



## ***In vitro* Anti-Herpes simplex Type-1 Virus Evaluation of Extracts from Kenya Grown Pyrethrum (*Chrysanthemum cinerariaefolium*)**

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### **Authors' contributions**

*This work was carried out in collaboration between all authors. Authors SA and FMT designed the study, approved protocol, performed the laboratory work, the statistical analysis and wrote the first draft of the manuscript. Authors NA, PGM, NKJ and AOM read, corrected the manuscript and approved its submission. All authors read and approved the final manuscript.*

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### **ABSTRACT**

**Objective:** To evaluate *in vitro* anti-Herpes simplex type 1 activity of methanol and aqueous crude extracts of pyrethrum (*Chrysanthemum cinerariaefolium*) plant grown in Kenya.

**Methods:** Cytotoxic effect of methanol and aqueous extracts was determined on vero cells (African green monkey kidney cells) by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-DiphenylTetrazolium Bromide) reduction colorimetric assay. Antiviral effect of pyrethrum extracts was evaluated; before (post-treatment infection) virus infection to the cells and after (pre-treatment infection) virus infection to cells.

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**Results:** Methanol extract exhibited higher cytotoxicity of  $CC_{50} = 42.23 \pm 0.320 \mu\text{g/ml}$  compared to aqueous extract of  $CC_{50} = 249 \pm 8.4 \mu\text{g/ml}$ . Methanol extract exhibited a higher anti HSV-1 potency of  $IC_{50} = 1.69 \mu\text{g/ml}$  (TI = 24.99) than the aqueous extract of  $IC_{50} = 38.13 \mu\text{g/ml}$  (TI = 6.53) in the post-treatment infection evaluations. This might be due to its effect on cellular receptors preventing virus entry, while the aqueous extract exhibited higher virus inhibitory potency in the pre-treatment infection, of  $IC_{50} = 23.21 \mu\text{g/ml}$  (TI=10.73) compared to the methanol extract of  $IC_{50} = 11.18 \mu\text{g/ml}$  (TI =3.78), this effect could be due to its effect on some stage during HSV virus replication process.

**Conclusions:** Crude methanol and aqueous extracts from Kenyan grown pyrethrum exhibited inhibition potency against HSV-1 *in vitro* on Vero cells.

**Keywords:** *Chrysanthemum cinerariaefolium*; anti HSV-1; cytotoxicity; *in vitro*; vero cells.

## 1. INTRODUCTION

*Herpes simplex* Viruses infections are common types of opportunistic infections [1]. They are type 1 and 2. *Herpes simplex* type one virus (HSV-1) could causes severe diseases to immunocompromised individuals [2]. This virus is acquired during early child hood [3]. If symptomatic, causes oral and ocular lesions and is the leading cause of viral caused corneal blindness and viral encephalitis [4,5]. Genital infections [6] are also reported. Whereas, *Herpes simplex* type two virus (HSV-2) is known to cause genital lesions and other serious diseases such as blindness, meningitis and encephalitis [7]. There is no cure for these viral infections. Drugs such as Acyclovir (ACV) [8] and Penciclovir [9] with their pro-drugs Valaciclovir [10] and Famciclovir [11] respectively, have been used for treatment and prophylaxis of these viral infections.

Acyclovir resistance by HSV strains [12] is a major public concern, a condition mainly observed in immunocompromised individuals due to long-term treatment or multiple treatments [13]. Therefore, there is a need to identify alternative therapies.

Traditionally plant extracts are used as a source of medicines [14]. Scientifically it is proven, that extracts derived from certain plants have anti-viral effects [15,16]. Pyrethrum plant (*C. cinerariaefolium*) belongs to the family of perennial plants *Asteraceae* and has insecticidal property. The active insecticidal components are known as pyrethrins [17], which is one of the main ingredients in many pesticide products [18].

Previous unpublished *in vivo* study of the Kenyan grown pyrethrum indicated a promising inhibitory effect against HSV-1. *In vitro* study indicated minimal activity of pyrethrins against HSV [19]. Pyrethrins are a fraction of the total extract from the pyrethrum plant. In this study

crude extract (methanol and aqueous extracts) derived from the flower of pyrethrum plant grown in Kenya was evaluated for its potential activity against HSV-1 to identify a new candidate drug.

## 2. MATERIALS AND METHODS

### 2.1 Ethical Consideration

The research was conducted in accordance to the Kenya Medical Research Institute (KEMRI) guidelines; the study was carried out upon receiving approval from KEMRI Scientific and Ethical Review Unit (SERU) KEMRI/SERU/CTMDR/005/3067.

### 2.2 Plant Material Collection and Preparation

Flower heads of *C. cinerariaefolium* were collected from Kiambogo, Nakuru County, Kenya (S 00°41' 56.3", E 036°25' 19.6") in February, 2015. It was air-dried at room temperature in a dark room and kept at the Center for Traditional Medicine and Drug Research (CTMDR) (KEMRI) storage facility with a voucher specimen number Tolo/Mwitari/Keter/001. It was grinded using laboratory mill (Christy & Norris Ltd., Chelmsford, England) and stored until extraction was performed.

### 2.3 Extraction and Preparation for *in vitro* Assay

#### 2.3.1 Aqueous extraction

Extraction process was carried out based on slight modification to previously published methods [20]. Powdered plant material was soaked in distilled water and placed in a water bath at 60°C. After 1 h it was decanted into a clean dry conical flask. Filtration was done through 2 layers of sterile gauze. The filtered extract was freeze dried using a Freeze Dryer (Edwards freeze dryer Modulyo). Dry extract was

weighed, labeled and stored at 4°C until use. During setup of the *in vitro* assay the plant material was dissolved in phosphate buffer solution (PBS). Sterilization was done using 0.22 µm Millex<sup>®</sup> syringe driven filter unit.

### **2.3.2 Methanol extraction**

Extraction process was carried out based on a slight modification to previously published methods [21]. Powdered plant material was soaked in methanol at room temperature for 3 days in a dark room. After 3 days it was filtered using sterile cotton gauze and concentrated using a rotary evaporator (Büchi Rota vapor R-114) at 60°C. Finally, the extract was weighed and stored at 4°C until use. During setup of the *in vitro* assay the plant material was dissolved in 0.1% dimethyl sulphoxide (DMSO) and topped with PBS to the desired volume. Sterilization was done using 0.22 µm Millex<sup>®</sup> syringe driven filter unit.

### **2.4 Herpes simplex Type-1 Virus Preparation and Titration**

Virus stock was obtained from the Center for Traditional Medicine and Drug Research (CTMDR) laboratory. Propagation was done on vero cells [22]. Briefly,  $2 \times 10^6$  cells were seeded in T 75 cell culture flask overnight and infected with the virus prepared in MEM (maintenance media with 2% serum) for 2 h to allow adsorption. After 2 h the media was aspirated and replaced with fresh media. Cells were observed periodically under microscope for a change in cellular morphology (Cytopathic effect) due to HSV-1 virus infection. After 100 percent cytopathic effect (CPE) was observed, the infected cells were frozen and thawed three times to lyse the cells and free the virus. The supernatant containing virus was then harvested and divided into small aliquots of 1 ml cryovials to be stored at -80°C until further use. Virus titer was determined by end point dilution assay on Vero cells. Briefly, Cell suspension in MEM were seeded at a density of  $1 \times 10^5$  cells/well in 96 microplates and incubated at 37°C under 5 percent CO<sub>2</sub> incubator for 24 h. Virus dilution of 1:10 was prepared in PBS from 1 ml virus stock and followed by a series 10 fold dilutions. Then the culture media was replaced with 100 µl PBS containing the virus dilutions. The first column contained 1:10 virus dilution. A positive cell control was included. After allowing the virus to adsorb for 2 h, it was replaced with 100 µl maintenance media. Cytopathic effect was

monitored under light microscope for 2 days and recorded. Fifty percent Tissue Culture Infectious Dose (TCID<sub>50</sub>/ml) was calculated by Spearman and karber algorithm method [23].

### **2.5 Cytotoxicity**

This assay was carried out with a slight modification to previously published methods [24]. The cytotoxic activity of both aqueous and methanol extracts of pyrethrum were determined in vero cells by the MTT (colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) reduction calorimetric assay. Briefly, vero cell were grown in 96-well plates at concentration of  $2 \times 10^4$  cells/well (rows 1, 2, 4, 5, 7, 8, 10, 11 filled with media containing cells, while rows 3, 6, 9 where used as controls filled with only the media and no cells) in a 5 % CO<sub>2</sub> incubator at 37°C for 24 h. After 24 h cell monolayers were treated with aqueous and methanol extracts at dilutions, briefly, row H of the plate contained the highest concentration of 1000 µg/ml and all the rows upward contained a serially 3 fold dilute concentration up to row B, while cell control contained only medium. The cells were incubated for 48 h at 37°C in a 5 percent CO<sub>2</sub> incubator. After 48 h days the culture medium was removed and 10 µl of MTT solution (5 mg/ml dissolved in PBS) was added to the cells in each well and incubated for 4 h at 37°C until crystals of formazan were observed. After removal of MTT, 100 µl DMSO was added to dissolve the formazan crystals. Optical densities (OD) were measured in spectrophotometer at 562 nm with a reference filter of 690 nm. The percentage cell viability was then calculated as  $[(A - B / C - B) \times 100]$ , where A, B and C indicate the mean of 2 optical density readings of treated cells, blank and control respectively. The experiment was done in a triplicate for each extract. Fifty percent cytotoxic concentration (CC<sub>50</sub>) is determined from the graph of percent cell viability against concentration. It is the extract concentration (µg/ml) that can reduce 50 percent of cell viability to compare with cell control.

### **2.6 In vitro Bioactivity of Pyrethrum Extracts against Herpes simplex Type 1 Virus**

The methods described here were carried out with a slight modification to the methods described previously [25]. The extracts could act by; 1) blocking receptors on the cell surface and prevent virus cell bonding that lead to penetration

in to the cell, 2) inhibit some steps of virus replication inside the cell. To attain this, the extract is incubated with the cells, before (post-treatment infection), and after (pre-treatment infection) virus infection of the cells. Briefly, Vero cells seeded at a density of  $1 \times 10^5$  in 96-well microplate and incubated overnight in humidified CO<sub>2</sub> incubator at 37°C. From each extract a maximum nontoxic concentration (MNC) derived from cytotoxicity experiments results was chosen as starting concentration and followed by a subsequent serial dilution. For both assays cell control (only cells) and virus control (virus infected cells) were added. The procedures are as follow.

### 2.6.1 Treatment before virus infection (Post-treatment infection)

Fifty µl of serially dilute extract was incubated with vero cells for 24 h at 37 °C in 5 percent CO<sub>2</sub> humidified incubator. After washing with PBS, the cells were incubated with 50 µl of 10<sup>6</sup> TCID<sub>50</sub> virus suspensions in 2 percent serum of MEM for 1 h. Then the cells were washed with PBS and incubated with fresh MEM.

### 2.6.2 Treatment after virus infection (Pre-treatment infection)

Virus suspension of 10<sup>6</sup> TCID<sub>50</sub> in MEM of 2 percent serum was incubated with cells for 1 h at 37°C in 5 percent CO<sub>2</sub> humidified incubator to allow adsorption. Then the cells were washed PBS and incubated with the serially diluted extracts for 24 h at 37°C in 5 percent CO<sub>2</sub> humidified incubator. The extract was removed and cells were washed with PBS. Fresh EMEM was then added.

Cytopathic effect was observed daily; after 72 h the cells ability to reduce MTT was measured

according the protocol described in the cytotoxicity assay. The percentage protection of the extract to the cells was calculated as  $[(A-B)/(C-B) \times 100]$ , where A, B and C 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 percent inhibitory concentration (IC<sub>50</sub>) was defined as the extract concentration that protects 50 percent of treated infected cells to compare with cell control. Therapeutic index (TI) of the extract was determined by calculating the ratio CC<sub>50</sub> to IC<sub>50</sub>.

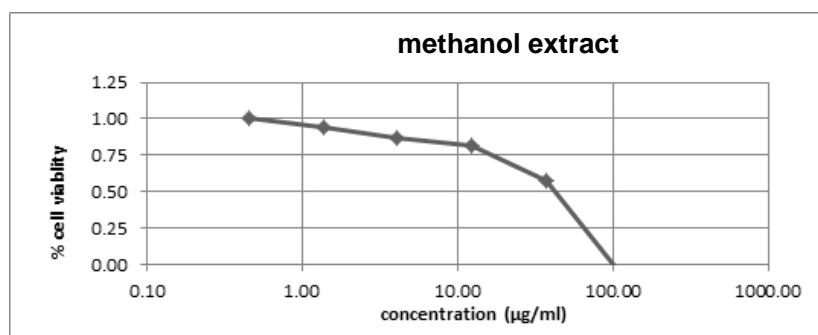
## 3. RESULTS

### 3.1 Cytotoxicity

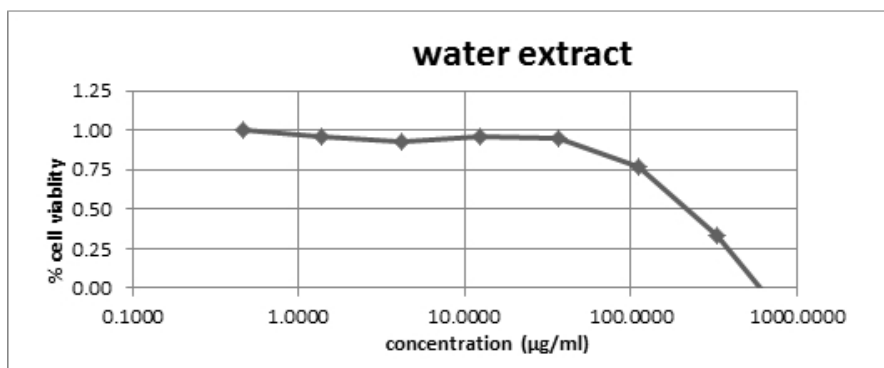
The methanol extracts of *C. cinerariaefolium* exhibited a higher toxicity when compared to the aqueous extract. The 50 percent cytotoxic concentrations (CC<sub>50</sub>) in µg/ml are  $42.23 \pm 0.32$  and  $249 \pm 8.4$  for methanol extract and aqueous extracts, respectively. It was obtained from the mean of 3 separate experiments Figs. 1 and 2.

### 3.2 In vitro Bioactivity of Pyrethrum Extracts

Anti HSV-1 activity of both methanol and aqueous extracts were evaluated using 2 different approaches. The therapeutic index of the extracts is the ratio of CC<sub>50</sub> to IC<sub>50</sub>. Methanol extract exhibited a higher inhibitory effect with IC<sub>50</sub> = 1.69 µg/ml (TI= 24.99) when incubated with the cells prior to infection, while the aqueous extract exhibited a higher inhibitory effect with IC<sub>50</sub> = 23.21 µg/ml (TI= 10.73) than the methanol extract IC<sub>50</sub> = 11.18 µg/ml (TI= 3.78) when incubated after the virus infection of the cells Table 1.



**Fig. 1. Interaction line plots of % cell viability against concentration (µg/ml) of methanol extract from *C. cinerariaefolium*. It indicates decline in cell viability as concentration of the extract increase**



**Fig. 2. Interaction line plots of % cell viability against concentration (µg/ml) of the aqueous extract from *C. cinerariaefolium*. It indicates decline in cell viability as concentration of the extract increase**

**Table 1. *In vitro* anti-virus evaluation of pyrethrum extracts**

|          | Pretreatment infection        |                               |                 | Posttreatment infection       |                 |
|----------|-------------------------------|-------------------------------|-----------------|-------------------------------|-----------------|
|          | CC <sub>50</sub> <sup>A</sup> | IC <sub>50</sub> <sup>B</sup> | TI <sup>C</sup> | IC <sub>50</sub> <sup>B</sup> | TI <sup>C</sup> |
| Methanol | 42.23 ± 0.32                  | 11.18 ± 3.81                  | 3.78            | 1.69 ± 0.219                  | 24.99           |
| Aqueous  | 249 ± 8.4                     | 23.21 ± 2.93                  | 10.73           | 38.13 ± 3.77                  | 6.53            |

A: Concentration of the extract that killed 50 % of the cells. B: Concentration of extract in µg/ml that Inhibits virus activity by 50%. C: Therapeutic index = CC<sub>50</sub>/IC<sub>50</sub>. Both CC<sub>50</sub> and IC<sub>50</sub> are the mean values of 3 experiments

#### 4. DISCUSSION

*Herpes simplex* infections (HSV) are among the main causes of morbidity and mortality in immunocompromised individuals [26]. These viruses also increase the chance of acquiring HIV [27,28]. *Herpes simplex* type one virus which is among the common type of infections [29] is usually acquired during early childhood [3]. Drug resistant strains of HSV-1 are a common phenomenon in immunocompromised individuals [12]. Plants have been found to contain important chemicals that can be used as antiviral agents [16,30]. Compounds of plant origin that contain anti HSV-1 activity as mentioned in other studies are alkaloids [31], flavonoids [32], saponins [33], terpenes [34] and tannins [35]. A previous unpublished qualitative study on crude extracts derived from the Kenyan pyrethrum indicated the presence of alkaloids, flavonoids, phenols, saponins, tannins and terpenoids.

Pyrethrins which are the active insecticidal components of pyrethrum [17] exhibited minimal *in vitro* anti HSV-1 effects [19]. But, in this study the crude extract is tested for its effect on HSV-1. Also, previous unpublished results of *in vivo* evaluations of the Kenyan pyrethrum exhibited a significant antiviral effect. Methanol and aqueous extracts were evaluated for their toxic effect on

cells and their inhibitory effect against HSV-1 *in vitro*. The methanol extract exhibited a higher toxicity (CC<sub>50</sub> = 42.23 ± 0.320 µg/ml) to cells than the aqueous extract (CC<sub>50</sub> = 249 ± 8.4 µg/ml). The toxicity of methanolic extract could be attributed to the presence of alkaloid which is toxic [36]. As indicated in both Figs. 1 and 2 there is a decrease in % cell viability as concentration increases.

The main target for anti-viral agents is the interruption of virus infection process which are, attachment to permissive cells [37], fusion of virus envelop to plasma membrane of cells [38], replication of viral proteins and genetic materials [39], envelopment [40] and viral escape mechanisms. In this study both extracts were evaluated for their effect in the pre-infection and post-infection to protect vero cells. Methanol extract exhibited higher inhibitory effect IC<sub>50</sub> = 1.69 µg/ml (TI = 24.99) than the aqueous extract IC<sub>50</sub> = 38.13 µg/ml (TI = 6.53) in the pre- infection treatment, this might possibly be by blocking the cell receptors preventing virus entry. This could be attributed to the presence of tannins that reportedly exhibit antiviral activity through inhibition of viral adsorption to cells [41]. While the aqueous extract exhibited a higher inhibitory effect of IC<sub>50</sub> = 23.21 µg/ml (TI = 10.73) than the methanol extract of IC<sub>50</sub> = 11.18 µg/ml (TI = 3.78)

when incubated with already infected cells, this could possibly be by interfering at some stage of virus replication process inside the cells. The results might demonstrate that the extracts also contain virucidal activity because there is increase in cell viability of treated cells when compared to infected untreated cells.

## 5. CONCLUSION

In this study methanol and aqueous extracts from *Chrysanthemum cinerariaefolium* exhibited higher inhibitory effect against HSV-1. Further studies are recommended to identify and extract specific bioactive compounds for such effect. It should also be tested on other members of the family *Herpesviridae* and other viruses of clinical significance.

## CONSENT

It is not applicable.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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