapeutic efficacy of *Carissa edulis* root bark extract in female BALB/c mice infected with 1 x 10^6 PFU wild type strain 7401H HSV-1. 85
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>(AP&lt;sup&gt;+&lt;/sup&gt;) HSV-1</td>
<td>Acyclovir resistant herpes simplex virus type 1</td>
</tr>
<tr>
<td>(TK&lt;sup&gt;-&lt;/sup&gt;) HSV-1</td>
<td>Thymidine kinase deficient herpes simplex virus type 1</td>
</tr>
<tr>
<td>ACV</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>ACVTP</td>
<td>Acyclovir triphosphate</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>CC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Cytotoxic concentration for 50% test</td>
</tr>
<tr>
<td>CCR</td>
<td>Centre for Clinical Research, KEMRI</td>
</tr>
<tr>
<td>CDC</td>
<td>Centres for Disease Control and Prevention</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRDR</td>
<td>Centre for Respiratory Diseases Research, KEMRI</td>
</tr>
<tr>
<td>CTMDR</td>
<td>Centre for Traditional Medicine and Drug Research, KEMRI</td>
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<tr>
<td>CVR</td>
<td>Centre for Virus Research, KEMRI</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Effective concentration for 50% test</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>HHV</td>
<td>Human herpes virus</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HSV-1</td>
<td>Herpes simplex virus type 1</td>
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<tr>
<td>HSV-2</td>
<td>Herpes simplex virus type 2</td>
</tr>
<tr>
<td>HW</td>
<td>Hot water</td>
</tr>
<tr>
<td>ICRAF</td>
<td>International Centre for Research in Agro-forestry</td>
</tr>
<tr>
<td>ICTV</td>
<td>International Committee on the Taxonomy of Viruses</td>
</tr>
<tr>
<td>JICA</td>
<td>Japan International Cooperation Agency</td>
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<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
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<tr>
<td>L</td>
<td>Leaves</td>
</tr>
<tr>
<td>LATs</td>
<td>Latency associated transcripts</td>
</tr>
<tr>
<td>MC</td>
<td>Methyl cellulose</td>
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<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
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<tr>
<td>PAA</td>
<td>Phosphonoacetic acid</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming units</td>
</tr>
<tr>
<td>RB</td>
<td>Root bark</td>
</tr>
<tr>
<td>S3</td>
<td>Sacral nerve root ganglia 3</td>
</tr>
<tr>
<td>S4</td>
<td>Sacral nerve root ganglia 4</td>
</tr>
<tr>
<td>SB</td>
<td>Stem bark</td>
</tr>
<tr>
<td>STD</td>
<td>Sexually transmitted disease</td>
</tr>
<tr>
<td>TI</td>
<td>Therapeutic index</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
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<td>UV</td>
<td>Ultra-violet</td>
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VZV - Varicella-zoster virus
CHAPTER ONE

1.0 INTRODUCTION

Herpes viruses are among the most common causes of viral infections of humans, resulting in a spectrum of illnesses ranging from asymptomatic to life-threatening disease. Most infections caused by these viruses are acquired early in childhood and are asymptomatic (with the exception of varicella). Clinical illness can occur throughout childhood and adulthood. These organisms establish latency and can reactivate periodically throughout a person's lifetime. The human herpes viruses include herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), human cytomegalovirus (CMV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), and human herpes viruses 6, 7 and 8 (HHV-6, HHV-7, and HHV-8). These viruses, with the exception of HHV-6, HHV-7 and HHV-8, cause significant morbidity and mortality in the immunocompromised host, especially in persons with human immunodeficiency virus (HIV) infection.

HSV-1 and HSV-2 are very common infections world-wide producing recurrent orofacial and genital infections, respectively (Dale, 1982; Nahmias et al., 1989; Roizman and Sears, 1990; Cynthia and Mertz, 1990). Transmission of infection requires intimate contact between individuals. Often, the transmitting party is an asymptomatic shedder of infectious virus. Using serology HSV-1
infection has been shown to occur during early childhood (Straus et al., 1985). Virtually all seropositive cases before age 10 years are due to HSV-1 (Nahmias et al., 1990). The prevalence of HSV-1 or HSV-2 antibody is higher in persons from lower socioeconomic groups than in those from middle or upper socioeconomic groups (Cynthia and Mertz, 1990; Nahmias et al., 1990). Antibodies to HSV-2 appears first in the population at puberty and the incidence increases with age and sexual experience (Corey, 1984; Straus et al., 1985; Johnson et al., 1989). However, because the incidence of HSV infection is lower in persons from the middle and upper socioeconomic classes, true primary genital herpes infections (those that occur in persons without pre-existing anti-body to HSV-1 or HSV-2) are more common in this group (Cynthia et al., 1990).

The two types of viruses cause clinically indistinguishable first episodes of genital herpes. Approximately 60-90% of the first episode of genital herpes infections are caused by HSV-2, and 10-40% by HSV-1 (Corey et al., 1983b; Kalinyak et al., 1977). However, patients with HSV-2 genital infections are much more likely to experience recurrences than those with HSV-1 genital infections (Reeves et al., 1981; Corey and Mindel, 1985; Mindel and Weller, 1986).

Reactivation of HSV infection occurs more frequently in patients with acquired immunodeficiency syndrome (AIDS), resulting in chronic, persistent mucocutaneous disease in many patients (Safrin et al., 1991a). The mucocutaneous disease manifest as large ulcerative lesions that can involve all areas of the body but more so in the genital and perirectal regions. Infection of the
central nervous system (CNS) can also occur in human immunodeficiency virus (HIV) infected persons. Bronchitis and pneumonitis due to HSV are AIDS-defining illnesses (CDC, 1987) but occur infrequently. Disseminated HSV disease, which occurs rarely in AIDS patients, may involve the lung, esophagus, liver, and adrenal glands (Connolly et al., 1989).

In the adult population of the United States, seroprevalences of HSV-1 and HSV-2 are greater than 80% and 20%-30%, respectively (Whitley, 1990). In selected populations, including homosexual men and drug injection users, the rates of HSV-1 infection are as high as 95%. Steve, (1985), demonstrated that HSV infection on abnormal skin such as that found in patients with eczema, abrasions and burns are more severe than that observed in the normal host.

Acyclovir (ACV), an acyclic guanosine analog released in the United States in 1982, is the drug of choice for HSV infection management (Dunkle et al., 1991). It is present as an oral, topical or intravenous formulation. For intracellular antiviral activity, acyclovir requires activation by phosphorylation which is accomplished in part by a virus-specified thymidine kinase. This then binds to viral DNA polymerase irreversibly preventing further DNA elongation, thereby stopping viral replication. However, the appearance of ACV-resistant HSV strains has become evident in immunosuppressed patients, such as organ transplant recipients and patients with AIDS (Birch et al., 1990; Coen, 1994). Those ACV-resistant viruses are also resistant to other nucleoside analogues (Nugier et al., 1992). Thus, development of an optimal strategy for prevention of
HSV infection or, the development of long-term suppressive treatment with an antiviral agent that would limit the emergence of acyclovir resistant strains is needed. To accomplish the former aim, the role of vaccination in the prevention of disease or, alternatively, the therapeutic use of vaccine to limit the number of recurrences should be investigated. Antiviral agents other than acyclovir need to be developed and evaluated for the treatment and suppression of HSV disease, especially disease caused by acyclovir-resistant viruses.

The search for new therapeutic agents for HSV infection management is an ongoing effort. Most researchers are now turning to medicinal plants as a source of these new agents (Kurokawa et al., 1993b; Hudson et al., 2000; Kofi-Tsekpo et al., 2001).

In this study twelve medicinal plants locally growing in Kenya were investigated for anti-HSV activity in vitro by determining their ability to inhibit formation of plaques in Vero E6 cells infected with wild type or resistant strains of HSV. The medicinal plant extract showing the best plaque inhibition in vitro was further evaluated for in vivo activity by determining its therapeutic efficacy in BALB/c mice, in a murine HSV infection model (Kurokawa et al., 1993b; 2001).
1.1 Justification of the Study

Treatment of HSV infections in humans is has become difficult due to resistance by the emerging strains of HSV to the present available drugs. New therapeutic agents with different modes of anti-HSV action are therefore desired, more so in this era of Acquired Immunodeficiency Syndrome (AIDS) in which HSV has emerged as a major opportunistic infection. Moreover, commercial drugs in the market for HSV management (e.g. acyclovir, drug of choice) are out of reach to most people in the developing world due to their high cost. There is, therefore, need to identify and develop alternative drugs which are effective, affordable and easily accessible to these people. Such an alternative had been observed by Kurokawa et al., (1999), where a medicinal plant of the orient *Rhus javanica* L. (gall) of family anacardiaceae is reported to have had both *in vitro* and *in vivo* efficacy against HSV. Medicinal plants are easily accessible to most people in sub-Saharan Africa.

1.2 The Hypothesis

The hot water extracts from some Kenyan medicinal plants used as herbal preparations for management of various diseases do contain agents with *in vitro* and *in vivo* anti-HSV activity.
1.3 Objectives of the Study

1.3.0 Main Objective

The main objective of the study was to assess the potential of herbal extracts from twelve medicinal plants growing in Kenya for anti-HSV activity.

1.3.1 Specific Objectives

The specific objectives of the study were:-

1. To determine, *in vitro*, the anti-HSV activities of aqueous extracts from twelve medicinal plants growing in Kenya.

2. To establish, *in vitro*, whether the extracts have biological activity against acyclovir resistant strains of HSV-1.

3. To determine, *in vivo*, the therapeutic efficacy of the medicinal plant extract(s) in mice infected with HSV.

4. To determine the type of phytochemical agents contained in the plants exhibiting anti-HSV activity.
CHAPTER TWO

2. LITERATURE REVIEW

2.0 Herpes Simplex Virus

2.0.1 History

Infections caused by herpes simplex viruses have troubled mankind since ancient times (Safrin, 1996). The disease resembling herpes was first described in biblical times with the terms ‘herpes’ (from the Greek word, to creep) around 100 A.D (Dale, 1982). Cold sores (herpes febrilis or oral-labial herpes) were described by the Roman physician Herodotus in 100 A.D. and genital herpes (herpes genitalis) was first described by the French physician Jean Astruc in 1736 (Corey, 1990). The viral etiology of herpes was first detected in experiments in 1912 when Gruter and Lowenstein (Dale, 1982) transmitted HSV infection by using material from human labial herpes and keratitis lesions to infect rabbit cornea. In 1920, a normal cornea of a blind man was infected by inoculation with HSV from a rabbit that had been previously experimentally infected with herpes (Adam, 1985). In 1921, Lipshutz (Corey, 1990) inoculated material from genital herpetic lesions into the skin of humans, eliciting infection within 48 to 72 hours in six persons and within 24 days in one person (Corey, 1990). In other experiments, Lipshutz (Corey, 1990) also observed that rabbits developed corneal infection
more readily with strains of herpes originating from genital sites than with those from oral-labial sites suggesting that there were epidemiological and clinical differences between oral and genital herpes (Corey, 1990). This was confirmed in the early 1960s when Dowdle et al., (1967) in the United states reported that HSV could be divided by neutralization tests into two antigenic types and that there was an association between the antigenic type and the site of viral recovery (Corey, 1990). With the discovery of tissue culture techniques in the second half of the 20th century, two main antigenic types of herpes simplex virus were distinguished: herpes simplex virus type 1 (HSV-1) as the causative agent of oral infections and herpes simplex virus type 2 (HSV-2) as the causative agent of genital infections (Holmes et al., 1990).

HSV was first grown in vitro in 1925 (Corey, 1990) and the first plaque assay for HSV was described in 1957 (Kaplan, 1957). Since that time, advances in viral technology have lead to the development of in vitro and in vivo methods of infection and recognition, increasing understanding of the mechanisms of latency and reactivation and development of the first successful antiviral agents for use against HSV.

2.0.2 Herpes simplex virus structure and composition

HSV is a large virus composed of a core of linear double-stranded DNA (Russell, 1962; Becker et al, 1968), in the form of a toroid, surrounded by a protein coat that exhibits icosahedral symmetry and has 162 capsomers. The
nucleocapsid is surrounded by an envelope that is derived from the nuclear membrane of the infected cell and contains viral glycoprotein spikes about 8-nm long. An amorphous, sometimes asymmetrical structure between the capsid and envelope is designated the tegument. The enveloped form measures 120-230 nm; the “naked” viron, 100 nm. The herpes simplex virus genome possess terminal and internal repeated sequences and undergoes genome rearrangements giving rise to different genome isomers. The HSV-1 genome is approximately 153 kilobase pairs (kbp) (Becker et al., 1968; McGeoch et al., 1988).

The HSV-1 and HSV-2 genomes show 50% sequence homology (Cynthia and Mertz, 1990). Every HSV-1 gene has a homologue encoded by HSV-2, and vice versa and all homologous genes occupy identical positions on each genome (Spear, 1990). However, they differ at the DNA level from base substitutions which may add or eliminate a restriction endonuclease cleavage sites and, on occasion, change an amino acid, or vary the number of repeated sequences present in a number of regions of the genome (Cynthia and Mertz, 1990).

Herpes simplex virus DNA have a coding capacity for over 50 different proteins and a molecular mass of 95 to 100 x 10³ kilodaltons. Virus-encoded enzymes including thymidine kinase and DNA polymerase participate in genome replication. These enzymes have unique substrate affinities and activities that distinguish them from similar cellular enzymes. Such properties have allowed the development of nucleoside analogs that interact with and specifically inhibit the
viral replicative processes in which they participate. Other viral proteins are involved in assembly and maturation of progeny virus.

The two types of herpes simplex viruses can be distinguished by restriction enzyme analysis of viral DNA (Buchman et al., 1978) or plaque forming characteristics in chicken embryo cells (Figueroa and Raws, 1969). HSV-1 tends to cause oral labial disease, and HSV-2 genital disease; however, each virus is capable of causing disease in both locations and produce clinically indistinguishable manifestations (Nahmias and Josey, 1982; Fife and Corey, 1990; Mindel and Carney, 1991).

2.0.3 Classification of herpes simplex virus

Herpes simplex virus (HSV) is classified as a member of the family Herpesviridae by the International Committee on the Taxonomy of Viruses (ICTV). The family Herpesvaridae is subdivided into three sub-families on the basis of biological properties. These include the alphaherpesvirinae, the betaherpesvirinae and the gammaherpesvirinae (Roizman et.al., 1992). HSV belongs to the alphaherpesvirinae sub-family. The alphaherpesvirinae sub-family members are classified on the basis of variable host range, relatively short reproductive circle, rapid spread in culture, efficient destruction of infected cells and capacity to establish latent infections in sensory ganglia. This sub-family contains the genera Simplexvirus and Varicellovirus. Herpes simplex virus types 1
and 2 belong to the genera *Simplexvirus*. Humans are the only "natural" hosts for herpes simplex virus.

Members of genera *Simplexvirus* exhibit a spectrum of diseases and have the ability to establish lifelong persistent infections in their hosts and to undergo periodic reactivation. This phenomenon, referred to as *latent infection* or *latency*, means that the genome of the virus is stably maintained by the infected cell (in the cell nucleus) with only limited expression of viral genes, no production of progeny virus, and no evident of virus induced cytotoxicity (Spear, 1990).

2.1 **Herpes simplex virus infection**

2.1.0 **Epidemiology**

2.1.0.1 **Incidence**

The frequency of symptomatic recurrence of HSV infection varies greatly among individuals but is greatest in the first few years after primary infection and decreases over time. About 85% of persons who present with a primary genital HSV-2 infection experience symptomatic recurrences over a 1-year period, with a mean rate of 0.3 recurrences/month (Corey *et al.*, 1983b; Corey and Spear, 1986). A primary HSV-1 infection at this location results in a comparable clinical presentation but yields considerably lower rates of recurrence; 55% of individuals experience recurrence, with a mean rate of 0.009 episodes/month (Corey and Spear, 1986; Corey *et al.*, 1983b). Oropharyngeal herpes have a lower rate of
symptomatic recurrence than genital infection (Spruance et al., 1977; Lafferty et al., 1987), with HSV-2 recurring less than HSV-1 at this location (Lafferty et al., 1987). Patients are rarely infected at one anatomic site with both HSV-1 and HSV-2 or with more than one strain of the same type (Buchman et al., 1979). Serial cultures of subsequent recurrences from most patients show the same virus type and strain (Schmidt et al., 1984). Sexual practices that involve oral-genital contact increase the risk of developing genital HSV-1 and orolabial HSV-2 infection (Lafferty et al., 1987).

2.1.0.2 Prevalence

The reported prevalence of HSV infections depends upon the demographic and clinical characteristics of the patient population studied and whether clinical or laboratory techniques, or both, are used for diagnosis (Nahmias and Josey, 1982). However, there is high prevalence of herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) in human populations worldwide (Nahmias et al., 1990). Seroepidemiologic studies have shown a wide disparity between antibody prevalence and clinical infection, indicating that many persons acquire asymptomatic infection (Nahmias, 1970a). In the United States, seroprevalence studies indicate that 22%-33% of the adult population is infected with HSV-2 (Fleming et al., 1997; Siegel et al., 1992). In the USA, it is estimated that 2-20 million people are infected with HSV (Mindel et al., 1991). The seroprevalence of HSV-2 in many developing countries is even higher, with evidence that 50%-80%
of individuals are infected in some highly sexually active populations (Nahmias et al., 1990). Because of this, it has been suggested that HSV-2 antibody could be used as a biologic marker of high risk sexual behavior since it is exclusively sexually transmitted (Nahmias et al., 1990; Obasi et al., 1999). In sub-Saharan Africa, studies of HSV have been scarce, owing to the lack of available laboratory tests and facilities (Mbopi-Keou et al., 2000). However, high seroprevalence rates in young adults (60%-80%) have been recorded in population based studies (Wagner et al., 1994; Gwanzura et al., 1998; Obasi et al., 1999). In Kenya, Mostad et al., (2000), studying the effects of hormonal contraception, pregnancy and vitamin A deficiency in cervical shedding of herpes simplex virus in human immunodeficiency virus-infected women attending a municipal STD clinic in Mombasa, reported that of the 314 HIV-1 seropositive women tested, 296 (94.3%) were HSV-1 seropositive, 293 (93.3%) were HSV-2 seropositive, and 275 (87.6%) were seropositive for both viruses. It has been suggested that the prevalence of HSV infection in AIDS patients equals or exceeds that of the general population (Erlich, 1989). HSV infection and recurrence is still expected to increase in frequency and severity in areas with high HIV prevalence (e.g. sub-Saharan Africa) because of immunosuppression (Safrin et al., 1991a; Mbopi-Keou et al., 2000).

HSV infection is a major cause of morbidity in immunosuppressed patients with AIDS or in organ transplant recipients (Chatis et al., 1989; Norri et al., 1988; Meyers et al., 1980 and Sibrack et al., 1982). Serologic studies have
shown that >95% of homosexual men with AIDS have been previously infected with both HSV-1 and HSV-2 (Rogers et al., 1983). Approximately 80-90% of HSV-seropositive patients undergoing bone marrow transplantation experience mucocutaneous herpes episodes, which are typically severe and may be associated with persistence of viral shedding, pain and lesions for periods of 3 to 4 weeks or more (Meyers et al., 1980; Saral et al., 1981; Wade et al., 1983; Wade et al., 1984; Gluckman et al., 1983). Studies have shown that HSV-2 antibody prevalence rates are about 5 to 10 percent higher in women than in men (Siegel et al., 1992).

2.1.1 Mode of transmission

Transmission of HSV infection most frequently occurs through close contact with a person who is shedding virus at a peripheral site, mucosal surface, or secretion. Contact may be genital to genital, mouth to genital, genital to anal, mouth to anal or mouth to mouth. Infection can occur when the source partner has clinical recurrence or more importantly, when they are shedding the virus asymptomatically (Mertz et al., 1985; Rooney et al., 1986; Mertz et al., 1992; Bryson et al., 1993). Direct human to human spread appears to be the only important method of transmission, as the virus is enveloped and requires a moist environment for survival. There are no known animal vectors for HSV (Corey, 1990). HSV cannot penetrate intact skin. Entry into the skin is usually aided by mechanical rubbing, resulting in abrasion of the skin. Since HSV is readily
inactivated at room temperature and by drying, aerosol and fomite spread are unusual means of transmission. Several studies have been done on transmission of HSV in couples (Mertz et al., 1992; Bryson et al., 1993), where one had the infection and the other did not. The largest study involved 144 couples who were followed up for a mean of 334 days (Mertz et al., 1992). During this period, studies on HSV transmission in couples gave a transmission rate of 10% and showed that the male to female (19%) transmission is greater than female to male (4.5%) transmission. These studies also indicated that most of these transmissions occurred when the source partner had no clinical recurrence or had clinically unrecognised infection or was an asymptomatic excretor of the infection (Mertz et al., 1992).

2.1.2 Pathogenesis

2.1.2.0 Anatomic site

It is difficult to define HSV-1 specific and HSV-2 specific aspects of pathogenicity in human disease, except perhaps for the differences in the frequency of recurrent lesions. Many features of infection caused by HSV-1 and HSV-2 are indistinguishable. Although HSV-1 and HSV-2 strains are isolated from different anatomic sites, either serotype can initiate and establish infection at whatever site they are inoculated. Although encephalitis in adults is most invariably caused by HSV-1 and meningitis is more frequently associated with genital infections (where HSV-2 isolations are more frequent than HSV-1
isolations), it cannot be assumed that intrinsic properties of each serotype solely determine whether encephalitis or meningitis can occur. The anatomic site at which inoculation occurs or the age of the host at the time of exposure at different sites may also be important factors in determining the nature of systemic disease.

2.1.2.1 Cell types infected with HSV

The pattern of tissue and organ damage caused by primary HSV infection and by reactivation of latent virus is in part determined by the types of cells in which the virus can replicate and in which latent infections can occur. Judging from studies of cultured cells and from the nature of disseminated disease in infants and immunocompromised adults, it appears that many human cell types are susceptible to HSV infection and can support viral replication. Despite this ability of HSV to infect many cell types, HSV disease is often localised to the body surface at the site of inoculation and to the sensory ganglia of nerves communicating with this site. Undoubtedly, an effective immune response is in part responsible for limiting the spread of infection. It has been observed that immunosuppressed patients, particularly those with impaired cell-mediated immunity, tend to manifest a greater frequency and severity of HSV infection (Cory and Spear, 1986). The possibility exists, however, that there are nonimmunologic barriers to the spread of infection in the normal adult. For example, certain cell types in fully differentiated tissues may not be able to support HSV replication, either because of inaccessibility or lack of required cell
surface receptors or because of lack of other factors for biosynthesis of viral components.

2.1.2.2 HSV entry into the body and its manifestation

HSV enters the body through damaged epithelium cell of the skin (broken skin) or susceptible mucosal surfaces (e.g., oropharynx, cervix, conjunctivae). Most primary infections go unrecognized or are subclinical. When studied histologically, a local lytic infection of the parabasal and intermediate epithelial cells is noted, with an accompanying local mononuclear inflammatory response. Together, these make up the characteristic vesicle of HSV infection (Dale, 1982). Multinucleated cells and occasional typical Cowdry type A intranuclear inclusions (cells with virus-induced eosinophilic nuclear inclusions) are seen (Naib et al., 1966), along with ballooning degeneration of the cells and chromatin migration. During primary infection, involvement of the lymphatics and regional lymph nodes is common, and viremia with further dissemination can occur in patients who do not have an intact immune system (Dale, 1982).

HSV becomes latent following a primary infection by travelling up the sensory nerves to the corresponding sensory ganglia. Human autopsy studies have demonstrated the presence of latent virus in sacral, trigeminal, and vagal ganglia (Bader et al., 1978; Baringer and Swoveland, 1973; Baringer, 1974b). The site of latency is felt to be within the neuron where viral DNA has been detected. HSV-2 (and rarely HSV-1) has been isolated from S3 and S4 sacral nerve root ganglia
(Baringer, 1974b) and HSV-1 from the trigeminal ganglion (Baringer and Swoveland, 1973). However, isolations of HSV-1 from the trigeminal ganglion and of HSV-2 from the sacral ganglion are accompanied by antibodies to the homologous type, indicating that latent infections with both types of virus and production of antigens occur in both sites (Forghani et al., 1974). The signal for reactivation is still a mystery, but various stimuli, such as fever, stress, sunlight and local trauma, may be recognized by individuals as precipitating factors. Following reactivation, the virus presumably travels back down the sensory nerve pathway and again establishes infection on the skin or mucous membranes. Antibody titre is not usually affected, and lesions may or may not be clinically evident.

Both humoral and cell-mediated immune mechanisms contribute to limitation of virus spread (Corey et al., 1978; Zweerick and Corey, 1982). Severe forms of disseminated HSV infection are more often seen in patients with depressed cell-mediated immunity. Viremia disseminates the virus in severe cases, resulting in infection that can involve the liver, lungs, kidneys and central nervous system. Infants and malnourished or immunosuppressed patients are at highest risk for this often lethal complication.
2.1.3 Life cycle and biology of HSV

2.1.3.0 Viral replication

Viral replication starts with adsorption of virus to a susceptible cell. Viral glycoproteins in the virion envelope bind to components of the cell surface in a cascade of interactions that culminates in penetration of the nucleocapsid into the cell cytoplasm. This penetration is effected by fusion of the virion envelope with the plasma membrane of the cell (Fuller and Spear, 1987). Attachment to the cell surface and fusion of the viral envelope with the plasma membrane involves viral surface proteins. Of the eleven known HSV membrane glycoproteins (gB, gC, gD, gE, gG, gH, gI, gK, gL, and gM) (Baines and Roizman, 1993; Spear, 1993), infection of susceptible cells is initiated by the attachment of virions, via glycoprotein C (gC) and/or gB, to cell surface heparan sulfate proteoglycans (WuDunn and Spear, 1989; Herold et al., 1991; Herold et al., 1994). This is followed by the interaction of gD with one of several cellular receptors. Then, pH-independent fusion occurs between the virus envelope and the host cell plasma membrane. Numerous studies have been unable to assign the responsibility for attachment to the cell surface to a sole viral glycoprotein (Cai et al., 1988; Centifanto-Fitzgerald et al., 1982). The reason for this is because HSV can utilise more than one attachment pathway since in the course of its normal life cycle, it must infect and replicate in two different cell types: epithelial cells and neurons. These cells are quite different from one another (Caplan and Martlin,
Cell surface receptor heparan sulfate, identified by WuDunn and Spear, 1989, is a major factor in binding of HSV to the cell surface. Heparan sulfate (HS), a viral receptor of carbohydrate nature, serves as an initial cell surface receptor for HSV-1 and HSV-2. In addition to providing sites for initial virus binding, HS has been reported to promote HSV-1-induced cell-to-cell fusion (Shieh and Spear, 1994). It is related in structure to heparin. Removal of heparan sulfate from cell lines either enzymatically or by use of mutant cell lines deficient in heparan sulfate synthesis reduces the levels of virus attachment to and infection of those cells by approximately 85% (Shieh and Spear, 1994).

Fusion between the viral envelope and the plasma membrane of the host cell leads to the penetration by the virus across the membrane through a multistep event which involves more than one viral glycoprotein (gB, gC, gD, gE, gG, gH and gI) (Ligas and Johnson, 1988; Campadelli-Fiume et al., 1988; Rodger et al., 2001). Upon entry into the cell, the capsids are transported to the nuclear pores where they uncoat and release viral DNA into the nucleoplasm where it immediately forms a circle (Batterson and Roizman, 1983a; Batterson et al., 1983b). Expression of the viral genome starts in a tightly regulated and sequential order in a cascade fashion (Honess and Roizman, 1974). The viral genes are transcribed by cellular RNA polymerase 11 under the control of viral regulatory components, some of which are brought into the cell along with the genome (Roizman and Batterson, 1985). Only a limited number of the viral genes are transcribed at first, then the immediate early or α-genes which encode mostly
regulatory genes. Optimal transcription of the α-genes requires the activity of a viral regulatory protein called α-TIF or VP16 (a protein designated by Spear and Roizman nomenclature and induces production of α-genes) (Batterson et al., 1983a,b; Campbell et al., 1984). This viral regulatory protein is a viron constituent localized between the capsid and the envelope and is one of the important viral proteins that is delivered to the cell along with the viral genome.

The transcription of α-genes reaches peak rates at approximately 2 to 4 hr post infection. The α-genes are then translated into the α-proteins and one or more of these proteins enables the next wave of transcription for expression of the early, or β, genes. The β-genes reach peak rates of synthesis at about 5 to 7 hr post infection. The β-genes encode enzymes and other proteins required for viral DNA replication, including thymidine kinase (TK) and DNA polymerase (both important targets of currently used drugs). The appearance of β-proteins signals the onset of viral DNA synthesis. Following α- and β-gene expression and DNA replication, the late, or γ, genes are expressed producing most of the virion proteins and glycoproteins which act as structural components (DelLuca and Schaffer, 1985; Fenwick and Walker, 1978; Read et al., 1993).

Viral DNA is transcribed throughout the replicative circle, synthesis of the viral DNA being carried out in a rolling-circle mechanism. Newly synthesized viral DNA is packed into preformed empty nucleocapsids in the cell nucleus (Newcomb et al., 1996;1999). Maturation occurs by budding of nucleocapsids
through the altered inner nuclear membrane, acquiring an envelop in the process (Homa and Brown, 1997). Enveloped viral proteins are then released from the cell through tubular structures that are continuous with the outside of the cell or from vacuoles that release their contents at the surface of the cell. The length of the replication cycle is about 18 hrs. Host macromolecular synthesis is shut off early in infection, normal cellular DNA and protein synthesis virtually stop as viral replication begins. The completion of HSV replication ultimately leads to death of the host cell.

2.1.3.1 The latent State.

The ability of the HSV to persist for life in their natural hosts depends in large part on their ability to establish latent infections. The latent state is one in which the viral genome is stably maintained in the cell nucleus with expression of only a limited subset of viral genes. The latently infected cell is not killed by the virus; viral gene products expression may even stimulate cell division. Virions or infectious virus cannot be recovered from latently infected tissue immediately after removal from an experimental animal or human cadaver, but reactivation of the latent virus to the replicating state, yielding infectious virus, can be achieved by in vitro cultivation of the explanted tissue.

Evidence of HSV latency has come principally from in vivo studies in animal models of HSV infection, primarily the mouse, rabbit and the guinea pig (Stevens and Cook, 1971; Baringer and Swoveland, 1974a; Stanberry et al.,
HSV latency in each of these models is similar to that in humans. However, various aspects of latency differ among models, and each model also differs from that in humans (Fraser et al., 1991). The HSV genomes present in the sensory ganglia of latently infected mice and humans have been shown to be episomal and circular (Rock and Fraser, 1983; Mellrick and Fraser, 1987), in contrast to viron associated genomes, which are linear.

As already mentioned, an initial mucocutaneous infection entails viral multiplication in epithelial cells that extends locally to involve the distal ramifications of sensory nerves. Upon entering the nerve, the viral nucleocapsid (Lycke et al., 1988) is transported centripetally through the axon at 5-10mm/h (Vallee et al., 1989) to the neuronal cell body residing in the sensory ganglia. Once in the ganglia, a limited amount of viral reproduction ensues (Margolis et al., 1989) and in some instances extends to neighbouring nonneuronal cells or interconnected neuronal cells (Baringer and Griffith, 1970; Margolis et al., 1989). Some of these infected cells may exhibit cytopathic effect (Baringer and Griffith, 1970; Margolis et al., 1989). Over several days there is a dramatic change in the pattern of gene expression, and all genes responsible for viral reproduction are shut off. However, one duplicated region of the genome remains transcriptionally active (Stevens et al., 1987; Croen et al., 1987; Croen et al., 1991), producing a family of RNAs referred to as the latency-associated transcripts (LATs). At this stage of infection the virus has operationally assumed a state of latency. An analyses of both animal and human tissues at this stage reveal the presence in
latently infected cells of transcripts from one specific region of the viral genome (Spivack and Fraser, 1987; Steiner, 1988; Stevens, 1988). The expression of these transcripts, is a good marker for the latent state induced by wild-type virus even though LATs are not absolutely essential for the establishment of latency in experimental animals or for reactivation of latent virus (Javier, 1988).

### 2.1.4 Clinical Course

The clinical course of an HSV infection can be of varying severity and duration. The primary infection may be asymptomatic or have a clinically manifest course. A recurrent clinical disease may follow known stimuli, such as upper respiratory infection, sunlight, or physical or emotional stress, or it may appear simultaneously with other conditions such as unspecified fever, menstruation, or trauma. Recurrent episodes are observed at different time intervals and without being linked to any established trigger factor.

A primary infection in a patient without pre-existing antibodies to HSV-1 or HSV-2 is usually clinically manifest and is more severe, being accompanied by fever and extensive lesions. The infection has a longer duration than in the individual with pre-existing antibodies and result in antibody production and establishment of latent infections in sensory ganglia (Braude et al., 1986). Recurrent infection is usually characterised by production of a small number of tightly clustered vesiculoulcerative lesions localised in a dermatome previously involved in the primary infection (Straus et al., 1985).
As already mentioned, there is no specific site tropism of the two types of HSV. In general terms, HSV-1 is the causative agent of oral infections and conditions above the waist, while HSV-2 causes genital disease or lesions below the waist. Nevertheless, HSV-1 infection has been associated with genital disease at rates varying from 12 to 50% of cases in some studies, while HSV-2 has been reported as the etiologic agent of non-genital lesions in a smaller percentage of cases (for example, oropharyngeal herpes in adults) (Dolin et al., 1975; Dowdle et al., 1967; Kaufman and Rawls, 1972; Kaufman et al., 1973). HSV infection could give rise to the following clinical conditions.

2.1.4.0 Oropharyngeal Disease

Primary HSV-1 infections are usually asymptomatic. Symptomatic disease occurs most frequently in small children (1-5 years of age) and involves the buccal and gingival mucosa of the mouth. The incubation period is short (about 3-5 days, with a range 2-12 days), and clinical illness lasts 2-3 weeks. Symptoms include fever, sore throat, vesicular and ulcerative lesions. Gingivitis (swollen, tender gums) is the most striking and common lesion. Primary infections in adults commonly cause pharyngitis and tonsillitis.

Recurrent disease is characterised by a cluster of vesicles most commonly localised at the border of the lip (herpes labialis, cold sores). Intense pain occurs at the onset but fades over 4-5 days. Lesions progress through the pustular and crusting stages, and healing without scarring is usually complete in 8-10 days.
The lesions may recur, repeatedly and at various intervals, in the same location. The frequency of recurrences varies widely among individuals.

2.1.4.1 Ocular Disease (Herpes keratitis).

The initial infection with HSV may be in the eye, producing severe keratoconjunctivitis. Recurrent episodes are also observed in this location, with lesions occasionally progressing to vesicular involvement of the eyelids or a dendritic ulcer in the cornea (Kaufman et al., 1968). The latter condition can lead to permanent blindness by destruction of stromal fibers and scar formation.

2.1.4.2 Involvement of the Nervous System

2.1.4.2.0 Encephalitis

Encephalitis (inflammation of the brain) in adults, if caused by HSV, is usually associated with type 1 infection (Nahmias and Roizman, 1973). The spread of the infection to the brain is facilitated by the inervation of the mucous membranes of the nasopharynx and eyes. Encephalitis, although uncommon, always produces a severe disease. Electrodiagnostic and perfusion studies usually reveal focal changes most frequently localised in the temporal or frontal lobe. There are no clinical symptoms on which the herpetic etiology of the disease can be established with certainty. During acute illness, only detection of the virus in a brain biopsy is diagnostic. The course of the untreated disease is fatal in the
majority of cases. In patients who survive, residual paralysis or mental impairment are frequent (Olson et al., 1967; Rennick et al., 1973; Whitley et al., 1977). Encephalitis may occur in individuals with prior evidence of HSV infection (Craig and Nahmias, 1973).

2.1.4.2.1 Aseptic Meningitis

Where HSV has been associated with acute aseptic meningitis (inflammation of the meninges of non bacterial origin) cases of adults, type 2 is primarily implicated (Craig and Nahmias, 1973). The route of dissemination of HSV-2 infection in humans is thought to be hematogenous, while HSV-1 in cases of encephalitis is believed to travel through the nervous system by direct cell-to-cell infection (Craig and Nahmias, 1973; Hevron, 1977).

2.1.4.3 Genital Herpes (Herpes Genitalis)

Data from several studies have shown that the highest incidence of herpes genitalis occurs in persons in the third decade of life (Ng et al., 1970; Nahmias et al., 1969).

2.1.4.3.0 Clinical course in females

Clinical manifestations of infection in females appear after an incubation period of 3-7 days following a sexual exposure with an infected individual. Itching and burning may precede the vesicular stage of the disease. Neurologic
pain radiating to the lower back and hips may occur as a prodromal symptom (Kaufman et al., 1973). The eruption of vesicles is observed on the libia majora and minora, vestibule of the vulva, and perineal area, as well as vaginal and ectocervical mucosa. A profuse discharge is frequently present. The vesicles, which are usually surrounded by red areola, may become confluent and form bullous lesions. The vesicles on the mucosal surfaces rupture early, while those on the outer skin surface last longer with ulceration occurring later. Ulcerations also occur on the vagina and ectocervix, although less frequently than on the vulva. In primary infection, fever can accompany other systemic symptoms such as headache and malaise (Kaufman et al., 1973).

The location of the lesions may vary. They may be confined to the cervix only or may extend to neighbouring areas such as the perineum, buttocks, thighs and rectum. The infection may even remain asymptomatic (Nash and Foley, 1970). The lesions heal within 2-4 weeks without apparent residual mucosal or skin defects. Recurrent episodes have a milder course, and the healing period is shorter, about 5-10 days (Adams et al., 1976).

The risk of development of genital herpes in a woman on exposure to an infected man is estimated to be 80% to 90% (Nahmias et al., 1969; Rawls et al., 1971).
2.1.4.3.1 **Clinical course in males**

There is not much difference between genital lesions in the male and those in the female in HSV-caused genital disease. In the male, herpetic lesions occur on the glans, prepuce, or shaft of the penis and spreads to the scrotum, thighs, or buttocks. The infection also involves the urethra. Chronic infection with erosion may occasionally occur in these areas (Logan *et al.*, 1971). Involvement of lymph nodes and neurogenic symptoms are similar to those seen in the infected female.

For both females and males, it is believed that a prior non-genital HSV infection modifies the course of the genital disease and that a milder clinical course and faster healing occur in recurrent disease. A primary HSV-1 infection is indistinguishable from an HSV-2 infection in both males and females.

2.1.4.4 **HSV Infection in Pregnant Women and the Neonate.**

Primary HSV infection in pregnancy has a more severe course than in the non-pregnant woman. Encephalitis in pregnant women, although a very rare event, has fatal outcome (Rawls *et al.*, 1966). The cervix is the most common site of infection. Occasionally pelvic pain and dysuria are the only clinical symptoms of the infection. Interestingly, HSV-2 infection is detected more often in pregnant than non-pregnant women of comparable age and socio-economic background (Ng *et al.*, 1970).

The source of infection of the neonate is the infected birth canal during delivery. The highest risk threatens the neonate if (1) a primary genital infection
occurs during the last month of pregnancy, (2) the virus is present at parturition, and (3) the infant is delivered vaginally more than 4 hr after rupture of membranes in the presence of genital disease (Nahmias et al., 1967b). Fetal monitoring is also a source of severe infection of the neonate (Parvey and Ch’ien, 1980). The disseminated form of the disease with CNS involvement is considered the most common clinical manifestation of HSV infection of the neonate. Localised involvement of other systems without dissemination or even an inapparent course of infection have also been observed. Skin involvement may be present or absent (Nahmias et al., 1970b). Transplacental antibodies do not protect the neonate from HSV infection (Nahmias et al., 1970b).

2.1.4.5 **Skin Infections.**

Localised lesions arising from abrasions on the skin that become contaminated with HSV give rise to traumatic herpes as seen on the fingers of dentists and hospital personnel (herpetic whitlow) and on bodies of wrestlers (herpes gladiatorum). Cutaneous infections are often severe and life threatening when they occur in individuals with disorders of the skin, such as eczema or burns, that permit extensive local viral replication and spread. Eczema herpeticum is a primary infection with HSV-1. There may be extensive vesiculation of the skin over much of the body and high fever. In rare instances, the illness may be fatal (Adam, 1985).
2.1.4.6 HSV Infections in Immunocompromised Hosts.

Persons with impaired or deficient cellular immunity often develop severe HSV infections. These include patients immunosuppressed by disease or therapy and individuals with malnutrition. Renal, cardiac and bone marrow transplant recipients are at particular risk for severe herpes infections. Patients with hematological malignancies and patients with acquired immunodeficiency syndrome (AIDS) suffer more frequent and more severe HSV infections. Most AIDS patients are infected with HSV before infection with human immunodeficiency virus (HIV), therefore recurrent herpes is a much more common problem than primary HSV infection in this population. In AIDS patients, HSV infection is a major opportunistic infection and large ulcerative HSV lesions (without visceral or cutaneous dissemination) are a frequent occurrence. Due to this, the Centres for Disease Control and Prevention (CDC) has included 'chronic mucocutaneous HSV infection present for longer than 1 month in an individual with no other cause of underlying immunodeficiency or with laboratory evidence of HIV infection as diagnostic of AIDS' (CDC, 1987).

In immunocompromised patients, herpes lesions may spread and involve the respiratory tract, esophagus and intestinal mucosa. Malnourished children are prone to fatal disseminated HSV infections. In most cases, the disease reflects reactivation of latent HSV infection.
2.1.5 Diagnosis

2.1.5.0 Collection of specimens

Proper specimen collection is done for any accurate infection diagnosis. In HSV, the highest rate of successful virus isolation is from the vesicles; with each subsequent stage, virus detection decreases. Material is obtained by aspiration of vesicular content with a tuberculin syringe and the needle and syringe are rinsed in the transport medium. Immediate transportation to the laboratory is done using a standard tissue culture medium (e.g. Eagle’s Minimum Essential Media supplemented with 2% fetal bovine serum). Cerebrospinal fluid is sent to the laboratory under sterile conditions. For cytologic examination, the cells are scraped from the herpetic lesions, transferred to a slide, and fixed with acetone. Biopsy specimens are immediately frozen and then dried, and acetone-fixed sections prepared. For electron microscope examination, vesicular fluid or cell-containing material is placed in a minimal amount of distilled water for immediate examination (Adam, 1985).

2.1.5.1 Detection of Virus.

2.1.5.1.0 Cell culture

Virus culture remains the gold standard for the diagnosis of active HSV infection. Isolation of the virus in appropriate cell culture is the most sensitive and
specific diagnostic method. Virus may be isolated from herpetic lesions (skin, cornea, or brain). It may also be found in throat washings, cerebrospinal fluid and stool, both during primary infection and during asymptomatic periods. Specimens are inoculated onto tissue culture cell lines (e.g. Vero cells from the kidneys of the African green monkey) growing under incubation at ambient temperature (discussed in detail under methods). With some high-titre virus specimens, a positive result can be obtained within 24 to 48 hours. However, if virus is present in low titre, up to 7 days may pass before the characteristic changes can be seen. Growth of the virus in the cells is associated with cytoplasmic granulation, ballooning degeneration, and multinucleated giant cells giving rise to the characteristic cytopathic effect due to HSV (Adam, 1985).

2.1.5.1.1 Other HSV detection techniques

Cytology and Biopsy (Tzanck test) is sometimes used for diagnosis of HSV infections but lacks sensitivity and specificity and does not differentiate between HSV and varicella-zoster virus (VZV) infection (Solomon et al., 1984). Cells obtained by the scraping of lesions from the base of a herpetic ulcer are stained with Wright’s or Giemsa’s stain (Tzanck smear) and examined under a microscope for the presence of multinucleated epithelial giant cells considered pathologic for viral infection (Nahmias et al., 1967a; Naib et al., 1966). Electron microscopy is also used and is very attractive because results can be available within 2 hours. Unfortunately, members of the herpesvirus group cannot be
distinguished with this technique. In addition, most medical centres lack the equipment and expertise.

Viral antibody detection (Serologic tests) is another virus detection technique. The tests detect the presence of HSV antibodies in the blood as an indication of infection. However, serodiagnosis of HSV infections is limited by the multiple antigens shared by HSV-1 and HSV-2. A particular problem is the cross-reactivity of antibodies to type 1 and type 2 virus. In persons with antibody to type 1, infection with type 2 virus frequently does not elicit a detectable change in complement-fixing antibody titre. Also, recurrences or re-infections often cannot be identified serologically. The presence of these antibodies can be measured by the enzyme linked immunosorbent assay (ELISA) (Vestergaard and Jensen, 1981). The latest virus detection technique involves the use of the Polymerase chain reaction (PCR) where the HSV DNA is detected. Detection of HSV by PCR method has proved useful for the diagnosis of herpes simplex encephalitis (Roeley et al., 1990) and for increased sensitivity in the monitoring of cutaneous virus shedding (Cone et al., 1991).

2.1.6 Measurement of HSV

Viruses are measured by several methods that can be divided into two categories. Viruses may be measured as either infectious units, that is, in terms of their ability to infect, multiply, and produce progeny or in terms of the total number of virus particles, irrespective of their function as infectious agents.
Measurement of amount of virus in terms of the number of infectious units per unit volume is known as titration. There are several ways of determining the titre of a virus suspension, all of them involving infection of host or target cells so that each particle that causes productive infection elicits a recognisable response. A series of monolayers of susceptible cells are inoculated with small aliquots of serial dilutions of the virus suspension to be titrated. Wherever virus particles infect cells, progeny virus particles are produced and released and immediately infect adjoining cells. This process is repeated until after a period of 2 to 12 days, there develops areas of infected cells that can be seen with the naked eye after a vital stain. These are called "plaques". In order to ensure that progeny virus particles liberated into the medium do not diffuse away and initiate separate (or secondary) plaques, agar or methyl cellulose is incorporated into the medium. Plaques represent dead or completely destroyed cells (cytopathic effect) which become detached from the surface on which they grow. There is a linear relationship between the amount of virus and the number of plaques produced. That is, the dose-response curve is linear. This indicates that each plaque is caused by a single virus particle. The virus progeny in each plaque, therefore, are clones. Titres are expressed in terms of numbers of plaque-forming units (PFU) per milliliter (Dale, 1982). The inhibition of the formation of plaques in Vero E6 cells is the basis of determination of anti-HSV activity in this study.
2.1.7 Chemotherapy of HSV infection

Many specific therapeutic approaches have been attempted to suppress this troublesome infection. These methods have focused primarily on the treatment of initial or recurrent infections.

2.1.7.0 Interferon-α

Various interferon preparations have been tested for HSV infections (Gresser et al., 1976; Spruance et al., 1982). One promising early trial showed a significant reduction in viral shedding and lesion formation in patients treated with interferon-α before and after trigeminal root operation. After discontinuation of therapy, though, the interferon-α and placebo groups had equal numbers of recurrences (Pazin et al., 1979). In renal transplant patients, prophylactic use of leukocyte interferon-α decreased the incidence of cytomegalovirus viremia but had no effect on the incidence of herpes simplex virus infections (Cheeseman and Rubin, 1979). O’Brien et al., (1998) also reported successful treatment of HSV-1 infected cells with interferon-α (IFN-α) by observing reduced levels of viral enzyme (ribonucleotide reductase) responsible for viral replication.
2.1.7.1 Antiviral Agents

2.1.7.1.0 Nucleoside Analogs

Nucleoside analogs compete with the nucleosides required for synthesis of virus DNA (Viral DNA polymerase). The nucleoside analogs are phosphorylated and incorporated into replicating DNA. This results in an abnormal DNA template for transcription and translation, as well as interference with enzyme functions necessary for DNA synthesis (Holmes et al., 1990). Some of the nucleosides analogs in use are Iodoxuridine, Vidarabine and Acyclovir.

2.1.7.1.0.1 Iodoxuridine (5-iodo-2'-deoxyuridine)

Iodoxuridine was the first agent shown to be efficacious in the treatment of herpes keratitis in humans (Kaufman et al., 1962). A topical administration of iodoxuridine on mucocutaneous disease shortens the time to cessation of viral shedding (Silvestri et al., 1982), however, it is too toxic for systemic use.

2.1.7.1.0.2 Vidarabine

Vidarabine inhibits viral DNA polymerase and has been used in the treatment of various herpesvirus infections, including herpes simplex encephalitis (Whitley et al., 1977), neonatal herpes (Whitley et al., 1980) and herpes simplex keratitis (Buchanan and Hess, 1980). However, topical and intravenous vidarabine treatments are ineffective for both primary and recurrent genital herpes, except in
immunodeficient patients (Adams et al., 1976; Goodman et al., 1975; Whitley et al., 1984). Also, vidarabine therapy has been associated with unexpectedly frequent and severe neurological toxicity in patients (Safrin et al., 1991b)

2.1.7.1.0.3 Acyclovir (9-[(2-hydroxyethoxy)methyl] guanine, Zovirax)

Acyclovir (ACV), a guanosine analog, has a selective action on HSV infected host cells and consequent low toxicity for normal uninfected host cells (Holmes et al., 1990). It is the drug of choice for management and treatment of HSV infections in humans (Dunkle et al., 1991). It was the first antiviral agent approved for treatment of mucutaneous herpes simplex in the United States and is available for intravenous, topical, and oral use (Elion, 1982). The compound itself is relatively inert, but HSV thymidine kinase present in infected cells preferentially phosphorylates acyclovir to its monophosphate form. Cellular thymidine kinase has a much lower affinity for the drug, so this step does not occur to any great extent in uninfected cells. Further phosphorylation to the triphosphate form is accomplished by cellular enzymes, and acyclovir-triphosphate then acts as an inhibitor of, and substrate for, the HSV DNA polymerase and effectively stops viral replication (Holmes et al., 1990). The triphosphate form is a substrate for viral DNA polymerase and as such is incorporated into newly formed viral DNA, resulting in termination of the DNA chain because acyclovir does not have a 3'-hydroxyl group on which to continue elongation (Furman et al., 1979; Derse et al., 1981).
For first episodes of genital herpes, all three formulations of ACV appear effective in stopping viral shedding. However, intravenous and oral acyclovir shows greater effect on the duration of symptoms and time to complete healing than topical preparation (Corey et al., 1983a). Oral ACV reduces the duration of viral shedding and the frequency of new lesion formation and slightly diminishes the time to complete healing, especially if therapy is initiated early (Nilsen et al., 1982). Acyclovir is effective only against actively replicating virus and does not eliminate latent herpes simplex genomes.

2.1.7.1.1 Non-nucleoside antivirals

2.1.7.1.1.0 Foscarnet (trisodium phosphonoformate)

Foscarnet is a pyrophosphate analog that directly inhibits viral DNA polymerase (Alenius et al., 1982; Wallin et al., 1979). It therefore does not need thymidine kinase for activity. Potential toxicities of foscarne which include nephrotoxicity, anemia, and gastrointestinal intolerance has reduced it use significantly (Youle et al., 1988; Cacoub et al., 1988).

2.1.7.1.1 Phosphonoacetic acid (PAA)

Phosphonoacetic acid (PAA) is an effective inhibitor of HSV growth in tissue culture (Overby et al., 1974) and significantly reduces mortality and morbidity of experimentally infected animals (Gerstein et al., 1975). It inhibits in
vitro activity of the virus-specific DNA polymerase of HSV (Overby et al., 1974). In vitro drug resistance has been encountered (Duff et al., 1978) and therefore in vivo resistance may develop.

2.1.8 Use of anti-HSV Vaccines

There are four major categories of virus vaccines:- live attenuated virus (Roizman et al., 1982), inactivated virus (replication-incompetent) (Thomson et al., 1983), virus sub-unit (Watson et al., 1982), and nucleic acid-based vaccines (Ulmer et al., 1993). Live attenuated virus provides an opportunity for the proliferation of the virus and concomitant stimulation of the immune system without the production of significant pathologic changes. Inactivated vaccines are recommended for diseases that stimulate a temporary immunity and for viruses with several antigenic types. Inactivation of the virus must be accompanied by retention of antigenicity. This is often accomplished by treatment of the virus with heat of formalin. Such inactivated vaccines contain nonreplicating virus antigenically capable of stimulating an immune response. Sub-unit vaccines consists of protein sub-units of the virus in question. This vaccine requires large amounts of virus for potent immunogenicity. Nucleic acid-based vaccines mainly consist of the viral nucleic acids whose gene sequence is altered through genetic engineering. However, as at the time of this writing, no successful HSV vaccine for human use is in the market.
2.2 Anti-viral Resistance

ACV-resistant HSV strains are evident in immunosuppressed patients, such as organ transplant recipients and patients with acquired immunodeficiency syndrome (Birch et al., 1990; Coen, 1994). Investigations of virus strains derived from clinical lesions has revealed the presence of acyclovir resistance mainly from immunocompromised patients and more so in AIDS patients (Safrin et al., 1990).

Treatment of cultures with low drug concentrations has identified herpes simplex viruses which are resistant to acyclovir, phosphonacetic acid, and foscarnet (Field, 1983). Studies of resistance in animals and humans are limited primarily to anti-herpes agent, acyclovir (Dunkle et al., 1991).

Drugs inhibit HSV infections by interacting with the virus-coded DNA polymerase so that subsequent viral replication is impaired. Some of the compounds active against the virus directly inhibit the DNA polymerase (for example, phosphonoacetic acid and foscarnet), whereas most nucleoside analogs can only inhibit DNA polymerase after phosphorylation by nucleoside kinases to their mono-, di-, and triphosphate forms. For some of these agents (such as acyclovir), the initial phosphorylation is selectively executed by virus-specified, thymidine kinase.

Three mechanisms of resistance of herpes simplex virus to acyclovir exist. The commonest mechanism in an alteration or deletion in the gene for thymidine kinase that renders herpes simplex unable to induce production of
thymidine kinase (Coen and Schaffer, 1980; Schnipper and Crumpacker, 1980). Such herpes mutants are termed thymidine kinase negative (deficient) or TK−, whereas mutants that induce thymidine kinase (naturally occurring) are termed TK+. Strains of TK− herpes simplex have reduced infectivity and less neurovirulence than wild-type, or naturally occurring, TK+ herpes simplex strains (Field and Wildy, 1978; Tenser et al., 1979).

Another mechanism of resistance of herpes simplex virus to acyclovir is an alteration in the substrate specificity of the DNA polymerase (pol) gene that renders herpes simplex DNA polymerase resistant to acyclovir (Coen and Schaffer, 1980; Schnipper and Crumpacker, 1980; Balfour, 1983). These mutants appear to be nearly as virulent as the wild type from which they derive (Field and Darby, 1980). Also, the site of the acyclovir DNA polymerase resistance marker is very close to the gene locus that codes for resistance to other antivirals such as phosphonoacetic acid (Coen and Scaffer, 1980; Schnipper and Crumpacker, 1980; Field et al., 1981). Therefore, some of these isolates are resistant to other antiviral agents besides acyclovir (Nugier et al., 1992). In HSV with mutant DNA polymerase (pol) gene, the altered enzyme can synthesise viral DNA in the presence of high concentrations of acyclovir triphosphate (ACVTP) (Furman et al., 1981).

The third mechanism of resistance comes as a result of altered substrate specificity of viral-induced thymidine kinase. This arises from a mutation in the structural gene for thymidine kinase. Such viral progeny induce 30% to 40% of
the normal amount of thymidine kinase but the enzyme is less efficient at phosphorylating acyclovir or the nucleoside analog drugs (Darby et al., 1981). Herpex simplex mutants with altered substrate specificity for thymidine kinase retain pathogenicity for mice with only a slight decrease of neurovirulence in mice.

The vast majority of acyclovir-resistant mutants from clinical lesions are based on thymidine kinase deficiency (TK\(^{-}\)) even though strains with thymidine kinase of altered specificity and DNA polymerase mutants have also been isolated from clinical lesions on rare occasions (Birch et al., 1990; Ellis et al., 1987; Parker et al., 1987).

With emergence of resistant strains of HSV, it is therefore, necessary to develop new therapeutic agents with different modes of anti-HSV action in order to control/manage the ever increasing HSV infections. One of the alternatives is to search for anti-HSV agents from medicinal plants.

2.3 Medicinal Plants and Traditional Medicine

2.3.0 Medicinal Plants

Medicinal plant extracts used as traditional medicines have been administered orally in the form of their hot water (HW)-extracts for various diseases in human. Information on their adverse reactions has been historically accumulated in traditional therapy (Jiangxu, 1978; Kokwaro, 1976; Gachathi,
1989). Thus traditional medicines appear to be useful sources to search for new antiviral agents (Herrmann, 1961; Chang and Yeung, 1988).

2.3.1 Traditional Medicine

Traditional medicines are medicines prepared from materials whose characteristics or properties are based on indigenous knowledge. They are prepared by people who have acquired the knowledge of their preparation from elder relatives or leaders in a community. In recent times, traditional medicines are more commonly prepared by traditional health practitioners (traditional doctors or traditional healers) who have taken it as a professional commercial practice. There are other categories of traditional medical practitioners like the divined and spirit mediums, who are more akin to contemporary psychiatrists in allopathic medicine.

Traditional medicines are used in the practice of traditional medicine by traditional medical practitioners who are composed of two main groups, namely, traditional doctors and traditional midwives (often referred to as traditional birth attendants). These two traditional medical practitioners use plant-derived medicines in their practice. The traditional practitioners who use plant-derived medicines are called herbalists.

Traditional medicines are used in the prevention and treatment of disease though they lack the chemical, pharmacological, toxicological, and
pharmaceutical specifications that are required to describe modern pharmaceuticals.

2.3.2 Plants as a source of anti-HSV agents

Natural products from medicinal plants have been shown to contain antiviral compounds *in vitro* (Hayashi *et al.*, 1992; Yao *et al.*, 1992; Kofi-Tsepo *et al.*, 2001; Craig *et al.*, 2001; Kuo *et al.*, 2001). Kurokawa *et al.*, (1993b), investigated 142 medicinal plant extracts (hot water extracts) for anti-HSV-1 activity using both tissue culture and laboratory animals. Thirty two of the extracts showed anti-HSV-1 activity with 12 (belonging to the families of *Caesalpiniaceae*, *Polygalaceae*, *Anacardiaceae*, *Punicaceae*, *Annonaceae*, *Combretaceae* and *Myrtaceae*) exhibiting both *in vitro* and *in vivo* activity. This indicated that extracts with therapeutic efficacy in the animal infection model are possible candidates for anti-HSV-1 traditional medicine. Kurokawa *et al.*, (1999), described a pure compound from *Rhus javanica* L. (gall), a medicinal herb of the orient, as having both *in vitro* and *in vivo* anti-HSV-1 activity. However, the mechanism of anti-HSV action of this compound needed further investigation since clear cut difference of anti-HSV activity to that of ACV had not been observed.

An investigation carried out by Bong-Joo *et al.*, (1998) in search for anti-HSV type 1 agents from Korean medicinal plants, revealed that out of the 57 medicinal plants examined, 6 boiled water extracts arising from four individual
plants and two complexes (more than one medicinal plant mixture) showed efficacy against HSV.

Nawawi et al., (1999), investigated the inhibitory effects of water and methanol extracts from 30 Indonesian traditional medicinal plants on the infection of HSV-1 and found that extracts from 8 plant species showed potent activity on the plaque reduction assay at a concentration of 100 μg/ml. Both hot water and methanol extracts from Punica granatum L. (punicaceae) inhibited plaque formation by 100%, an indication of potent anti-HSV activity.

Hudson et al., (2000), evaluated 16 extracts of Turkish medicinal plants for antiviral activities against HSV and Sindbis virus (SINV) and found that the extracts from Galanthus elwesii and Rheum ribes showed the most potent anti-HSV activities.

Based on these previous observations, twelve medicinal plants locally used as traditional medicines in Kenya, were investigated for their potential anti-HSV activity. Their hot water extracts were first examined for anti-HSV activity in vitro by plaque reduction assay (Kurokawa et al., 1993b; 2001). Those extracts that exhibited high antiviral activity were selected and examined further to determine the concentration which reduced plaque formation by 50% (EC50) and the cell cytotoxic concentration (CC50) which caused cell lysis. The extract which gave the best activity was then evaluated for in vivo therapeutic efficacy in BALB/c mice cutaneously infected with HSV (Kurokawa et al., 1993b; 2001).
2.3.3 Plants included in the study

Extracts from 12 medicinal plants were screened for anti-HSV activity. The plants were selected from ethno-medical use and literature search (Kokwaro, 1976; Gachathi, 1989). The plants were; *Toddalia asiatica* (L.) Lam. (Rutaceae), *Rhus natalensis* Krauss (Anacardiaceae), *Clausena anisata* (Willd.) Benth. (Rutaceae), *Vernonia jugalis* Oliv. & Hiern (Compositae), *Maytenus heterophylla* (Eckl. & Zeyh.) Robson (Celastraceae), *Rhamnus prinoides* L’Herit. (Rhamnaceae), *Carissa edulis* (Forssk.) Vahl (Apocynaceae), *Teclia simplicifolia* (Engl.) Verdoorn (Rutaceae), *Periploca linearifolia* Dill & A. Rich. (Asclepiadaceae), *Albizia coriaria* Oliv. (Leguminosae), *Fagaropsis angolensis* (Engl.) Dale (Rutaceae) and *Zanthoxylum usambarense* (Engl.) Kokwaro (Rutaceae). Out of the twelve medicinal plants three of them (*Carissa edulis*, *Maytenus heterophylla* and *Periploca linearifolia*) showed anti-HSV activity (Chapter 4). The background of the three medicinal plants are described below.

2.3.3.0 Carissa edulis

*Carissa edulis* belongs to the family *Apocynaceae* (Beentje, 1994). It is an evergreen shrub, occasionally scrambling, common in most districts of Kenya in bush and forest edges 0-2000m above sea level. It tolerates most soils, including black cotton. The shrub is spiny and about 1-5m. It has a grey bark with straight woody spines of up to 5 cm which are in pairs and rarely forked. The bark produces milky latex on cutting. The leaves are opposite, leathery, dark green and
shiny and up to 5cm long. The tip is pointed, base rounded and a very short stalk. The plant bears flowers which are pink-white with terminal clusters. It also bears fruit which are rounded berries about 1 cm, purple-black when ripe, sweet and edible containing 2-4 seeds. The plant can be grown from seedlings. In addition to being of medicinal value, this plant is used as a live fence, for fuelwood, food seasoning and soup making. The roots are the most widely used medicinal part (ICRAF, 1992).

A study of the *Apocynaceae* species used in traditional medicine in Kenya revealed that the most common category of diseases treated with this plant are skin and ectoparasitic diseases followed by abdominal diseases, diseases of the head and venereal diseases (Omino and Kokwaro, 1993). *Carissa edulis* is also traditionally used for the treatment of malaria, indigestion and general chest illness (Kokwaro, 1976). El-Fiky *et al.*, (1996), has reported that ethanolic extracts of *C. edulis* leaves can lower blood glucose levels in diabetic rats.

The chemistry of this species has shown that its members usually have indole alkaloids in their roots (Omino and Kokwaro, 1993). Bentley *et al.*, (1984) have reported the presence of 2-Hydroxyacetophenone in the roots of *C. edulis* from East Africa. Lindsay *et al.*, (2000) have isolated carissone, dehydrocarissone and carindone from dichloromethane extracts of a *Carissa* species, *Carissa lanceolata* R.Br. They have also shown that these natural products from members of this family have antibacterial activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, with dehydrocarissone and
carindone having a minimum inhibitory concentration of less than 0.5 mg/ml against *S. aureus* and *E. coli*.

### 2.3.3.1 *Maytenus heterophylla*

The plant belongs to the family *Celastraceae* (Beentje, 1994). It is a shrub or small tree 1.5-6m long and contains spines 1-6cm long. Leaves fasciculate on short shoots and have a cuneate base with a rounded apex. The plant produces white, cream or yellow flowers with yellow or red fruit obovoid in shape 4-10mm long. It grows along the coast 1-350m and inland 1150-2700m above sea level in dry upland and lowland forests. *Maytenus heterophylla* is a medicinal plant whose roots are boiled and liquid drunk as an anthelminic, cure for hernia and syphilis (Kokwaro, 1976). The roots are boiled as a vegetable by the Masai (Beentje, 1994).

*Maytenus heterophylla* ethanol extracts have been reported to contain dihydroagarofuran alkaloids and triterpenes with antimicrobial activity (Orabi *et al.*, 2001). Wagner and Burghart, (1977) have also reported the presence of spermidine alkaloids and triterpenes in this plant. Members of the species (*M. disticha* and *M. boaria*) have been reported to have insecticidal activity (Avilla *et al.*, 2000; Cespedes *et al.*, 2001). Kennedy *et al.*, (2001) have reported a new sesquiterpenes from the roots of *M. magellanica* and *M. chubutensis* with antiparasitic activity.
2.3.3.2 *Periploca linearifolia*

*Periploca linearifolia* belongs to the family *Asclepiadaceae* (Beentje, 1994). The plant is a climbing herb of up to 10m long. It has linear or narrow elliptic leaves with cuneate or obtuse base and a gradually tapering apex. The plant produces flowers which are cream or greenish-yellow and cylindric fruits. It grows 1700-2400m above sea level on forest margins, riverine forest and secondary bushland derived from forest. The plant is ceremonial for the Kalenjin tribe (Beentje, 1994) and a medicinal plant used in soup and for treatment of chest complaints and fevers (Gachathi, 1989).

The chemistry of the members of this species has shown the presence of triterpenes in the stems of *P. aphylla* with anti-bacterial activity (Mustafa *et al.*, 2000), steroid glycosides from the barks of *P. sepium* BGE (Umehara *et al.*, 1995), pregnane glycosides with antitumor activity from *P. sepium* (Itokawa *et al.*, 1988) and cardiac glycosides from *P. sepium* BGE (Ding and Lou, 1966).
## MATERIALS AND METHODS

### Medicinal plants

The following 12 medicinal plants were investigated for anti-HSV activity.

<table>
<thead>
<tr>
<th>Name</th>
<th>Family</th>
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<tbody>
<tr>
<td><em>Albizia coriaria</em></td>
<td><em>Leguminosae</em></td>
</tr>
<tr>
<td><em>Toddalia asiatica</em></td>
<td><em>Rutaceae</em></td>
</tr>
<tr>
<td><em>Rhus natalensis</em></td>
<td><em>Anacardiaceae</em></td>
</tr>
<tr>
<td><em>Vernonia jugalis</em></td>
<td><em>Compositae</em></td>
</tr>
<tr>
<td><em>Maytenus heterophylla</em></td>
<td><em>Celastraceae</em></td>
</tr>
<tr>
<td><em>Clausena anisata</em></td>
<td><em>Rutaceae</em></td>
</tr>
<tr>
<td><em>Teclea simplicifolia</em></td>
<td><em>Rutaceae</em></td>
</tr>
<tr>
<td><em>Rhamnus prinoides</em></td>
<td><em>Rhamnaceae</em></td>
</tr>
<tr>
<td><em>Carissa edulis</em></td>
<td><em>Apocynaceae</em></td>
</tr>
<tr>
<td><em>Periploca linearifolia</em></td>
<td><em>Asclepiadaceae</em></td>
</tr>
<tr>
<td><em>Fagaropsis angolensis</em></td>
<td><em>Rutaceae</em></td>
</tr>
<tr>
<td><em>Zanthoxylum usambarense</em></td>
<td><em>Rutaceae</em></td>
</tr>
</tbody>
</table>

The medicinal plants were chosen from information obtained from ethnomedical use and literature search (Kokwaro, 1976; Kokwaro, 1993; Gachathi, 1989).
3.0.1 Collection of the medicinal plants

The medicinal plants used in this study were collected from their natural habitats in different geographical zones of Kenya. *Rhus natalensis*, *Maytenus heterophylla*, *Clausena anisata*, *Carissa edulis*, *Periploca linearifolia*, *Fagaropsis angolensis*, *Zanthoxylum usambarense*, *Rhamnus prinoides* and *Teclea simplicifolia* were collected from Gitoro forest, Meru, in Eastern Province, Kenya. *Toddalia asiatica* and *Albizia coriaria* were collected in Rangwe and Migori areas of Nyanza Province, Kenya. *Vernonia jugalis* was collected from Ngong forest in the Rift Valley Province, Kenya. The plants were identified on the ground before collection by a plant taxonomist who was part of the collection team. The plant parts were collected with bio-conservation aspects in mind. Voucher specimens are on deposit at the East African Herbarium, Nairobi, Kenya.

3.0.2 Initial processing of the medicinal plant parts

Either roots, stems or leaves from *T. asiatica*, *A. coriaria*, *R. natalensis*, *V. jugalis*, *M. heterophylla*, *C. anisata*, *T. simplicifolia*, *R. prinoides*, *C. edulis*, *P. linearifolia*, *F. angolensis* and *Z. usambarense* were used. The roots and stems were harvested and their barks peeled off while still fresh and cut into small portions and then dried at room temperature for 1 week. The leaves were collected while green and dried similarly. The root bark, stem bark and the leaves were separately ground when completely dry using an electric mill (Christy and Norris
Ltd., England). The powdered plant materials were kept separately in closed plastic containers at room temperature (23°-25°C).

3.0.3. Preparation of the aqueous extracts

One hundred grams of each powdered plant material was boiled in 1 liter of distilled water for 1 hr. At the end of the boiling time, the extract was decanted into a 3 litre clean dry conical flask and filtered through folded cotton gauze (Harley’s chemist medical grade) into another dry clean 3 liter conical flask. The filtered extract was then freeze-dried in 200 ml portions using a Modulyo Freeze Dryer (Edwards, England) for 48 hr. The freeze-dried powder was then pooled together in an air tight container, weighed and stored at room temperature (≈23°C approx.) until used in the experiments (Kofi-Tsekpo et al., 1985).

3.0.4 Preparation of stock solutions of the extracts

A 1 g/ml stock solution of the extracts was aseptically prepared by dissolving 10 g of the freeze-dried powder in sterile double distilled water and made to the 10mls mark of a volumetric flask. The solution was centrifuged at 3,000 rpm for 15 min (Kubota centrifuge, Japan) to separate any undissolved matter and the supernatant collected and filtered through a 0.45 μm membrane filter. The sterile filtrate was stored at 4°C and used as a stock solution within a week (Kurokawa et al., 1993b).
For *in vitro* experiments, a working solution of 1 mg/ml was aseptically prepared by diluting 1 µl stock with 0.999 ml (999 µl) Eagle's Minimum Essential Medium (MEM) (GIBCO BRL, Scotland) supplemented with 2% Fetal Bovine Serum (FBS) (GIBCO BRL, Scotland). For the initial *in vitro* screening, the test concentrations of 50 µg/ml and 100 µg/ml was achieved by adding 50 µl or 100 µl working solution to 950µl or 900µl of MEM containing 2% FBS and 0.8% nutrient methyl cellulose (MC) (BDH, England) respectively for treatment of infected cells.

For the *in vivo* experiments, a working solution of 20 mg/ml was aseptically prepared from the stock solution by diluting 20 µl stock solution in 0.98 ml (980 µl) sterile double distilled water. 0.25 ml of the working solution was then orally administered to a 20 g body weight mouse to give 5 mg/20g (250 mg/kg - experimentally determined as therapeutic).

3.1 Acyclovir (ACV)

Acyclovir was purchased as tablets (Zovirax 200, Wellcome) from Nippon Wellcome K. K. Japan through the Japan International Co-operation Agency (JICA), by the Kenya Medical Research Institute (KEMRI) liaison office. A stock suspension of 10mg/ml for *in vitro* assay was prepared by carefully powdering a tablet (200 mg ACV) in a clean mortar using a pestle and directly adding 20 ml of sterile double distilled water, under aseptic conditions. The mixture was vortexed for 1 min and the milky suspension kept at 4°C. For *in vitro* assays, a working
solution of 1 mg/ml was achieved by diluting 100 μl stock solution with 0.9 ml (900 μl) MEM supplemented with 2% FBS. 5 μl of the working solution was added in 995μl MEM containing 2% FBS and 0.8% MC for a standard test concentration of 5 μg/ml for treatment of HSV infected Vero E6 cells.

For *in vivo* assays a working concentration of 0.4 mg/ml was prepared by diluting 40 μl of stock solution with 0.96 mls (960 μl) of sterile double distilled water and 0.25 ml of the working suspension was then administered to a 20 g body weight mouse to give 100 μg/20g (5 mg/kg) (Kurokawa *et al.*, 1993b).

### 3.2 Viruses and cells.

#### 3.2.0 Cells

The Vero E6 cells were donated by the Virology department, Toyama Medical and Pharmaceutical University, Toyama, Japan, through JICA, KEMRI liaison office. The cells were grown under 5% CO<sub>2</sub> at 37°C in MEM supplemented with 5% FBS for cell growth, and 2% FBS for cell maintenance after growth.

#### 3.2.1 Viruses

The herpes simplex virus (HSV) strains used in the study were wild-type 7401H HSV-1 (Kurokawa *et al.*, 1993b), wild-type HSV-2 (Ito-1262) (Kurokawa *et al.*, 1995b), thymidine kinase deficient (TK<sup>−</sup>) B2006 HSV-1(Dubbs and
Kit, 1964) and acyclovir resistant (AP$^3$) 7401H HSV-1 (Kurokawa et al., 1995b). These viruses were donated by the Virology Department, Toyama Medical and Pharmaceutical University, Toyama, Japan, through JICA, and received by KEMRI liaison office. The virus stocks were prepared from infected Vero E6 cells. The infected cultures were frozen and thawed three times to lyse the cells, and centrifuged at 3,000 rpm for 15 min. Their supernatants, containing HSV, were harvested and stored at -80 °C until use (Kurokawa et al., 1993b).

3.2.2. Virus titration

200 μl of neat virus stock was used to infect Vero E6 cells in each 60 mm diameter petri-dish and left to adsorb for 1 hr. The infected cells were then incubated at 37 °C in 5% CO$_2$ for 24 hr. At the end of the incubation time, the cell cultures were frozen at -80 °C and thawed at 37 °C for 1 hr. This procedure was repeated 3 times to ensure that all the infected cells were lysed to enable the harvesting of the virus. The lysed cultures were collected in 15 ml tubes and centrifuged for 10 min at 3000 rpm. The virus titre of the supernatant was then determined by the plaque assay on Vero E6 cells as explained below.

A serial dilution of the supernatant was prepared in MEM supplemented with 2% FBS (serial dilutions of 1:10, 1:100, 1:1000, 1:10000, 1:100000 and 1:1000000). 200 μl of each serial dilution was then used to infect a monolayer of Vero cells in duplicate. After viral adsorption for 1 hr, 5 ml of nutrient methylcellulose (MC) was added into each dish and incubated for 3 days at 37 °C.
in 5% CO₂. At the end of the incubation period, the cells were fixed with 5% formalin solution for 1 hr and media aspirated off. 2 ml of 0.03% methylene blue solution was added in each dish and left to stand for 1 hr. The dye was then washed off with tap water and plates dried at room temperature. The plaques were counted under a dissecting microscope and plaque forming units (PFU) determined. The virus titre was then calculated using the formula below (Payment and Trudel, 1993).

\[
\text{PFU/ml} = \text{Dilution} \times \frac{p^1 + p^2 + \ldots + p^n \times \frac{1}{n}}{v}
\]

Dilution = virus dilution from stock e.g., 10²

\( p = \) number of plaques counted in all the dishes at this dilution

\( n = \) number of dishes at this dilution

\( v = \) volume inoculated in each dish (in millilitres)
3.3  *In vitro* Assays

3.3.0 Plaque reduction assay

The herbal extracts were examined for the extent of inhibition of plaque formation on HSV infected tissue culture as an indication of anti-viral activity as described by Kurokawa *et al.*, (1993b; 2001).

Vero E6 cells were cultured to a confluent monolayer in 5 ml MEM supplemented with 5% FBS in 60 ml sterile petri-dishes in a 5% CO$_2$ incubator (Hirasawa Works, Japan) at 37 °C for 3 days. The culture medium was then aspirated off aseptically and the cells infected with 100 plaque forming units (PFU) of HSV. The virus was left to adsorb for 1 hr at room temperature on a tray shaker. The cells were then overlaid with 5 ml of MEM containing 2% FBS and 0.8% MC and various concentrations of herbal extract (50 or 100 μg/ml) or acyclovir at 5 μg/ml. Each concentration was done in duplicate. The cells were then incubated at 37 °C in 5% CO$_2$ for 3 days. Acyclovir was used as a reference drug. At the end of the incubation period on the third day, the infected cells were fixed by adding 2 ml of 5% formalin (Sigma, USA) solution into each dish and left to stand for 1 hr. The media in each plate was then decanted and the cells gently washed with running tap water without disturbing the cells. Two millilitres of 0.03% methylene blue solution (Wako Pure Chemical Industries Ltd., Japan) was then added into each plate and left to stand for 1 hr. The stain was then washed off with tap water (cells which had not been infected by the virus pick the
stain while the ones which had been killed by virus do not). The plates were then
dried at room temperature and observed under a dissecting microscope. The plaques, appearing as transparent dots, were counted with the aid of an electronic maker pen and the percent plaque formation calculated. The per cent plaque inhibition was then also calculated from the formula (Kuo et al., 2001):

\[
\% \text{ plaque formation} = \frac{\text{Number of experimental plaques}}{\text{Number of control plaques}} \times 100
\]

\[
\% \text{ plaque inhibition} = \frac{\text{Number of plaques (control – experimental)}}{\text{Number of control plaques}} \times 100
\]

Where, control = number of plaques in untreated dishes

experimental = number of plaques in treated dishes

3.3.1 EC\textsubscript{50} Determination

The effective concentration which inhibits the formation of plaques by 50% (EC\textsubscript{50}) was determined for medicinal plant extracts which exhibited high anti-HSV activity. Plaque reduction assay was run for the extracts at various extract concentrations (10, 40, 60, 80, 100 and 200\textmu g/ml). A graph was then drawn for concentration verses % plaque inhibition from which the EC\textsubscript{50} values were obtained (appendix 1). The EC\textsubscript{50} determination was independently done three times for each plant extract and the mean and standard deviation (S.D) calculated.
3.3.2 Cell cytotoxicity assay (CC$_{50}$)

The cytotoxic concentration which causes 50% cell lysis and death (CC$_{50}$) was determined for the extracts showing high anti-HSV activity in the plaque reduction assay by the method described by Kurokawa et al., (2001). The CC$_{50}$ was evaluated by counting the number of viable cells using the trypan blue exclusion test after incubation of Vero E6 cells in various concentrations of the plant extracts.

Vero E6 cells were grown in 25 cm$^2$ flasks in MEM supplemented with 5% FBS to a logarithmic phase. The cells were then harvested by trypsinization and pooled in a 50 ml tube. The cells were centrifuged at 1000 rpm for 5 min and the supernatant aspirated off and cell stock prepared by re-suspending the cells in 40 ml fresh MEM supplemented with 5% FBS. A 1:10 dilution of the cells was then made by diluting 0.1 ml stock with 0.9 ml MEM supplemented with 5% FBS. The number of cells in the diluted suspension was then counted by the trypan blue exclusion test (as detailed below). A 1 ml cell suspension containing 6 x 10$^4$ cells was then added into each well of a 24 well plate. The cells were incubated at 37 $^\circ$C in 5% CO$_2$ for 2 days (for growth to the logarithmic phase). The media was then aspirated off. One millitre of various concentrations of the test samples (100, 200, 300, 400, 600, 700, 800, 900 and 1000 µg/ml prepared in MEM supplemented with 5% FBS) was then added into each well (3 wells per sample) and the cells were further incubated at 37 $^\circ$C in 5% CO$_2$ for 2 days. The
media containing the various concentrations of the extracts was then decanted. The viable cells were then counted by the trypan blue exclusion test.

### 3.3.2.1 Trypan blue exclusion test

Two hundred and fifty microliters of 0.3% trypsin solution in Phosphate buffered saline (PBS) (Nissui Seiyaku Pharmaceutical Co., Ltd., Japan) was added into each well and left to stand for 5 min. Fifty microliters of 0.03% trypan blue solution in PBS was separately added in a 96 well plate and an equal amount of cell suspension added and mixed. The number of viable cells was then counted on a hemocytometer. The cytotoxic concentration (CC₅₀) was then determined from a graph relating percentage of cell viability to the concentration of the extracts (appendix 2). The total viable cells were calculated according to the formula below (Sigma, 1997):

\[
\text{Total viable cell count} = a \times DF \times b \times 10^4
\]

Where
- \( a \) = average viable cell count from hemocytometer (4 counts)
- \( DF \) = dilution factor (e.g. 50 μl in 100 μl is 2 as in the exp.)
- \( b \) = Original volume of cells (e.g. 250 μl as in the exp.)
- \( 10^4 \) = hemocytometer cell concentration per ml. (a constant value)
3.3.3 Virus yield reduction assay

The *C. edulis* root bark extract was compared for its antiviral activity with acyclovir on the growth of the wild HSV-1, AP′ HSV-1, TK− HSV-1 and wild HSV-2 in the virus yield reduction assay as described by Kurokawa *et al.*, (1998).

Vero E6 cells were grown to a confluent monolayer in MEM supplemented with 5% FBS in 60mm diameter dishes at 37 °C in 5% CO₂. Three dishes of the prepared cells were harvested by trypsinization and separately pooled in 50 ml tubes. The harvested cells were centrifuged at 1000 rpm for 5 min and the supernatant aspirated off. A cell stock for each of the three was prepared by re-suspending the cells in 40 ml fresh MEM supplemented with 5% FBS. A 1:10 dilution of the cells was then made by diluting 0.1 ml stock with 0.9 ml MEM supplemented with 5% FBS. The number of cells in each of the suspensions was counted by the trypan blue exclusion test. The average number of cells was found to be 5 x 10⁶ cells/60mm diameter dish. This cell number was used in the next stage of the assay to determine the amount of virus to be used to infect the cells having confluent growth in the remaining 60 mm dishes prepared at the start of the experiment.

The medium was aspirated from the remaining dishes with confluent monolayer of cell growth and 200 µl of HSV at multiplicity of infection of 5 (M.O.I 5) (i.e. 5 x 5 x 10⁶ PFU HSV, same as 5PFU for each cell) was added into each dish. The virus was spread on the cells by gentle shaking. The virus was then left to adsorb for 1hr on a tray shaker. The cells were washed 3 times with MEM.
Five milliters of MEM supplemented with 2% FBS and containing various concentrations of the test sample (5, 15, 20, 60, 100, and 200 μg/ml) were added into each labelled dish. Acyclovir, at various concentrations (0.25, 0.5. 1.0, and 2.0 μg/ml) was also separately added. The cells were then incubated at 37 °C in 5% CO₂ for 24 hr. The cell cultures were frozen at -80 °C and thawed at 37 °C for 1 hr. This procedure was repeated 3 times. The lysed cultures were then collected in 15ml tubes and centrifuged at 3000 rpm for 10min. A serial dilution of each supernatant was prepared in MEM supplemented with 2% FBS (serial dilutions of 1:10, 1:100, 1:1000, 1:10000, 1:100000 and 1:1000000). Two hundred microlitres of each serial dilution was then used to infect a monolayer of Vero cells. The virus titre of each supernatant was then determined by the plaque assay.

3.4 In Vivo Assays

3.4.0 Therapeutic efficacy of Carissa edulis root bark extract in male and female BALB/c mice cutaneously infected with HSV

The determination of the therapeutic efficacy of the C. edulis root bark extract was carried out using BALB/c as described by Kurokawa et al., (1993b; 2001).

Specific-pathogen free female and male BALB/c mice 7-weeks old weighing between 19-20 g were purchased from Nairobi University, Kabete Campus, Nairobi, Kenya. The mice were housed five per cage with food (Mice
cubes, Unga feeds, Kenya) and water ad libitum. The mice were acclimatised for 3 days before the start of the experiments.

After 3 days of acclimatisation the mid flank of each mouse was shaved using an electric hair trimmer (Wahl super taper, England). The hair was then completely removed by applying a chemical hair remover (Shiseido, Co., Ltd., Tokyo, Japan) on the shaved area and washed off with warm water. The animals were left to adjust to the shaved state at room temperature for 2 days.

On the day of the experiment, the mice were randomly divided into four groups each comprising of 5 mice. The first group of mice were the control group. This group was not infected even though the skin was scratched and they received no treatment. The second group was also a control group but they were scratched, infected and received no treatment. The third group was the test group. The mice were scratched, infected, and received the plant extract treatment. The final group, the fourth, was the reference group. In this group mice were scratched, infected and received ACV treatment. An initial oral dose of the test extract or ACV contained in 0.25 ml of double distilled water, was administered to the mice accordingly before infection (the mice were initially orally administered with 125, 250, and 375 mg/kg of the freeze dried C. edulis root bark extract accordingly or 5 mg/kg ACV. In subsequent experiments after determination of the best therapeutic dose, 250 mg/ml extract and 5 mg/ml ACV was administered). The two control groups were administered with distilled water. Four hours after the oral administration, the shaved mid flank of each mouse was scratched using a
bundle of G27 needles and the sacrificed area infected with $1 \times 10^6$ PFU/2.5μl HSV apart from the control group 1. Four hours after infection, the second oral administration was given followed with the final administration for the day 12 hrs after infection. Oral administration of the test sample and reference drug were given three times a day at 8 hr intervals for seven consecutive days. The control mice were administered with distilled water at the same time. The development of skin lesions and mortality were continuously monitored every 8 hrs daily and scored as follows: 0-no lesion, 2-vesicles in local region, 4-erosion and/or ulceration in the local region, 6-mild zosteriform, 8-moderate zosteriform, 10-severe zosteriform and death. The scoring of 0, 2, 4, 6, 8 and 10 was selected to avoid transition points where the score could either be 0 or 2, 2 or 4, 4 or 6, 6 or 8, 8 or 10. The infected mice were fed and observed for 30 days to determine their mortality.

3.4.1 Determination of acute toxicity of the *Carissa edulis* root bark extract in uninfected mice

The determination of *in vivo* toxicity for *Carissa edulis* root bark extract was performed as described by Kurokawa et al., (1993b; 2001). Specific-pathogen free 7 week old 20 female and 20 male BALB/c mice were used for the determination of acute toxicity. Two hundred and fifty milligrams per kilogram of freeze-dried *C. edulis* root bark extract, the concentration found to exhibit therapeutic efficacy or 5mg/kg ACV were orally administered to the uninfected
mice three times daily for 7 days following the same schedule as under the determination of therapeutic efficacy (3.4.0). The mice were weighed every day in the morning for 21 days to determine any change in weight. The mortality of the uninfected mice was calculated on the 30th day. The weights and mortality were used for the determination of toxicity.

3.5 Phytochemical screening

A phytochemical screening for *C. edulis*, *M. heterophylla* and *P. linearifolia* total aqueous root bark extracts was investigated to determine the class of compounds present in these extracts by the methods described by Harborne, (1984).

3.5.0 Thin Layer Chromatography (TLC) of the total extracts

An aliquot of a 1 g/ml solution of a total root bark extract was sported 2 cm from the bottom of a silica gel (MN-Silica Gel G/UV254, MACHEREY-NAGEL GmbH & Co. KG, Germany) plate. A capillary tube was used for spotting the extract on the 1.0 mm thick silica gel and evaporated to dryness with a hair dryer. The sported plate was then placed in a TLC tank lined with a Whatman filter paper no. 1(Whatman, U.K) and containing a solvent system composed of butanol (40 mls): acetic acid (10 mls): water (10 mls) in the ratio of 4:1:1. The tank was covered and the gel left to absorb the solvent system upto a convenient point on the plate (solvent front) that was marked and the plate
removed from the tank. The plate was left to dry for 15 min at room temperature and observed under UV-light. The plate was then sprayed with a specific reagent to show the presence of a particular chemical compound. Different reagents were used to identify the presence of different chemical compounds. For identification of each compound a fresh sported plate was run in the appropriate solvent system, dried and sprayed with the particular reagent.

3.5.0.1 Determination for the presence of nitrogenous compounds

Plants contain nitrogenous compounds, mainly amino acids and alkaloids. Amino acids are colourless ionic compounds and are soluble in water. Alkaloids are basic substances which contain one or more nitrogen atoms combined to form a cyclic system (Harborne, 1984).

3.5.0.1.1 Alkaloids

To assess for the presence of alkaloids, the TLC plate was sprayed with Dragendorff reagent. The Dragendorff reagent is composed of; (a) Stock solution A, prepared by adding 0.6 g bismuth subnitrate in 2 ml concentrated hydrochloric acid (HCl). The mixture is then added to 10 ml water. (b) Stock solution B, prepared by adding 6 g potassium iodide in 10 ml water. The stock solutions (A and B) are mixed together and the mixture diluted to 400mls with water just before spraying the plate. The presence of orange-brown spots on a yellow background was indicative of alkaloids.
3.5.0.2 Determination for the presence of phenolic compounds

3.5.0.2.1 General presence of phenolics

To assess for the presence of phenolics, the TLC plate was sprayed with ferric ferricyanide reagent. This reagent is composed of equal volumes of freshly prepared 1% ferric chloride (FeCl₃) solution in water and 1% potassium ferricyanide \( \{K₃Fe(CN)₆\} \) solution in water. Observation of blue spots was indicative of the presence of phenolic compounds.

3.5.0.2.2 Presence of specific phenolics

3.5.0.2.2.1 Flavonoids

To assess for the presence of flavonoids, the TLC plate was exposed to 1% ammonia in a fume chamber. The appearance of a yellow, blue, dark brown, red, orange or green spot was indicative of flavonoids.

3.5.0.2.2.2 Anthraquinones

To assess for the presence of anthraquinones, the TLC plate was sprayed with 10% methanolic potassium hydroxide solution. This reagent is composed of 10g potassium hydroxide (KOH) crystals dissolved in analar grade (absolute) methanol upto the mark of a 100 ml volumetric flask. If there were visible spots of yellow and yellow-brown colours in daylight on the developed plate which
changed to red, violet, green or purple colour on spraying, this was indicative of anthraquinones.

The presence of anthraquinones was further confirmed by the method described by Rukunga, (1984). Two grams of the powdered plant material was shaken with 10 ml of benzene and filtered. 5 ml of a 10% ammonium hydroxide solution was added and the mixture shaken. The presence of a violet colour in the ammoniacal phase was indicative of the presence of anthraquinones.

3.5.0.3 **Determination for the presence of Terpenoids**

3.5.0.3.1 **General presence of terpenoids**

To assess for the presence of terpenoids, the TLC plate was sprayed with Vanillin and concentrated (conc.) Sulphuric acid (H$_2$SO$_4$). The spray was composed of 10% vanillin in absolute ethanol and conc. H$_2$SO$_4$ in the ratio of 2:1 (v/v). This was followed by heating the plate at 100°C for 15 min. The presence of terpenoids was indicated by the appearance of blue, brownish or purplish spots.

3.5.0.3.2 **Presence of specific terpenoids**

3.5.0.3.2.1 **Cardiac glycosides**

To assess for the presence of cardiac glycosides, the TLC plate was sprayed with Kedde (Carr-Price) reagent. This reagent is composed of 20%
antimony chloride in chloroform. The plate was heated for 10 min at 100°C. The appearance of brown spots was indicative of cardiac glycosides.

3.5.0.3.2.2 Steroids

To assess for the presence of steroids, the TLC plate was sprayed with Liebermann-Burchard reagent. This reagent is composed of a mixture of 1 ml conc. H₂SO₄, 20ml acetic anhydride and 50ml chloroform. The plate was heated at 90°C for 15 min. The appearance of grey, yellow or pink spots was indicative of steroids.

3.5.0.3.2.3 Saponins

To assess for the presence of saponins, 1.0 g of the freeze dried plant extract was shaken with distilled water in a test tube. The occurrence of frothing which persisted for at least half an hour was taken as a positive test for saponins (Rukunga, 1984).
3.6 Data analysis

In the *in vivo* experiments the student's t-test was used to evaluate the significance of differences between mean survival times and the mean times at which skin lesion were initially scored 2 or 6 after infection. The significance of differences in mean weights between control and extract treated mice in the toxicity experiment was also evaluated by the Student's t-test. The repeated measures ANOVA was used to analyse the interaction between *Carissa edulis* root bark extract or acyclovir and water (control) in mean skin lesion for 3 to 10 days after infection. Statistical differences in the mortality were evaluated using the Bonferroni/Dunn test method (Kurokawa *et al.*, 2001). A *p*-value of less than 0.05 was statistically defined as significant.

In the *in vitro* assays the number of plaques were used to calculate the percent inhibition of plaque formation thereby giving an indication of possible anti-HSV action. The number of viable cells in the cytotoxicity assay was used for the graphical determination of the cytotoxic concentration (CC$_{50}$).

The 1998 StatView computer software by SAS Institute Inc. USA was used for statistical analysis.
CHAPTER FOUR

4. RESULTS

4.0 *In vitro* assays

4.0.1 Plaque reduction assay for all medicinal plants

Table 1 shows the *in vitro* anti-HSV activity of the medicinal plant extracts on wild type strain 7401H HSV-1 in the plaque reduction assay. The results show that *C. edulis* RB extract had the highest anti-HSV activity with 100% plaque inhibition at both 50 and 100 µg/ml. This was followed by *P. linearifolia* RB extract, inhibiting plaque formation by 58.7% at 50 µg/ml and 100% at 100 µg/ml. *M. heterophylla* RB extract had an anti-HSV activity of 71.4% at 50 µg/ml and 87.7% at 100 µg/ml. The *A. coriaria* SB extract caused cell lysis at 50 and 100 µg/ml indicating cytotoxicity. All the other extracts gave a low anti-HSV activity with *T. asiatica* SB extract showing no activity at all at 50 µg/ml. The extracts from *Carissa edulis*, *M. heterophylla* and *P. linearifolia* which showed high anti-HSV activity were selected for further analysis (Table 1).
Table 1:  *In vitro* anti-HSV activity of medicinal plant extracts on wild type 7401H strain herpes simplex virus type 1 (HSV-1).

<table>
<thead>
<tr>
<th>Medicinal plant</th>
<th>Part used</th>
<th>% Plaque inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Control (Double distilled water)</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Toddalia asiatica</td>
<td>RB</td>
<td>27.6</td>
</tr>
<tr>
<td>Toddalia asiatica</td>
<td>L</td>
<td>14.4</td>
</tr>
<tr>
<td>Toddalia asiatica</td>
<td>SB</td>
<td>0</td>
</tr>
<tr>
<td>Rhus natalensis</td>
<td>L</td>
<td>9.7</td>
</tr>
<tr>
<td>Albizia coriaria</td>
<td>SB</td>
<td>Cytotoxic</td>
</tr>
<tr>
<td>Vernonia jugalis</td>
<td>L</td>
<td>5.2</td>
</tr>
<tr>
<td>Rhamnus prinoides</td>
<td>RB</td>
<td>34.5</td>
</tr>
<tr>
<td>Maytenus heterophylla</td>
<td>SB</td>
<td>4.0</td>
</tr>
<tr>
<td>Maytenus heterophylla</td>
<td>RB</td>
<td>71.4*</td>
</tr>
<tr>
<td>Clausena anisata</td>
<td>SB</td>
<td>17.5</td>
</tr>
<tr>
<td>Carissa edulis</td>
<td>RB</td>
<td>100*</td>
</tr>
<tr>
<td>Periploca linearifolia</td>
<td>RB</td>
<td>58.7**</td>
</tr>
<tr>
<td>Fagaropsis angolensis</td>
<td>SB</td>
<td>1.7</td>
</tr>
<tr>
<td>Zanthoxylum usambarense</td>
<td>RB</td>
<td>4.3</td>
</tr>
<tr>
<td>Teclea simplicifolia</td>
<td>SB</td>
<td>17.5</td>
</tr>
</tbody>
</table>

SB - Stem bark  
RB - Root bark  
L - Leaves

* - High degree of plaque inhibition  
** - Moderate degree of plaque inhibition

4.0.2 Determination of EC$_{50}$ and CC$_{50}$ for the three medicinal plant extracts with high anti-HSV activity.

When the EC$_{50}$ of the three aqueous plant extracts were assessed, *C. edulis* RB extract exhibited the best anti-HSV activity with an EC$_{50}$ of 6.9 µg/ml for HSV-2, 8.1 µg/ml for AP', 11.1 µg/ml for TK- HSV-1 and 15.1 µg/ml for HSV-1. This extract had a CC$_{50}$ of 480 µg/ml. The standard deviation of ±5.66 for TK- HSV-1 was high because there was no consistency in the three determinations. *P. linearifolia* RB extract also gave a good anti-HSV activity with an EC$_{50}$ of 16.0
μg/ml for TK HSV-1, 17.3 μg/ml for AP HSV-1, 18.0 μg/ml for HSV-2 and 28.5 μg/ml for HSV-1. *M. heterophylla* RB extract gave an EC$_{50}$ of 18.0 μg/ml for TK HSV-1, 20.2 μg/ml for AP HSV-1, 24.5 μg/ml for HSV-2 and 60.8 μg/ml for HSV-1. Acyclovir (ACV) had no significant effect on the resistant strains because the anti-HSV activity for the highest investigated dose of 10μg/ml was below 50% and therefore the EC$_{50}$ could not be calculated (Table 2).

Table 2: EC$_{50}$ and CC$_{50}$ determination for the three medicinal plant extracts.

<table>
<thead>
<tr>
<th>Extract / Drug</th>
<th>EC$_{50}$ ± S.D$^b$ (μg/ml)</th>
<th>CC$_{50}$ $^c$ (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSV-1</td>
<td>HSV-2</td>
</tr>
<tr>
<td><em>Carissa edulis</em> Root bark</td>
<td>15.1±0.57</td>
<td>6.9±1.27</td>
</tr>
<tr>
<td><em>Periploca linearifolia</em> Root bark</td>
<td>28.5±0.71</td>
<td>18.0±0.00</td>
</tr>
<tr>
<td><em>Maytenus heterophylla</em> Root bark</td>
<td>60.8±1.13</td>
<td>24.5±0.71</td>
</tr>
<tr>
<td>Acyclovir (Zovirax)</td>
<td>0.91±0.46</td>
<td>0.87±0.44</td>
</tr>
</tbody>
</table>

$^a$ ND – Not determined; $^b$ Effective concentration for 50% plaque reduction; $^c$ Cytotoxic concentration causing 50% cell lysis. The results are a mean of three independent experiments.

4.0.3 Plaque assay results for *Carissa edulis* RB extract.

In the plaque reduction assay experiments, *Carissa edulis* RB extract completely inhibited plaque formation at 20, 30, and 50 μg/ml for HSV-2, AP$^r$
HSV-1, TK- HSV-1 and HSV-1. The resistant strains (AP HSV-1 and TK HSV-1) were not susceptible to acyclovir at 5 μg/ml as formation of plaques were clearly evident (Plate 1).

Since C. edulis root bark extract had the best in vitro anti-HSV activity (Table 2), it was selected for further analysis.

4.0.4 Virus yield reduction assay

The activity of the Carissa edulis RB extract on the growth of wild type and resistant strains of HSV was compared to that of acyclovir in the virus yield reduction assay at concentrations greater than the EC$_{50}$ due to the high virus titre used in the assay. The cells were infected with 5 PFU/cell HSV ensuring that at least each single cell was infected. The unabsorbed virus was washed off. Since the number of cells were known, this could reasonably be taken to represent the number of virus units. Treatment of these cells with different concentrations of extract or acyclovir and then recounting the virus showed whether the virus number had reduced, increased or remained static. Table 3 shows the activity of the Carissa edulis RB extract at 60, 100, and 200 μg/ml in Vero E6 cells infected with HSV in the virus yield reduction assay. At 200 μg/ml, the extract significantly reduced the virus yields of AP HSV-1 by 100%, HSV-2 by 99.5%, HSV-1 by 97.8% and TK- HSV-1 by 96.3% (p < 0.05 verses control by student’s
Plate 1: Anti-HSV activity of *Carissa edulis* RB extract. Vero E6 cells were infected with 100 PFU HSV and treated with 20-50μg/ml extract.

The clear dots in the dishes are plaques.
t-test). Table 4 shows that Acyclovir at 5 μg/ml reduced the virus yields by 100% for both HSV-1 and HSV-2 (p < 0.05, student’s t-test) but was not effective on the resistant strains of HSV (AP<sup>e</sup> HSV-1 and TK<sup>-</sup> HSV-1) at the same concentration.

Table 3: **In vitro activity of Carissa edulis** RB extract on the growth of HSV in Vero E6 cells.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Control</th>
<th>Treatment (μg/ml)</th>
<th>%&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1</td>
<td>(6.0±0.14) x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>(2.38±0.25) x 10&lt;sup&gt;5&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(1.8±0.32) x 10&lt;sup&gt;6&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>HSV-2</td>
<td>(6.08±0.11) x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>(3.0±0.14) x 10&lt;sup&gt;5&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(8.0±0.04) x 10&lt;sup&gt;4&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ap&lt;sup&gt;e&lt;/sup&gt; HSV-1</td>
<td>(6.65±0.14) x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>(2.8±0.11) x 10&lt;sup&gt;5&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(8.0±0.04) x 10&lt;sup&gt;4&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>TK&lt;sup&gt;-&lt;/sup&gt; HSV-1</td>
<td>(6.73±0.11) x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>(2.63±0.32) x 10&lt;sup&gt;5&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(1.60±0.28) x 10&lt;sup&gt;6&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are mean ± S.D of two independent experiments

<sup>b</sup> Values are % virus yield reduction at 200μg/ml of extract

<sup>c</sup> p < 0.05 verses control, by Student’s t-test
Table 4: *In vitro* activity of acyclovir (Zovirax) on the growth of wild type and resistant HSV in Vero E6 cells.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Control</th>
<th>Treatment (µg/ml)</th>
<th>% ( ^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(6.0±0.14) x 10^6</td>
<td>(3.50±0.71) x 10^5</td>
<td>0^c 100</td>
</tr>
<tr>
<td>HSV-1</td>
<td>(6.08±0.11) x 10^6</td>
<td>(3.50±2.12) x 10^3</td>
<td>0^c 100</td>
</tr>
<tr>
<td>HSV-2</td>
<td>(6.65±0.14) x 10^6</td>
<td>(6.63±0.18) x 10^6</td>
<td>(6.63±0.11) x 10^6</td>
</tr>
<tr>
<td>Ap^HSV-1</td>
<td>(6.65±0.14) x 10^6</td>
<td>(6.63±0.18) x 10^6</td>
<td>(6.63±0.07) x 10^6</td>
</tr>
<tr>
<td>TK^HSV-1</td>
<td>(6.65±0.14) x 10^6</td>
<td>(6.63±0.18) x 10^6</td>
<td>(6.63±0.35) x 10^6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(7.5±0.30) x 10^3</td>
<td>(7.5±0.35) x 10^3</td>
</tr>
</tbody>
</table>

^a Values are mean ± S.D of two independent experiments

^b Values are % virus yield reduction at 5µg/ml of acyclovir

^c p < 0.05 versus control, by Student's t-test

### 4.1 *In vivo* assays

#### 4.1.0 Progression of infection in untreated BALB/c mice following a cutaneous wild type 7401H HSV-1 infection.

Plate 2 shows the progression of HSV-1 infection in untreated male and female BALB/c mice cutaneously infected with 1x10^6 HSV-1 (lethal dose). Vesicles appeared on the local region between the 3^rd^ and 4^th^ day (score 2) and
erosion and ulceration between the 5\textsuperscript{th} and 6\textsuperscript{th} day (score 4). Mild zosteriform lesion was observed between the 5\textsuperscript{th} and the 6\textsuperscript{th} day (score 6). Moderate zosteriform lesion in the local region was observed between the 8\textsuperscript{th} and the 10\textsuperscript{th} day (score 8). Severe zosteriform lesion and death due to infection (score 10) was observed between the 8\textsuperscript{th} and 10\textsuperscript{th} day after infection.

4.1.1 Therapeutic efficacy of *Carissa edulis* root bark extract in male BALB/c mice infected with wild type 7401H strain HSV-1.

The therapeutic efficacy of *Carissa edulis* root bark (RB) extract on cutaneous HSV-1 infection in male BALB/c mice was examined after an oral administration of 125, 250 and 375mg extract per kg body weight of mouse respectively. Acyclovir (ACV) was used as a standard control and each mouse in its group were administered with 5 mg ACV per kg body weight. Table 5 shows the results of the effects of *C. edulis* root bark extract on cutaneous wild type 7401H strain HSV-1 infection in male BALB/c mice. At the onset of infection, mean lesion score of 2 (vesicles in the local region) was observed on the 4\textsuperscript{th} day for the treated mice receiving 125, 250, 375 mg/kg *C. edulis* RB extract or 5 mg/kg ACV, while the untreated mice (control-2) score 2 was observed on the 3\textsuperscript{rd} day. Both *C. edulis* RB extract and ACV significantly delayed the onset of the HSV-1 infection (p < 0.005 verses control by student’s t-test). The progression of infection was slow for treated mice with the mean lesion score 6 (mild zosteriform lesion) being observed on the 4\textsuperscript{th} day for the untreated control-2 and
on the 5th day for the extract or ACV treated mice. *Carissa edulis* RB extract at 250 and 375 mg/kg concentrations or ACV at 5 μg/kg again significantly prolonged the mean survival time of the treated mice (p < 0.005 verses control by student's t-test). However the mean survival time for mice treated with 250 mg/kg extract was higher than in those treated with 375 mg/kg extract. The Mean survival time represents the number of days the experimental mouse lived from the day of infection to the day of death as a result of progression of infection. One hundred percent mortality was observed in both the untreated infected mice (control-2) and those on 125 mg/kg *C. edulis* RB extract. The extract at 250 and 375mg/kg or ACV at 5mg/kg significantly lowered the mortality rate of the treated mice relative to both the infected untreated and 125mg/kg extract treated mice (p < 0.05 verses control by repeated measures ANOVA). Mortality was calculated on day 30. No uninfected and untreated mice (Control-1) died. *C. edulis* RB extract at an administrative dose of 250 mg/kg was selected for the subsequent determinations of therapeutic efficacy in both male and female mice since it was the smallest dose providing the highest mean survival time (Table 5).

4.1.2 Therapeutic efficacy of *Carissa edulis* root bark extract in female BALB/c mice infected with wild type 7401H strain HSV-1.

Table 6 shows the results of the determination of therapeutic efficacy of the *C. edulis* RB extract in the female BALB/c mice at 250mg/kg body weight concentration. The *C. edulis* RB extract and ACV significantly delayed the onset
of infection (score 2 observed on 5th day for *Carissa edulis* or ACV) as opposed to the untreated control-2 (score 2 observed on 3rd day) (p < 0.005 verses control by student's t-test). *C. edulis* RB extract and ACV had significant low mortality of 30% and 10% respectively as opposed to the untreated control-2 which had 100% mortality (p < 0.0005 verses control by repeated measures ANOVA). No mortality was observed in the untreated uninfected mice (control-1). Plate 3 shows the pictorial view of one of the female BALB/c mice infected with the wild type HSV-1 and treated with *Carissa edulis* RB extract at 250 mg/kg 3 times daily for 7 days. The prolonged delay in the onset of infection (score 2 observed on the 5th day) and the suppression of that infection (no score 6 observed in this particular mouse) is evident.

Both male and female mice responded similarly to treatment with ACV at 5 mg/ml or extract at 250 mg/ml (Tables 5 and 6) after HSV-1 infection.
Plate 2: Progression of HSV infection in untreated BALB/c mice following a cutaneous wild type strain 7401H HSV-1 (1 x 10^6 PFU) infection. The scoring of infection.

- Scoring:
  - 0 Day after infection (Score 0)
  - 3-4 Days after infection (Score 2)
  - 5-6 Days after infection (Score 4)
  - 5-6 Days after infection (Score 6)
  - 8-10 Days after infection (Score 8)
  - 8-10 Days after infection (Score 10)
### Table 5: Effect of *Carissa edulis* root bark extract on cutaneous wild strain 7401H HSV-1 (1 x 10^6 PFU) infection in male BALB/c mice.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Treatment</th>
<th>Mean time (Days ± S.D)</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Score 2(^a)</td>
<td>Score 6(^a)</td>
</tr>
<tr>
<td>Control-1</td>
<td>0mg/kg</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>Control-2</td>
<td>0 mg/kg</td>
<td>3.00±0.00</td>
<td>4.00±0.00</td>
</tr>
<tr>
<td>ACV</td>
<td>5 mg/kg(^d)</td>
<td>4.00±0.00(^e)</td>
<td>5.20±0.45(^e)</td>
</tr>
<tr>
<td><em>C. edulis</em></td>
<td>375 mg/kg(^d)</td>
<td>4.25±0.50(^e)</td>
<td>5.40±0.55(^e)</td>
</tr>
<tr>
<td><em>C. edulis</em></td>
<td>250 mg/kg(^d)</td>
<td>4.00±0.00(^e)</td>
<td>5.40±0.55(^e)</td>
</tr>
<tr>
<td><em>C. edulis</em></td>
<td>125 mg/kg</td>
<td>4.20±0.45(^e)</td>
<td>5.20±0.45(^e)</td>
</tr>
</tbody>
</table>

\(^a\) Mean times at which score 2 or 6 was first observed

\(^b\) Surviving mice were not included for the calculation of mean survival times.

\(^c\) Number of dead mice / number of mice tested.

\(^d\) \(p < 0.05\) verses control by repeated measures ANOVA (Bonferroni/Dunn)

\(^e\) \(p < 0.005\) verses control by Student's t-test

NO – score not observed since mice were not infected

NC – not calculated because all the mice survived

Control-1 – uninfected untreated mice

Control-2 – infected treated mice.
Table 6: Effect of *Carissa edulis* root bark extract on cutaneous wild strain 7401H HSV-1 (1 x 10^6 PFU) infection in female BALB/c mice.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Treatment</th>
<th>Mean time (Days ± S.D)</th>
<th>Mortality&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Score 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Score 6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control-1</td>
<td>0 mg/ml</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td></td>
<td>Not infected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control-2</td>
<td>0 mg/kg</td>
<td>3.30±0.68</td>
<td>5.70±0.82</td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACV</td>
<td>5 mg/kg&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.67±0.58&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.33±0.58</td>
</tr>
<tr>
<td><em>C. edulis</em></td>
<td>250 mg/kg&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.00±1.10&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.40±0.55</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean times at which score 2 or 6 was first observed

<sup>b</sup> Surviving mice were not included for the calculation of mean survival times

<sup>c</sup> Number of dead mice / number of mice tested. Mortality was calculated on day 30

<sup>d</sup> p < 0.0005 verses control by repeated measures ANOVA (Bonferroni/Dunn)

<sup>e</sup> p < 0.005 verses control by Student's t-test

<sup>f</sup> less than 2 mice died so S.D could not be calculated.

NO – score not observed since mice were not infected

NC – not calculated because all the mice survived

Control-1 – uninfected untreated mice

Control-2 – infected untreated mice
Plate 3: Therapeutic efficacy of *Carissa edulis* root bark extract in female BALB/c mice infected with $1 \times 10^6$ PFU wild type strain 7401H HSV-1.

- Infection site 3 days after infection, still no sign of vesicles.
- 5 days after infection, the infection site is healing, slight appearance of vesicles.
- 10 days after infection, the infection site has healed. No signs of HSV-1 infection.
4.1.3 Therapeutic efficacy of *Carissa edulis* root bark extract in female BALB/c mice infected with acyclovir resistant strain (AP') HSV-1.

Table 7 shows the therapeutic efficacy of the *Carissa edulis* RB extract at 250 mg/kg on cutaneous acyclovir resistant (AP') HSV-1 infection in female BALB/c mice. The results show that *Carissa edulis* RB extract significantly delayed the onset of infection of the resistant HSV in the treated mice (p < 0.005 verses control by student’s t-test). The vesicles in the local region (score 2) appeared on the 5th day as opposed to ACV treated mice and the infected untreated mice (control-2 group) where the vesicles appeared on the 3rd day. No mild zosteriform lesion (score 6) was observed in mice treated with the extract or ACV. There was 50% mortality for the infected untreated mice and no mortality for the extract or ACV treated mice.

4.1.4 Therapeutic efficacy of *Carissa edulis* root bark extract in male BALB/c mice infected with acyclovir resistant strain (AP') HSV-1.

Table 8 shows the effect of the *Carissa edulis* root bark extract on cutaneous acyclovir resistant (AP') infection in male BALB/c mice. The results show that *Carissa edulis* RB extract significantly delayed the onset of infection of the resistant HSV-1 strain in the male mice (p < 0.05 verses control by student’s t-test). The onset of infection, score 2 (vesicles in local region) was observed on the 4th day as opposed to the infected untreated mice and those receiving ACV treatment at 5 mg/kg where score 2 was observed on the 3rd day. Mild zosteriform lesion was observed on day 7 for the extract treated mice and on the 6th day for
the ACV treated and infected untreated (control-2) mice. The mean survival time could not be calculated since there was no mortality in both the untreated (control-2) and the treated (extract or ACV) infected mice groups.

Table 7: Effect of *Carissa edulis* root bark extract on cutaneous acyclovir resistant (AP') HSV-1 (1 x 10⁶ PFU) infection in female BALB/c mice.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Treatment</th>
<th>Mean time (Days ± S.D)</th>
<th>Mortality&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Score 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Score 6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control-1</td>
<td>0 mg/ml</td>
<td>NO&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NO&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Not infected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control-2</td>
<td>0 mg/kg</td>
<td>3.63±0.74</td>
<td>8.00±1.73</td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACV</td>
<td>5 mg/kg</td>
<td>3.89±0.60</td>
<td>NO&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>C. edulis</td>
<td>250 mg/kg</td>
<td>5.50±1.31&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NO&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean times at which score 2 or 6 was first observed;  <sup>b</sup> Surviving mice were not included for the calculation of mean survival times;  <sup>c</sup> Number of dead mice / number of mice tested. Mortality was calculated on day 30;  <sup>d</sup> p < 0.005 verses control by Student’s t-test;  NO<sup>e</sup> – score not observed since mice were not infected;  NO<sup>f</sup> – score not observed since infection did not progress to that stage;  NC – not calculated because all the mice survived;  Control-1 – uninfected untreated mice;  Control-2 – infected untreated mice.
Table 8: Effect of *Carissa edulis* root bark extract on cutaneous acyclovir resistant (AP') HSV-1 (1 x 10^6 PFU) infection in male BALB/c mice.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Treatment</th>
<th>Mean time (Days ± S.D)</th>
<th>Mortality e</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Score 2^a</td>
<td>Score 6^a</td>
</tr>
<tr>
<td>Control</td>
<td>0 mg/kg</td>
<td>3.20±0.45</td>
<td>6.00±0.00</td>
</tr>
<tr>
<td>ACV</td>
<td>5 mg/kg</td>
<td>3.20±0.45</td>
<td>6.60±0.55</td>
</tr>
<tr>
<td><em>C. edulis</em></td>
<td>250 mg/kg</td>
<td>4.50±0.58^d</td>
<td>7.00±0.00</td>
</tr>
</tbody>
</table>

^a Mean times at which score 2 or 6 was first observed.

^b Surviving mice were not included for the calculation of mean survival times.

^c Number of dead mice / number of mice tested. Mortality was calculated on day 30.

^d p < 0.05 verses control by Student's t-test.

NC - not calculated because all the mice survived

Control – infected untreated mice

4.1.5 Therapeutic efficacy of *Carissa edulis* root bark extract in male BALB/c mice infected with thymidine kinase deficient (TK-) HSV-1.

Table 9 shows the effect of the *Carissa edulis* root bark extract on cutaneous thymidine kinase deficient (TK-) HSV-1 infection in male BALB/c mice. The results indicate that there was no infection observed in all the mice cutaneously infected with the TK^- HSV-1, with or without treatment. There was
no mortality in both the treated and untreated mice at the end of the experiment. Similar results were observed with female BALB/c mice.

Table 9: Effect of *Carissa edulis* root bark extract in male BALB/c mice cutaneously infected with thymidine kinase deficient (TK<sup>−</sup>) strain HSV-1 (1 x 10<sup>6</sup> PFU)

<table>
<thead>
<tr>
<th>Animals</th>
<th>Treatment</th>
<th>Mean time (Days ± S.D)</th>
<th>Mortality&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Score 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Score 6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>0 mg/kg</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>ACV</td>
<td>5 mg/kg</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td><em>C. edulis</em></td>
<td>250 mg/kg</td>
<td>NO</td>
<td>NO</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean times at which score 2 or 6 was first observed.

<sup>b</sup> Surviving mice were not included for the calculation of mean survival times.

<sup>c</sup> Number of dead mice / number of mice tested. Mortality was calculated on day 30

NO – score not observed

NC – mean survival time not calculated because all the mice survived.

Control – infected untreated mice
4.1.6 Therapeutic efficacy of *Carissa edulis* root bark extract in male BALB/c mice infected with wild type strain HSV-2.

Table 10 shows the effect of the *C. edulis* root bark extract on cutaneous wild strain HSV-2 infection in male BALB/c mice following treatment with 250 mg/kg extract. The results show that the *C. edulis* RB extract delayed the onset of HSV-2 infection in the treated mice by 2 days when compared to the untreated control (score 2 appearing on 3\(^{rd}\) day for infected untreated mice and 5\(^{th}\) day for infected extract treated mice). ACV significantly delayed the onset of HSV-2 infection by 2.5 days (\(p < 0.005\) verses control by student’s t-test.) The progression of infection was relatively fast for extract treated mice since mild zosteriform lesion (score 6) were observed on the 6\(^{th}\) day the same as that of infected untreated mice. No mild zosteriform lesion was observed in the ACV treated mice. The mean survival time for mice treated with the extract or ACV was the same (8 days). The reduced mortality rate of mice treated with the extract or ACV was 20% and 40% respectively as opposed to 100% mortality for the infected untreated mice (\(p < 0.05\) verses control by repeated measures ANOVA).

4.1.7 Determination of acute toxicity of the *Carissa edulis* root bark extract in uninfected mice.

Table 11 shows the evaluation of acute toxicity for an oral dose of 250 mg/kg *Carissa edulis* root bark extract on uninfected male and female BALB/c mice. The results indicate that there was no significant difference in the mean weights of the treated and untreated mice in both sexes. The initial mean weight
of mice on day 0 was between 19 and 20g for both treated and untreated mice and the final weight on day 21 was between 20 and 21g. After the final weights on day 21, the animals were observed daily for death until the 30th day. Mortality rates were calculated on the 30th day after the first administration of *C. edulis* root bark extract.

**Table 10:** Effect of *Carissa edulis* root bark extract on cutaneous wild type strain HSV-2 (1 x 10^6 PFU) infection in male BALB/c mice.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Treatment</th>
<th>Mean time (Days ± S.D)</th>
<th>Mortality c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Score 2^a</td>
<td>Score 6^a</td>
</tr>
<tr>
<td>Control</td>
<td>0 mg/kg</td>
<td>3.00±0.00</td>
<td>6.400±0.89</td>
</tr>
<tr>
<td>ACV</td>
<td>5 mg/kg^d</td>
<td>5.50±0.71^e</td>
<td>NO</td>
</tr>
<tr>
<td><em>C. edulis</em></td>
<td>250 mg/kg^d</td>
<td>5.00±0.00</td>
<td>6.00±0.00</td>
</tr>
</tbody>
</table>

^a^ Mean times at which score 2 or 6 was first observed.

^b^ Surviving mice were not included for the calculation of mean survival times.

^c^ Number of dead mice / number of mice tested.

^d^ p < 0.05 verses control by repeated measure ANOVA (Bonferroni/Dunn).

^e^ p < 0.005 verses control by Student's t-test.

NO – score not observed.

NC – not calculated since only one mouse died.
Table 11: Toxicity of *Carissa edulis* root bark extract in uninfected BALB/c mice following treatment with 250 mg/kg extract.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Treatment</th>
<th>Mean weight ± S.D on Day</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Female BALB/c mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0 mg/kg</td>
<td>19.30±1.34</td>
<td>20.00±1.16</td>
</tr>
<tr>
<td>C. <em>edulis</em></td>
<td>250 mg/kg</td>
<td>19.50±2.01</td>
<td>19.50±1.41</td>
</tr>
<tr>
<td>Male BALB/c mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0 mg/kg</td>
<td>19.40±1.58</td>
<td>20.60±2.07</td>
</tr>
<tr>
<td>C. <em>edulis</em></td>
<td>250 mg/kg</td>
<td>20.80±1.48</td>
<td>21.50±1.65</td>
</tr>
</tbody>
</table>

* Mean weight ± S.D. of ten mice in each group.

b Number of dead mice/number of mice tested.

c Accidental deaths arising from faults in drug administration were not included in the statistical analysis.

d p<0.001 versus control by repeated measure ANOVA (Bonferroni/Dunn).
4.2 Phytochemical Screening

4.2.0 Phytochemical screening for Carissa edulis, Maytenus heterophylla and Periploca linearifolia aqueous root bark extracts

Table 12 shows the class of compounds present in the Carissa edulis, Maytenus heterophylla and Periploca linearifolia aqueous root bark extracts. The results show that Carissa edulis aqueous root bark extract contained phenolic compounds in which flavonoids were detected. The extract also contained terpenoidal compounds in which steroids were detected. Maytenus heterophylla aqueous root bark extract contained phenolic and terpenoidal compounds. The terpenoidal compounds consisted of saponins. The Periploca linearifolia aqueous root bark extract contained phenolic compounds in which flavonoids were detected and terpenoidal compounds in which cardiac glycosides were detected in trace amounts. No alkaloids or anthraquinones were detected in all the three aqueous extracts.
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<th>+AG</th>
<th>-AG</th>
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<th>+AG(1,9°C)</th>
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<tr>
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<td>-AG</td>
<td>-AG</td>
<td>+AG</td>
</tr>
</tbody>
</table>

Σημείωση: Η Μεταλλάθημα μεταλλάθημα μεταλλάθημα στην ΚΒ θα αφαιρεθεί. Η ΚΒ θα αφαιρεθεί από τα Συννάγματα.
CHAPTER FIVE

5. DISCUSSION

The results of this study indicate that out of the 12 medicinal plants evaluated for in vitro anti-HSV activity by the plaque inhibition assay, three aqueous extracts from the root barks of the medicinal plants; *Carissa edulis*, *Periploca linearifolia* and *Maytenus heterophylla*, had in vitro anti-HSV activity. These three aqueous extracts at 50 $\mu$g/ml and 100 $\mu$g/ml respectively reduced the formation of plaques by between 58% and 100% in Vero cells infected with 100 PFU wild type strain 7401H HSV-1. *Carissa edulis* root bark extract gave the best activity since it reduced plaque formation by 100% at both 50 and 100 $\mu$g/ml concentrations. These extract concentrations of 50 and 100 $\mu$g/ml were selected as starting points for screening for anti-HSV activity based on what had been used earlier by other investigators (Kurokawa *et al.*, 1993b; Nawawi *et al.*, 1999; Kuo *et al.*, 2001; Kofi-Tsekpo *et al.*, 2001). Kurokawa *et al.*, (1993b) while examining 142 hot water (HW) extracts from medicinal plants used as traditional medicines in China, Indonesia and Japan had used 100 $\mu$g/ml concentration as the starting point. Nawawi *et al.*, (1999) while examining 30 medicinal plants of Indonesia for inhibitory effects on infection of HSV-1, used 100 $\mu$g/ml extract concentration as a marker of activity. Kuo *et al.*, (2001) while examining ethanolic extracts from seven Chinese herbs for antitherpetic activity used 50 $\mu$g/ml and 100 $\mu$g/ml concentrations as part of their initial investigative concentrations. HSV-1 was
used as the screening virus and any extract reducing plaque formation by 50% at 50 μg/ml was chosen for further analysis. The aqueous plant extracts showing low anti-HSV activity in vitro could either have or not have therapeutic value in vivo. They could have therapeutic value in vivo if they are prodrugs and therefore require metabolic activation or biological modification to act antivirally. Because of this reason, the other 9 medicinal plants whose extracts had low anti-HSV action by the plaque method could still be potential candidates for anti-HSV chemotherapy. The aqueous plant extract (stem bark extract of Albizia coriaria) which caused cell lysis and hence was cytotoxic at 50 and 100 μg/ml in vitro could be assumed to have therapeutic value at lower concentrations. However, an aqueous stem bark extract from another similar species, Albizia gummifera (J F Gmel.) CA. Sm. has been shown to have low in vitro anti-HSV-1 activity by the plaque assay method at 50 and 100 μg/ml (Kofi-Tsekpo et al., 2001). This therefore casts some doubt as to whether aqueous extracts from members of this species have anti-HSV activity in vitro.

The EC_{50} for Carissa edulis root bark extract for both the wild and resistant strain virus of between 6.9 and 15.1 μg/ml which was below the cell cytotoxic concentration (CC_{50}) of 480 μg/ml (Table 2) were an indication that this extract had a good margin of safety at the active concentration because of the big therapeutic index (TI). The TI (CC_{50}/EC_{50}) was between 31.7 and 69.6. The other two medicinal plant extracts were also safe with of a TI of between 7.4 and 25 for Maytenus heterophylla root bark and between 10.5 and 18.8 for Periploca
*linearifolia* root bark on both wild and resistant virus strains. That all the three aqueous medicinal plant extracts had slightly lower EC$_{50}$ values for the resistant HSV strains (AP$^r$ HSV-1 and TK- HSV-1) than for wild type strains could be attributed to lose of virulence for the resistant viruses. The acyclovir resistance virus (AP$^r$) used in the study was plaque purified in the presence of high concentrations of acyclovir from strain 7401H HSV-1 infected Vero E6 cell cultures (Kurokawa *et al.*, 1995b). On the other hand, thymidine kinase deficient (TK$^{-}$) HSV-1 strains usually have low virulence with attenuated pathogenesity in animal model systems (Efstathious *et al.*, 1989; Field and Darby, 1980). The susceptibility of the resistant HSV strains to acyclovir was demonstrated to be low as expected (Plate 1). The sensitivity of the resistant strains to the aqueous extracts from the three medicinal plants suggests that there exists a difference in the mechanism of action between the extracts and acyclovir. Exploitation of this difference could result in identification of novel bio-active compounds from any of these medicinal plants with a completely different mechanism of anti-HSV action. The EC$_{50}$ for acyclovir for both wild type strains of HSV-1 and HSV-2 fell within the acceptable *in vitro* documented values of 0.1 to 3 µg/ml (Darby, 1993). That the resistant viruses (AP$^r$ HSV-1 7401H and TK$^{-}$ HSV-1 B2006) used in this study were not susceptible to 5µg/ml acyclovir when cultured in Vero E6 cells *in vitro* was as expected (Kurokawa *et al.*, 1995a; 1995b).

The results showing that *Carissa edulis* root bark extract at 200 µg/ml completely reduced the virus yield of AP$^r$ HSV-1 by 100% and the other viruses
by between 96 and 98% and that acyclovir at 5 mg/kg reduced the virus yields of the wild type strains (HSV-1 and HSV-2) by 100% but could not reduce the virus yields of the resistant strains (AP' HSV-1 and TK- HSV-) above 3%, confirmed the observations made in the plaque reduction assay. It also further indicated that the *Carissa edulis* root bark extract had a different mechanism of anti-HSV action to that of acyclovir.

In the *in vivo* assays, when BALB/c mice were cutaneously infected with wild type strains of HSV-1 or HSV-2 at 1 x 10⁶ PFU/mouse, all the infected and untreated mice ultimately died. In the cutaneous wild type strain HSV-1 infection, the significant reduction of the mortality rate by 60% for acyclovir or *Carissa edulis* aqueous extract (250 and 375 mg/kg), the delayed onset of infection and its slow progression and the longer mean survival time for male mice following treatment indicated the therapeutic efficacy of this extract. Incidentally, the 250 mg/kg extract concentration corresponded to the conventional doses of dried traditional herbal medicines for human use in China (Kurokawa *et al.*, 1995a). That the uninfected untreated mice did not show any infection and no mortality confirmed that the observed deaths in the infected untreated mice arose from HSV infections. That *Carissa edulis* aqueous extract at 250mg/kg in female mice showed significant delay in the onset of infection following treatment and an increased mean survival time and a low mortality rate of 30% for the extract treated mice indicated again the therapeutic efficacy of the extract.
The therapeutic efficacy of the *Carissa edulis* root bark extract following cutaneous infection in male and female BALB/c mice with the acyclovir resistant strain (AP) HSV-1 (Tables 7 and 8) was evident even though marred with slight lose of virulence. There was a significant low mortality rate in female mice treated with extract even though this was not clear in the male group where the infected untreated and the extract treated mice behaved similarly. What was clear was the inability of acyclovir to delay the onset of infection in both male and female mice while the *Carissa edulis* root bark extract did, indicating that the extract had some *in vivo* effect on the resistant virus. That no mortality was observed in both the male and female groups treated with acyclovir could not be fully attributed to treatment since the infected untreated mice had behaved similarly.

The loss in virulence for the thymidine kinase deficient (TK-) strain HSV-1 was very remarkable since no infection was observed in the infected untreated as well as the treated mice in both male and female groups (Table 9). Lack of virulence in the TK- strains of HSV-1 in animal models has been observed by other researchers (Efstathious et al., 1989; Field and Darby, 1980). No conclusive *in vivo* results could therefore be obtained in the TK- strain HSV-1 cutaneous infection in mice in this study.

The fact that infection and death was observed in HSV-1 or HSV-2 cutaneously infected untreated mice confirms that there is no specific site tropism for HSV-1 or HSV-2 infection. At 250 mg/kg, the *Carissa edulis* root bark extract
exhibited a therapeutic efficacy in the wild type strain HSV-2 infected mice by significantly delaying the onset of infection by 3 days and lowering the mortality rate by 20%. Infection never progressed to mild zosteriform lesion in mice treated with 5 mg/ml acyclovir following HSV-2 infection. However, 2 mice in the group died due to unexplained reasons.

The aqueous extract of *C. edulis* root bark was not toxic *in vivo* in both male and female BALB/c mice at 250 mg/kg oral administrative dose. A similar method has been used in the determination of acute toxicity in mice (Kurokawa *et al.*, 1993b; 2001).

*C. edulis, M. heterophylla* and *P. linearifolia* aqueous root bark extracts that had shown anti-HSV activity, contained terpenoidal and phenolic compounds. Omino and Kokwaro, (1993) had reported that the roots of the members of the *Apocynaceae* species with medicinal value contained alkaloids. Absence of alkaloids in the aqueous extract from *C. edulis* root bark could have arisen due to a difference in geographical localities of collection.

The *P. linearifolia* aqueous root bark extract contained terpenoidal compounds in which cardiac glycosides were faintly detected. This agreed with Ding and Lou, (1966) who had reported a similar finding in a member of the same genus, *P. sepium*. Cardiac glycosides have pronounced effect on the heart. Even though therapeutic concentrations of cardiac glycosides increase the contractility of the cardiac muscle and form the main stray of the drug treatment of congestive heart failure, they also can be potent poisons to the same organ at higher doses.
(Bowman and Rand, 1980). However, the presence of trace amounts of cardiac glycosides in the *Periploca linearifolia* extract may not raise any serious safety considerations. On the other hand, one of the reasons why this plant was selected for anti-HSV screening was due to its ethno-medical use for management of other diseases. No human toxicity has been recorded in its use as a herbal medicine.

### 5.0 Conclusions and Recommendations

Three aqueous extracts each from a medicinal plant have shown significant anti-HSV activity. The three extracts from *Carissa edulis*, *Maytenus heterophylla* and *Periploca linearifolia* clearly demonstrated their ability in reducing plaque formation on Vero E6 cells infected with HSV *in vitro*, *Carissa edulis* extract being the most potent of the three. An important significant finding was the *in vitro* susceptibility of the resistant HSV strains to these extracts, indicating a possibility of difference in the mechanism of anti-HSV action to that of acyclovir. It was not possible to predict the mode of anti-HSV action. However, compounds in each of these aqueous extracts perhaps work together synergistically and therefore have an advantage of low resistance development by the sensitive viruses to them.

The *Carissa edulis* root bark extract has *in vivo* therapeutic efficacy against the wild type strains of HSV (HSV-1 and HSV-2). The acyclovir resistant (AP') strain of HSV-1 susceptibility to the *Carissa edulis* root bark extract *in vivo* was not clear due to lose of virulence even though the extract significantly
delayed the onset of infection while acyclovir did not. The thymidine kinase 
deficient (TK-) strain HSV-1 was unable to illicit any noticeable infection in the 
experimental mice also due to lose of virulence and therefore no in vivo activity 
could be reported.

Arising from the phytochemical investigations of the three aqueous 
extracts, it appears that the anti-HSV activity observed in the in vitro assays and 
in the in vivo experiments for the Carissa edulis extract, was arising from 
terpenoidal and phenolic compounds detected in these extracts.

From these results, the hypothesis put forward of finding anti-HSV agents 
from medicinal plants is justified and the objectives of this study have been 
fulfilled. I therefore would like to recommend the following:-

1. The determination of the mechanism of anti-HSV action for the three 
medicinal plant extracts from Carissa edulis, Maytenus heterophylla and 
Periploca linearifolia is necessary. This would open a new chapter in the 
therapeutics of HSV infection.

2. The in vivo experiment seems to provide a prophylactic treatment since 
treatment is commenced before infection (this is the design of the murine 
HSV infection model). In order to establish whether the Carissa edulis 
extract can suppress an already established HSV infection arising from a 
wild type strain virus (HSV-1 or HSV-2), the murine HSV infection model 
can be adjusted so that the administration of the extract is commenced when 
the skin lesions occur (vesicles in local region, score 2). However,
Kurokawa and colleagues who have widely used this murine technique have explained that in the murine model by the time the HSV infection in noticeable, without any prior treatment, it would be too late to render any treatment since the infection would take its full course killing the mouse in the process.

3. The development of the hot water extract from *Carissa edulis* root bark as an alternative herbal remedy for HSV infection is ideal. *C. edulis* is a medicinal plant known by various ethnic communities. The local names for this plant as reported by ICRAF, (1992); Kokwaro, (1976; 1993) and Beentje, (1994) are: KAMASAI (Nyamuha tribe), KIKAWAM MUKAWA (Kamba tribe), MFUMBWE (Hehe tribe), OCHUOGA (Luo tribe), LAMURIEI (Samburu tribe), MUKAWA (Kikuyu tribe), OLAMURIAKI (Masai tribe), KAMURIA (Meru tribe), LEGETEUET (Nandi tribe), LAKATETUET (Pokot tribe), LEGETETWET (Kipsigis and Tugen tribes) and MTANDA-MBOO (Swahili).

4. A follow up of the other two medicinal plant extracts from *Maytenus heterophylla* and *Periploca linearifolia* to determine their anti-HSV activity *in vivo* is necessary since such results could be contributory to their development as herbal remedies for HSV infection. The *in vivo* determinations of therapeutic efficacy against the resistant HSV-1 strains (AP\textsuperscript{T} and TK\textsuperscript{T}) should include the determination of the viral loads in the brain of the treated and untreated mice. This viral load in the brain of the
treated mice would give an indication of anti-HSV action. It is also necessary to look at organic solvent extracts (for example chloroform and methanol extracts) from all the three plants and see how they compare with observations made from the water extracts.

5. The three medicinal plants should be screened against the local HSV strains, since there is evidence now that they posses anti-HSV activity following screening with the standard strains of wild and resistant HSV. Even though similar observations would be expected, it is important to register results of the local strains also.

6. Efforts should be made to isolate and identify bio-active compounds which are present in the three extracts. Such an effort could lead to the identification of a new range of compounds for management of HSV infections.

7. Finally, preclinical studies on higher animals prior to human clinical trials should be conducted to authenticate the use of these medicinal plant extracts as herbal remedies for HSV infection.
REFERENCES


Appendix 1

**EC$_{50}$ determination for *Carissa edulis* root bark extract**

Note: The graph was drawn from the 1st experiment of the three independent experiments.
Appendix 2

The cell cytotoxic concentrations (CC<sub>50</sub>) of the most active medicinal plant extracts

![Graph showing cell cytotoxic concentrations (CC<sub>50</sub>) of various medicinal plant extracts.]

Carissa edulis (RB) CC<sub>50</sub> = 480μg/ml
Maytenus heterophylla (RB) CC<sub>50</sub> = 450μg/ml
Periploca linearifolia (RB) CC<sub>50</sub> = 300μg/ml

Note:- The graph was drawn from a mean % viable cell count of three independent experiments.
Appendix 3

Determination of therapeutic index (TI)

Formula:

\[
\text{Therapeutic index} = \frac{\text{Cytotoxic concentration for 50\% test (CC}_{50})}{\text{Effective Concentration for 50\% test (EC}_{50})}
\]

<table>
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<th>Medicinal plant</th>
<th>Therapeutic index (TI)</th>
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<tr>
<td></td>
<td>7401H HSV-1</td>
</tr>
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</tr>
<tr>
<td>M. heterophylla</td>
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</tr>
<tr>
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