Antiviral Activity of Dicrocephala Integrifolia (Kuntze) Against Herpes Simplex Type 1 Virus: An in vitro Study

Abdi Hussein Hadun\textsuperscript{1*}, James Mucunu Mbaria\textsuperscript{1}, Gabriel Oluga Aboge\textsuperscript{1}, Mitchell Otieno Okumu\textsuperscript{1,2} Antony Letoyah Yaiele\textsuperscript{1,3}

\textsuperscript{1}Department of Public Health, Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Nairobi, P. O. Box 29053-00625, Nairobi, KENYA.
\textsuperscript{2}Department of Pharmacy, Jaramogi Oginga Odinga Teaching and Referral Hospital, PO Box 849-40100, Kisumu, KENYA.
\textsuperscript{3}Department of Pharmacy, University Health Services, Masai Mara University, P. O. Box 861-20500, Narok, KENYA.

\textbf{ABSTRACT}

\textbf{Introduction:} At present, acyclovir is commercially available as the drug of choice for managing herpes simplex type I (HSV-1) viral infection. However, the high prevalence of the infection coupled with the emergence of resistant viral strains has limited its effectiveness. Thus, the development of novel antiviral agents is crucial. Practitioners of herbal medicine in Kenya make use of Dicrocephala integrifolia (DI) for the management of several diseases including viral infections. However, information on the efficacy of this plant against HSV-1 viral infection is not available. The aim of the present study was to determine the \textit{in vitro} antiviral activity of crude extracts of DI against HSV-1.

\textbf{Methods:} Leaves, roots, flowers and stems of DI were extracted using water (W) and methanol (ME) and qualitatively screened to identify the phytoconstituents present. Furthermore, the anti-HSV-1 activity of the obtained extracts was evaluated on Vero cell lines using the 3-[4, 5 dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide] assay. The 50\% cytotoxic concentration (CC\textsubscript{50}) and 50\% effective concentration (EC\textsubscript{50}) of each extract was determined using regression analysis. The effects of crude DI extracts on adsorption and post-adsorption stages of the HSV-1 replication cycle was evaluated against acyclovir using a cytopathogenic inhibition assay.

\textbf{Results:} Alkaloids, glycosides, flavonoids, phenols, saponins, tannins and terpenoids were found to be present in the extracts. The CC\textsubscript{50} values of the aqueous DI extracts was in the range 71.31 ± 2.65 to >100 µg/ml compared to >100 µg/ml of acyclovir. The EC\textsubscript{50} values of crude extracts of DI on the pre-adsorptive phase of HSV-1 activity was in the range 54.45±3.45 to >100µg/ml compared to 4.772±781µg/ml of acyclovir whilst the EC\textsubscript{50} value of the crude extracts of DI on the post-adsorptive phase of HSV-1 activity was in the range 45.270±4.31 to >100µg/ml compared to >100µg/ml of acyclovir.

\textbf{Conclusion:} The results suggest that crude extracts of DI may be a reservoir of phytochemicals with potentially good efficacy against HSV-1.

\textbf{Key words:} Dicrocephala integrifolia, \textit{in vitro}, Cytotoxicity, Antiviral activity, HSV-1.

\textbf{INTRODUCTION}

Herpes simplex virus type 1 (HSV-1) is a common pathogen with a wide global prevalence.\textsuperscript{1} The virus has been shown to reside in the trigeminal ganglia of man\textsuperscript{2} making it a lifelong infection.\textsuperscript{3} Although infected individuals seldom exhibit clinical manifestations, oral and genital lesions have been identified as clinical symptoms of HSV-1 infection.\textsuperscript{4,5} The mainstay of treatment of HSV-1 infections is the nucleoside, acyclovir.\textsuperscript{6} However, the high prevalence of HSV-1 infection coupled with the emergence of resistant strains makes the quest to develop novel antiviral agents extremely important.\textsuperscript{6,7} Several authors have reported on plant derived lead molecules efficacious against various stages of viral development.\textsuperscript{8,9} \textit{Dicrocephala integrifolia} is a flowering plant that belongs to the Asteraceae family.\textsuperscript{10} In Kenya, it is used in the treatment of skin infections,\textsuperscript{11} induction of emesis, purgative, antitumor agent as well as in the management of liver, spleen, kidney, bladder, bone and joint diseases.\textsuperscript{12} However, there is no scientific data to support its use as an antiviral agent. The aim of this study was to determine the antiviral activity of crude extracts of DI against herpes simplex virus type 1.

\textbf{MATERIALS AND METHODS}

\textbf{Materials}

Fetal bovine serum (FBS), acyclovir, Modified Eagle's Medium (MEM), penicillin, streptomycin, trypan blue and the 3-(4, 5-dimethylthiazol-2-ly)-2,5-diphenyl tetrazolium bromide (MTT) assay kit were procured from Sigma chemical company (St Louis., MO). Vero cell lines (African green monkey kidney cells) and the clinical isolate of HSV-1 were obtained from the centre for traditional medicine and drug research unit of the Kenya Medical Research Institute (KEMRI-CTMDR). All other chemicals were of analytical grade and high purity.

\textbf{Sample collection}

Whole plant material of DI (Figure 1) was collected from Mabaririri, Nyamira county, Kenya (S 00° 31. 367', E 034° 56. 426') by Dr. Abdi Hussein Hadun in December 2015. Taxonomic identification was done by Mr. Antony Mutiso, a botanist at the University of Nairobi’s School of Biological Science. A voucher number AHH2015/01 was deposited at the University of Nairobi herbarium for future reference.

\textbf{Sample preparation}

Plant material was carefully washed and allowed to dry in a clean, well-ventilated room at the Department of Public Health, Pharmacology and Toxicology, University of Nairobi. Dried plants were then ground to a fine powder using an electric grinder and packed in zip-locked plastic bags awaiting further use.

\textbf{Extraction of different plant parts}

The method of Mwitari \textit{et al.}\textsuperscript{13} was used with minor modifications. Briefly, 350g of dry powder material of different parts of DI were accurately weighed on an analytical balance, poured into separate conical flasks and soaked in 1 liter of distilled water and methanol separately for 48 h with frequent shaking. The resultant mixtures were then filtered. The aqueous filtrates were freeze-dried to a lyophilized powder which was then weighed and transferred into clean sample bottles and stored at -20°C awaiting further use. The organic filtrates were transferred to a rotavapor operating at 40°C to remove residual solvent. The resulting product was then weighed and transferred to clean sample bottles, labelled and stored at -20°C awaiting further use.
Qualitative phytochemical screening
The methods of Harborne and Trease and Evans were used to qualitatively screen the extracts for plant secondary metabolites. A qualitative scale was used to evaluate the phytochemical composition of the different plant extracts. In this scale, +++++ signified a very high concentration of phytochemical, ++ signified a high concentration of phytochemical, + signified a low concentration of phytochemical while − signified the absence of the phytochemical.

Test for alkaloids
Approximately 0.5 g of the extract was dissolved with about 10 ml of 1% hydrochloric acid. The mixture was then boiled for 5 min then filtered. The filtrate was put in two test tubes of 2 ml each. Mayer’s reagent was added to the first test tube. Thereafter, three drops of Dragendorff’s reagent was added to the second test tube. Positive identification of alkaloids was demonstrated by the formation of an orange or orange red precipitate after addition of Dragendorff’s reagent.

Test for cardiac glycosides
One fifty milligrams of each extract was mixed with 1.5 ml of glacial acetic acid containing ferric chloride (FeCl₃) solution. To this solution, 0.5 ml of concentrated sulphuric (H₂SO₄) acid was added to the sides of the test tube. Positive identification of cardiac glycosides was demonstrated by the development of a red-brown colour at the boundary of the layer between FeCl₃ and H₂SO₄ which turned to a blue-green colour after 5 min.

Test for flavonoids
One gram of each of the crude plant extracts were seperately dissolved in 10 ml distilled water and then filtered using Whatman filter paper (No 41). By use of a pipette, 0.5 ml of the filtrate was then added to 6 mg of magnesium turnings, followed by the addition of 0.05 ml concentrated sulphuric acid. A pink or red colour was indicative of the presence of flavonoids.

Test for phenolics
Approximately one gram of ground crude extracts was dissolved in two milliliters of 2% iron (III) chloride. The formation of a red, blue, green or purple colouration signified the presence of phenolics.

Test for saponins
The presence of saponins was determined by dissolving approximately one gram of the plant extracts in boiling water and allowed to stand for 15 min. The mixture was then agitated until a persistent froth was formed. Positive identity of saponin was confirmed by formation of a stable emulsion after addition of olive oil to the froth.

Test for tannins
Approximately 0.8 g of each extract was dissolved in 15 ml distilled water, boiled and later filtered. A few drops of ferric chloride (FeCl₃) were then added to the resultant filtrate. A deposit of a red precipitate confirmed the presence of tannins.

Test for terpenoids
Four milliliters of each of the crude extracts were mixed with 2 ml of chloroform solution and then evaporated to dryness in a water bath. A few drops of concentrated H₂SO₄ was then slowly added on the sides of the test tube. A red brown coloration at the interface of the two liquids was indicative of the presence of terpenoids.

Cell culturing and virus propagation
The cell were propagated in Modified Eagle's Medium (MEM) supplemented with 1% fetal bovine serum (FBS), 100 units/ml of penicillin and 100 µg/ml streptomycin in a 5% CO₂ incubator (Thermo Fisher Scientific, USA) at 37°C. The medium was removed after 24 hrs, the cells washed with phosphate buffer saline and new medium added. The cells were then incubated to attain confluence. A clinical isolate of HSV-1 virus purchased from the center for viral research at the Kenya Medical Research Institute (KEMRI) was propagated in Vero cells in a T75 flask at 37°C and 5% CO₂. The virus titre was determined as the 50% tissue-culture infective dose (TCID₅₀) by the method of Reed and Mench with modifications described by Kohn and others. Briefly, Vero cells were seeded at a density of 1.0 × 10⁶ cells/ml in 96-well culture plates and incubated at 37°C in a humidified atmosphere comprising of carbon dioxide (CO₂) for a period of 24 h. Serial dilutions of HSV-1 were prepared and cells were infected accordingly. A further 48 h was allowed for incubation and the cytopathic effect was recorded. 50% of the tissue culture infective dose (TCID₅₀) per ml was calculated by the method as described by Reed and Mench.

Cytotoxicity assay
Prior to evaluating the anti HSV-1 activity of DI, the cytotoxic effects of each of the extracts was determined by the method of Mosmann with minor modifications. Briefly, 100µl of Vero cells were seeded onto 96-well plates at a density of 1.0×10⁵ cells per well. The plates were then incubated at 37°C/5% CO₂ for 24 h to attain at least 95% confluence. The cell culture medium was then aspirated and washed with physiological buffer saline (PBS). The cells were then treated with 100µl of serial dilutions (0.14-100 µg/ml) of aqueous and methanol extracts of different plant parts of DI and incubated at 37°C/5% CO₂ for 48 h in a humidified atmosphere. Twenty microliters of 5mg/ml 3-[4, 5 dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) solution was then added to each well and cells further dissolved in 100µl of dimethyl sulfoxide (DMSO). Cell viability was examined by the ability of the cells to cleave the tetrazolium salt of MTT. The resulting optical density was read on a multi well reader under a spectrophotometer at 562nm. The
Table 1: Phytochemical profile of crude extracts of different plant parts of *Dicrocephala integrifolia*.

<table>
<thead>
<tr>
<th>Phytoconstituents</th>
<th>Leaves</th>
<th>Root</th>
<th>Flowers</th>
<th>Stem</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>W</td>
<td>ME</td>
<td>W</td>
<td>ME</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phenolics</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>


![Figure 2: Effects of extracts of different plant part extracts of *Dicrocephala integrifolia* on cell viability.](image)

Pharmacognosy Communications, Vol 8, Issue 2, Apr-Jun, 2018

Antiviral activity assay

Antiviral activity of crude extracts of different plant parts of *D. integrifolia* was determined using the cytotoxic effect inhibition assay as described by Kohn and co-workers with modifications as described by Moradi and others. Initially, Vero cells were seeded in a 96-well microtiter plate at a density of 1.0×10⁴ cells per well and incubated for 48 h at 37°C/5% CO₂ in a humidified incubator to reach at least 95% confluence. Next, three separate experiments were run on the confluent cell cultures as follows: 1) pre-treated prior to infection (1 h at 37°C), 2) treatment only during the virus adsorption period (adsorption), 3) treatment after the adsorption period (post-adsorption). The 95% confluent cells were pre-treated with the extract prior to infection for 1 hour at 37°C (pre-treatment of cell lines) then the cells were infected with 50TCID₅₀ (50µl/well) of HSV-1

![Figure 2: Effects of extracts of different plant part extracts of *Dicrocephala integrifolia* on cell viability.](image)

absorbance values were recorded and the percentage cell viability calculated by the formula as described by Moyo and Mukanganyama. From these results the 50% cytotoxic concentration (CC₅₀) was determined using regression analysis.

Table 2: Antiviral activity (EC₅₀), cytotoxicity (CC₅₀) and selectivity index (SI) of crude extracts of different plant parts of *Dicrocephala integrifolia* against herpes virus type-1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>CC₅₀ (µg/ml)</th>
<th>EC₅₀ (µg/ml)</th>
<th>Selectivity index (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-W</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>N/A</td>
</tr>
<tr>
<td>F-ME</td>
<td>71.31 ± 2.65</td>
<td>45.27 ± 2.41</td>
<td>1.58</td>
</tr>
<tr>
<td>S-W</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>S-ME</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>N/A</td>
</tr>
<tr>
<td>L-W</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>N/A</td>
</tr>
<tr>
<td>L-ME</td>
<td>&gt;100</td>
<td>30.53 ± 4.51</td>
<td>N/A</td>
</tr>
<tr>
<td>R-W</td>
<td>&gt;100</td>
<td>0.333 ± 1.23</td>
<td>&gt;300.3</td>
</tr>
<tr>
<td>R-ME</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>N/A</td>
</tr>
<tr>
<td>Acyclovir</td>
<td>&gt;100</td>
<td>24.51 ±3.57</td>
<td>&gt;4.080</td>
</tr>
</tbody>
</table>


Table 3: Inhibition of HSV-1 cytopathic effects on pre-treated and post-treated vero cells by different plant parts of *D. integrifolia*.

<table>
<thead>
<tr>
<th>Plant parts</th>
<th>Extracts</th>
<th>Pre-treatment IC₅₀ (µg/ml)</th>
<th>Post-treatment IC₅₀ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. integrifolia Stem</td>
<td>Water</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>D. integrifolia Stem</td>
<td>Methanol</td>
<td>63.95±5.36</td>
<td>&gt;100</td>
</tr>
<tr>
<td>D. Integrifolia Flowers</td>
<td>Methanol</td>
<td>&gt;100</td>
<td>45.270±4.31</td>
</tr>
<tr>
<td>D. Integrifolia Flowers</td>
<td>Water</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>D. Integrifolia Roots</td>
<td>Water</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>D. Integrifolia Roots</td>
<td>Methanol</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>D. Integrifolia Leaf</td>
<td>Methanol</td>
<td>54.45±3.45</td>
<td>&gt;100</td>
</tr>
<tr>
<td>D. Integrifolia Leaf</td>
<td>Water</td>
<td>86.20±7.56</td>
<td>82.44±7.92</td>
</tr>
<tr>
<td>Acyclovir</td>
<td></td>
<td>4.772±7.81</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

Key: a = Cytotoxic concentration 50 (CC₅₀)
b = Inhibitory Concentration 50 (IC₅₀)
virus suspension in the presence or absence of 50µl of serial dilutions (0.14-100 µg/ml) of the extracts and further incubated at 37°C for 1 h, allowing the adsorption of the viral particles into the cells (adsorption). Subsequently, the supernatant was removed and medium containing 1.5% physiological buffer saline (PBS) with or without inclusion of the extract. This was incubated for 3 days at 37°C/CO₂. Cell viability was assessed using the MTT assay as described previously. These tests were carried out in triplicate. The 50% effective concentration (EC₅₀) and selectivity index (CC₅₀/EC₅₀) were determined for each test.

**RESULTS**

**Qualitative phytochemical screening**

Phytochemical analysis of crude extracts of different plant parts of *DI* revealed the presence of alkaloids, flavonoids, glycosides, phenols, saponins, tannins and terpenoids. Table 1.

**Cytotoxicity assay**

We identified a dose dependent decrease in the growth of the Vero cells in the presence of the extract (Figure 2). Further, all crude extracts of *DI* had a CC₅₀ value of >100µg/ml except for the methanol flower extract (Table 2).

**Antiviral activity assay**

Based on the results of the antiviral assay of *DI*, we established that the aqueous extract of *DI* had a better selectivity index than acyclovir (Table 2).

**Mechanism of activity**

From our results, we found that the methanol flower extract of *DI* inhibited HSV-1 infection more strongly post-adsorption than during other treatment durations (Table 3). However, the methanol stem and leaf extracts of *DI* inhibited the HSV-1 viral infection more strongly pre-adsorption than during other treatment durations (Table 3). Moreover, the methanol flower, leaf and aqueous root extracts of *DI* prevented the attachment of HSV-1 into Vero cells (Table 3).

**DISCUSSION**

The potential utility of medicinal products derived from nature is vast. However, in evaluating the viability of medicinal herbs, it is important to first qualitatively screen medicinal plants for phytoconstituents before further claim validation is undertaken. Active principles from a variety of medicinal plants have been used for a multitude of infectious viral diseases. In the current study, we have identified the presence of alkaloids, flavonoids, glycosides, phenols, saponins, tannins and terpenoids. This is in agreement with the findings of previous workers. Moreover, other workers have attributed antiviral activity of medicinal plants to be due to the presence of anthraquinones, phenolics, terpenes and flavonoids. There is a general misguided perception that herbal preparations are safe and thus the documentation of the toxic potential of herbal medicine and nutrients remains elusive. A majority of the reports touching on the toxicity of herbal medicines are often associated with hepatotoxicity yet toxic effects may also manifest at cellular level in the various body systems. In the current study, we sought to determine the cytotoxic effects of crude extracts of *D. integrifolia* on Vero cell lines using the MTT assay. Based on our results, we identified a dose dependent decrease in Vero cell line growth post extract testing. This is in agreement with the findings of Vijayarathna and Sasidharan who studied the cytotoxic effects of *Elaeis guineensis* in MCF-7 cancer cell lines. Further, Loomis and Hayes developed a scale for classifying the cytotoxicity of crude medicinal plant extracts. Based on this scale, a cytotoxic concentration at 50% (CC₅₀) value of less than 1 is considered as extremely toxic while a CC₅₀ value of greater than 50 but less than 500 is considered as moderately toxic. Thus, in view of the results we obtained, it may be suggested that crude extracts of *D. integrifolia* are moderately toxic.

To evaluate the mechanism of antiviral activity *vis-à-vis* the step of the viral cycle where replication of HSV-1 was inhibited, different treatment protocols were followed. Pre-treatment studies involved treating the HSV-1 virus with each of the crude extracts of *D. integrifolia* before infecting the cells. This was done to evaluate the virus inactivation capacity of these extracts. Further, adsorption studies involved adding each of the crude extracts of *D. integrifolia* to the cells before HSV-1 infection. Post adsorption studies involved first infecting the cells with the HSV-1 virus followed an hour later by the addition of crude extracts of *D. integrifolia*. From our results, we found that the methanol flower extract of *D. integrifolia* inhibited HSV-1 infection more strongly post-adsorption than during other treatment durations. This may suggest that this extract may not prevent the entry of HSV-1 into the Vero cell, but it acts after the virus has already penetrated the cell. This is in agreement with the findings of Moradi and co-workers who studied the anti-viral activity of *Melissa officinalis*. However, the methanol stem and leaf extracts of *D. integrifolia* inhibited the HSV-1 viral infection more strongly pre-adsorption than during other treatment durations. This suggests that these extracts may interact with HSV-1 viral particles and inactivate them. Moreover, the methanol flower, leaf and aqueous root extracts of *D. integrifolia* prevented the attachment of HSV-1 into the Vero cells. This is in agreement with what was reported on *Swertia mussotii*, *Dracocephalum heterophyllum*, *Dracocephalum tanguaticum* and *Lagotis brevituba* by Zhang and colleagues.

**CONCLUSION**

The results of this study suggest that crude methanol leaf and flower extracts, as well as the aqueous root extracts of *D. integrifolia* have promising *in vitro* anti HSV-1 activity. However, further studies on the *in vivo* efficacy and active components of these extracts may be important in identifying useful molecules in the development of novel antiviral agents.

**ACKNOWLEDGEMENT**

The authors would like to acknowledge the contribution of Mr. Antony Mutiso, a botanist of the University of Nairobi, School of Biological Sciences and Dr. Peter Mwitari of the Centre for Traditional Medicine and Drug Research.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**REFERENCES**

• The methanolic flower extracts of *Dicrocephala integrifolia* had a better selectivity index against HSV-1 infection than acyclovir (1.58 compared to >4.08).

• The methanolic leaf and flower extracts of *Dicrocephala integrifolia* were better inhibitors of HSV-1 infection post-treatment than acyclovir, with IC50 values of 82.44±7.92µg/ml and 45.27±4.31µg/ml respectively compared to >100µg/ml.

• All crude extracts of *Dicrocephala integrifolia* studied were moderately toxic on Vero cell lines, with CC50 values of between 50µg/ml and 500µg/ml.

• Methanolic stem and leaf extracts of *Dicrocephala integrifolia* were better inhibitors of HSV-1 viral infection in the pre-treatment stage as compared to the post-treatment stage, with IC50 values of 63.95±5.36µg/ml and 54.45±3.45µg/ml respectively compared to 100µg/ml.

### ABOUT AUTHORS

**Dr. Hadun:** Is a Research Scholar at the Department of Public Health, Pharmacology and Toxicology at the University of Nairobi. His research interests include natural product chemistry, environmental and industrial toxicology. He is currently working on nano therapeutic approaches in the management of malaria.