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Original Article

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

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ABSTRACT Introduction: At present, acyclovir is commercially available as the drug of choice for managing herpes simplex type I (HSV-1) viral infection. However,

terpenoids the aqueous were DI extracts found to was be in present the range in 71.31 the extracts. ± 2.65 to The >100 CC

50 µg/ml values com- of

the high prevalence of the infection coupled with the emergence of resistant viral strains has limited its effectiveness. Thus, the development of novel

pared the pre-adsorptive to >100 µg/ml phase of acyclovir. of HSV-1 The activity EC

50

values was in of the crude range extracts 54.45±3.45 of DI on to antiviral agents is crucial. Practitioners of herbal medicine in Kenya make >100µg/ml compared to 4.772±7.81µg/ml of acyclovir whilst use of Dicrocephala integrifolia (DI) for the management of several diseases of the crude extracts of DI on the post-adsorptive phase of 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 in vitro antiviral activity of crude extracts of DI against HSV-1. 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 dimeth- ylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide] assay. The 50% cytotoxic concentration (CC

50

Sample collection Whole plant material of DI (Figure 1) was collected from Mabariri, Nyamira county, Kenya (S 000 31. 367s, E 0340 56. 426s) 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.

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 parts were then ground to a fine powder using an electric grinder and packed in zip-locked plastic bags awaiting further use.

Extraction of different plant parts The method of Mwitari et al.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 -20oC 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 -20oc awaiting further use.

the HSV-1 EC

50 activity value

was in the range 45.270±4.31 to >100µg/ml compared to >100µg/ml of acyclovir. Conclusion: The results suggest that crude extracts of DI may be a reservoir of phytochemicals with potentially good efficacy against HSV-1. Key words: Dicrocephala integrifolia, Iin vitro, Cytotoxicity, Antiviral activity, HSV-1.

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) and 50% effective concentration (EC

50

) of each extract was determined using regression analysis. The effects of crude DI extracts

Department of Public Health, Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Nairobi, P.O. Box 29053-00625, Nairobi, KENYA. on adsorption and post-adsorption stages of the HSV-1 replication cycle

Tel:+254722119150 was evaluated against acyclovir using a cytopathogenic inhibition assay.

E-mail: hadun2017@gmail.com Results: Alkaloids, glycosides, flavonoids, phenols, saponins, tannins and

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INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a common pathogen with a wide global prevalence.1 The virus has been shown to reside in the trigeminal ganglia of man 2 making it a lifelong infection.3 Although infected individuals seldom exhibit clinical manifestations, oral and genital lesions have been identified as clinical symptoms of HSV-1 infection.4,5 The mainstay of treatment of HSV-1 infections is the nucleoside, acyclovir.3 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.6,7 Several authors have reported on plant derived lead molecules efficacious against various stages of viral develop- ment.8,9 Dicrocephala integrifolia is a flowering plant that belongs to the Asteraceae family.10 In Kenya, it is used in the treatment of skin infec- tions,11 induction of emesis, purgative, antitumor agent as well as in the management of liver, spleen, kidney, bladder, bone and joint diseases.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.

MATERIALS AND METHODS 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.

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 were then added to the resultant filtrate. A deposit of chloride a red precipitate

(FeCl

3

)

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 the test tube. A red brown H

coloration 2

SO

4

was then at the slowly interface added of the on the two sides liquids of

was indicative of the presence of terpenoids.

Qualitative phytochemical screening

Cell culturing and virus propagation The cells were propagated in Modified Eagle's Medium (MEM) supple- The methods of Harborne 14 and Trease and Evans 15 were used to quali-

mented with 1% fetal bovine serum (FBS), 100

units/ml of penicillin tatively screen the extracts for plant secondary metabolites. A qualitative scale was used to evaluate the phytochemical composition of the different

and 100 µg/ml streptomycin in a Scientific, USA) at 37°C. The medium 5% was CO

removed 2

incubator (Thermo Fisher after 24hrs, the cells plant extracts. In this scale, +++ signified a very high concentration of

washed with phosphate buffer saline and new medium

added. The cells phytochemical, ++ signified a high concentration of phytochemical,

were then incubated to attain confluence. A clinical

isolate of HSV-1 virus + signified a low concentration of phytochemical while – signified the

purchased from the center for viral research at the

Kenya Medical Research absence of the phytochemical.

Institute (KEMRI) was propagated in Vero cells in a T75 flask at 37°C and

Test for alkaloids Approximately 0.5 g of the extract was dissolved with about 10ml of

5% dose as described CO (TCID

2 . The 50

virus by) titre was determined as the 50% tissue-culture infective by the method of Reed and Mench 16 with modifications Kohn and others.17 Briefly, Vero cells were seeded at a 1 % hydrochloric acid. The mixture was then boiled for 5 min then filtered. The filtrate was put in two test tubes of 2ml each. Mayer's reagent was added to the first test tube. Thereafter, three drops of

Dragendorrf ’s

density of 1.0 ×105 cells/ml in 96-well culture plates and incubated at 37°C in a humidified for a period of 24 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 Dragendorrf ’s reagent.

Test for cardiac glycosides One fifty milligrams of each extract was mixed with 1.5ml of glacial acetic acid containing ferric chloride (FeCl

3

atmosphere comprising h. Serial dilutions of HSV-1 of were carbon prepared dioxide and (CO

cells

2

)

were infected accordingly. A further 48 h was allowed for incubation and the cytopathic effect was recorded. 50% of the tissue culture infec- tive dose Reed and (TCID

Mench.16

50

) per ml was calculated by the method as described by

Cytotoxicity assay

) solution. To this solution, 0.5 ml of concentrated the test tube. Positive identification sulphuric (H 2

SO of 4

cardiac) acid was glycosides added to was the demon- sides of

strated by the development of a red-brown colour at the boundary of the layer between FeCl

3

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 18 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

and after 5 min.

incubated The cell H

2

SO

4

which turned to a blue-green colour

at culture 37°C/5% medium CO

was 2

for 24 then h to attain at least 95% confluence. aspirated and washed with physi- ological buffer saline (PBS). The cells were then treated with 100µl of Test for flavonoids One gram of each of the crude plant extracts were seperately dissolved

serial dilutions (0.14-100 µg/ml) of aqueous and methanol extracts of different plant parts of DI and incubated at 37°C/5% CO

2

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 6mg 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.

for 48 h in a humidified atmosphere. Twenty microliters of 5mg/ml 3-[4, 5 dimeth- ylthiazol-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

Figure 1: Aerial parts of Dicrocephala integrifolia.

Table 1: Phytochemical profile of crude extracts of different plant parts of Dicrocephala integrifolia.

Phytoconstituents Plant parts

Leaves Root Flowers Stem

W ME W ME W ME W ME

Alkaloids + + + + + + + +

Flavonoids + + + - + + + +

Glycosides + + - + + + + +

Phenolics + + + + + + + +

Saponins + + + + + + + +

Terpenoids + + + + + + + +

Key: F-W: water extract of the flower, F-ME: methanol flower extract, S-W: water extract of the stem extract, S-ME: methanol stem extract, L-W: water extract of the leaf, L-ME: methanol leaf extract, R-W: water extract of the root, R-ME: methanol root extract, +: present, -: absent

Table (SI) of 2: crude Antiviral extracts activity of different (EC

50

), cytotoxicity plant parts (CC

of Dicrocephala 50

) and selectivity integrifolia index

against herpes virus type-1.

Sample CC

50

(µg/ml)a EC

50

(µg/ml)b Selectivity index (SI)c

F-W >100 >100 N/A

F-ME 71.31 ± 2.65 45.27 ± 2.41 1.58

S-W >100 >100 >100

S-ME >100 >100 N/A

L-W >100 >100 N/A

L-ME >100 30.53 ± 4.51 N/A

R-W >100 0.333 ± 1.23 >300.3

R-ME >100 >100 N/A

Acyclovir >100 24.51 ±3.57 >4.080

F-W; water extract of the flower, F-ME; methanol flower extract, S-W; water extract of the stem, S-ME; methanol stem extract, L-W; water extract of the leaf, L-ME; methanol leaf extract, R-W; water extract of the root, R-ME; methanol root extract, index

CC

50

; cytotoxic concentration, EC

50

; effective concentration, SI; selectivity

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

Plant parts Extracts Pre-treatment

Post- IC

50

(µg/ml)

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

absorbance values were recorded and the percentage cell viability calcu-

D. Integrifolia Flowers Water >100 >100 lated by

the formula as described by Moyo and Mukanganyama.19 From

D. Integrifolia Roots Water >100 >100 these results

using regression the 50% analysis.

cytotoxic concentration (CC

50

) was determined D. Integrifolia Roots Methanol D. Integrifolia Leaf Methanol

>100 54.45±3.45 >100 >100 Antiviral activity assay

D. Integrifolia Leaf Water 86.20±7.56 82.44±7.92

Antiviral activity of crude extracts of different plant parts of DI was

Acyclovir 4.772±7.81 >100 determined using

the cytopathic effect inhibition assay as described by

Key: a = Cytotoxic concentration 50 (CC

50 Kohn and co-workers 20 with modifications as described by Moradi and others.21 Initially, Vero cells were seeded in a 96-well microtiter plate at a in density a humidified of 1.0×105 incubator cells per to reach well and at least incubated 95% confluence. for 48 h at 37°C/5CO Next, three 2

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

) b = Inhibitory Concentration 50 (IC50)

the virus adsorption period (adsorption), 3) treatment after the adsorp- tion 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 50TCID

50

(50µl/well) of HSV-1

Figure 2: Effects of extracts of different plant part extracts of Dicrocephala integrifolia on cell viability. DIFM: Methanol extract of the flower of Dicrocephala integrifolia, DIFW: Water extract of the flower of Dicrocephala integrifolia, DISM: Methanol extract of the stem of Dicrocephala integrifolia, DISW: Water extract of the stem of Dicrocephala integrifolia, DIRM: Methanol extract of the root of Dicrocephala integrifolia, DIRW: Water extract of the root of Dicrocephala integrifolia, DILM: Methanol extract of the leaves of Dicrocephala integrifolia, DILW: Water extract of the leaves of Dicro- cephala integrifolia.

virus suspension in the presence or absence of 50µl of serial dilutions

systems.31 In the current study, we sought to determine the cytotoxic (0.14-100 µg/ml) of the extracts and further incubated at 37°C for 1 h,

effects of crude extracts of D. integrifolia on Vero cell lines using the MTT allowing the adsorption of the viral particles into the cells (adsorption).

assay. Based on our results, we identified a dose dependent decrease in Subsequently, the supernatant was removed and medium containing

Vero cell line growth post extract testing. This is in agreement with the 1.5% physiological buffer saline (PBS) with or without inclusion of the

findings of Vijayarathna and Sasidharan who studied the cytotoxic extract. This was incubated for 3 days assessed using the MTT assay as described at 37°C/CO

previously. 2

. Cell viability was These tests were

effects of Elaeis guineensis in MCF-7 cancer cell lines.32 Further, Loomis and Hayes 33 developed a scale for classifying the cytotoxicity of crude carried out in triplicate. selectivity index (CC

50

The 50% effective concentration (EC

50

) and

medicinal at 50% plant extracts. Based on this scale, a cytotoxic concentration /EC

50

) were determined for each test.

Statistical analysis

a ately CC

50 toxic. value (CC

Thus, 50 of) value greater in view of than less of than 50 the but results 1 is less considered than we obtained, 500 as extremely toxic while is considered as moder- it may be suggested The (EC 5.02. 50

50%) The values cytotoxic results were were concentration calculated obtained using from (CC

Graph triplicate 50

) and Pad 50% assays Prism effective using Software concentration

eight 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.20,21 Pre-treatment studies involved treating the HSV-1 virus with each of the crude extracts of D. integrifolia before infecting the cells.20 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.20 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.21 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 mussoti, Dracocephalum heterophyllum, Dracocephalum tanguticum and Lagotis brevituba by Zhang and colleagues.34

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 prom- ising 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 lead molecules in the development of novel antiviral agents.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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version extract concentrations (0.14-100µg/ml). The percentage cell viability (cytotoxicity) was calculated as absorbance of sample/absorbance of control×100. The selectivity index was calculated as CC

50

/EC

50

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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 (Table a CC 2).

50

value of >100µg/ml except for the methanol flower extract

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). More- over, 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.22,23 Active principles from a variety of medicinal plants have been used for a multitude of infectious viral diseases.17,24,25 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.26 Moreover, other workers have attributed antiviral activity of medicinal plants to be due to the presence of anthraquinones, phenolics, terpenes and flavonoids.21,27,28 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.29 A majority of the reports touching on the toxicity of herbal medicines are often associated with hepatotoxicity30 yet toxic effects may also manifest at cellular level in the various body

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PICTORIAL ABSTRACT HIGHLIGHTS OF PAPER

• The methanolic flower extracts of Dicrocephala integrifolia had a better selectivity index against HSV-1 infection than acyclovir (1.58 compared to >4.080)

• The methanolic leaf and flower extracts of Dicrocephala integrifolia were better inhibitors of HSV-1 infection post-treatment than acyclovir, with compared IC

50

values to >100µg/ml.

82.44±7.92µg/ml and 45.27±4.31µg/ml respectively

• All crude extracts of Dicrocephala integrifolia studied were moderate- ly 500µg/ml.

toxic on Vero cell lines, with CC

50

values of between 50µg/ml and

• Methanolic stem and leaf extracts of Dicrocephala integrifolia were bet- ter inhibitors of HSV-1 viral infection in the pre-treatment stage as com- pared 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 indus- trial toxicology. He is currently working on nano ther- apeutic approaches in the management of malaria.