PHYTOCHEMICAL COMPOSITION, ANTIVIRAL ACTIVITY AND TOXICITY OF *DICHROCEPHALA INTEGRIFOLIA* KUNTZE. CRUDE EXTRACTS

By

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DECLARATION

This thesis is my original work and has not been presented in any other institution for examination or any other purposes.

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DEDICATION

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LIST OF ABBREVIATIONS AND ACRONYMS

KEMRI	Kenya Medical Research Institute
OECD	Organization for economic co-operation and development
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
UDP	Up-and-Down-Procedure
DMSO	Dimethyl sulfoxide
OD	Optical density
PBS	Phosphate buffered saline
EMEM	Eagle's minimum essential media
ATCC	American Type Culture Collection
ELISA	Enzyme-linked immunosorbent assay
CO_2	Carbon (IV) oxide
CC_{50}	Median cytotoxic concentration dose
IC_{50}	Median inhibitory concentration dose
LCD_{50}	Median Lethal Dose
SARS	Severe respiratory syndrome
LAT	Latency Associated Transcript
NAHWES	A National health and Nutrition Examination Survey
HSV	Human Simplex Virus
HIV	Human Immunodeficiency Virus
PCR	Polymerase Chain Reaction

EGCG	Epigallo Catechin – 3 – Gallate
ECG	Epicatechin Gallate
EGC	Epigallocatechin
HCMV	Human Cytomegalovirus
MNC	Minimum non-toxic concentration
CTMDR	Centre for Traditional Medicine and Drug Research
CVR	Centre for Viral Research
$TICD_{50}$	Tissue Culture Infectious Dose
MEM	Minimum Essential Medium

ABSTRACT

Human herpes simplex viruses are among the world's most ubiquitous human infections. Generally, there are two types of Human Herpex simplex viruses (HSV); HSV-1 and HSV-2. Worldwide over 90% of people are infected by either one or both. HSV-1 is a viral disease known to cause genital and oral lesions. Currently, a major antiviral drug is acyclovir that is used for the treatment of HSV infections has proven unsatisfactory as resistance to this drug has been frequently reported. Furthermore, severe side effects have been witnessed in pregnant mothers and infants. High prevalence of HSV, lack of vaccines and limited treatments options warrants an urgent need for more effective anti-HSV agents. The aim of this study was to investigate phytochemical composition, *in vitro* anti-HSV, *in vivo* and *in vitro* toxicity of *D. integrifolia* crude extracts. Leaves, roots, flowers, and stem of *D. integrifolia* were collected from the plants' natural habitat in Mabariri village in Nyamira County. The identification of the plants was done by a botanist in the school of biological sciences, University of Nairobi. The materials were extracted with methanol and water using standard methods. Qualitative chemical tests were carried out to determine the presence or absence of alkaloids, flavonoids, saponins, tannins, glycosides, and terpenoids. The MTT assay was used to investigate the *in vitro* cytotoxic activity of the crude extracts using Vero cell lines obtained from Kenya Medical Research Institute (KEMRI) in the center for viral research. Antiviral activity was assessed based on the ability of the extract to protect normal cells (Vero cell lines) from HSV attack. *In vivo* toxic effects of the crude extracts were evaluated in female Swiss albino mice using standard oral acute toxicity protocols described by Organization for Economic Co-operation and Development. The qualitative phytochemical analysis results showed that the test extracts contained tannins, flavonoids, alkaloids, terpenoids, phenols, glycosides, and saponins. The extracts were not

cytotoxic to Vero cells except the methanolic extract of the flower which had CC_{50} value of 71.31± 2.65µg/ml. The extracts demonstrated interference in the adsorption step of HSV-1 may be by blocking the epitopes for the virus on the cell's membrane. Methanolic extracts of the stem, leaves and aqueous extracts of leaves inhibited the ability of HSV-1 virus to cause a cytopathic effect when pre-treatment was done, with IC_{50} values of 63.95±5.36 µg/ml, 54.45±3.45 µg/ml, 86.20±7.56 µg/ml respectively. The methanolic extract of flower, aqueous extract of the root and the methanolic extract of leaves showed virucidal activity with IC_{50} values of 45.27±2.41 µg/ml, 0.333±1.23 µg/ml, and 30.53±4.51 µg/ml respectively. Oral administration of the extracts to mice at both 300mg/kg and 2000mg/kg did not result in any toxic effects or mortality. In all the groups, no major behavioral or appearance changes were observed. Generally, the presence of pharmacologically important phytochemicals such as flavones, phenols, terpenoids, and tannins that have been implicated in antimicrobial action support the use of this plant in the management of these pathologies. Results on cytotoxicity, efficacy, and acute oral toxicity depict that the plant has no major toxicity. Therefore, the preparation of antiviral herbal remedy from the plant parts may be safe for use in patients. However further research is required to elucidate the mechanisms of actions of the extracts and isolate the bioactive agents for the treatment of viral infections. It is recommended that the toxic effects of the use of the plant's products to be studied on formulated products for pharmacological aspects.

Keywords: *Dichrocephala integrifolia,* antiviral, phytochemical, cytotoxicity, Herpes Virus simplex, *In vivo* safety

CHAPTER ONE

INTRODUCTION

1.1 Background information

Traditional medicine has been practiced since time immemorial (Dery *et al.,* 1999). It is still a vital component in health care systems to date especially in low-income communities in developing nations. It is used against diseases like Acquired Immune Deficiency Syndrome (AIDS), Herpes simplex viruses (HSV), Ebola, influenza, malaria, cancer, diabetes, tuberculosis (TB), Human Immunodeficiency Virus (HIV) among others (Balick and Cox, 1996). A large population of people especially those in developing countries majorly rely on drugs from the natural origin for their healthcare requirement (Cunningham, 1993; Balick and Cox, 1996; Mworia, 2000). The World Health Organization (WHO) approximates that close to 6 billion of people around the world uses plants derived products for their primary health care needs (Choudhry *et al.,* 2004). Approximately 84% of the population in Peru prefers traditional medicine in comparison to conventional drugs for their primary healthcare. They believe that herbal products are less toxic, easily accessible and well tolerated when used, thus traditional medicines are regarded as safe and at times combine both (Bussmann *et al.,* 2007). In Africa, many communities use medicinal plants for treatment of many human diseases mainly due to unstable economies (Fakung *et al*., 2011). In Ethiopia, up 80% of the people relies on natural products for prophylaxis and treatments different human ailments. In Kenya, almost 90% of the population uses complementary and alternative medicine from natural sources at least once in their life for various health conditions (Chirchir *et al.,* 2006). Various plants or their parts harbor active principles that are responsible for their medicinal properties.

Despite the wide scope of clinically active synthetic and semi-synthetic antibiotics, the search for new efficacious anti-infective drug remains a paramount quest due to resistance and emergence of new parasites strains or new therapeutic targets (Fair and Tor, 2014). Currently, the need for substances with antiviral activity is high, since the drugs used for the treatment of viral infections are not readily available. A major problem has been the emergence of mutant viral strain not responding to the available antiviral drugs. The greatest drawback in the fight against human simplex virus infections is the drug resistance menace that is rapidly evolving increasing the cost of treatment. Virus resistance to acyclovir which has been allied to mutations at TK gene has been documented (Morfin and Thouvenot, 2003). Herpes simplex virus strains resistant to acyclovir have been isolated from normal hosts and often in patients with recurrent infections. Immune compromised people such as the HIV-AIDS patients are at great risk of attack by these strains (Morfin and Thouvenot, 2003). These drawbacks call for multifaceted approach to curb the disease.

Plants have been used since ancient times in the fight against various diseases including viral diseases (Kinghorn *et al*., 2011). Natural product based approach could help in the discovery of new leads which are safe and have diverse targets to the virus hence reducing chances of resistance development. The ethnomedical studies and bioprospecting of medicinal plants may therefore facilitate the extraction of vital compounds that could be potent, cost effective, with fewer side effects. Diseases of viral origin have been treated with natural products from plants for many decades (Kinghorn *et al*., 2011; Newman and Cragg, 2007). However, on few studies have been conducted to evaluate the plants with antiviral activities and number active compounds have been isolated from higher plants (David *et al*., 2015; Kingstone, 2011; Farnsworth *et al.*, 1981). These studies suggested that selection of plant materials on the basis of ethnomedical use

gives a higher lead generation compared to screening programs for search from general synthetic products (Kingstone, 2011). Most of the plants used for medicinal purpose by different communities have not been investigated thoroughly using scientific techniques. In this study, *D. integrofolia* was selected for scientific validation based on its ethnomedical uses. This was a qualitative screening of the phytochemicals present in plants crude extracts was done using standard procedures (Evans, 2009). *In-vitro* antiviral (against HSV) properties, as well as cytotoxicity of the extracts, were performed to ascertain its potency and safety in the management of HSV.

1.2 Statement of the Problem

Infection with HSV and other viral infections are a public health problem in both developing and developed countries. In Kenya, the overall prevalence of HSV is estimated at 26.6%. Adults are the highest affected populations estimated at 31.5% while the adolescents at 10.7% (Akinyi et al., 2017). Herpes simplex virus causes genital herpes and is also one of the most highly sexually transmitted viral diseases. Herpes simplex virus is a major opportunistic infection associated with HIV (Tolo *et al.,* 2006; Bharti *et al.,* 2009). The use of antiviral drugs against HSV has been the mainstay method in the fight against its infection. The development of resistance towards the available antivirals by some pathogenic viruses and serious side effects with current antiviral drugs has resulted in a continuous search for alternative agents for use as antivirals. Currently, Acyclovir is the drug of choice in the management of HSV; however, the emergence of resistance by some strains has been reported, especially in immune compromised patients (Tolo *et al.*, 2006). According to Frobert *et al*. (2014), less than 1% immunocompetent HSV patients, exhibit resistance against Acyclovir while patients whose immunity is compromised have increased resistance levels estimated between 4-30% worldwide. The resistance level in Kenya has not yet been established to the best of our knowledge but we estimate it to be

significantly high. Given other reasons such as intolerance of side effects by patients, limited accessibility to conventional health care services and the high cost of treatment and lack of suitable alternative, the search for more antiviral drugs is inevitable and medicinal plants could provide novel arsenals in the fight against this virus.

1.3 Study justification

Globally, HSV accounts for up to 25 % emerging infections (Khan, 2005; Xu *et al.,* 2006). The development of resistance towards the available antivirals and serious side effects has resulted in a continuous search for alternative agents for use as antivirals. Currently Acyclovir is the drug of choice in the management of HSV; however, the emergence of resistance by some strains has been reported especially in immune compromised patients (Tolo *et al.,* 2006). The drawbacks being experienced with the current methods of HSV treatment have led many turns to a search of more potent drug from natural sources for treatment of majority human ailments. Plants have been used as sources medicine for many decades in the treatment of various diseases including viral infections, however, the efficacy and safety for most of these herbs remains undeciphered. The plant, *D. integrifolia* has been used traditionally for the cure of various infections among them being viral infections. Despite the wide traditional use efficacy of the plant against various diseases including bacterial infection, fungal infection and non-communicable diseases in a laboratory setting is scanty. Scientific justification of its antiviral activity and safety remains unknown. The current study therefore sought to evaluate the antiviral activity and safety levels of the *D. integrifolia* crude extracts.

1.4 Study objectives

1.4.1 General objective

To screen for the phytochemical constituents and determine antiviral properties and toxic effects of *Dichrocephela integrifolia* crude extracts in mice.

1.4.2 Specific objectives

i. To screen for the phytochemical compounds, present in the crude extracts of *D. integrifolia*
ii. To determine the *in vitro* antiviral activity of extracts of *D. integrifolia* against HSV
iii. To determine the *in-vitro* cytotoxic effects of *D. integrifolia* extract against Vero cell lines
iv. To determine the *in-vivo* mammalian toxicity of the methanolic and aqueous extracts of *D. integrifolia* using Swiss albino mice.

CHAPTER TWO

LITERATURE REVIEW

2.1 Ethnobotany and Ethnopharmacology

Ethnobotany is simply defined as the study of different plants in a region and documenting their specific traditional uses by the people inhabiting an area. Ethnobotany involves studies of the relationships between the various uses of plants and various tribes as medicine (Martin, 1995; Cotton, 1996). The study of Ethnobotany mainly focuses on drug discovery from the traditional uses of plants and eventually development of new drugs to treat human diseases. The field of Ethnobotany is vital for economic growth and developments of any country, therefore, conservation of biodiversity is key to preventing the loss of plants more so medicinal plants. Currently, research on ethnobotany has doubled in the recent years but loss of biodiversity due climate change, encroachment of forest and other habitat may slow down the search for plant-based medicines (Balick and Cox 1995). Apart from food, shelter, and medicine the relationship between plants and man has other important uses such as making clothes, decoration, religious ceremonies and much more (Choudhary, 2008).

Ethnopharmacology is a study of ethnic groups and their use of drugs. Enthnopharmacological research by pharmaceutical industry is not yet innovative as expected because many plants remain unexplored. The main challenge has been technology intensity and increased cost as a result of post-approval and post-marketing withdrawals (Patwardhan, 2005). This has limited drug discovery from plants as many companies dedicate most of their resources to discovery of new targets for drug interactions. Such a move will promote microbes and parasite survival in the human body creating more suffering and deaths. However, bioprospecting of new medicines has been a culture by Ayurveda and Chinese medical systems (Patwardhan, 2005). It is therefore

necessary for African countries especially Kenya to follow soot and contribute to development of newer, safer and affordable medicines. According to Patwardhan and colleagues (2004), Ayurvedic medicinal plants have been actively engaging in pharmacognosy, chemistry, pharmacology and clinical therapeutics research. As a matter of fact, Ayurvedic experimental base has discovered several active molecules for different illnesses. Some of the molecules include *Rauwolfia* alkaloids for hypertension, withanolides and steroidal lactones and their glycosides as immunomodulators, psoralens for vitiligo, guggulsterons as hypolipidemic agents and phyllantins as antivirals. Others include *Holarrhena* alkaloids for amoebiasis, Mucuna pruriens for Parkinson's disease, baccosides for mental retention, curcumines for inflammation, piperidenes as bioavailability enhancers and picrosides for hepatic protection (Patwardhan, 2000).

2.2 Traditional Medicine and Medicinal plants

2.2.1 Natural products and drug development

Medicinal compounds from plant kingdom have continually played a great role in improving the health status and quality of life of mankind for millions of years. According to the WHO, about eighty percent of the people living in the world use extracts or active ingredients from plants for prevention and treatment of the majority of their ailments. It is now known that nearly 50% of the modern drugs used for treatments of infectious diseases are directly or indirectly of natural product origin (Kirbag *et al*., 2009).

Traditional medicine includes all subsistances that are used as medicines that are from plants, animal, and minerals for the treatment of various diseases (Kokwaro, 2009). Through the ages, mankind has been improving on the search for new drugs for treatment and preventions of

ailments to improve the quality of life (Kumar *et al.*, 2006; Kumar, 2014). Every society in particular region of the world has systems of treatments which can be formal or informal (Sindiga, 1995).

Medicinal plants are used as medicine in both developed and developing countries across the world. The global market for plant-derived medicinal products is expanding rapidly with more than $79 billion USD is sold annually (Tilburt and Kaptchuk 2008). Furthermore, approximately 25% of conventional drugs and more than 70% of drugs used for cancer treatment are from natural origin. According to WHO (2001), majority of people prefer the use of natural products due to their availability and accessibility. The development of new products from natural sources is also encouraging because less 25 % of the plant's species in the world which are close to 350,000 plants have been investigated for their safety and pharmacological potential (WHO, 2013). According to Sahoo *et al.* (2010) of the 225 drugs on the WHO essential drugs list, at least 25 are completely from plant base. the widespread use of drugs from plants have necessitated WHO to published selected plants Monographs volume one to five to provide information on medicinal plants quality control, their safety and efficacy for reference (Gisesa, 2004; WHO, 2007).

Traditional medicine is a common practice all over Africa and different communities have different practices and beliefs (Rukangira, 2001). Due to the rich biodiversity in all parts of Africa, the accessibility of medicinal plants by the community for health care reasons is easy. In addition, herbal remedy is the most preferred therapeutic option among patients due to beliefs of not causing any risk (Rukangira, 2001; WHO, 2005). Indeed, Africa has rich diversity plants and plant species is approximately between 40 and 45,000 species and those used medically are over 5,000 species (Van Wyk, 2008). The tropical and subtropical climate in Africa has a greater

impact in phytochemical compositions of plants. The active substance in plants comes as a result of interaction between plants and its surrounding environments (Peñuelas *et al*., 1997). The certain phytochemical component can be high under specific environmental conditions such as altitude, temperature and the length of exposure of sunlight to the plants (Peñuelas *et al.*, 1997). Studies have shown that same plants growing in different geographical locations may have different quantities of active components. This may be attributed to different morphological and genetic variation (Dong *et al*., 2011). In Africa, tropical conditions of extreme sunlight make plants produce a protective chemical compound that will protect the plants from ultraviolet rays and also microorganisms (WHO, 2013). This means that these plants can have a myriad of uses against various diseases as they confer diverse properties including antiviral activity.

2.2.2 Active principles in medicinal plants

Phytochemical compounds in plants play a vital role in therapeutic property of a plant. Secondary metabolites are available in most of the plant and the most common secondary metabolites in plants include; alkaloids, steroids, tannins, and phenolic compounds. These compounds are stored at specific parts of the whole plant (Joseph and Raj 2010). The productions of these phytochemical compounds protect plants from stress such as diseases. They also contribute to the plant's color, aroma, and flavor. Generally, these phytochemicals are believed to play a vital role of protecting plant cells from harsh environmental conditions such as pollution, stress and pathogenic attack (Milter, 2006). Recently, these phytochemicals have been demonstrated to improve human health when taken as dietary supplements (American Cancer Society, 2000). Out of over 3900 phytochemical compounds are categorized and documented, 160 have been well investigated (American Cancer Society, 2000; Meagher and Thomson, 1999).

The leaves are a storage site for most of the phytochemicals compounds. Fruits also have a good number of active ingredients, and therefore are mainly taken orally as fluid concoctions through mouth to achieve the desired biological action. Some other parts of plants included are whole roots, buds, flowers heads, seeds or fruits, leave stalk, barks etc. also can use for extracting active phytochemicals (Chan *et al.*, 2012). Secondary metabolites of plants are also used for synthesis of nutraceuticals, drugs and also cosmetics (Bourgaud *et al.*, 2001). Phytochemicals are considered as potential sources of new drugs and insecticides (Crozier *et al.*, 2006). This is due to their pharmacological importance and potential health effects such as antioxidant, anticancer, anti-aging, anti-atherosclerotic, antimicrobial and anti-inflammatory activities. The active compounds in plants that confer various biological activities include; alkaloids, terpenoids, flavonoids, saponins, polyphenols, carotenoids, vitamins and enzymes among other pigments (Madhuri and Pandey, 2009).

The particular function of most phytochemical components in plants is not well understood, however, a good number of research have shown compounds involvement in the interaction of diseases and pests in plants. Phytochemical and antimicrobial screening of crude extracts from plants provide a benchmark in search for novel drugs. Phytochemical studies have attracted the most attentions of scientists in the search of lead compound from natural sources in recent years due to the development of new and highly sophisticated techniques that facilitate their identification, determination, and isolation. New techniques such as combinatory chemistry, high performance liquid chromatorgraphy and mass spectroscopy have enhanced drugs discovery from natural sources for pharmaceutical industries (Shakeri *et al*., 2012).

Flavonoids have exhibited various pharmacological activities such as antimicrobial, antioxidant, anti-inflammatory, antineoplastic, antiulcers, antihepatotoxic, activities and cytotoxicity in

different studies (Cushnie and Lamb, 2005; Pengelly, 2004). An example is Lupinifolin, which is a prenylated flavanone, a major compound in stem and stem bark of *Derris reticulata* (Mahidol *et al.,* 1997). This compound has been isolated from other plants like *Myriopteron extension, Eriosema chinense* and *Albizia myriophylla* (Joycharat *et al.,* 2013; Prasad *et al.,* 2013). It has been demonstrated to possess antimicrobial activities against several pathogens such as HSV-1 (Soonthornchareonnon *et al.,* 2004)

2.3 Chemotherapy of Viral diseases

2.3.1 Aetiology and Classification

Viral diseases are highly prevalent and lead to great morbidity and mortality across the globe.Among the viral diseases which have been of great threat to humans for thousands of years include influenza, polio, smallpox, yellow fever, infectious hepatitis, AIDS, HSV, rabies, Ebola, SARS, and Marburg (Tolo *et al.,* 2006). Diseases caused by viruses are infectious and highly complex due to their complex pathology. Emerging viruses are those that were not known to man previously but only appeared recently through transmission animal reservoir this is due changing the landscape and environmental conditions. Good examples of emerging viruses include Ebola, SARS, Marburg virus and some strains of influenza virus (Becker *et al.,* 2009). The category of viruses is the re-emerging type. Reemerging is the resurge of microorganism and diseases that had been successfully eliminated or controlled through medicines, better living conditions and vaccines like dengue and polio (National Institute of Allergy and Infectious Disease, 2009).

Many viral infections result in no disease or long term life threatening consequences in the health of humans as in the case of Human cytomegalovirus (HCMV) while others may cause mild to severe diseases like HIV-AIDS or Ebola hemorrhagic fever (Tolo *et al.*, 2006; NAID 2009).

Viral diseases are among the prominent life-threatening infectious diseases globally. For instance, smallpox was the leading cause of deaths compared to all other infectious diseases together before it was eradicated in the 20th Century by worldwide vaccination. Massive international campaigns against the deadly polio virus by the public health officials may also have contributed significantly to the eradication of the virus (NAID, 2009).

Viruses have caused two major worldwide pandemics; for instance in between the year 1918 and 1919 Spanish flu caused the death of 20 million to 40 million people and HIV-AIDS caused about 2 million deaths in 2008. Table 1 shows the number of cases of viral infections that had been reported by the year 2010. These viral infections are still high and most of them lack potent cures (Ruwali *et al.*, 2013).

Table 1: Number of cases of some viral infections in the world in the year 2010

Region	No of cases				
	Rubella	**Yellow fever**	**Measles**	**Mumps**	**Congenital syndrome**
Africa	2,754	714	186,675	-	-
America	12	23	208	24,608	00
South East Asia	-	-	50,265	-	-
Europe	10,551	00	30,625	27,013	02
East Mediterranean	1,398	-	10,072	-	-
West Pacific	45,966	-	49,460	486,449	-

Source: NAID, 2009

Besides HSV causing viral infections, they also are known to be associated with other diseases including cancers and many autoimmune conditions such as multiple sclerosis, rheumatoid arthritis, and diabetes (Table 2).

Table 2: Cancer and associated viral infections

Cancer	Viral infection
Hepatocellular carcinoma	HBV, HCV
Cervical and Laryngeal cancer	HPV
T- Cell Leukemia	HTLV-1
Kaposi's sarcoma	HIV and HHV-8
Nasopharyngeal carcinoma	Epstein-Barr Virus

Source: Chien-Jen, *et al.,* 2014

2.3.2 Herpes Viruses and their pathology

HSV-1 is a pathogen that is commonly transmitted through direct contact with infected person or contaminated body secretions like saliva, blood, and serum. Herpes simplex virus type 1 and type 2,varicella-zoster virus, cytomegalovirus Epstein-Barr virus and human herpes viruses six, seven, and eight are all herpes viruses within *Herpesviridae* that infect human beings. HSV-1 is most frequently linked to oral lesions while HSV-2 is well known mainly linked to genital lesions but they are interchangeable. It is estimated that HSV-1 is responsible for about one-third of the reported new genital herpes cases (Anzivino, *et al*., 2009).

HSV-1 infections are widespread among the human population with a sero-prevalence ranging from 60 to over 95% in some places (Faral-Tell *et al*., 2012). The high prevalence of HSV-1 infections may be due to the virus's ability to be asymptomatic in its host during the latent stage, where the virus is dormant in the host's body for some period of time before eliciting notable symptoms (Laing *et al*., 2012). Besides leading to cold sores, more serious consequences of Herpes virus infections include encephalitis, aseptic meningitis, and corneal scarring leading to brain injury and blindness (Bradley *et al*., 2014). HSV-1 is also concern among HIV patients, as the virus is opportunistic on the immunocompromised patient, leading to pneumonia, esophagitis, hepatitis or meningoencephalitis thus worsening the already debilitating condition (Piret and Boivin, 2011). In some instances, mother-to-child transmission of the herpes virus occurs during birth, causing oral/ocular infections, central nervous system diseases, and possibly other fatal infections to the young ones (Pepose *et al*., 2006). All herpes viruses set up latency and may reactivate periodically throughout the life of the subject.

2.3.3 Structure and Life Cycle of Herpes Simplex Virus

Structurally, the HSV-1 is made up of linear, double-stranded DNA that is packed inside its icosahedral shaped capsid. On the outermost of the herpes virus, surrounding the capsid is an envelope made of a lipid bi-layer with glycoproteins and other embedded proteins that are bound to it (Akhtar and Shukla, 2009). HSV-1 uses these proteins, such as glycoprotein C, to bind to a receptor called heparan sulfate that is found on the surface of the host cells (Geraghty *et al*., 1998). Heparan sulfate is a glycosaminoglycan present on cell surfaces and extracellular matrix of virtually every animal cell. Both glycoprotein C and heparan sulfate are not required for virus entry, but the efficiency of entry is reduced if either is absent or faulty (Xu and Esko, 2014; Spear *et al*., 2000).

After attaching to a surface receptor on the host cell, the virus fuses its envelope with the host cell membrane so that viral DNA can be released and transported downstream to the host nucleus. Once viral DNA is inside the host nucleus, it is replicated by using the host machinery. The viral DNA after replication is then transcribed and translated into capsids and proteins that assemble and package the replicated viral genome. New herpes virions (See Fig. 2.1) then bud out of the host cell to obtain attachment glycoproteins and infect other cells. Due to this, the herpes virus can spread from cell to cell, and eventually infect the neurons in the central nervous system where it remains latent until an opportune time (Akhtar and Shukla, 2009).

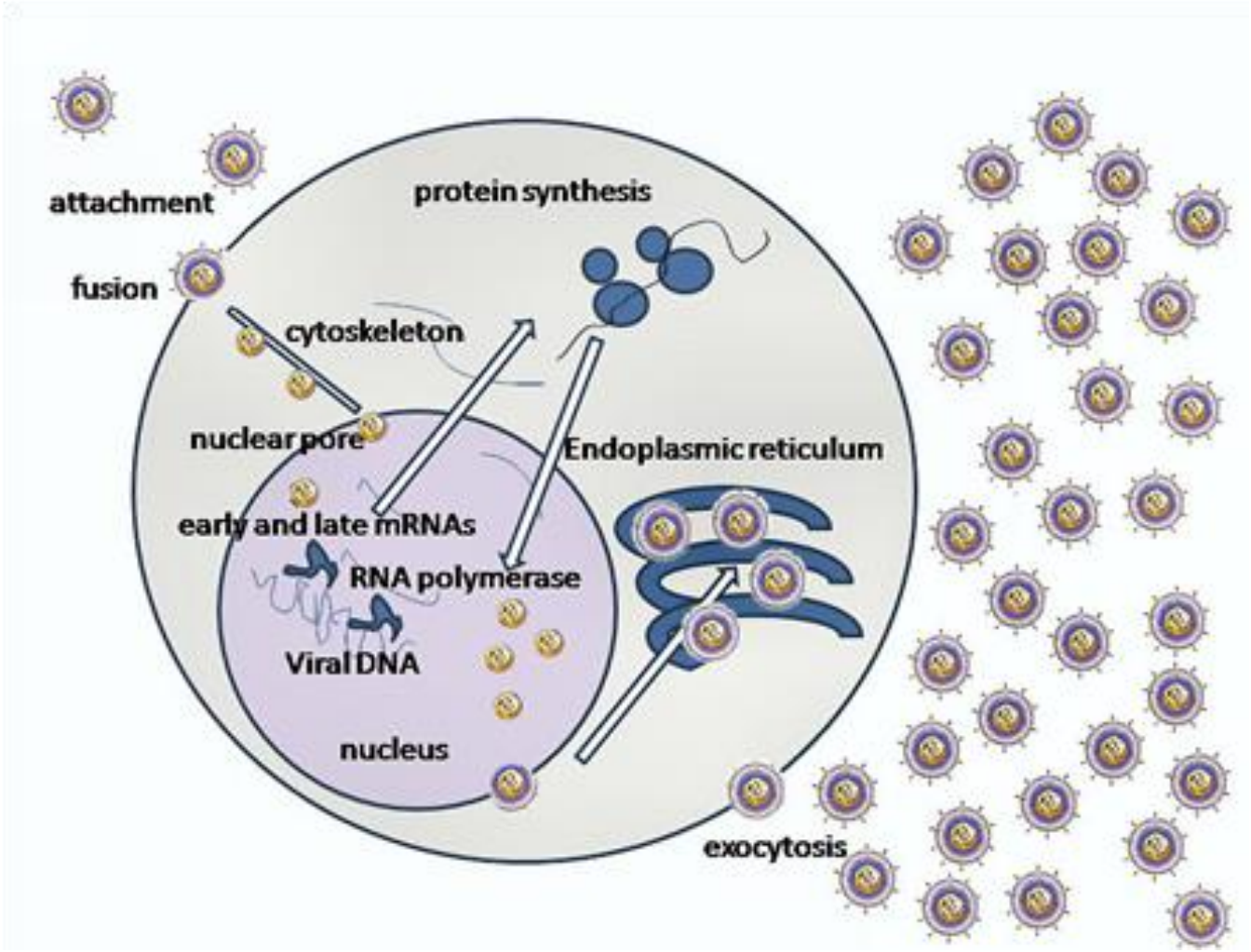

Figure 1: A diagram showing HSV-1 life cycle and Mechanism of Infection (Source: Akhtar and Shukla, 2009)

Hsv-1 can cause a continuum of diseases worldwide and is especially troublesome for immune-compromised individuals and neonates (Anzivino *et al*., 2009; Kimberlin, 2007). After attaching to its specific host cell receptor, HSV-1 commonly enters the cell through receptor-mediated endocytosis or by fusion. The virus then enters the nucleus and begins replication by utilizing the host's replication machinery. New virions are assembled and eventually bud out of the cell to spread the infection to other cells. The virus may become latent in the body like in the neurons.

The HSV-1 life cycle presents many opportunities where the virus could be inhibited (Akhtar *et al*., 2009).

All herpes viruses are able to remain dormant/ or latent and may reactivate periodically throughout the lifetime of the individual. Reactivation of HSV-1 and 2 is linked to oral or genital lesions or may be asymptomatic (Piret and Boivin, 2011). After an infection, HSV-1 enters the nucleus of trigeminal ganglia sensory neurons through rapid axonal transport and becomes gains latency (Li *et al*., 2015). HSV-2 is usually found in the lumbosacral ganglia in its latent (Anzivino, *et al*., 2009). Once HSV-1 has entered the neurons, majority viral gene expression is stopped, with the exception of latency-associated transcript (LAT) gene. During latency, viral gene expression required for productive infection does not take place. It is hypothesized that LAT RNA is expressed in large quantities during latency and is used to inhibit productive infection by HSV-1. LAT is good for reactivation of latency cycle since it inhibits gene expression, active infection, and apoptosis since it is the only gene that is highly expressed during this stage (Jones, 2003). A variety of factors, such as stress, hormones, ultraviolet radiation, or an immunocompromised state can increase the risk of reactivation (Oakley *et al*., 1997).

2.3.4 Epidemiology of HSV infections

HSV infections make a larger part of the most virulent viruses in the globe. The infections are mostly asymptomatic or frequently undiagnosed, however serological data shows that more than half of the world population is seropositive and more than 20% are positive for HSV-2 (Kriebs, 2008). Studies have shown that most neonatal HSV infections are as a result of undiagnosed HSV infection in mothers.

According to a National Prevention Information Network, 45 million people in the USA have genital HSV and up to 0.5 million new cases are recorded annually. In US, National Health and Nutrition Examination Survey (NAHNES) depict a decrease in both HSV type 1 and 2 in between 1994-2004. According to NAHNES, HSV type 1 reduced by 7% while HSV 2 reduction was 19%. The reduction of HSV 1 is noteworthy given the health stress burden it causes to humans. The rates of HSV are higher among women (23.1%) compared to Men (11.2%) (Xu *et al.,* 2006). Contrary to the commonly held belief that HSV 2 is genital herpes, both HSV-1 and HSV-2 have been known to cause genital lesions and shedding. Meanwhile, the rates of genital infections caused by HSV-1 are increasing faster compared to those associated with HSV-2. Generally, infections caused by HSV-1 account for one-third of all new genital infections (Xu *et al.,* 2006). According to Robert *et al.* (2003), infections caused by HSV-1 had up to 78% increase rate among youths. However, recurrent HSV-1 outbreaks are less frequent compared to HSV-2. Both HSV-1 and 2 are believed to be transmitted by sexually active women. According to Xu *et al.* (2006), 72% of all infected persons were pregnant women, affirming the notion that sexual intercourse is the common route of exposure to genital infections.

Undiagnosed or asymptomatic HSV infection high rates hinder the virus prevention. However, studies have demonstrated that use of a condom could decrease the risk of heterosexual transmission of herpes virus (Wald *et al.,* 2001; Kriebs, 2008). Herpes viral shedding can occur both during active outbreaks and at asymptomatic stages. Interestingly, most symptomatic recurrences have been associated with sub-clinical or asymptomatic shedding. This has complicated the fight against HSV hence making prevention a hard nut to crack among scientists (Wald *et al.,* 1995; Crespi *et al.,* 2007). Herpes infections lack accurate methods of diagnosis

(Kriebs, 2008). However, genital ulcers are more often associated to HSV than any other known disease.

Lack of awareness of HSV symptoms aggravates the problem further especially in developing countries. Antibody test, cultures, and PCR techniques are some of the most common methods for HSV diagnosis. On the other hand, some scientists have recommended lesions in the genital area to be treated as an HSV infection until otherwise proven (Simmons, 2002).

HSV-1 is said to be a common causative agent of orofacial lesions and to be predominantly present in trigeminal ganglia, while on the other hand, HSV-2 is common in lumbosacral ganglia. However, the apparent prevalence of these two types of HSV into a particular region is often misleading since they can infect any region of the body (Kriebs, 2008).

HSV infections possess a great risk to pregnant women and children. Mother-to-child transmission has been associated with neonatal HSV. Neonatal HSV infection has detrimental consequences including encephalitis and disseminated disease which can be fatal. In fact, neonatal HSV infection has been rated to cause over 50% of infected children even upon treatment (Brown *et al.,* 1996; Kriebs, 2008).

2.4 Antiviral chemotherapy

Specific treatments for most viruses are lacking and in most cases treatment is often if necessary to assuage the symptoms and in few cases by immunization like in the case of influenza, polio, measles, mumps and rubella viruses. Lack of appropriate vaccines for many viruses make the search for antiviral leads inevitable. Many antiviral drugs inhibit viral replication and development by interfering with cellular metabolic pathways also present in the mammalian host and therefore impacting a negative effect on the host cell. Antivirals in principle should be designed to target either viral protein or cellular protein (Clercq, 2002).

Acyclovir and valacyclovir have been found to be effective in reducing symptomatic and subclinical viral shedding by 76-82% (Corey *et al.,* 2004). Although acyclovir has proven to be successful in the treatment of HSV various side effects have been associated with it. Oral administration of acyclovir has been known to cause gastrointestinal upset, rashes, and headache while intravenous acyclovir is associated with severe inflammation, phlebitis and vesicular eruption hence lead to cutaneous necrosis at the point of injection (Kimberlin and Whitley, 2007). The current widespread of drug resistance against the majority of the currently available antiviral drugs make the need for better chemotherapeutic agents necessary and urgent (Evans, 2009; Clercq, 2002).

HSV1 and 2 are important widespread human viral pathogens throughout the world (Khan, 2005; Xu *et al.,* 2006). The mainstay in the management of herpes virus infections is antiviral drugs if taken on the onset of the disease these drugs will reduce 70% of the disease. The anti-herpes drugs are still underdeveloped only 11 anti-herpetic drugs that are licensed (De Clercq *et al.*, 2006). The nucleoside analog is most commonly used such as acyclovir and its derivatives (Elion, 1993). These drugs and their therapeutic potential and pharmacology are well documented. It has been established that continuous therapy with these agents leads to the emergence of resistant viral strains (Bacon *et al.,* 2003). Data from previous research indicate the existence of mutant clinical strains, with cross-resistance and double-crossed resistance against these antiviral drugs (Sarasini *et al.,* 1995). This has prompted relentless efforts in the search for new therapeutic agents to counter these drawbacks.

2.5. Plants as sources of antiviral medicines

Significant work has been done on plants as potential source of antifungal and antibacterial but antiviral potential still remains underexplored. The advantages of some natural compounds over

the synthetic drugs are that the natural products develop less resistance and their action is delayed as a result of the complex structure and their lower cytotoxicity. A wide number of bio-active compounds have been identified and isolated globally from natural sources (Harvey, 2000; Istatkova *et al.,* 2012). Therefore, products from natural origin such as traditional medicinal plants have high potential as sources of new potent antiviral drugs. Many of compounds isolated from various species of plants have shown antiviral activity (Hudson, 1990). Plants and their derived compounds are typically appropriate as antiviral candidates due to the following:

i. These plants or their derived compounds have a long history of use against various diseases including infectious diseases.
ii. Plants synthesize a wide variety of phytochemical compounds to adapt themselves to environmental stressors including invasion by microorganisms These substances include the Indoles, Phytosterols, Polysaccharides, Alkaloids, Tannins, Glucans, phenolics among others.
iii. Plants are natural, thus, it is believed that they cause less damage to host cells infected by viruses, than the synthetic pharmaceutical antiviral agents (Dixon *et al*., 2005).

Although the study and screening of antiviral potential of various promising plants were difficult in the past, in the last three decades, scientific strategies for the *in vitro* evaluation of plant natural products with biological activity have progressed remarkably. This has been attributed to the development of highly automated antiviral bioassay screening techniques like colorimetric quantification of the proliferating cell cultures (Ho *et al.,* 2001; Hu *et al*., 1994).

Tea (*Camellia sinensis*) extracts have been screened and exhibited a remarkable antiviral activity against rotavirus and enterovirus *in vitro* (Mukoyama *et al.,* 1991). The active principle in this

ointment is a mixture of catechins extracted from green tea that possesses immunomodulatory and antiviral properties (William, 2009).

In the recent years, green tea crude extract and variously derived catechins have shown antiviral activities against different influenza virus strains (Vlietinck *et al*., 1991, 1997). Similarly, it has been reported that pomegranate (*Punica granatum*) components blocked the interaction between HIV-1 viral envelope glycoproteins with cell receptors and inhibits *in vitro* infection. This could be attributable to the high concentration of polyphenolic compounds in pomegranate fruit. More recently, it was found that pomegranate components are directly virucidal for influenza viruses and also act at the intracellular level to inhibit influenza virus replication (Rukunga *et al*., 2002). Phytochemicals including tannins, flavones, and alkaloids have been reported to have *in vitro* activity against numerous viruses (Ngono *et al.,* 2011). Some of the medicinal plants that have been described for management of viral diseases include *Costus afer*, *Enantia chlorantha*, *Momordica charantia*, *Ptleopsis hylodendron*, *Sphatodea campanulata* and *Carissa edulis* (Ngono *et al.*, 2011).

Plant-based products with potential activity are marketed as a complementary treatment to antiviral therapy, providing safe, well-tolerated herbal medicines for the treatment of opportunistic infections, offsetting side effects from antiviral medication, boost the immune system, raise appetite and treatment of sexually transmitted diseases, thus potential candidates in reducing the risks HSV infections and re-infections. In Kenya, previous studies have demonstrated significant activity of plant extracts against viral strains including *Carissa edulis*, *Prunus Africana* and *Melia azedarach* against human cytomegalovirus (HCMV) and *Carissa edulis* against the Human simplex virus (Tolo *et al.*, 2006; Tolo *et al.*, 2007). This study focused

on antiviral properties of *Dichrocephala integrifolia* (Asteraceae), which was selected based on its' ethnobotanical use.

2.6 Asteraceae family

2.6.1 Taxonomic Classification of *D. integrifolia*

The family Asteraceae (Compositae) is one of the eight families of the order campanula le's. The flowering plants and contains more than 960 genera and close to 19000 species (Evans, 2009). Compared with other larger families of flowering plants the numbers of important economic products derived from this family are relatively few. Pharmacological research has raised medical interests in the family with many of the indigenous plants having been forgotten by the communities due to related side effects.

Many of the plants in this family have been proved to have antitumor activity, antibacterial activity, and others forming commercial sources of cooking oil (Evans, 2009). Stevioside, anent-kaurene Glycoside from *Stevia rebaudiana* has been used as a sweetener and at times as antidepressant (Miosonge *et al.*, 2015).

The plant *D. integrifolia* (Asteraceae) has been used for a number infectious disease in Kenya and around the world. Despite the wide traditional use plant, its pharmacological value as an antiviral agent remains unknown (Ogoche, 2013).

Table 3: Taxonomic classification

Kingdom	Plantae
Phylum	Charophyta
Class	Equisetopsida
Order	Asterales
Family	Compositae/Asteraceae
Genus	*Dichrocephala*
Species	*Intergrifolia*

Source: Kokwaro, 2009

D.integrifolia (L.f.) Kuntze (Asteraceae) synonym *D. latifolia*. (Lam.). D. Grangealatifolia (Lam.). ex poir, *D. bicolor* (Roth.). The plant is an annual herb that is erect or spreading, it is either smooth or hairy weed; it is a tropical plant with height ranging 30 to 60 cm. The plant has large leaves that are entire, ovate or lanceolate. *D. integrifolia* is taller than other species and has purple flowers (Ashirley, 1994).

Figure 2: Dichrocephala *integrifolia* flowers

Figure 3: Dichrocephala integrifolia leaves

In Africa, it is growing in grasslands and tropical rainforest. The plants are safe for animals and grazed by animals mostly cattle in Kenya. *D. integrifolia* thrives well at altitudes of between 500-3000 m in sub-tropic and tropical regions. The plant produces flowers the whole year except in dry and cool areas where it produces flowers in April and November. It is native and abundantly grows in tropics of Africa, Asia, South Europe, entire Malaysia, Australia and the Pacific. It is wild crafted but is naturalized in Italy and Turkey. (Schmelzer *et al.,* 2001)

2.6.2 Phytochemical Compounds isolated from *D. integrifolia*

The identification and later purification of six phytochemical compounds were done and this include; stearic acid, 22-dien-3-ol, tri tetracontane, α-amyrin, stigmasta-72, epifriedelanol, methyl stearate, and fatty acids (Zhangx *et al.,* 2010). Phytochemical screening on *D. Integrifolia* extract carried out earlier by (Mohamed *et al.,* 2012; Kapkarich *et al.,* 2013) has shown the presence of alkaloids, saponins, tannins and phytosterols. A study conducted by Mohammed and Teshale (2012) reported several chemical composition using different solvents. Phytosteroids were detected in ethyl acetate, chloroform and ethanol extracts. In addition carotenoids were detected in petroleum ether extracts. They also found presence of alkaloids, saponins and tannins which is consistent to the findings of other previous studies as described above. This analysis shows the need for further search of other compounds from this plant using different solvents such as methanol and water. There may be a variety of compounds in this plant that have not been identified to date which warrants similar research.

2.6.3 Ethnomedicinal information of *D. integrifolia*

In many ethnic communities *D. integrifolia* is used for a number of conditions such as an analgesic, anesthetic, anti-inflammatory, antibiotic, diuretic, dyspepsia, indigestion, mouth

ulcers, eye infections (conjunctivitis), traumatic tooth extraction, anthrax and antiemetic. Parts of the plants used include; flower buds, young shoots, leave (Chifundera, 1998; Jiofack *et al.,* 2010; Ashu *et al.,* 2011). In Kenya *D. integrifolia* (leaves, flower, and stems) are commonly used in the treatment of skin infections (Kokwaro, 2009). A decoction of the plant has been used as emetics, purgatives, antitumor and also for the management of liver, spleen, kidney, bladder, bone and joint diseases (Ogoche, 2013).

2.6.4 Biological activity of *D. integrifolia*

Antimicrobial activity of the ethanolic-water extract of *D. integrifolia* has been demonstrated (Kamgang *et al.,* 2015; Mohamed *et al.,* 2012). Anticancer and antioxidant activity, as well as ovicidal and larvicidal activities against *Heligmosomoides bakeri*, have also been reported (Mothana *et al.,* 2009). Antiplasmodial and antiprotozoal activities of methanolic extracts of *D. integrifolia* have been reported Mothana and colleagues (2014). The antiviral activity has been studied on species *Integrifolia* but from other genus including Tessaria, Holoptela (Sharma *et al*., 2009), Rhus (Kinsley and Nguyen, 2016), Gallesia (Arunachalam *et al*., 2016), Magnolia and Artocarpus (Akkouh *et al*., 2015) among others. To the best of our knowledge there is limited or no evidence from the literature about antiviral activity of the genus *Dichrocephala*. Therefore, there is a gap about antiviral activities of genus *Dichrocephala* which forms the basis of the current study.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

The study was carried out using laboratory based *in vitro* and *in vivo* models to ascertain the antiviral potential and safety of the plant extracts. Vero cells were used as the *in vitro* cell model while Swiss albino mice were used as the *in vivo* animal model. Qualitative phytochemical analysis was carried out using standard chemical tests to determine presence or absence of different active ingredients such as alkaloids, saponin, tannins, flavonoids, glycosides and terpenoids.

3.2 Study site

The study activity was done at the University of Nairobi Campus of Agriculture and Veterinary Science Nairobi, Kenya in the Department of Public Health, Pharmacology, and Toxicology laboratories. Plant materials were collected from Mabariri (S 00^0 31. 367', E 034^0 56. 426', Nyamira County, Kenya). Laboratories were well equipped with good facilities and a clean bench. *In vivo* work was done in the animal house laboratory.

Figure 4: Map of Nyamira County

Source: Kenya Mpya, 2012 (http://www.kenyampya.com/index.php?county=Nyamira)

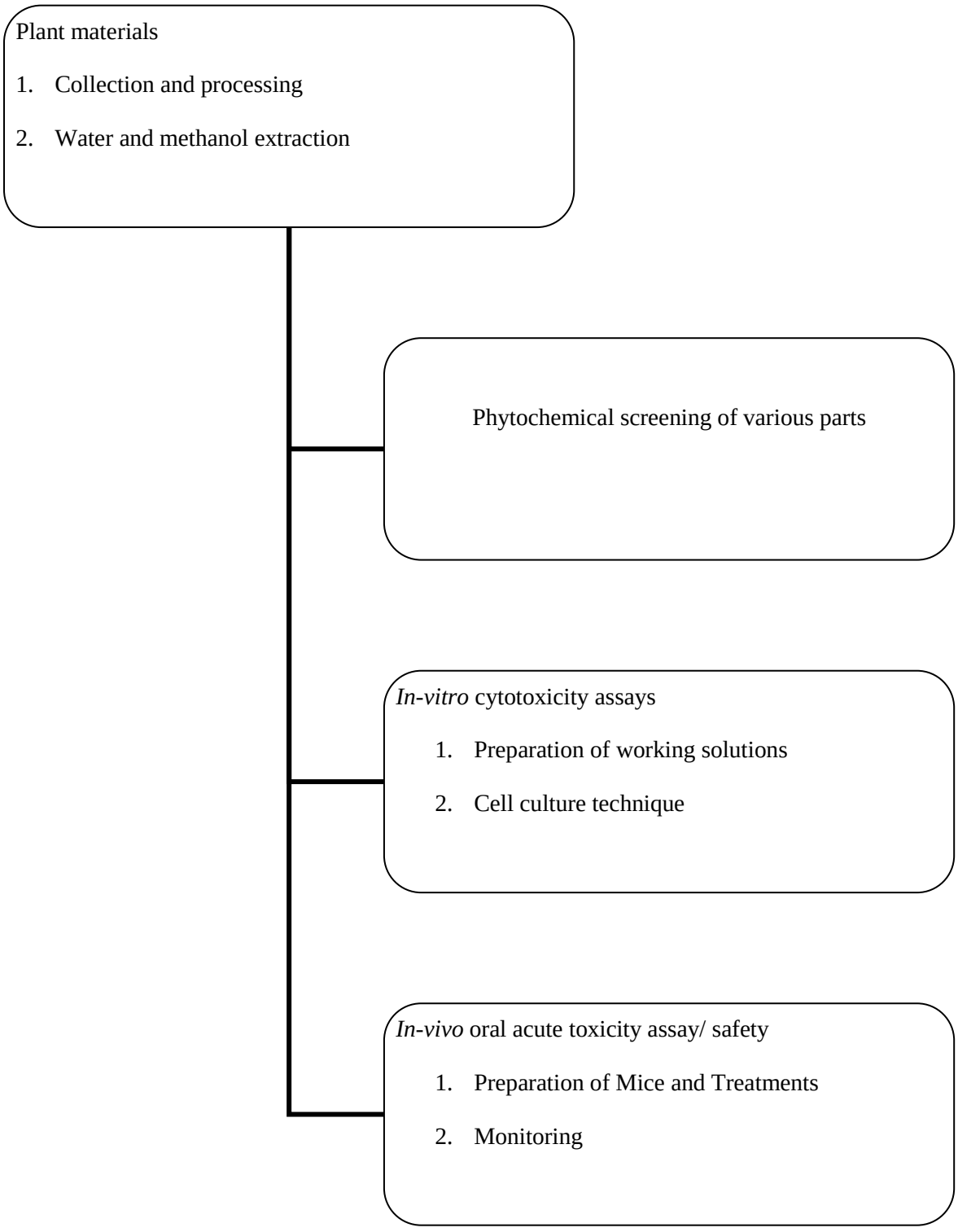

Figure 5: A scheme showing the work design

3.3 Medicinal plant collection

The plant, *D. integrifolia* parts namely the leaves, roots, flowers and stems were collected from its natural habitation in Mabariri S 00^0 31. 367', E 034^0 56. 426', Nyamira County, Kenya (Figure 4). The plants were collected from a single location due to its availability. Whole plant was uprooted which weighed approximately weighed 4000g wet matter. They were placed in a khaki envelop and immediately transferred to the University of Nairobi laboratories where they were stored under shade at room (25 ^{0}C). Taxonomic identification and authentication were done by Mr. Antony Mutiso a botanist at the school of biological sciences, the University of Nairobi. The Voucher specimens number AHH2015/01 was then deposited for future reference at the University of Nairobi herbarium in Chiromo campus.

3.4 Preparation of plant parts

The plant materials were immediately washed separately and allowed to dry its own at room temperature in a clean, well-ventilated room at the departments of public health pharmacology and toxicology. The dried parts were then ground to a fine powder using Gibbons electric grinding machine (Wood-Rolfe Road Tollesbury, Essex, UK). The samples were packed in translucent paper bags and stored at room temperature until use.

3.5 Reagents, chemicals, and assay kits

Dimethyl sulfoxide (DMSO), ethanol, fetal bovine serum/ heat-inactivated newborn calf serum, acyclovir, Eagle minimum essential medium (EMEM), trypsin, penicillin, streptomycin ,trypan blue and 3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyl tetrazolium bromide (MTT) were procured from Sigma (USA) according to the Nairobi University procurement policies and stored in standard condition as stated on their labels during the study period. Briefly, DMSO was stored in

a well ventilated and cool place (25 ^{0}C) (Ashe, 2016), MTT at 4 ^{0}C in the dark (American Type Culture Collection, 2011) and EMEM at 4^{0}C in the dark when not in use (ATCC, 2016).

3.6 Extraction of plant materials

3.6.1 Aqueous extraction

Leaves, flowers, stem and roots were used to prepare crude extracts. Extraction was carried out based on modification to the method previously described by Awoyinka et *al.,* 2007. The ground plant materials were weighed on an analytical balance (Mettler PM 4600) (200g) and extracted by maceration using 1000ml of water. The samples were submerged into the water and extraction allowed to proceed for 48 hours with manual shaking. The samples were then filtered using the filter on a funnel. The water extracts were freeze-dried using Edwards freeze dryer, Modulyo, to obtain a dry powder that was transferred to clean sample bottles, weighed and stored in a freezer at -20 ^{0}C until use

3.6.2 Methanol extraction

Three hundred and fifty grams (350g) of the grounded roots, stem, leaves and flowers were extracted by maceration using methanol as solvent (Parekh *et al*., 2005). The samples were submerged into the 800ml of methanol in a flat-bottom flask plugged with cotton gauze and extraction allowed to proceed for 48 hours with frequent shaking. The samples were then filtered using filter paper on a funnel. The extracts were concentrated under vacuum using a rotary evaporator B-480 (Búchi-technik IK AG, Switzerland) at 40 ^{0}C to concentrate the samples. The extracts were then transferred to clean sample bottles, weighed, labeled and stored in a freezer at -20 ^{0}C until use.

3.7 Evaluation of Phytochemical constituents of *D. integrifolia*

Different qualitative chemical tests were carried out to determine the prsence or absence of different phytochemicals including flavonoid, tannins, saponins, alkaloids, glycosides, phenols, and terpenoids in crude extracts of *D. integrifolia.* The results were evaluated by visual inspection as a change in color or precipitation. Qualitative chemical tests for detection of bioactive compounds proceeded as follows:

3.7.1 Test for Tannins

Approximately 0.8g of the dried methanolic and aqueous extracts were dissolved in 15 ml distilled water and boiled then later filtered. Few drops of ferric chloride were added to the resultant filtrate. Bluish- Green precipitate indicates the presence of tannins (Evans, 2009; Segelman *et al*., 1969).

3.7.2 Test for Saponins

The presence of saponins was determined by dissolving approximately one gram (1g) of the plant extracts in boiling water for 5 minutes and allowed to stand for 15 minutes. The formation of the Stable froth of more than 2cm and persisting for at least 40 minutes was indicative of saponins (Evans, 2009; Kapoor *et al*., 1969).

3.7.3 Test for Alkaloids

The presence of alkaloid was confirmed by dissolving approximately 0.5g of the extract(s) with about 10ml of 1 % hydrochloric acid. The mixture was boiled for 5 minutes then followed by filtering. The filtrate was put in two test tubes of 2ml each. Mayer’s reagent was added to the first test tube with 2ml of the filtrate and the appearance of cream colored precipitate was a positive confirmation of alkaloids presence. Several drops of Dragendorff’s reagent were added

to the second test tube with 2ml of the filtrate and the Reddish-brown precipitate is a confirmation of presence of alkaloids (Evans, 2009; Salehi-Surmaghi *et al*., 1992).

3.7.4 Test for Glycosides

3.7.4.1 Cardiac glycosides- Keller-killiani test

Keller-kiliani test was used to confirm the presence of cardiac glycosides in the extract. One fifty milligrams (150mg) of each extract was mixed with 1.5ml of glacial acetic acid containing some element ferric chloride ($FeCl_3$) solution. To this solution, 0.5 ml of concentrated sulphuric acid was added to the side of the test tube. The appearance of a brown ring at the interface of the two layers with the lower acidic layer turning blue-green is a positive presence of cardiac glycosides (Ajaiyeobu, 2002).

3.7.4.2 Modified Borntrager's test

One gram (1g) of crude plant extract was boiled 3ml of 10% hydrochloric acid in a test tube for 4 minutes. It was then filtered while still hot, cooled and then shaken with 3ml of chloroform. Then the upper layer of chloroform was removed and shaken with half of its volume with dilute ammonia. A rose pink to red coloured produced in the ammonia layer is an indication of the presence of glycosides (Evans, 2009).

3.7.4.3 Keddie test

One gram (1g) of the crude extract was dissolved in chloroform and evaporated to dryness, then 2 drops of concentrated alcohol and 3 drops of benzoic acid. The presence of purple color indicates the presence of glycosides whose aglycone moiety has unsaturated lactone ring (Evans, 2009).

3.7.5 Tests for flavonoids

One gram of the crude plant extracts was dissolved in 10 ml distilled water and then filtered using Whatman filter. 0.5 ml of the filtrate was then mixed with 6mg of magnesium turnings this was followed by the addition of 0.05 ml concentrated sulphuric acid. The presence of magenta red observed after five minutes confirmed the presence of flavonoids (Brain and Turner, 1995).

3.7.6 Test of Phenols

Approximately one gram (1 g) of grounded crude extracts was dissolved in two milliliters of 2% of iron (111) chloride and appearance blue- green precipitate indicates the presence of phenols (Evans, 2009).

3.7.7 Test for terpenoids

Four milliliter of the crude extracts was mixed with 2 mL of chloroform solution and then evaporated to dryness in a water bath. Few drops of concentrated H_2SO_4 were added slowly on the wall of a test tube. Formation reddish brown coloration and green color in the upper layer of the test tube indicates presence of terpenoids (Evans, 2009).

3.8 *In vitro* assay

3.8.1 Cell line and culture media

Vero cell E6 obtained from CTMDR/KEMRI were used in this study. The cells were cultured and maintained using Minimum Essential Medium (MEM) with 2% of fetal bovine serum, two antibiotics (streptomycin100 µg/ml and penicillin 100 units /ml) and retained in 5% CO_2 incubator (Thermo Fisher Scientific, Toll-Free, USA) at 37^0C. The media was removed after 24hrs and the cells were washed with phosphate buffer saline (PBS) and thereafter new medium

was added. The cells were then incubated to attain confluence and upon formation of 100% confluence supernatant was harvested and stored at -85 ^{0}C (Tolo *et al.*, 2010).

3.8.2 Cytotoxicity determination on Vero cell lines

Vero cell E6 were seeded at a concentration of 50,000 cells/well (in 100 µl of maintenance media) into a flat bottom microtiter cell culture enabled 96-well plates (Sigma, USA) and incubated in 5% CO_2 incubator at 37 ^{0}C. Crude methanol and aqueous extracts of *D. Integrifolia* in the concentration range of 0.45-100 µg/ml were exposed to the Vero cell lines and incubated in 5% CO_2 incubator of 37 ^{0}C in humidified air for 48 hrs. The cell was then washed with phosphate buffer saline (PBS) and ten micrograms (10 µl) of tetrazolium dye (5mg/ml) was then added and incubated in 5% CO_2 incubator of 37 ^{0}C in humidified air for 2 hrs. Mitochondrial dehydrogenase which is a biomarker of a living cell interacts with MTT dye, reducing it to insoluble formazan. The formazan formed is corresponding to the number of life cells. Trypan blue exclusion method was used for determining cell viability according to Tolo *et al*. (2007). Formazan formation was confirmed using an inverted light microscope and then solubilized with 50µl of 100% DMSO and optical density (OD) read at 562 nm in a 96-well microtiter plate multiplex reader.

3.8.3 HSV-1 isolate culture

A clinical isolate of HSV 1 virus was obtained from the center for viral research (KEMRI) was propagated in Vero cells in a T75 flask and allowed to grow in 5% CO_2 incubator at 37°C until the complete cytopathic effect was seen. The virus was harvested through freeze-thaw technique, suspended in phosphate buffer saline (PBS) and centrifugation was done at 3,000 rpm for 15

minutes. The virus stock (supernatant) was stored at 35% sorbitol at -80°C until use (Tolo *et al*., 2010).

3.8.4 Determination of Antiherpetic activity of *Dichrocephala integrifolia* in Vero cells

The study was carried out using the method previously described by Alem *et al*. (2016). To characterize the effect of the extracts on various stages of virus replication and probable mechanisms of actions different experimental approaches were employed. The targeted replication cycles important to virus growth were: attachment, a fusion of virus envelope to the plasma membrane of cells and replication of viral proteins (Alem *et al*., 2016). The following procedures were followed namely; post –treatment studies, pre-treatments studies and virucidal effects.

3.8.5 Treatment after virus infection (Post-infection Treatment)

Virus suspension in serum free media at 10^6 $TCID_{50}$ was incubated with cells in 5% CO_2 incubator at a temperature of 37 ^{0}C for I hour. Then the cells were washed with PBS and incubated with the serially diluted extracts were grown in 5% CO_2 incubator at 37 ^{0}C for 24 hours. The extract was removed and then all cells were washed with PBS. Fresh media was then added. Cytopathic effect was observed daily. After 48 h, the cells ability to reduce MTT dye to formazan as earlier described was determined. The percentage protection of the extract to the cells was calculated as [(A-B)/(C-B) x 100], where A, B, and C (A- untreated cells, B- blank, C- treated cells) indicate the optical densities (OD) measured in spectrophotometer at 562 nm with a reference filter of 690 nm of the tested extract with virus infected cells, virus and cell controls. The 50% half maximum inhibitory concentration (IC_{50}) is defined as the extract concentration that protects 50% of treated infected cells to compare with cell control using regression analysis.

The therapeutic index (TI) of the extract for the antiviral activity was determined by calculating the ratio CC_{50} divided by IC_{50} (Alem *et al*., 2016). The same procedure was done for acyclovir (positive control).

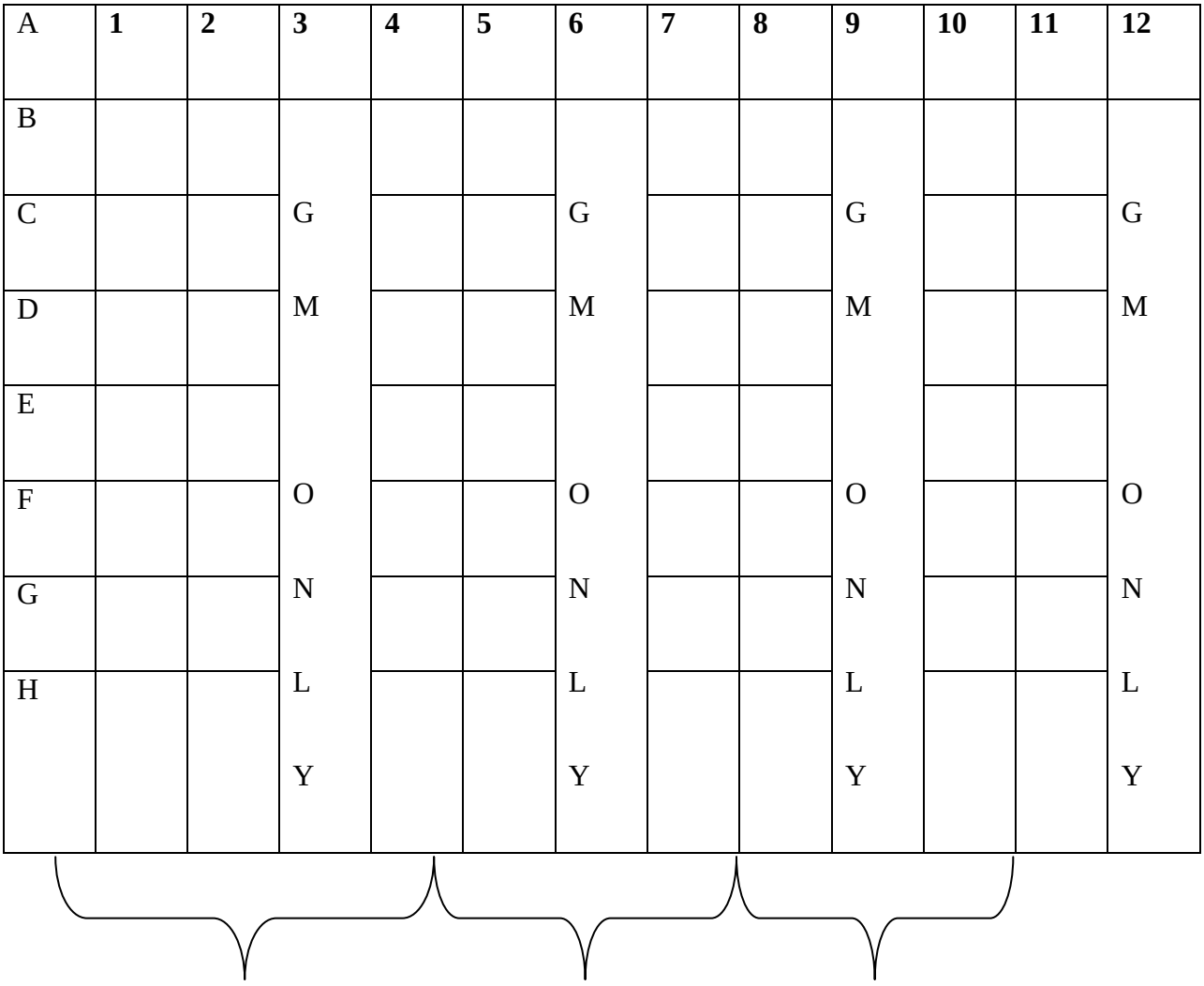

Figure 6: 96 well-plate cell suspensions setting

3.8.6 Treatment before virus infection (Pre-infection treatment)

Fifty (50) µl of the serially diluted extract was incubated in 5% CO_2 incubator with Vero cells for 24 hours. After washing with PBS, the cells were incubated with 50 µl of 10^6 $TCID_{50}$ virus suspensions in serum-free MEM for 1 h, washed with phosphate buffer saline (PBS) and grown with fresh media. Cytopathic effect was observed and the same protocol for cell viability

followed as mentioned in the post treatment experiment. The same procedure was done for acyclovir (positive control).

3.8.7 Investigation of Virucidal activities

Different non-toxic concentrations of crude extract were tested for antiviral property by virucidal assay. Fifty µl of 10^6 $TCID_{50}$ of HSV-1 virus suspensions were incubated with various concentrations of the crude extracts at 37^0C in 5% CO_2 incubator for 1 hour (crude extracts + virus suspension). Solvents (used to dissolve crude extracts) along with virus suspension were kept a blank and cells alone as control. After 1h 100µl of each mixture (crude extracts + virus suspension) were added to monolayer cultures grown in 96 well plates and incubated for 48hrs. The cytopathic effect was observed under light microscope. The effect of the extracts of the cells was evaluated using the MTT assay method. The percentage cell protection/inhibition was calculated by formula shown in MTT assay (Section 3.7.5). The same procedure was done for acyclovir (positive control).

3.9 *In-vivo* assay

3.9.1 Handling of the animals during experimentation and Personal Protective Equipment

The female Swiss mice were handled as per guidelines and protocols established for Laboratory Animals by the organization for economic development and co-operations (OECD, 2001). The study was carried out at Pharmacology and Toxicology Laboratories on the campus of agriculture and veterinary sciences, University of Nairobi. The experimental laboratories were suitable for animal biosafety level two (ABSL/BSL) with a strict aseptic technique using NaOCL at 10% and alcohol at 70%. The mice were restrained using universal mice restrainer for ease of identification, weighing and drugs administration. The laboratory was well ventilated to avoid breathing dust, fumes, gas, mist, vapours, and spray. Principal investigator and personal

assistance all the time used latex gloves, lab coat, and face mask while anti-tetanus and anti-rabies vaccines were made available in the refrigerator in case of injuries. Animals were disposed off through incineration after anesthesia.

3.9.2 Animal model

Adult fifty-seven, female Swiss albino mice aged 8 weeks and weighing 20-25 g were used to investigate the acute toxicity of active crude extract(s).The animals were obtained from the University of Nairobi Kabete animal facility. Ethical approval was obtained from faculty of veterinary medicine Biosafety, Animal Care and Use Committee (BACUC), University of Nairobi Reference BACUC/J56/74093/2014.

3.9.3 Housing and feeding conditions

The mice were housed in a group of three in polycarbonate cages of 35cm length× 25cm width × 18cm height fitted with wire mesh tops. They were kept in cages for ten days prior to dosing to allow adjusting to the laboratory conditions. Cages were cleaned once a week and bedding husk replaced two times a week. The Temperature of the study room was maintained at 23 - 27˚c, relative humidity of between 50-60% .A cycle of 12-hour light and 12-hour dark was maintained. Standard mice pellet diet (Unga Feeds) was given in plenty and water was also provided with automatic water dispensers *ad libitum* during acclimatization and in the experiment period.

3.9.4 Determination of *in-vivo* toxicity of the extract of *D. integrifolia* using Swiss albino mice

This experiment was carried out according to OECD/OCDE guideline 423 (2001) with adaptation. After acclimatization, identification of the mice was achieved by marking at the tail with a permanent marker for ease of identification. The identified mice were weighed using weighing balance (mettle PM 4600) and weight recorded and released back to their cages.The

extracts were prepared in double distilled water to the desired concentration. Prior to dosing animals fasted for four hours and weighed before oral administration of a single dose that was initiated at 300 mg/kg of the test subsistence was selected. The food was further withheld for another 3 hours after administration of test subsistence. Based on the result a subsequent higher dose of 2000mg/kg body of the tests substance was administered to each mouse with an oral gavage needle. Physiological saline was administered to the control group. Individual observations of signs of toxicity wellness parameters such as change of fur, lacrimation, and inflammation of mucous membrane, excessive salivation, drowsiness, convulsions, tremors, body weight, morbidity, and mortality were observed and recorded. The weight was recorded on day 0, 7 and 14. The data obtained was presented in tables and the LD_{50} values were determined statistically (OECD /OCDE, 2001).

3.10 Data analysis

Data obtained from the study was put as a mean ± standard error of the mean (SEM) of the three independent experiments. Data was transferred onto a graph pad prism version 7 and paired t test was used to compare change of weights pre and post treatment. Selectivity indices were determined as the ratio of CC_{50} to IC_{50} (CC_{50}/IC_{50}). *P* value less than 0.05 was considered as statistically significant. The concentration that inhibited 50 % viability of the cells and cytotoxic concentration 50 were evaluated by linear regression curve. The dosage required to kill 50% of the animals (LD_{50}) was calculated using Acute Oral toxicity guidelines as described by (OECD, 2001). Briefly, sequential design was used to determine the needed doses in relation to the body weight and shown in table 4.

Table 4: Working estimate of LD_{50} for use in Stage 2 of the sequential design derived from mortality in a limit dose test at 2000 mg/kg-bwt

Mortality (%)	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**
Working Estimate of LD_{50}	3606	2944	2541	2244	2000	1782	1574	1358	1109

Source: OECD, 2010

3.11 Disposal of cells and experimental animals and Ethical considerations

All the used cell lines were disposed off in accordance with the protocols that are set by University of Nairobi Ethical Committees. Briefly, the liquid waste containing cells was autoclaved and disposed in the sanitary drain followed by water. Animal remains shall be tagged in outer bags or container with prominent poison tags. The mice carcasses were placed in transparent and sealable polyethylene bags after confirming the death. They were disposed of by incineration as per the University of Nairobi Faculty of Veterinary Sciences disposal protocol (AVMA, 2016).

The study was carried out at the University of Nairobi at the department of public health pharmacology and toxicology. Permission to carry out the study and ethical clearance were granted by the biosafety, animal care and use committee (BACUC) University of Nairobi at the Faculty of Veterinary Medicine reference BACUC/J56/74093/2014. The research was conducted according to the University of Nairobi guidelines on the laboratory animal use and care.

CHAPTER FOUR

RESULTS

4.1 Phytochemical composition of different extracts of *Dichrocephala integrifolia* plant

The plant was screened for secondary metabolites using standard procedures and found to contain various pharmacologically important compounds including phenols, flavonoids, tannins, glycosides, terpenoids, alkaloids and saponins. Flavonoids were absent in the methanolic root extract and glycosides were absent in the aqueous extract of the root and methanolic extract of flowers. The result is shown in Table 5 and 6.

Table 5: Phytochemical composition of methanolic extracts of *Dichrocephala integrifolia* plant

Phytochemical	***D. integrifolia* leaves**	***D. integrifolia* root**	***D. integrifolia* flowers**	***D. integrifolia* leaves**
Saponins	+	+	+	+
Alkaloids	+	+	+	+
Phenols	+	+	+	+
Flavonoids	+	-	+	+
Glycosides	+	+	+	+
Terpenoids	+	+	+	+
Tannins	+	+	+	+

Key: (+) - Present, (-) – Absent

Table 6: Phytochemical composition of aqueous extracts of *D. integrifolia* plant

Phytochemical	***D. integrifolia* leaves**	***D. integrifolia* stem**	***D. integrifolia* flowers**	***D. integrifolia* root**
Saponins	+	+	+	+
Alkaloids	+	+	+	+
Phenols	+	+	+	+
Flavonoids	+	+	+	+
Glycosides	+	+	+	-
Terpenoids	+	+	+	+
Tannins	+	+	+	+

Key: (+) - Present, (-) – Absent.

4.2 *In vitro* cytotoxicity of extract of *D.integrifolia* against Vero cell lines

All extract were not toxic to Vero cells except the methanolic extract of the flower which showed slight toxicity with a CC_{50} value of 71.31 ± 2.65. According to national cancer, Institute CC_{50} values greater than 100µg/ml are deemed safe. The result is shown in Table 7.

Table 7: Cytotoxic effect of *D. integrifolia* extracts on Vero cells (normal).

Study extract	Solvent	CC_{50} Values (µg/ml)
D. integrifolia Stem	Water	>100
D. integrifolia Stem	Methanol	>100
D. Integrifolia Flowers	Methanol	71.31 ± 2.65
D. Integrifolia Flowers	Water	>100
D. Integrifolia Roots	Water	>100
D. Integrifolia Roots	Methanol	>100
D. Integrifolia Leaf	Methanol	>100
D. Integrifolia Leaf	Water	>100
Acyclovir	N/A	>100

>100: Depicts that the CC_{50} value of the sample tested was above 100µg/ml, therefore, could not be obtained within the concentrations exposed to the cells.

4.3 *In vitro* antiviral activity of extract of *Dichrocephala integrifolia* against Herpes simplex virus

Pre-treatment of cells results obtained shows that *D. integrifolia* methanolic extracts of the stem, leaves and aqueous extracts of leaves inhibited the ability of HSV-1 virus to cause cytopathic effects Vero cells with IC_{50} values of 63.95±5.36, 54.45±3.45, 86.20±7.56 respectively. Post-treatment of cells, only the methanolic extract of the flower and the aqueous extract of the leaves protected the cell from cytopathic effects caused by the virus at IC_{50} values 86.20±7.56 and 82.44±7.92 respectively. The other extracts had IC_{50} values greater than 100µg/ml.

Table 8: The IC_{50} values of *D. integrifolia* extracts from pre-treated and post-treated Vero cells

Plant parts	Extracts	Pre-treatment IC_{50} (µg/ml)	Post-treatment IC_{50} (µg/ml)
D. integrifolia Stem	Water	>100	>100
D. integrifolia Stem	Methanol	63.95±5.36	>100
D. Integrifolia Flowers	Methanol	>100	45.270±4.31
D. Integrifolia Flowers	Water	>100	>100
D. Integrifolia Roots	Water	>100	>100
D. Integrifolia Roots	Methanol	>100	>100
D. Integrifolia Leaf	Methanol	54.45±3.45	>100
D. Integrifolia Leaf	Water	86.20±7.56	82.44±7.92
Acyclovir	Water	4.772±7.81	>100

IC_{50} = Inhibitory Concentration 50 (IC_{50})

4.4 *In vitro* virucidal activity of extract of *D. integrifolia* against Herpes simplex virus

The methanolic extract of flower, aqueous extract of the root and the methanolic extract of leaves showed direct inactivation of virus when the extracts were incubated with the virus prior to incubation with the cells at IC_{50} values of 45.27±2.41, 0.333±1.23 and 30.53±4.51 respectively. The plant extracts selectively inhibited the growth of the virus. This is shown by the selectivity indices obtained as shown in Table 9.

Table 9: The potential of various plant parts extracts and Acyclovir (positive control) to prevent cell damage by HSV virus

Plant parts	Extracts	CC^{a}_{50} (µg/ml)	IC^{b}_{50} (µg/ml)	Selectivity index (SI^{c})
D. integrifolia Stem	Water	>100	>100	N/A
D. integrifolia Stem	Methanol	>100	>100	N/A
D. Integrifolia Flowers	Methanol	71.31± 2.65	45.27±2.41	1.58
D. Integrifolia Flowers	Water	>100	>100	N/A
D. Integrifolia Roots	Water	>100	0.333±1.23	>300.3
D. Integrifolia Roots	Methanol	>100	>100	N/A
D. Integrifolia Leaf	Methanol	>100	30.53±4.51	>3.28
D. Integrifolia Leaf	Water	>100	>100	NA
Acyclovir	N/A	>100	24.51 ±3.57	>4.080

Key: a = Cytotoxic concentration 50 (CC_{50})

b = Inhibitory Concentration 50 (IC_{50})

c = Selective index = CC_{50}/IC_{50}

NA – Not applicable - the data presented spectacle means (± standard error) of three independent experiments performed.

4.5 *In-vivo* toxicity of the extract of *Dichrocephala integrifolia* using Swiss albino mice

Tables 10 & 11 shows control and treated animals body weights and general behavior and appearance of mice at both 300 and 2000mg/kg body weight. From the results, no mortality or gross changes in animal behavior and appearance were at both 300 and 2000 mg/kg. All animals depicted normal increment in weight and no significant differences between both control and test groups. This indicates that the extract does not affect the growth of the mice. No mortality was

observed at 300mg and 2000mg/kg body weight doses of different extracts from different parts of the plant indicating an LD_{50} of >2000 mg/Kg body weight.

Table 10: Changes in body weight of Swiss albino mice following administration of crude extracts of D. integrifolia at 300 mg/kg

Study extract	Day 0	Day 7	Day 14	*P* value
Leaf extract $_{(aq)}$	21.53±2.12	23.91±2.15	25.35±2.00	0.693
Flower extract $_{(aq)}$	20.11±1.45	23.65±2.45	25.00±2.13	0.554
Root extract $_{(aq)}$	25.10±3.64	25.31±3.73	27.85±4.45	0.156
Root extract $_{(Me)}$	24.50±1.73	24.57±2.01	25.74±1.69	0.213
Stem extract $_{(aq)}$	23.27±2.96	23.86±1.89	25.00±2.52	0.076
Flower extract $_{(Me)}$	21.53±0.67	22.06±2.43	24.04±0.78	0.092
Leave extract $_{(Me)}$	26.30±4.77	26.16±2.11	27.72±0.15	0.108
Stem extract $_{(Me)}$	21.73±0.68	22.06±0.53	22.27±2.00	0.074
Negative control	22.49±0.48	22.00±2.00	22.65±0.35	0.726

Key:

All mice groups (n = 3) were administered with the crude extracts at 300mg/kg, aq = Water extract, Me = Methanol extract

Table 11: Changes in body weight of swiss albino mice following administration of crude extracts of D. integrifolia at 2000 mg/kg

Study extract	Day 0	Day 7	Day 14	*P* value
Leave extract (aq)	26.12±0.12	26.71±2.00	26.20±0.37	0.866
Flower extract (aq)	23.19±0.38	23.51±2.11	23.07±2.05	0.594
Root extract (aq)	25.48±0.39	25.95±0.61	25.24±0.45	0.646
Root extract (me)	24.61±0.33	24.97±2.13	24.65±0.33	0.918
Stem extract (aq)	23.32±0.19	23.50±2.22	23.89±0.40	0.080
Flower extract(me)	22.51±1.90	22.68±1.57	22.95±0.45	0.671
Leave extract (me)	25.84±0.19	25.79±2.11	25.64±0.22	0.336
Stem extract (me)	28.44±0.43	28.15±2.15	28.18±0.17	0.423
Negative control	25.16±0.32	25.00±2.57	25.10±0.20	0.857

Key:

All mice groups (n = 3) were administered with the crude extracts at 2000mg/kg, aq = Water extract, Me = Methanol extract

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Phytochemical compounds from plants have been used in treatment of different diseases including diseases of viral origin (Kohn *et al.*, 2015, 2016; Li *et al.*, 2009; Abad *et al*., 1999). The following phytochemical compounds from plants have been reported to have antiviral activity in different previous studies alkaloids (Martin 1987, McMahon *et al*., 1995), flavonoids (Lin *et al*.,1999, Pengsuparp *et al.*, 1995), saponins (Sindambiwe *et al*., 1998), terpenes (Bourne *et al*., 1999), tannins (Ferrea *et al*., 1993) . In this study phytochemical component of aqueous and methanol crude extracts of a *D. integrifolia* screened using qualitative analysis have tested positive for the presence of alkaloids, flavonoids, phenols, saponins, tannins and Terpenoids except root methanol and root acqueous that lacked flavonoids and glycoside respectively. This result is consistent with other with previous studies (Mohammed and Teshale 2012). The current antiviral activity could be associated with these important pharmacological compounds present in *D. integrifolia* crude extracts especially the glycosides, flavonoids, phenolics, terpenoids, and tannins. This can be further investigated by isolating the bioactive compounds.

The study reports on cytotoxicity of different extracts from different parts of the plant on normal monkey kidney cells (Vero). Methanol extract of flower has exhibited moderate toxicity while other parts of the plant were safe. Decoctions from plants have been widely used in the treatment of various diseases traditionally without scientific justification on its safety (Okumu *et al*., 2016). Majority of the reports on toxicity of plants based crude drugs are often associated with liver toxicity (Agbor *et al*., 2010). The wide traditional use of *D. integrifolia* warrants evaluation for

its toxicity properties putting into consideration public health protection to the plant extracts which could cause undesirable effects to the consumers. Cytotoxicity activities of alkaloids and flavanoids have also been reported (Ozcelik *et al.*, 2011) therefore the the cytotoxicity effects of flower might be due the phytochemical such as flavanoids present in the extracts. Further study need to be done to identify the exert bioactive compounds causing cytotoxicity in leaves.

In the current study plants, *in vitro* antiviral activity of four part *D. integrifolia* crude extracts evaluated against herpes simplex virus revealed that all the investigated parts had antiviral against herpes simplex virus. Previous studies have reported antiviral activity in different medicinal plants. Despite reports on antiviral and general antimicrobial activity of plants in the Asteraceae family studies on *D. integrifolia* antiviral activity are lacking. When the virus was exposed to the cell before treating with the extracts different part of the plant exhibited moderate inhibitory effect that could possibly be due to blocking of cellular receptor cellular receptors preventing virus entry in the cell. Plant extracts that possess antiviral activity may inhibit the virus through several mechanisms such as blocking specific proteins for viral entry or by working directly on the virus itself (Yang, 2013). The methanol of flower and aqueous extract of leaves exhibited a moderate inhibitory effect of when incubated with infected cells; this could possibly be due to its interference at some stage of virus replication process inside the cells. Aqueous extracts of root and methanol extracts of leaves exhibited highest antiviral activity were incubated with the virus prior to incubation with the cells. This is for the first time the antiviral activity of this plant is reported

All animals experienced normal increment in weight and no drastic differences between both control and test groups were observed. Therefore, the absence of these toxicity indicators means that the extracts were safe at these doses and that their lethal doses are much higher. Oral acute

toxicity has been used widely in the evaluation herbal remedies safety (Rang *et al.,* 2001). Administration of *D. integrifolia* extract to ethanol administered mice brought transaminases towards normal values after significant increase after alcohol dosage. The aqueous extract of the plant prevented the development of hepatic tissue abnormalities and improved hepatic function in ethanol-induced hepatic damage (Florence *et al.,* 2016). Additionally, Franco *et al.* (2015) found *Chresta martii* a plant in the Asteraceae family to have no acute toxicity neither were any mortalities recorded. Still, debates rumors on the rational of extrapolating animal model results to humans. However previous studies have proved that mice model is a better predictor for human lethal dosage as compared to rats (Walum *et al.,* 1995).

5.2 Conclusions

The results obtained depict that different roots and flower extracts of *D. integrifolia* could be a good candidate in the search for new leads for anti-HSV. The safety observed in mice is recommendable given the wide use of the plant in ethnobotanical medicine. The current study lays the basis for further research on the isolation of bioactive compounds and further evaluation of the mechanisms of action of the plants bioactive at the molecular level. This study provides a partial scientific justification on the use *D. integrifolia* in HSV infections. Given the high cost, unbearable side effects and unavailability of the current anti-viral drugs targeted towards HSV to most of the people in rural areas, the current study provides hope that new cheap antiviral drug could be obtained from this plant.

5.3 Recommendations

1. Purification of the crude extracts should be done to determine and quantify individual the pure active compounds.

2. Further research should be done on acute and chronic toxicity on mammals.

3. Further research should be carried out to determine the mode of action of the identified compounds.

4. The extracts should be tested against other viruses.

5. Further research should be done to determine the effects of the extracts on DNA replication.

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APPENDICES

Appendix I: Ethical Approval Letter from Nairobi University – School of veterinary Science (BAUEC)

UNIVERSITY OF NAIROBI
FACULTY OF VETERINARY MEDICINE
DEPARTMENT OF VETERINARY ANATOMY AND PHYSIOLOGY

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Dr Abdi Hussein Hadun
C/o Dept of Public Health, Pharmacology & Toxicology

REF:FVM BAUEC/2016/118

Dear Dr Hadun,

17/10/2016

RE: Approval of Proposal by Biosafety, Animal use and Ethics committee
A Study of antiviral activity and toxic effects of *Dichlocephala integrifolia* KUNTZE
By Abdi Hussein Hadun (Reg. No: J56/74093/2014)

We refer to the above revised proposal that you submitted to our committee for review. We have now reviewed the proposal and have noted that you have addressed the issues to do with proposed Animal husbandry, welfare, euthanasia and disposal satisfactorily. This approval however, is restricted to the use of Swiss albino mice for testing of extracts of the plant *Dichlocephala integrifolia* for toxicity.
Furthermore, it is understood that the proposed antiviral studies on the same plant will be carried out at the KEMRI where an appropriate permit will be sought particularly for the use of the monkey cell lines and the viruses stated in your proposal.
We hereby approve your study on the determination of toxicity of extracts of *Dichlocephala integrifolia* as per your revised proposal

Rodi O. Ojoo BVM MSc PhD
Chairman,
Biosafety, Animal Use and Ethics Committee,
Faculty of Veterinary Medicine

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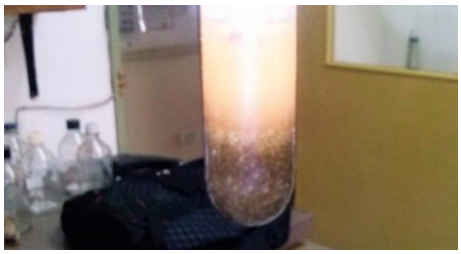
Appendix II: Photos of Secondary Metabolites

a. Test for alkaloids of methanol and aqueous extract.

b. Test for flavonoids.

Aqueous extract



c. Test for tannins.

Organic extract

d. Test for Terpenoids

Methanol extract

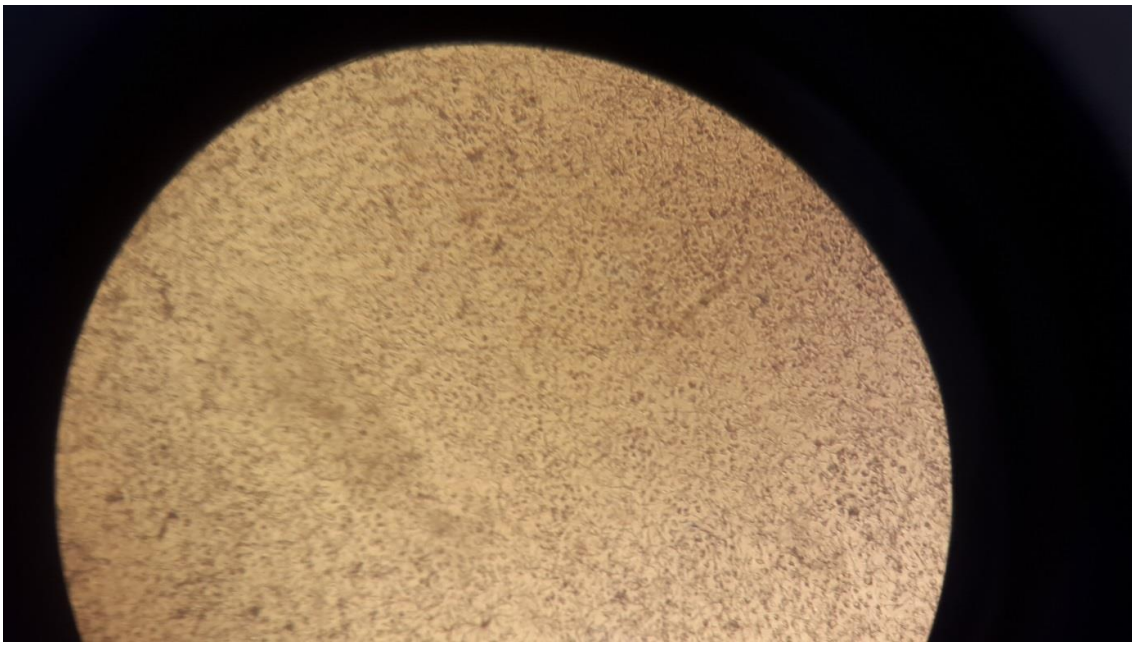
e. Test for Saponins

Aqueous extract

Appendix III: Photograph of Female Albino Swiss Mice

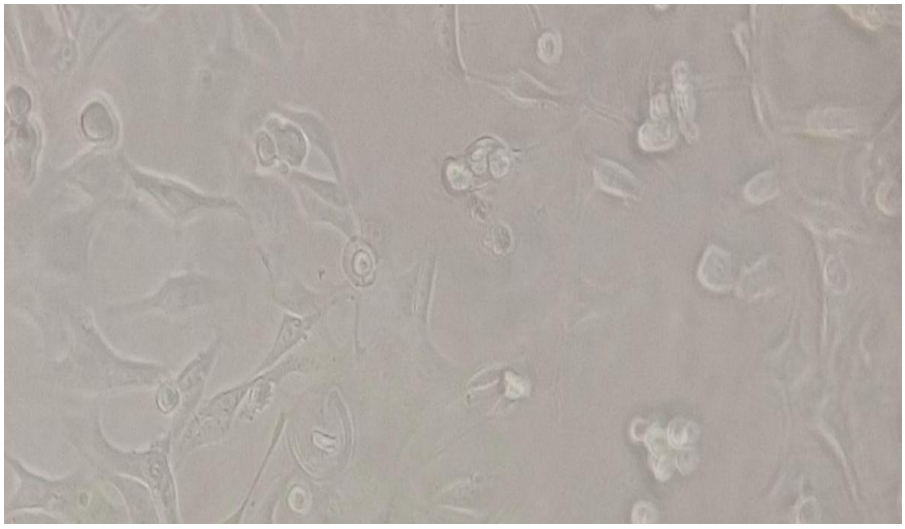
Female Swiss Albino mice marked at tail for identification

Appendix IV: Photo showing fully confluent monolayer cells

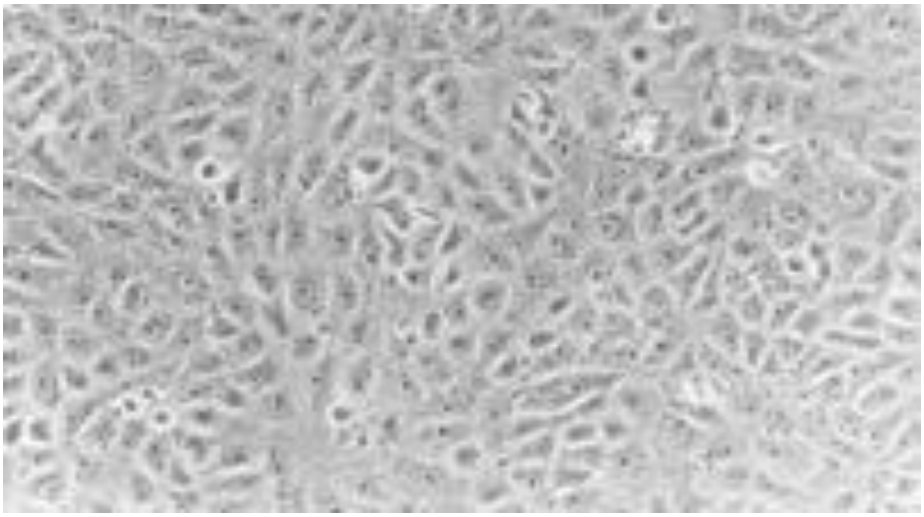


An image of confluent monolayer cells observed microscopically

Appendix V: Vero cells

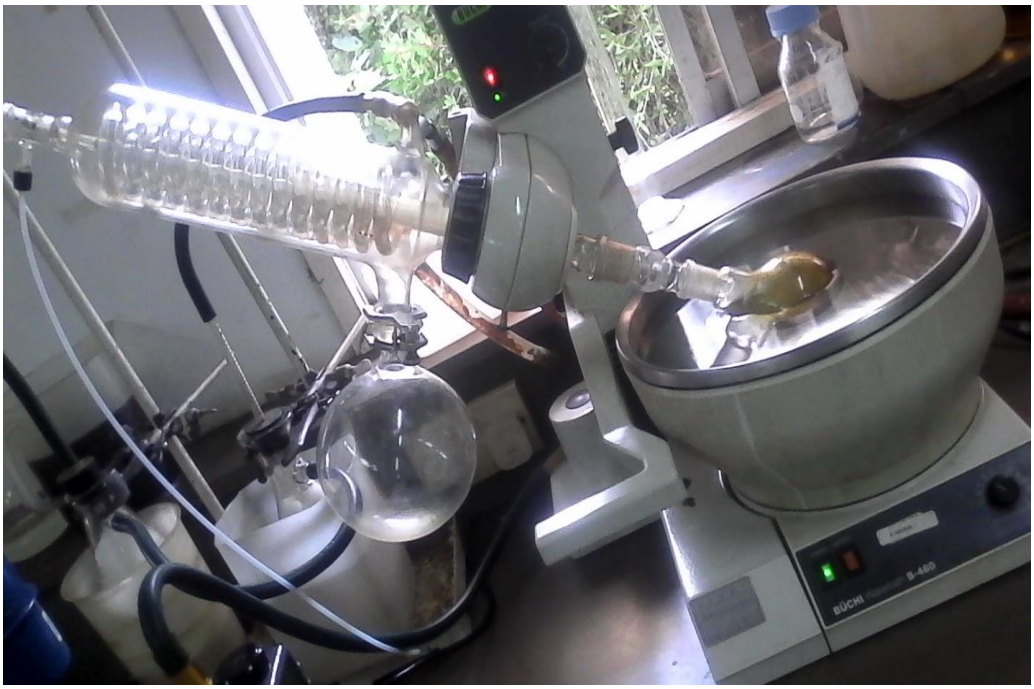


A. Uninfected vero cells



B. Vero Cells infected with HSV

Appendix VI: Photograph of Methanol Extraction process



Methanol crude extract concentration under vacuum using rotary evaporator

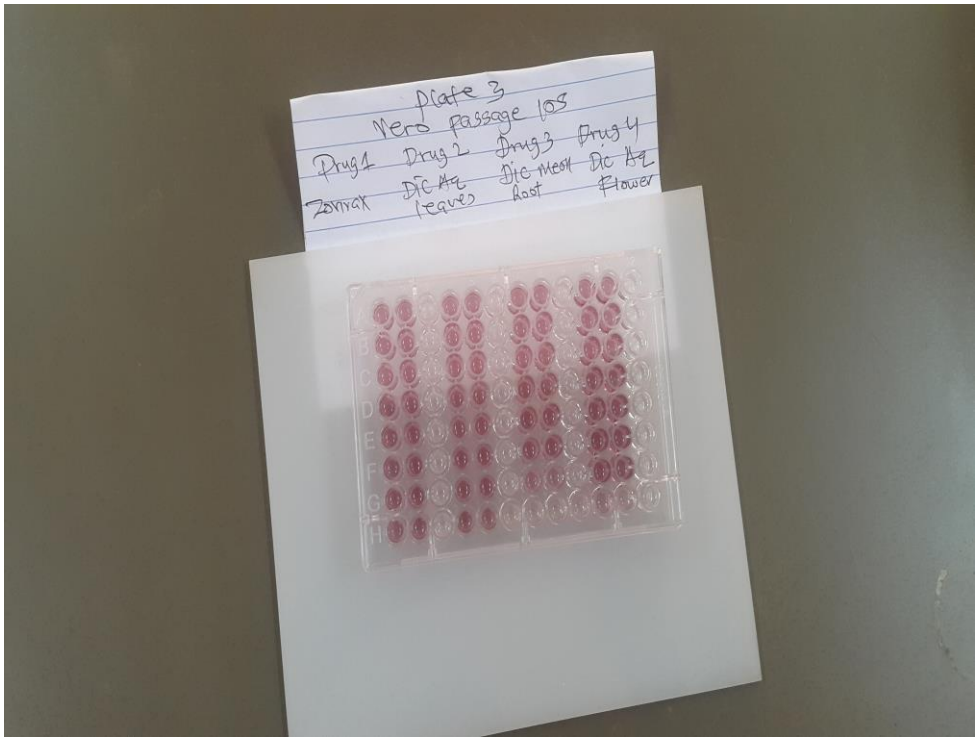
Appendix VII: Freeze drying photograph of water Extracts

Freeze drying of aqueous crude extract using Edwards Freeze dryer, Modulyo

Appendix VIII: Laboratory grinding mill photograph

Grinding machine used to grind dried plant materials

Appendix IX: Cytotoxicity test using Vero E6 cells



An image of 96 well plate with Vero cells treated with plant extracts and stained with MTT

Appendix X: Photograph of Female Swiss Mice Oral drug administration

Test extract administration to Female Swiss Albino mice

Appendix XI. Turnitin Originality Report

Turnitin Originality Report

Phytochemical, Antiviral Potential and Toxic Effects Of Dichrocephala Integrifolia Kuntze. Extract. by Abdi Hadun

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