ASIAN J TROP BIOTECHNOL
Volume 19, Number 2, December 2022
Pages: 72-81

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biotek/c190204

Phytochemical composition of *Dichrocephala integrifolia* crude extracts, antiviral activity and toxicity

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Manuscript received: 4 August 2022. Revision accepted: 11 September 2022.

Abstract. *Hadun AH, Mbaria JM, Aboge GO. 2022. Phytochemical composition of* Dichrocephala integrifolia *crude extracts, antiviral activity and toxicity. Asian J Trop Biotechnol 19: 72-81.* 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, infecting over 90% of people with either one or both. HSV-1 is a viral disease to cause genital and oral lesions. As a major antiviral drug to treat HSV infections, Acyclovir has proven unsatisfactory as resistance, and severe side effects in pregnant mothers and infants have been frequently reported. The high prevalence of HSV, lack of vaccines, and limited treatment options warrant an urgent need for more effective anti-HSV agents. The aim of this study was to investigate phytochemical composition, in vitro anti-HSV, and in vivo and in vitro toxicity of *Dichrocephala integrifolia* (L.fil.) Kuntze crude extracts. Leaves, roots, flowers, and stems of *D. integrifolia* were collected from Mabariri Village in Nyamira County, Kenya. The identification by a botanist in the school of biological sciences, University of Nairobi, Kenya. The materials were extracted with methanol and water, and Qualitative tests were conducted to determine alkaloids, flavonoids, saponins, tannins, glycosides, and terpenoids presences. The MTT assay investigated in vitro cytotoxic activity using Vero cell lines from the center for viral research Kenya Medical Research Institute (KEMRI). Antiviral activity has assessed the ability to protect normal cells (Vero cell lines) from HSV attack. In vivo toxic effects in female Swiss albino mice using oral acute toxicity protocols by OECD. The qualitative phytochemical showed the extracts contained tannins, flavonoids, alkaloids, terpenoids, phenols, glycosides, and saponins. It was not cytotoxic to Vero cells except the flower's methanolic extract, which had a CC_{50} value of 71.31± 2.65 µg/mL. The extracts interference adsorption step of HSV-1 blocks the virus's epitopes on the cell's membrane. Methanolic extracts of the stem, leaves and aqueous extracts of leaves inhibited HSV-1 virus from causing a cytopathic effect, with IC_{50} values of 63.95±5.36 µg/mL, 54.45±3.45 µg/mL, 86.20±7.56 µg/mL. Methanolic flower, aqueous root, and methanolic leaves extract show virucidal with IC_{50} values of 45.27±2.41 µg/mL, 0.333±1.23 µg/mL, and 30.53±4.51 µg/mL. Oral administration to mice at 300 mg/kg and 2,000 mg/kg did not result in any toxic effects or mortality. In all the groups, no major behavioral or appearance changes were observed. Pharmacologically phytochemicals such as flavones, phenols, terpenoids, and tannins in antimicrobial action support this plant's pathologies management. The cytotoxicity, efficacy, and acute oral show no major toxicity; preparing antiviral herbal remedies may be safe for patients. Further research into plant mechanisms and isolating the bioactive agents are needed; studying toxic effects is also recommended to formulate pharmacological products.

Keywords: Antiviral, cytotoxicity, *Dichrocephala integrifolia*, Herpes Virus Simplex, in vivo safety, phytochemical

INTRODUCTION

Traditional medicine has been practiced since immemorial times (Dery et al. 1999). It is still a vital component in health care systems, 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), and Human Immunodeficiency Virus (HIV), among others (Balick and Cox 1995a). Many people, especially those in developing countries, mostly rely on drugs of natural origin for their healthcare requirements (Cunningham 1993; Balick and Cox 1995b; Mworia 2000). The World Health Organization (WHO) approximates that nearly 6 billion people use plant-derived products for their primary healthcare needs (Choudhry et al. 2004). For example, approximately 84% of Peru's population prefers traditional medicine to conventional drugs for their primary healthcare. They believe that herbal products are less toxic, easily accessible, and well-tolerated; thus, traditional medicines are considered safe and combine conventional drugs (Bussmann et al. 2007). In Africa, many communities use medicinal plants to treat 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 of different human ailments. In Kenya, almost 90% of the population uses complementary and alternative medicine from natural sources at least once for various health conditions (Chirchir et al. 2006), and various plants or parts harbor active principles responsible for their medicinal properties.

Despite the wide scope of clinically active synthetic and semi-synthetic antibiotics, the search for new efficacious anti-infective drugs remains paramount due to resistance and the emergence of new parasite strains or new therapeutic targets (Fair and Tor 2014). Currently, the need for substances with antiviral activity is high since the drugs used to treat viral infections are not readily available. A

major problem has been the emergence of mutant viral strains not responding to the available antiviral drugs. The greatest drawback in the fight against human simplex virus infections is the rapidly evolving drug resistance, increasing the cost of treatment. Virus resistance to Acyclovir which has been allied to mutations at the 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 HIV-AIDS patients, are at great risk of attack by these strains (Morfin and Thouvenot 2003). These drawbacks call for a multifaceted approach to curb the disease.

Plants have been used since ancient times to fight against various diseases, including viral ones (Kinghorn et al. 2011). A natural product-based approach could help discover safe new leads with diverse targets to the virus, 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 and cost-effective with fewer side effects. Diseases of viral origin have been treated with natural products from plants for decades (Newman and Cragg 2007; Kinghorn et al. 2011). However, few studies have been conducted to evaluate the plants with antiviral activities, and the number of active compounds has been isolated from higher plants (Farnsworth and Kaas 1981; Kingstone 2011; David et al. 2015). These studies suggested that selecting plant materials based on ethnomedical use gives a higher lead generation than screening programs for search from general synthetic products (Kingstone 2011). However, most of the plants used for medicinal purposes by different communities have not been investigated thoroughly using scientific techniques. In this study, *Dichrocephala integrifolia* (L.fil.) Kuntze was selected for scientific validation based on its ethnomedical uses.

Furthermore, a qualitative screening of the phytochemicals in plant crude extracts was done using standard procedures (Evans 2009). In vitro antiviral (against HSV) properties and cytotoxicity of the extracts were performed to ascertain their potency and safety in managing HSV. The study aimed to screen for the phytochemical compounds present in the crude extracts of *D. integrifolia*; to determine the in vitro antiviral activity of extracts of *D. integrifolia* against HSV; to determine the in vitro cytotoxic effects of *D. integrifolia* extract against Vero cell lines, and to determine the in vivo mammalian toxicity of the methanolic and aqueous extracts of *D. integrifolia* using Swiss albino mice.

MATERIALS AND METHODS

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. In addition, qualitative phytochemical analysis was carried out using standard chemical tests to determine the presence or absence of different active ingredients such as alkaloids, saponin, tannins, flavonoids, glycosides, and terpenoids.

Study site

The study activity was done at the University of Nairobi Campus of Agriculture and Veterinary Science in Nairobi, Kenya. 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.

Medicinal plant collection

The parts of plant *D. integrifolia*, 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 1). The plants were collected from a single location due to their availability. The whole plant was uprooted, weighing approximately 4,000 g of wet matter. They were placed in a khaki envelope and immediately transferred to the University of Nairobi laboratories, where they were stored under shade in the room (25°C). Mr. Antony Mutiso, a botanist at the school of biological sciences at the University of Nairobi, did taxonomic identification and authentication. The Voucher specimen number AHH2015/01 was then deposited for future reference at the University of Nairobi herbarium on the Chiromo campus.

Preparation of plant parts

The plant materials were immediately washed separately and allowed to dry 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). Finally, the samples were packed in translucent paper bags and stored at room temperature until use.

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°C) (Ashe 2016), MTT at 4°C in the dark (American Type Culture Collection, 2011), and EMEM at 4°C in the dark when not in use (ATCC 2016).

Figure 1. Map of Nyamira County, Kenya. Source: Kenya Mpya 2012 (http://www.kenyampya.com/index.php?county=Nyamira)

Extraction of plant materials

Aqueous extraction

Leaves, flowers, stems, and roots prepared crude extracts. Extraction was performed based on a modification to the method previously described by Awoyinka et al. 2007. The ground plant materials were weighed on an analytical balance (Mettler PM 4600) (200 g) and extractedby maceration using 1,000mL water. The samples were submerged in the water, and extraction was allowed to proceed for 48 hours with manual shaking. The samples were then filtered using the filter on a funnel. Finally, the water extracts were freeze-dried using Edwards freeze dryer, Modulyo, to obtain a dry powder that was transferred to clean sample bottles, weighed, andstored in a freezer at -20 °C until use.

Methanol extraction

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

Evaluation of phytochemical constituents of *Dichrocephala integrifolia*

Different qualitative chemical tests were conducted to determine the presence or absence of different phytochemicals, including flavonoids, 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 the detection of bioactive compounds proceeded as follows:

Test for tannins

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

Test for saponins

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

Test for alkaloids

The presence of alkaloid was confirmed by dissolving approximately 0.5 g of the extract(s) with about 10 mL of 1% hydrochloric acid. The mixture was boiled for 5 minutes then, followed by filtering. The filtrate was put in two test tubes of 2 mL each. Mayer's reagent was added to the first test tube with 2 mL of the filtrate, and the appearance of a cream-colored precipitate was a positive confirmation of the alkaloids' presence. Several drops of Dragendorff's reagent were added to the second test tube with 2 mL of the filtrate, and the Reddish-brown precipitate confirmed the presence of alkaloids (Salehi-Surmaghi et al. 1992; Evans 2009).

Test for glycosides

Cardiac glycosides-Keller-killiani test. Keller-killiani test was used to confirm the presence of cardiac glycosides in the extract. First, one hundred and fifty milligrams (150 mg) of each extract was mixed with 1.5 mL of glacial acetic acid containing some element of ferric chloride ($FeCl_3$) solution. Next, this solution added 0.5 mL of concentrated sulphuric acid to the side of the test tube. As a result, 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).

Modified Borntrager's test. One gram (1 g) of crude plant extract was boiled in 3 mL of 10% hydrochloric acid in a test tube for 4 minutes. Next, it was filtered while still

hot, cooled, and shaken with 3 mL 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 color produced in the ammonia layer indicates the presence of glycosides (Evans 2009).

Keddie test. One gram (1 g) 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 purple color indicates the presence of glycosides whose aglycone moiety has an unsaturated lactone ring (Evans 2009).

Tests for flavonoids

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

Test of phenols

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

Test for terpenoids

Four milliliters of the crude extracts were mixed with 2 mL of chloroform solution and then evaporated to dryness in a water bath. Then, a few drops of concentrated H_2SO_4 were added slowly to the test tube wall. The formation of reddish brown coloration and green color in the test tube's upper layer indicates the presence of terpenoids (Evans 2009).

In vitro assay

Cell line and culture media

Vero cell E6 obtained from CTMDR/KEMRI was 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°C. The media was removed after 24hrs, the cells were washed with phosphate buffer saline (PBS), and a new medium was added. The cells were then incubated to attain confluence, and upon formation of 100% confluence, the supernatant was harvested and stored at -85°C (Lamorde et al. 2010).

Cytotoxicity determination on Vero cell lines

Vero cell E6 was 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 a 5% CO_2 incubator at 37°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 a 5% CO_2 incubator of 37°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 (5 mg/mL) was then added and incubated in 5% CO_2 incubator of 37 °C in humidified air for 2 hrs. Mitochondrial dehydrogenase, a biomarker of a living cell, interacts with MTT dye, reducing it to insoluble formazan. The formazan formed corresponds to the number of live cells. According to Tolo et al. (2007), the trypan blue exclusion method was used for cell viability. Formazan formation was confirmed using an inverted light microscope and then solubilized with 50 µL of 100% DMSO, and optical density (OD) was read at 562 nm in a 96-well microtiter plate multiplex reader.

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 a 5% CO_2 incubator at 37°C until the complete cytopathic effect was seen. Then, the virus was harvested through the freeze-thaw technique, suspended in phosphate buffer saline (PBS), and centrifugation was done at 3,000 rpm for 15 minutes. Finally, the virus stock (supernatant) was stored at 35% sorbitol at -80°C until use (Lamorde et al. 2010).

Determination of the antiherpetic activity of Dichrocephala integrifolia *in Vero cells*

The study was carried out using the method previously described by Alem et al. (2016). Different experimental approaches were employed to characterize the effect of the extracts on various stages of virus replication and probable mechanisms of action. 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). In addition, the following procedures were followed: post-treatment studies, pre-treatment studies, and virucidal effects.

Treatment after virus infection (Post-infection treatment)

Virus suspension in serum-free media at 10^6 $TCID_{50}$ was incubated with cells in a 5% CO_2 incubator at a temperature of 37°C for I hour. Then the cells were washed with PBS, incubated with the serially diluted extracts, and grown in a 5% CO_2 incubator at 37°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 a 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 the extract concentration that protects 50% of treated infected cells to compare with cell control using regression analysis. The extract's therapeutic index (TI) 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).

Treatment before virus infection (Pre-infection treatment)

Fifty (50) µL of the serially diluted extract was incubated in a 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).

Investigation of virucidal activities

Different non-toxic concentrations of crude extract were tested for antiviral properties 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°C in a 5% CO_2 incubator for 1 hour (crude extracts + virus suspension). Solvents (used to dissolve crude extracts) and virus suspension were kept blank, and cells alone as the control. After 1 h, 100 µL of each mixture (crude extracts + virus suspension) were added to monolayer cultures grown in 96 well plates and incubated for 48 hrs. The cytopathic effect was observed under a light microscope. The effect of the extracts of the cells was evaluated using the MTT assay method. The cell protection/inhibition percentage was calculated using the MTT assay formula. The same procedure was done for Acyclovir (positive control) (Figure 2).

In-vivo assay

Handling of the animals during experimentation and personal protective equipment

The female Swiss mice were handled per guidelines and protocols established for Laboratory Animals by the organization for economic development and co-operations (OECD/OCD 2001). The study was carried out at Pharmacology and Toxicology Laboratories on the agriculture and veterinary sciences campus, 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 drug administration. The laboratory was well-ventilated to avoid breathing dust, fumes, gas, mist, vapors, and spray. The principal investigator and personal assistant always used latex gloves, lab coats, and face masks. At the same time, anti-tetanus and anti-rabies vaccines were made available in the refrigerator in case of injuries. Animals were disposed of through incineration after anesthesia.

Animal model

Fifty-seven adult 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 the faculty of veterinary medicine Biosafety, Animal Care and Use Committee (BACUC), University of Nairobi Reference BACUC/J56/74093/2014.

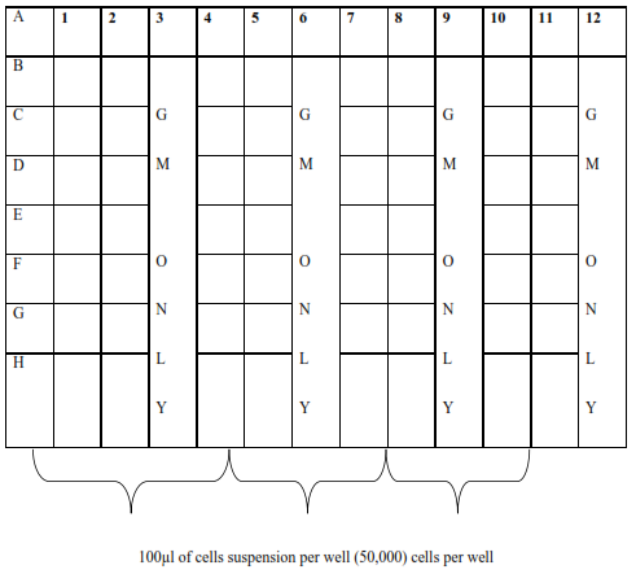

Figure 2. 96 well-plate cell suspension setting

Housing and feeding conditions

The mice were housed in a group of three in polycarbonate cages of 35 cm in length × 25 cm width × 18cm height fitted with wire mesh tops. They were kept in cages for ten days before dosing to allow adjusting to the laboratory conditions. Cages were cleaned once a week, and the bedding husk was replaced two times a week. The Temperature of the study room was maintained at 23 - 27˚C, with a 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 plenty, and water was also provided with automatic water dispensers ad libitum during acclimatization and the experiment period.

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, the mice were identified by marking the tail with a permanent marker for easy identification. The identified mice were weighed using a weighing balance (mettle PM 4600), and their weight was recorded and released back to their cages. The extracts were prepared in double distilled water to the desired concentration. Before dosing, animals fasted for four hours and weighed before oral administration of a single dose 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 2,000 mg/kg body of the test substance was administered to each mouse with an oral gavage needle. Physiological saline was administered to the control group. Individual observations of toxicity wellness parameters such as change of fur, lacrimation, mucous membrane inflammation, excessive salivation, drowsiness, convulsions, tremors, body weight, morbidity, and mortality were recorded. The weight was recorded on days

0, 7, and 14. The data obtained were presented in tables, and the LD_{50} values were determined statistically (OECD /OCDE 2001).

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 the change of weights pre and post-treatment. Selectivity indices were determined as the ratio of CC_{50} to IC_{50} (CC_{50}/IC_{50}). A *p-value* less than 0.05 was considered 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 (OECD/OCD 2001). Briefly, the sequential design was used to determine the needed doses concerning the body weight, as shown in Table 1.

Disposal of cells and experimental animals and Ethical considerations

All the used cell lines were disposed of following the protocols that the University of Nairobi Ethical Committees set. Briefly, the liquid waste containing cells was autoclaved and disposed of in the sanitary drain, followed by water. Animal remains shall be tagged with prominent poison tags in outer bags or containers. After confirming the death, the mice carcasses were placed in transparent and sealable polyethylene bags. Finally, they were disposed of by incineration per the University of Nairobi Faculty of Veterinary Sciences disposal protocol (AVMA 2013).

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 was granted by the biosafety, animal care, and use committee (BACUC) at the 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 laboratory animal use and care.

RESULTS AND DISCUSSION

Phytochemical composition of different extracts of *Dichrocephala integrifolia* plant

The plant was screened for secondary metabolites using standard procedures and contained 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 flowers' root and methanolic extract. The result is shown in Tables 2 and 3.

***In vitro* cytotoxicity of extract of *Dichrocephala integrifolia* against Vero cell lines**

All extracts were not toxic to Vero cells except the flower's methanolic extract, which showed slight toxicity with a CC_{50} value of 71.31 ± 2.65. According to the national cancer Institute, CC_{50} values greater than 100µg/mL are deemed safe. The result is shown in Table 4.

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

Pre-treatment of cells results 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 100 µg/mL (Table 5).

In vitro virucidal activity of extract of *Dichrocephala integrifolia* against Herpes Simplex Virus

The methanolic extract of the flower, aqueous extract of the root, and the methanolic extract of leaves showed direct inactivation of the virus when the extracts were incubated with the virus before incubating with the cells at the cells IC_{50} values of 45.27±2.41, 0.333±1.23 and 30.53±4.51 respectively. In addition, the plant extracts selectively inhibited the growth of the virus. That is shown by the selectivity indices obtained in Table 6.

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

Tables 7 and 8 show the control and treated animals' body weights and mice's general behavior at both 300 and 2,000 mg/kg body weight. The results showed no mortality or gross changes in animal behavior and appearance at 300 and 2,000 mg/kg. All animals depicted normal increments in weight and no significant differences between the control and test groups, which indicates that the extract does not affect the growth of the mice. No mortality was observed at 300 mg and 2000 mg/kg body weight doses of different extracts from different parts of the plant, indicating an LD_{50} of >2000 mg/kg body weight.

Table 1. 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 /OCD (2001)

Table 2. Phytochemical composition of methanolic extracts of *Dichrocephala integrifolia* plant

Phytochemical	*D. integrifolia*			
	Leaves	Root	Flowers	Leaves
Saponins	+	+	+	+
Alkaloids	+	+	+	+
Phenols	+	+	+	+
Flavonoids	+	-	+	+
Glycosides	+	+	+	+
Terpenoids	+	+	+	+
Tannins	+	+	+	+

Note: +: Present, -: Absent

Table 3. Phytochemical composition of aqueous extracts of *Dichrocephala integrifolia* plant

Phytochemical	*D. integrifolia*			
	Leaves	Stem	Flowers	Root
Saponins	+	+	+	+
Alkaloids	+	+	+	+
Phenols	+	+	+	+
Flavonoids	+	+	+	+
Glycosides	+	+	+	-
Terpenoids	+	+	+	+
Tannins	+	+	+	+

Note: +: Present, -: Absent

Table 4. Cytotoxic effect of *Dichrocephala 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

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

Table 5. The IC_{50} values of *Dichrocephala integrifolia* extract 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

Note: IC_{50}: Inhibitory Concentration 50 (IC_{50})

Table 6. 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} (µg/mL) 50	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

Note: 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

Table 7. Changes in body weight of Swiss albino mice following administration of crude extracts of *Dichrocephala integrifolia* at 300 mg/kg

Study extract	Day 0	Day 7	Day 14	*P* value
Leave 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

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

Table 8. Changes in body weight of swiss albino mice following administration of crude extracts of *Dichrocephala 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

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

Discussion

Phytochemical compounds from plants have been used to treat different diseases, including diseases of viral origin (Abad et al. 1999; Li et al. 2009; Kohn et al. 2015). 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 (Pengsuparp et al. 1995; Lin et al. 1999), saponins (Sindambiwe et al. 1998), terpenes (Bourne et al. 1999), tannins (Ferrea et al. 1993). In this study, the 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 for root methanol and root aqueous that lacked flavonoids and glycoside respectively. This result is consistent with previous studies (Mohammed and Teshale 2012). Therefore, 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, which can be further investigated by isolating the bioactive compounds.

The study reports on the cytotoxicity of different extracts from different parts of the plant on normal monkey kidney cells (Vero). Methanol extract from the flower exhibited moderate toxicity, while other parts of the plant were safe. Decoctions from plants have traditionally been widely used in treating various diseases without scientific justification for their safety (Okumu et al. 2016). Most reports on the toxicity of plants based crude drugs are often associated with liver toxicity (Agbor et al. 2010). The broad traditional use of *D. integrifolia* warrants evaluation for its toxicity properties considering public health protection to the plant extracts, which could cause undesirable consumer effects. Cytotoxicity activities of alkaloids and flavonoids have also been reported (Özçelik et al. 2011); therefore, flower cytotoxicity effects might be due to phytochemicals such as flavonoids present in the extracts. Further study must be done to identify the exerted 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 the antiviral and general antimicrobial activity of plants in the Asteraceae family, studies on *D. integrifolia*'s antiviral activity are lacking. When the virus was exposed to the cell before treatment with the extracts, different parts of the plant exhibited moderate inhibitory effects could be due to the blocking of cellular receptor cellular receptors preventing virus entry in the cell. Plant extracts with antiviral activity may inhibit the virus through several mechanisms, such as blocking specific proteins for viral entry or working directly on the

virus itself (Yang et al. 2013). The methanol of the flower and aqueous extract of leaves exhibited a moderate inhibitory effect when incubated with infected cells; this could be due to its interference at some stage of virus replication inside the cells. Aqueous extracts of root and methanol extracts of leaves that exhibited the highest antiviral activity were incubated with the virus before incubation with the cells, which is for the first time the antiviral activity of this plant is reported.

All animals experienced a normal increment in weight, and no drastic differences between the 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 evaluating herbal remedies' safety (Rang et al. 2001). For example, the administration of *D. integrifolia* extract to ethanol-administered mice brought transaminases toward normal values after a significant increase in alcohol dosage. In addition, 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. 2017). Additionally, Franco et al. (2015) found *Chresta martii* (DC.) H.Rob., a plant in the Asteraceae family, to have no acute toxicity and no mortalities recorded. Still, debates rumors on the rationale of extrapolating animal model results to humans. However, previous studies have proved that the mice model is a better predictor for human lethal dosage than rats (Walum et al. 1995).

The results show that different roots and flower extracts of *D. integrifolia* could be good candidates in searching for new anti-HSV leads. 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 for using *D. integrifolia* in HSV infections. Given the high cost, unbearable side effects, and unavailability of the current antiviral drugs targeted towards HSV to most people in rural areas, the current study provides hope that new cheap antiviral drugs could be obtained from this plant.

REFERENCES

Abad MJ, Bermejo P, Gonzales E, Iglesias I, Iruruzun A, Carrasco L. 1999. Antiviral activity of Bolivian plant extracts. Gen Pharmacol 32: 499-503. DOI: 10.1016/S0306-3623(98)00214-6.

Agbor GA, Vinson JA, Oben JE, Ngogang JY. 2010. Antioxidant effect of herbs and spices on copper-mediated oxidation of lower and very low density lipoprotein. Chinese J Nat Med 8 (2): 114-120. DOI: 10.3724/SP.J.1009.2010.00114.

Ajaiyeobu EO. 2002. Phytochemical and antibacterial activity of *Parkia biglobosa* and *Parkia bicolor* leaf extracts. Afr J Biomed Res 5: 125-129.

Alem S, Tolo FM, Adipo N, Mwitari PG, Japheth KN, Makokha AO. 2016. In vitro Anti-Herpes Simplex Type-1 Virus evaluation of extracts from Kenya Grown Pyrethrum (*Chrysanthemum cinerariaefolium*). Eur J Med Plants 17 (2): 1-8. DOI: 10.9734/EJMP/2016/28967.

American Type Culture Collection. 2011. MTT Cell Proliferation Assay ATCC® 30-1010K. Available at: https://www.atcc.org/~/media/DA5285A1F52C414E864C966FD78C9A79.ashx

Ashe CG. 2016. Safety Data Sheet: Dimethyl Sulfoxide (DMSO). Available at: https://www.dmso.com/PDF/MSDS.pdf

AVMA. 2013. Guidelines for the Euthanasia of Animals 2013: Edition.

Awoyinka OA, Biologun IO, Ogunnowa AA. 2007. Phytochemical screening and in vitro bio activity of *Cnidoscolus asconiti* folus (Europhiaceae). J Med Plant Res 3: 63-65.

Balick MJ, Cox PA. 1995. Ethnobotanical research and traditional healthcare in developing countries. In: Bodeker G, Vantomme P (Eds). Medical Plants for Forest Conservation and Health Care. Non-wood forest product No. 11. FAO, Rome.

Balick MJ, Cox PAR. 1995. Plants, People, and Culture. The Science of Ethnobotany, Scientific American Library, New York, USA.

Bourne KZ, Bourne N, Reising SF, Stanberry LR. 1999. Plant products as topical microbicide candidates: assessment of in vitro and in vivo activity against herpes simplex virus type 2. Antiviral Res 42: 219-226. DOI: 10.1016/S0166-3542(99)00020-0.

Brain KR, Turner TD. 1995. Practical Evaluation of Phytopharmaceuticals, 1st Ed. Wright Scientechnica, Bristol.

Bussmann RW, Sharon D, Lopez A. 2007. Blending traditional and western medicine: Medicinal plant use among patients at Clinica Anticona in El Porvenir, Peru. Ethnobot Res Appl 5: 185-199. DOI: 10.17348/era.5.0.185-199.

Chirchir J, Mungai G, Kariuki P. 2006. Indigenous Knowledge and Conservation of Natural Resources: Resource Medicinal Plants Utilisation in Eastern Africa; Proceedings of National Museums of Kenya First Scientific Conference, 15-17 November 2006.

Choudhry RP, Acharyu R, Nair AGC, Reddy AVR, Garg AN. 2004. Availability of essential trace elements in medicinal plants used for diabetes mellitus and their possible correlations. J Radioanal Nucl Chem 120 (2): 85-93. DOI: 10.1007/s10967-007-0414-8.

Cunningham AB. 1993. African Medicinal Plants. Setting Priorities at the Interface between Conservation and Primary Health Care. UNESCO Paris.

David B, Wolfender JL, Dias DA. 2015. Pharmaceutical industry and natural products: Historical status and new trends. Phytochem Rev 14 (2): 299-315. DOI: 10.1007/s11101-014-9367-z.

Dery BB, Ofsynia R, Ngatigwa C. 1999. Indigenous Knowledge of Medicinal Trees and Setting Priorities for their Domestication in Shinyanga Region: International Center for Research in Agroforestry, Nairobi, Kenya.

Evans WC. 2009. Trease and Evans' Pharmacognosy. (15th Edition), WB Saunders Company Ltd., London.

Fair RJ, Tor Y. 2014. Antibiotics and bacterial resistance in the 21st Century. Perspect Med Chem 6: 25-64. DOI: 10.4137/PMC.S14459.

Fakung C, Ndikum V, Tabi O, Jiofack R, Ngameni B, Gueove N, Kamsu-Kom. 2011. Traditional medicine; the past, present and future research and development prospects and integration in National Health System of Cameroon. Afr J Tradit Complement Altern Med 8 (3): 284-295. DOI: 10.4314/ajtcam.v8i3.65276.

Farnsworth NR, Kaas CJ. 1981. An approach utilizing information from traditional medicine to identify tumor inhibiting plants. J Ethnopharmacol 3: 85-100. DOI: 10.1016/0378-8741(81)90014-3.

Ferrea G, Canessa A, Sampietro F, Cruciani M, Romussi G, Bassetti D. 1993. In vitro activity of a Combre-tum micranthum extract against herpes simplex virus types1 and 2. Antiviral Res 21: 317-325. DOI: 10.1016/0166-3542(93)90010-G.

Florence NT, Huguette STS, Hubert DJ, Raceline GK, Desire DDP, Pierre K, Theophile D. 2017. Aqueous extract of *Peperomia pellucida* (L.) HBK accelerates fracture healing in Wistar rats. BMC Complement Altern Med 17 (1): 188. DOI: 10.1186/s12906-017-1686-3.

Franco ES, Melo ME, Militao GC, Rocha RE, Silva LT, Jatoba BJ, Silva PB, Santana AL, Silva AA, Silva TG, Nascimento MS, Maia MB. 2015. Evaluation of the Acute toxicity, cytotoxicity, and genotoxicity of *Chresta martii* (Asteraceae). J Toxicol Environ Health A 78 (17): 1083-1093. DOI: 10.1080/15287394.2014.1004007.

Kapoor LD, Singh A, Kapoor SL, Shrivastava SN. 1969. Survey of Indian medicinal plants for saponin, alkaloid, and flavonoids. Lloyd 32: 297-302.

Kinghorn AD, Pan L, Fletcher JN, Chai H. 2011. The relevance of higher plants in lead compound discovery program. J Nat Prod 74: 496-511. DOI: 10.1021/np200391c.

Kingstone DG. 2011. Modern natural products drug discovery and its relevance to biodiversity conservation. J Nat Prod 74: 496-511. DOI: 10.1021/np100550t.

Kohn LK, Foglio MA, Rodrigues RA, Sousa IM de O, Martini MC, Padilla MA, de Lima Neto DF, Arns CW. 2015. In vitro antiviral activities of extracts of plants of the Brazilian Cerrado against the Asian Metapneumovirus (aMPV). Braz J Poult Sci 17 (3): 275-280. DOI: 10.1590/1516-635X1703275-280.

Lamorde M, Tabuti JRS, Obua C, Kukunda-Byobona C, Lanyero H, Byakika-Kibwika P, Bbosa GS, Lubega A, Ogwal-Okeng J, Ryan M, Waako PJ, Merry C. 2010. Medicinal plants used by traditional medicine practitioners for the treatment of HIV/AIDS and related conditions in Uganda. J Ethnopharmacol 130 (1): 43-53. DOI: 10.1016/j.jep.2010.04.004.

Li Q, Maddox C, Rasmussen L, Hobrath JU, White LE. 2009. Assay development and highthroughput antiviral drug screening against Bluetongue virus. Antiviral Res 83: 267-273. DOI: 10.1016/j.antiviral.2009.06.004.

Lin YM, Flavin MT, Schure R, Chen FC, Sidwell R, Barnard DL, Huffman JH, Kern ER. 1999. Antiviral activities of biflavonoids. Planta Med 65: 120-125. DOI: 10.1055/s-1999-13971.

Martin SF. 1987. The amaryllidaceae alkaloids. Alkaloids 30: 251-253. DOI: 10.1016/S0099-9598(08)60208-4.

McMahon JB, Currens MJ, Gulakowski RJ, Buckheit RWJ, Lackman-Smith C, Hallock YF, Boyd MR, Michellamine B. 1995. A novel plant alkaloid inhibits human immune deficiency virus-induced cell killing by at least two distinct mechanisms. Antimicrob Age Chemother 39: 484-488. DOI: 10.1128/AAC.39.2.484.

Mohammed T, Teshale C. 2012. Preliminary phytochemical screening and evaluation of antibacterial activity of *Dichrocepala integrifolia* (L.f) O. kuntze. J Intercult Ethnopharmacol 1 (1): 30-34. DOI: 10.5455/jice.20120401054654.

Morfin F, Thouvenot D. 2003. Herpes simplex virus resistance to antiviral drugs. J Clin Virol 26: 29-37. DOI: 10.1016/S1386-6532(02)00263-9.

Mworia AG. 2000. Status, Value, and Management of Indigenous Plants of Upper Imenti Forest Reserve, Meru District, Kenya. [Msc Thesis]. Kenyatta University. [Kenya]

Newman DJ, Cragg GM. 2007. Natural products as sources of new drugs over the last 25 years. J Nat Prod 70: 461-477. DOI: 10.1021/np068054v.

Okumu MO, Mbaria JM, Kanja LW, Gakuya DW, Kiama SG, Ochola FO. 2016. Phytochemical profile and antioxidant capacity of leaves of *Moringa oleifera (Lam)* extracted using different solvent systems. J Pharmacogn Phytochem 5 (4): 302-308.

Organization of Economic Cooperation and Development (OECD /OCD). 2001. Guidelines for acute toxicity of chemicals. No. 420 (Adopted: 17 December 2001).

Özçelik B, Kartal M, Orhan I. 2011. Cytotoxicity, antiviral and antimicrobial activities of alkaloids, flavonoids, and phenolic acids. Pharmac Biol 49 (4): 396-402. DOI: 10.3109/13880209.2010.519390.

Parekh J, Nair R, Chanda S. 2005. Preliminary screening of some folklore plants from Western India for potential antimicrobial activity. Ind J Pharmacol 37: 408-409. DOI: 10.4103/0253-7613.19085.

Pengsuparp T, Cai L, Constant H, Fong HH, Lin LZ, Kinghorn AD, Pezzuto JM, Cordell GA, Ingolfsdottir K, Wagner H. 1995. Mechanistic evaluation of new plant- derived compounds that inhibit HIV-1 reverse transcriptase. J Nat Prod 58: 1024-1031. DOI: 10.1021/np50121a006.

Rang HP, Dale M, Ritter J. 2001. Pharmacology. 4th ed. Churchill Livingstone, New York, NY, USA.

Salehi-Surmaghi MH, Aynehchi Y, Amin GH, Mahmoodi Z. 1992. Survey of Iranian Plants for saponins, alkaloids, flavonoids, and Tannins. DARU J Pharmac Sci 2 (2-3): 1-11.

Segelman AB, Farnsworth NR, Quimby MD. 1969. False negative saponin results induced by the presence of tannins. Lloydia 32: 52-58.

Sindambiwe JB, Calomme M, Geerts S, Pieters L, Vlietinck AJ, Vanden Berghe DA. 1998. Evaluation ofbiological activities of triterpenoid saponins from Maesalanceolata. J Nat Prod 61: 585-590. DOI: 10.1021/np9705165.

Tolo FM, Rukunga GM, Muli WF, Ochora J, Yoshito E, Muthaura NC, Kimani CW, Mungai GM, Kofi-Tsekpo MW. 2007. In the vitro antiviral activity of aqueous extracts of Kenyan *Carissa edulis*, *Prunus Africana* and *Melia azedarach* against human cytomegalovirus. Afr J Health Sci 14: 143-148. DOI: 10.4314/ajhs.v14i3.30861.

Walum E, Nilsson M, Clemedson C, Ekwall B. 1995. The MEIC program and its implications for the prediction of acute human systemic toxicity. In: Goldberg AM, van Zutphen LFM (Eds). Alternative Methods in Toxicology and the Life Sciences Mary Ann Liebert, New York, NY, USA.

Yang J, Li M, Shen X, Liu S. 2013. Influenza a virus entry inhibitors targeting the hemagglutinin. Viruses 5 (1): 352-373. DOI: 10.3390/v5010352.