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Full Length Research Paper

Preclinical anti-HSV-1 activity of aqueous and methanol extracts of Kenya grown pyrethrum (*Chrysanthemum cinerariaefolium*)

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Due to the opportunistic nature of Herpes simplex viral infections, it is of great public concern in sub-Saharan Africa. To date, there is no vaccine or cure for this viral infection. In this study, anti-viral activity of the Kenyan *Chrysanthemum cinerariaefolium* (Pyrethrum) against Herpes simplex virus (HSV-1) was evaluated *in vivo* (using Swiss mice). Phytochemical screening for presence of secondary metabolites of both methanol and aqueous extract of the plant material (flowers) indicated positive, for presence of alkaloids, flavonoids, phenols, saponins, tannins and terpenoids. The extracts were given orally after acute oral toxicity results (LD50 >2000 mg/kg of body weight) indicated both extracts are safe to be given orally. Upon induction of topical infection with HSV-1 virus, 2 dose levels (10 mg/kg and 25 mg/kg of methanol extract and 25 mg/kg and 50 mg/kg of aqueous extract) of both extracts were administered, 2 times per day for 7 successive days. Results showed Acyclovir (ACV) at 5 mg/kg and organic extract at 10 mg/kg delayed onset of lesion in local regions significantly ($p \leq 0.05$ test vs. control by student *t* test). Also, both the organic extract (at a concentration of 10 mg/kg and 25 mg/kg) and aqueous extract (at a concentration of 50 mg/kg) delayed progression of infection significant ($p \leq 0.05$ test vs. control by repeated measures ANOVA). The results indicate extracts from C. *cinerariaefolium* are active against Hsv-1. So, further investigation is recommended in the Kenya grown *C. cinerariaefolium,* on its bio-active compounds, safety, and activity on other members of the family *Herpesviridae*.

Key words: Anti-Hsv, phytochemical screening, acute toxicity, *Chrysanthemum cinerariaefolium*.

INTRODUCTION

Herpes simplex viruses are DNA viruses. The virus belongs to the subfamily *Alphaherpesvirinae*, in the family *Herpesviridae.* There are two strains of the virus; HSV-1 which is known to cause oral and ocular lesions is a leading cause of viral caused corneal blindness and viral encephalitis (Herpetic Eye Disease Study Group, 1998;

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Shoji et al., 2002). Whereas, HSV-2 is known to cause genital lesions and other serious diseases such as blindness, meningitis, and encephalitis (Connolly et al., 2011), with global estimates of 536 million infected persons with an annual incidence of 23.6 million cases among persons aged 15 to 49 years (Tronstein et al., 2011).

In sub-Saharan Africa, the prevalence in adults ranges from 30 to 80% of females and 10 to 50% of males. A considerable rate of prevalence is indicated in different regions of the world (Anzivino et al., 2009). Acyclovir (ACV) has been used for treatment and prophylaxis of this viral infection (Stranska et al., 2005), but until now, there is no vaccine or cure for HSV infections.

Currently, there is a growing public health concern due to emerging new diseases and the development of resistance by pathogens to the available therapies; such as Acyclovir resistance by HSV strains (Chen et al., 2000**;** Christophers et al., 1998; Coen et al., 1980; Schnipper et al., 1980; Looker et al., 2008). Therefore, there is a need to identify alternative therapies. Pyrethrum plant (*C. cinerariaefolim*) that belongs to the family of perennial plants *Asteraceae*, genus *Chrysanthemum* and species *C. cinerariaefolim* has insecticidal property. Its active insecticidal components are known as Pyrethrins (Morris et al., 2006).

This plant is cultivated widely in Kenya, Australia, Japan, India, New Guinea, Uganda and other countries (Greenhill, 2007). It is used abundantly in many pesticide products in or around buildings (Cox, 2002), crops, ornamental plants, pets and live stocks. Its toxicity is relatively low in humans and other mammals; toxicity in rats is indicated as 4000 times lower than Pyrethrins toxicity to the houseflies (Klaassen et al., 1996).

According to the study by Stanberry et al. (1986), Pyrerhrins exhibited *in vitro* anti HSV activity, the objective of this study was to determine the effect of total extract (methanol and aqueous extracts) derived from the flower of Pyrethrum plant against HSV-1 to identify a new candidate drug. This virus is more virulent and common (Xu et al., 2002; Whitley *et* al., 1998) than HSV-2. Moreover, discoveries from this study would be opening a gate for further research on other members of the family *Herpesviridae*.

MATERIALS AND METHODS

Ethical considerations

The research was conducted in accordance with Kenya Medical Research Institute (KEMRI) guidelines on the international accepted conduct of experimental research and the internationally accepted principles for laboratory research. Approval was obtained from KEMRI Scientific and Ethical Review Unit (SERU) and Animal Care and Use Committee (ACUC) (KEMRI/ACUC/02.07.15).

Plant collection and preparation

Flower heads of *C. cinerariaefolium* were collected from Kiambogo, Nakuru County, Kenya (S 000 41 563', E 0360 25 196') in February 2015. It was air dried at room temperature in a dark room and deposited in the Center for Traditional Medicine and Drug Research (CTMDR) (KEMRI) herbarium with a voucher specimen deposited number Tolo/Mwitari/Keter/001. The plant material was then grinded using laboratory mill (Christy & Norris Ltd., Chelmsford, England) at the Center for Traditional Medicine and Drug research, KEMRI. The plant material was then packed in air tight polyethylene bags to prevent moisture and kept away from direct sunlight until extraction was performed. Percentage yield was calculated as follows.

Extraction

Aqueous extraction

Extraction process was carried out based on slight modification to the method indicated by Awoyinka et al. (2007). Two hundred grams (200 g) of the powdered plant material were soaked in 2000 ml of distilled water and placed in a water bath at 60°C. After 1 h it was decanted into a clean, dry 600 ml conical flask. Filtration was done through 2 layers of sterile gauze. The filtered extract was freeze dried in 200 ml portions using a Freeze Dryer (Edwards freeze dryer Modulyo). Finally, the powder was weighed, labeled and stored in an air tight 50 ml centrifuge tube at 4°C until use.

Methanol extraction

Extraction process was carried out based on a slight modification to the methods used by Parekh et al. (2005). Five hundred grams (500 g) of the dried powder plant material were soaked with 1700 ml methanol in a flat-bottomed 3 L conical flask at room temperature for 3 days in a dark room covered by cotton gauze. After 3 days, it was filtered using sterile cotton gauze and concentrated using a rotary evaporator (Büchi Rota vapor R-114) at 70°C. Finally, the extract was weighed and stored in a cap tight round bottom flack at 4°C until use.

Phytochemical screening

Qualitative analysis in a tube test was carried out to screen for the presence of Alkaloids, Flavonoids, Phenols, Saponins, Tannins and Terpenoids according to the procedures indicated by Wagner (1993), Sofowora (1993), Mace (1963), Kokate (1994) and Segelman et al. (1969), and according the procedures indicated in the *Salkowski test* respectively.

HSV-1 virus

Virus stock was obtained from CTMDR laboratory. It was propagated *in vero* cells (kidney cells of African green monkey) and stored in -80°C freezer until use. Virus titer was determined by end point dilution assay *in vitro*, in Vero cells. Cells were seeded at a density of 1×10^5 cells/well in 96 well plates and were grown in minimum essential media (MEM), at 37 °C under 5 % CO2 incubator for 24 h. After 24 h, the cells were 90% confluent and a virus dilution of 1:10 was prepared in PBS from the original 1 ml virus stock. Then the culture media was replaced with 100 µl PBS containing the virus dilution. The first column contained 1:10 virus dilution and a series of 3 fold dilution was transferred to the subsequent columns. The last 6 columns were used as cell controls. After allowing the virus to adsorb for 2 h, it was replaced with 100 µl maintenance media. Cytopathic effect (CPE) was monitored under light microscope for 2 days and recorded.

Table 1. Design to test efficacy of *C. cinerariaefolium* in HSV-1 infected mice.

Treatment (mg/kg)		Number of mice	Outcome
Placebo	0	8	Not infected/not treated/given water
Control	0	8	Infected /not treated/given PBS with < 10% tween 80
Acyclovir	5	8	Infected/treated
Methanol extract	10	8	Infected/treated
	25	8	
Aqueous extract	25	8	Infected /treated
	50	8	
Total number of animals		56	-

The $TCID_{50}$ (50% Tissue Culture Infectious Dose) was calculated by Spearman and karber algorithm method. A pilot study was done on mice to determine infectious titer; groups of mice were infected with the virus topically starting with highest dilution and infectious titer was chosen rather than the lethal titer.

Acute oral toxicity

The toxicological study was conducted in KEMRI animal house laboratory. It was carried out according to the method described in Organization for Economic Co-operation and Development (OECD) guideline 423 which was adopted in 2001. The experiment was carried out on Swiss mice. Five male and female mice weighing 20 g ± 2 g each were obtained from Kenya Medical Research Institute (KEMRI) animal house and left to acclimatize with the experimental room for 3 days. Each group contained 3 mice from the same sex. The animals were fed with standard feed and water in the course of the study. Both methanol and aqueous extracts of the plant material were administered by oral route in a single dose. The methanol extract was dissolved with Tween 80 < 10% and topped with PBS (Phosphate buffer solution). All the experimental mice received the initial dose of methanol and aqueous extracts at a concentration of 2000 mg/kg of body weight according to the guideline. Two control groups for each extract, a group of one sex were given PBS with < 10% Tween 80. The symptoms and weight were recorded before administration of the extract and during the first 30 min after administration and regularly during the first 24 h after treatment for 14 days daily**.**

Anti-HSV-1 efficacy of *C. cinerariaefolium* in mice

The experiment was performed in KEMRI animal house. A Total of fifty six female Swiss mice (for 2 sets of experiment), aged seven/six weeks from the KEMRI animal house were used. Mice were assigned into 7 groups; each group contained 8 mice. The mice were left to acclimatize to the experimental room housed in a clean shoebox cage, being provided adequate animal feed and clean water. A 2 cm^2 area on each mouse was shaved with an electric hair shaver and the remaining fur on the shaved area was completely removed by a chemical depilatory VEET ® hair removal cream (ingredients declared are; Thioglycolic acid and Potassium hydroxide). Each mouse was assigned randomly into a group. This anti-viral evaluation was done according to the method described by Li et al. (1997).

The shaved area of all mice was wiped with 70% alcohol, and scratched superficially with a bunch of 27 gauge needle to breach the skin slightly to give scarified area of 1cm^2. Then 5 µl suspension of 10^3 tissue culture infectious dose (10^3 $Tcid_{50}$/ml) wild type HSV-1 was applied to the scarified area of the 48 mice. The remaining 8 mice were used as a placebo group; which were not infected nor given any drug or the extract and were only given water. Of the 48 mice 16 mice were used as a control, divided into two groups. The first group, a group of 8 mice, was used as negative control; the negative controls were handled in the same manner as the test mice, but were not treated with the extract or the reference drug ACV and only given PBS with < 10% Tween 80. The second group was given ACV at a concentration of 5 mg/kg. It was used as a reference drug (positive controls) and was given to 8 mice.

Then the remaining 32 mice were divided into 4 groups, each group containing 8 mice. Two groups were given the methanol extract at a concentration of 10 and 25 mg/kg. The other two groups were given the water extract at a dose of 25 and 50 mg/kg of body weight. Experiment design is indicated in Table 1. The extracts and acyclovir were administered orally using oral gavage of 22 gauges (0.72 mm in diameter, 3.8 cm in length), starting 4 h after the initial infection for 7 days 2 times daily. The development of skin lesions was monitored every day for 10 days and mortality was monitored over a period of 30 days. Lesions were scored as follows; 0- no lesion, 2 vesicles in the local region, 4- erosion and/or ulceration in the local region, 6- mild zoster form, 8-zosteriform lesion and 10-serious zoster form lesion and death. The scoring of 0-2-4-6-8-10 is selected to avoid transition points where the score is 0 or 2, 2 or 4, 4 or 6, 6 or 8, 8 or 10. The infected mice were fed and observed for 30 days to determine mortality. In this experiment, the dose levels were chosen based on the cytotoxicity results, which indicated the aqueous extracts to be less toxic.

Data analysis

The student *t* test was used to evaluate the significance of difference between control and treated mice in mean times at which skin lesion was initially scored 2 (vesicles in local region) or 6 (zosteriform lesion) after infection. The repeated measures ANOVA (Benferroni) was used to analyze the interaction between extracts and ACV treatment in mean skin lesion for 3 to 10 days after infection (Li et al., 1997). A p-value ≤ 0.05 was defined as statistically significant. All analysis was done using statistical analysis software SPSS (V. 17)

RESULTS

Percentage yield of aqueous and methanol extracts

The results of the yield of both aqueous and methanol extracts from Pyrethrum flowers are presented in Table 2.

Table 2. Percentage yield of aqueous and methanol extracts.

Medicinal plant	Dry weight (g)	Freeze dry weight (g)	Percentage yield (%)
Aqueous extract	200	13	6.5
-	-	Rotary-evaporated weight (g)	-
Methanol extract	500	65	13

Table 3. Phytochemical screening of aqueous and methanol extract.

Secondary metabolites	Aqueous extract	Methanol extract
Alkaloids	++	+++
Flavonoids	+++	+++
Phenols	+++	+++
Saponins	+++	++
Tannins	+++	+++
Terpenoids	+++	+

+++= deep color, ++= slight deeper, += faint color.

Table 4. Efficacy of aqueous and methanol extracts in mouse model.

Treatment (mg/kg)		Mean days ± S.D		Mortality [B]
		Score 2 [A]	score 6 [A]	
Control	0	3.25±0.35	5.27±0.44	0/8
Acyclovir	5	4.75*± 0.75	7.83±1.51	0/8
Organic extract	10	4.58*±0.57	7.5 ±2.0	0/8
Organic extract	25	3.83±0.83	6.83±1.83	0/8
Water extract	25	4.25±0.5	5.0 ±0.0	0/8
Water extract	50	3.67±0.57	7.5±2.44	0/8

*= $p \leq 0.05$ (test vs. control by student *t* test). A= Mean times at which score 2 or 6 were first observed. B=Number of dead mice against total surviving mice, it was calculated on day 30.

Obtained yield for the aqueous extract was 6.5 and 13% for the methanol extract.

Phytochemical screening of aqueous and methanol extract

Results for the phytochemicals present are represented in Table 3.

Acute oral toxicity results (LD_{50} Determination, OECD 423 guideline)

The results of acute oral toxicity tests are presented in Table 4. Upon receiving both extracts at a concentration of 2000 mg/kg, no mortality was observed. According to the results, the LD_{50} for both extracts in both the sexes is > 2000 mg/kg.

Anti hsv-1 efficacy of aqueous and methanol extracts in mice model

After the initial infection, onset of the infection was observed as development of vesicles in a local region (score 2) on Day 3 for the controls and Day 4 for those given the ACV 5 mg/kg. For the test groups, vesicles on the local region (score 2) was observed on days 4 and 3 for those given the organic extract at a concentration of 10 and 25 mg/kg, respectively. For those mice that were given the aqueous extract, vesicles were observed on days 4 and 3 at a concentration of 25 and 50 mg/kg, respectively.

Mild zosteriform lesions (score 6) were observed on Day 5 and 7 for the controls and for those treated with ACV at 5 mg/kg, respectively, for the test groups, mild zosteriform lesions (score 6) were observed on Days 7 and 6 for those given organic extract at a concentration of 10 and 25 mg/kg, respectively. For the groups which were

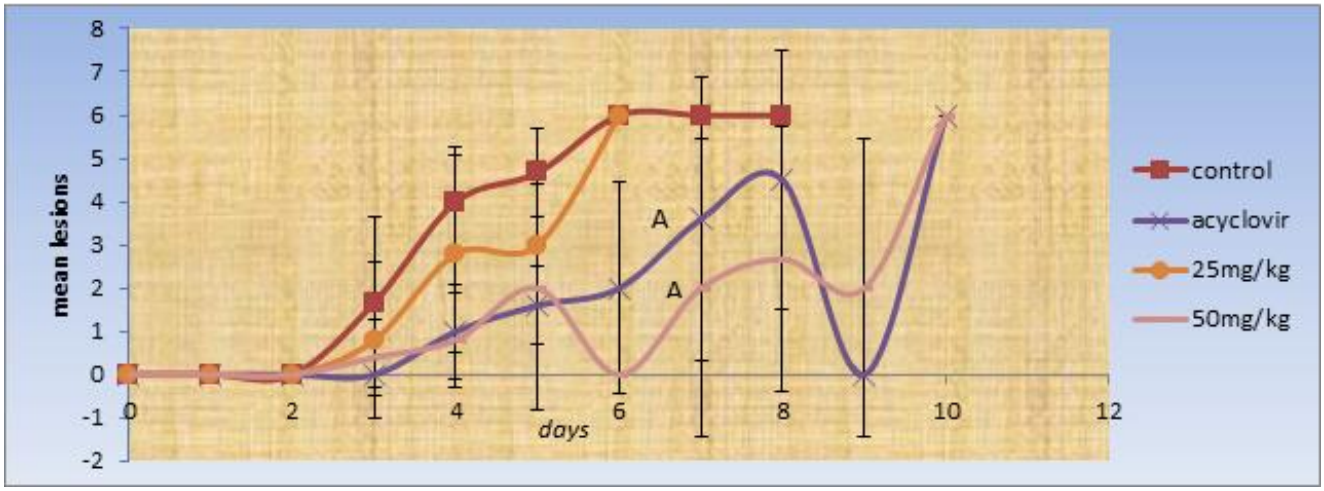

Figure 1. Interaction line plots for progression of HSV-1 infection in Swiss albino mice following oral treatments with aqueous extract of *Chrysanthemum Cinerariaefolium* (10 mg/kg, 25 mg/kg) and Acyclovir 5 m/kg, $p \leq 0.05$ Test Vs control by repeated measures ANOVA (Benferroni). Error bars are deviations within the mean of 8 mice in each group. A= significant.

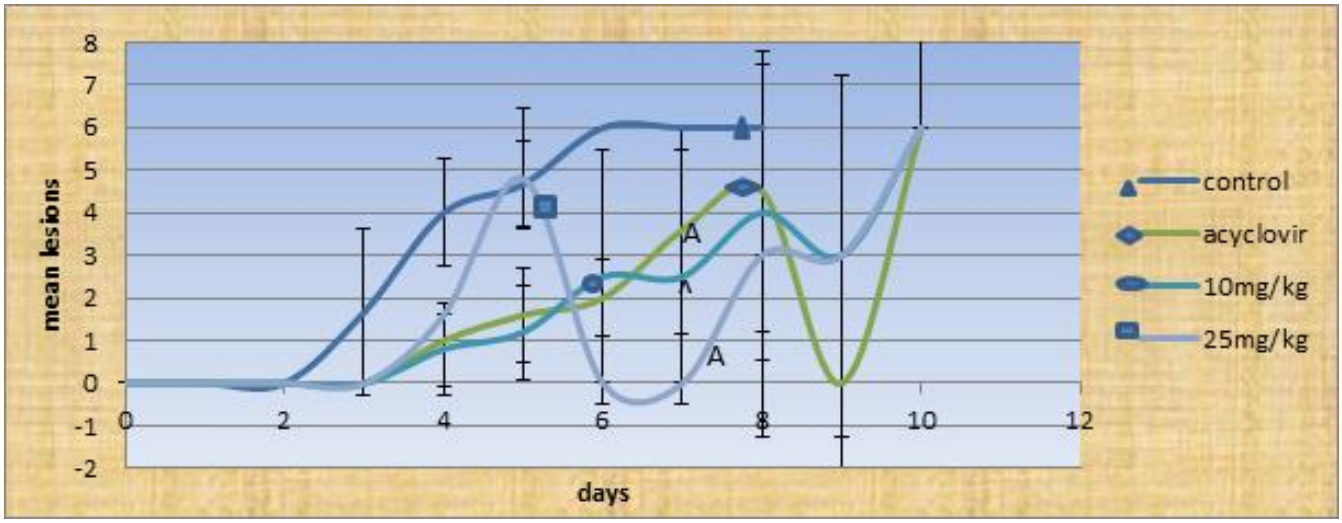

Figure 2. Interaction line plots for progression of HSV-1 infection in Swiss albino mice following oral treatments with methanol extract of *Chrysanthemum Cinerariaefolium* (10 mg/kg, 25 mg/kg) and Acyclovir 5 m/kg, ($p \leq 0.05$ Test Vs. control by repeated measures ANOVA (Benferroni). Error bars are deviations within the mean of 8 mice in each group. A= Significant..

were given the aqueous extract at a concentration of 25 mg/kg mild zosteriform lesions was observed in Day 5 and for those which were given 50 mg/kg mild zosteriform lesions was observed on day 7. In both experiments there was no progression to a Sever mild zosteriform lesion and no mortality was observed up to the 30th day.

Figure 1 displays the interaction of the line plots for progression of HSV-1 infection in mice, following oral treatments with aqueous extract at 25 and 50 mg/kg. The progression of infection in mice treated with 25 mg/kg was similar to that of control. The 50 mg/kg treatment kept the progression of infection the same as those which were given Acv at 5mg/kg and the influence was significant ($p \leq 0.05$ test Vs control by repeated measures ANOVA (Benferroni), but when the treatment was stopped at the 7th day there was an upsurge in the progression of the infection. Figure 2 displays the interaction of the line plots for progression of HSV -1 infection in mice. Following oral treatment with the methanol extract at a concentration of 10 and 25 mg/kg, there was significant delay ($P \leq 0.05$) in the progression of the infection in comparison to the control. By repeated measures ANOVA (Benferroni) as does the acyclovir at 5 mg/kg. But upon withdrawal on the 7th day infection

progressed.

DISCUSION

C. cinerariaefolim (Pyrethrum) plants are well known for their insecticidal properties. It contains active insecticidal compound known as Pyrethrins (Morris et al., 2006). There are different varieties in the world, in this study the Kenya grown variety was tested for its anti-viral activity against HSV-1 in mice. According to previous studies (USEPA, 1994), Pyrethrins which are extracted from this plant showed no observable adverse effects at a concentration of 686 and 834 mg/kg in both male and female mice, respectively.

In this study, mice were given orally the total extract of both aqueous and methanol at a concentration of 2000 mg/kg, and no mortality was observed. Increase in heart beat in some mice was noticed during the first 20 min. According to a previous study (Rao et al., 1973; Sashida et al., 1983) flavonoid, fatty acids (Head, 1968) and essential oils (Saggar et al., 1997) were identified in Pyrethrum plant.

In this study aqueous and methanol extracts of Pyrethrum were screened for the presence of secondary metabolites even though the technique used in this experiment is qualitative analysis, both extracts were tested positive for the presence of secondary metabolites: alkaloids, flavonoids, phenols, saponins, tannins and terpenoids. These secondary metabolites from other plants have shown bioactivity, as it is indicated elsewhere were flavonoids (Pengsuparp et al., 1995) and alkaloids (McMahon et al., 1995) contain anti-HSV activity.

According to the study done by Sassi et al. (2008), *C. trifurcatum* grown elsewhere exhibited *in vitro* anti-HSV activity, also, Stanberry et al. (1986) indicated, Pyrerhrins the active insecticide from C. *cinerariaefolim* exhibited *in vitro* anti HSV activity. In this study also both extracts when tested for their effect on HSV-1 *in vivo* in mouse model at different concentrations, showed an activity that might be attributed to the presence of the secondary metabolites, the methanol extract at 10 mg/kg delayed onset of infection significantly, as does the reference drug (ACV 5 mg/kg). Both dose levels of the methanol extract delayed progression of infection significantly as does the reference drug, and kept the infection to its minimum level, but upon withdrawal of the extract on the 7th day there was progression of infection unlike the reference drug which was seen to contain the infection. The Aqueous extract at 50 mg/kg was also observed to contain progression of infection significantly, but upon withdrawal of the extract on the 7^{th} day the infection progressed. For both extracts, the initial onset of lesions were seen to be delayed at the lower concentration, while there was a delay on the initial score of zoster formation at the higher concentration for both extracts. The reason for this cannot be predicted in this study. In this experiment no mortality was observed in all the groups, this is because the infectious titer of the virus was chosen rather than the lethal titer.

Conclusion

In this study, *C. cinerariaefolim* tested positive for the presence of secondary metabolites. It also demonstrated a significant anti HSV -1 activity. It is therefore recommended that, further study is required for its mode of action. Also, this plant extract should be tested on those emerging Acyclovir resistant strains and on other members of the family *Herpesviridae* which are also concern for a public health.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

DNA damages promoted by the essential oil from leaves of *Casearia sylvestris* Sw. (Salicaceae)

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The *Casearia* species (Salicaceae) occur in the tropics and subtropics and their extracts are rich in clerodane-type diterpenes, known as casearins. According to the literature, extracts from *Casearia sylvestris* exhibit cytotoxic and genotoxic effects in different tumor cell lines, possibly related to the casearins. On the other hand, there are few studies related to the DNA damages of the essential oils from this species. This study is aimed at evaluating DNA damages promoted by the essential oil from leaves of *C. sylvestris* collected in Rio de Janeiro. The essential oil was obtained from fresh leaves (1.5 kg) by hydrodistillation for 2 h in a Clevenger-type apparatus, and analyzed both by gas chromatography coupled to a mass spectrometer (GC-MS) and gas chromatography coupled to a flame ionization detector (GC-FID). These analyses revealed a very diversified (*n* = 21 compounds) volatile fraction composed mainly of non-oxygenated sesquiterpenes (72.1%), and the major component was identified as α-humulene (17.8%). Genotoxicity was evaluated by the comet assay, showing DNA damages, mainly of classes 3 and 4 at 4.0 µg/ml (*p* < 0.05) according to the damage index (DI). This is the first demonstration of DNA damages in response to the essential oil of *C. sylvestris.*

Key words: A549, α-humulene, comet assay, sesquiterpenes.

INTRODUCTION

Casearia sylvestris Sw. (Salicaceae) can be found throughout the Brazilian territory, including the State of Rio de Janeiro (Marquete and Vaz, 2007; Marquete and Mansano, 2013), and is popularly known as "guaçatonga". This species presents several medicinal properties, according to ethnobotanical surveys (Tomazi

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et al., 2014). It is one of the 71 plants of interest of the Brazilian Public Health System (Sistema Único de Saúde (SUS)), due to its wide use by the population to treat different conditions, such as herpes and tumors (Pereira et al., 2016; Ferreira et al., 2016).

Recent studies reported that the essential oils of *Casearia* genus are rich in sesquiterpenes, while monoterpenes are rare and arylpropanoids are absent (Esteves et al., 2005; Tininis et al., 2006; Sousa et al., 2007; Silva et al., 2008). In addition, the sesquiterpenes showed cytotoxic activity against different tumor cell lines (Silva et al., 2008; Bou et al., 2013) as well as genotoxic effects (Péres et al., 2009; Maistro et al., 2010; Ortiz et al., 2016). However, although this species is widely used by the Brazilian population, possible genotoxic effects promoted by the essential oil from leaves have not been investigated so far.

MATERIALS AND METHODS

Plant collection

C. sylvestris Sw. (Salicaceae) was collected in Tijuca National Park (S22°57'05.04" W43°17'10.09"), Rio de Janeiro, Brazil (SISBIO license n. 38765-1 / CGEN license n. 010105/2014-0). Plant identification was performed by Dr. Ronaldo Marquete, and the herbarium voucher was deposited in the Botanical Garden Herbarium of Rio de Janeiro, with registration number RB 570651.

Essential oil extraction and analysis

Fresh leaves of *C. sylvestris* (1.5 kg) were collected in June 2014, chopped into to small pieces and led to hydrodistillation in a modified Clevenger-type apparatus for 2 h. Essential oil was directly separated from the aqueous phase yielding 1.2% (w/v), transferred to amber flasks and kept at low temperature (-20°C) until analysis. Essential oil was subjected to analysis by gas chromatography coupled to flame ionization detector (HP-Agilent 6890, GC-FID), and by gas chromatography coupled to mass spectrometry (HP Agilent GC 6890 – MS 5973), at the Analytical Platform of Institute of Pharmaceutical Technology, Farmanguinhos, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. Briefly, the essential oil was diluted in dichloromethane (1.0 mg/ml) and analyzed by GC-MS to obtain the mass spectra and to perform chemical characterization. Concomitantly, another sample of essential oil (0.5 mg/ml) was analyzed by GC-FID for quantification of chemical constituents and to determine the retention indices (RI). The relative abundance of each essential oil component in the sample was quantified based in the individual component's relative peak area in the chromatogram. The substances in the essential oil were identified by comparing their mass spectra with database registration (WILEY7n) and by comparison of calculated Retention Indices (RI) with those from the literature (Adams, 2001; Pereira et al., 2016). RI were calculated from GC data of a homologous series of saturated aliphatic hydrocarbons within C8 to C20 (Sigma-Aldrich), performed using the same column and conditions used in the GC analysis for the essential oils, and with the equation proposed by Van dendool and Kratz (1963).

Evaluation of cell viability by the clonogenic assay

The A549 cell lineage was obtained from Microbiology Department from the Rio de Janeiro State University (Brazil). The cytotoxic potential of the essential oil was measured by the mitogenic capacity of treated cells using the clonogenic assay (Franken et al., 2006).

Comet assay

The procedure of comet assay was performed as described by Tice (2000), Cestari et al. (2004), and Miyaji et al. (2004) with modifications. Briefly, for each slide, 50 nuclei were randomly observed, always from the left to the right side, and total scoring (TS) was by obtained multiplying the number of cells in each class (nx) by the damage class (TS = (0 × n0) + (1 × n1) + (2 × n2) + (3 × n3) + (4 × n4)), ranging from 0 to 150 (Dantas et al., 2002). Nuclei were visually classified according to the migration of fragments (tail size) to one of five classes as follows: class 0 (undamaged nuclei, absence of tail), class 1 (nuclei with short tails, smaller than their diameter), class 2 (tail length one to two times the diameter of the nuclei), class 3 (tails twice or the diameter of the nuclei), and class 4 (nuclei maximally damaged).

Statistical analysis

Statistical analysis was performed using the ANOVA and Tukey–Kramer multiple comparison tests by the statistical program InStat 3.01 version (GraphPad Software, San Diego, CA, USA). The significance level of $p < 0.05$ was adopted to compare data within the same experiment.

RESULTS

Essential oil chemical profile

According to GC-MS, GC-FID and Retention Indices (RI) analysis, it was possible to characterize 21 compounds, comprising 98.2% of the essential oil from leaves of *C. sylvestris* (Table 1).

Cytotoxic and genotoxic activities

The essential oil exhibited cytotoxic activity against A549 tumor cells, with EC_{50} of 4.0 µg/ml, and a dose dependent pattern ($r = -0.79$, $p = 0.03$), as determined by linear regression test. It is interesting to note that no cytotoxic effect was detected on Vero cells (African green monkey kidney, maximum nontoxic concentration ≥ 250 µg/ml) (Table 2). The essential oil also presented suppressive effects on the colony-forming ability on A549 cells after the treatment. The results of the comet assay are shown in Table 3. All types of damages could be observed, especially classes 3 and 4 ($p < 0.05$), when compared with the control.

DISCUSSION

The comet assay was performed to evaluate potential genotoxic effects of essential oils, considering that it is a

Table 1. Chemical composition of the essential oil from fresh leaves of *C. sylvestris*.

Components	RI_{calc}	RI_{lit}	%
Non-oxygenated sesquiterpenes	**n = 14**		**72.1**
α-Cubebene	1354	1351	7.2
α-Copaene	1382	1376	8.5
β-Cubebene	1394	1390	1.7
β-Elemene	1396	1391	3.8
(*E*)-Caryophyllene	1414	1418	7.6
γ-Elemeno	1426	1433	4.8
α-Humulene (α-caryophyllene)	1451	1454	17.8
Seichellene	1455	1460	2.4
γ-Muurolene	1474	1477	0.1
Germacrene D	1476	1480	3.1
Byciclogermacrene	1491	1494	3.1
γ-Cadinene	1508	1513	2.5
7-*epi*-α-Selinene	1513	1517	2.1
Germacrene B	1555	1556	7.4
Oxygenated sesquiterpenes	**n = 7**		**25.6**
Sphatulenol	1570	1576	11.8
Caryophyllene oxide	1575	1581	3.5
Humulene epoxide II	1600	1606	4.1
1-*epi*-Cubenol	1620	1627	1.8
γ-Eudesmol	1629	1630	2.6
14-Hydroxy-9-*epi*-β-caryophyllene	1664	1663	0.5
α-Bisabolol	1681	1683	1.8
Total of identified compounds n, %	**n = 21**		**98.2**

RI_{cal} = Retention Index values calculated;RI_{lit} = Retention index values from literature data.

Table 2. Cytotoxicity activity of the essential oil from fresh leaves of *C. sylvestris* on A549 cell line.

Samples	MNTC(µg/ml)	CC_{50} µg/ml (Vero cell)	EC_{50} µg/ml (A549)	CC_{50} µg/ml (A549)
Essential oil	≥250	>250	4.0	10.0
Doxorrubicina	0.05420	-	0.01358	0.02168

MNTC: Maximum non-toxic concentration; CC_{50}: 50% cytotoxic concentration; EC_{50}: effective concentration; A549: human lung carcinoma.

Table 3. Comet assay scores for the essential oil of *C. sylvestris* on A549 tumor cell line.

Samples	Number of cells		Classes				
	Analyzed	Tailed	0	1	2	3	4
Control	100	40	2	58	20	15	5
Essential oil (4 µg/ml)	100	79	6	15*	22	30*	27*

Class 0: Undamaged nucleus, no tail; Class 1: nucleus with a short tail; Class 2: tail length is 1 and 2 times the diameter of the head; Class 3: tail is longer than the diameter of the nucleus; Class 4: nucleus totallydamaged; *p<0.05.

rapid and sensitive method to measure DNA damages (Tice et al., 2000; Almeida et al., 2012). Evaluation of cytotoxicity by clonogenic assay, and DNA damage of the essential oil have also been investigated. Considering

that *C. sylvestris* is widely used in folk medicine, it is important to verify the toxic effects of its volatile fractions, especially considering that the National Sanitary Surveillance Agency (Agência Nacional de Vigilância Sanitária - ANVISA) recommends the assessment of possible mutagenicity of medicinal plants (Balbino and Dias, 2010). *C. sylvestris* essential oil is composed exclusively by sesquiterpenes and the main identified compounds were α-humulene (17.8%), spathulenol (11.8%), and α-copaene (8.5%) (Pereira et al., 2016). For instance, sesquiterpenes were also identified as the major compounds in the essential oils from the leaves of *C. sylvestris* collected in the Brazilian states of São Paulo, Santa Catarina and Minas Gerais (Tininis et al., 2006; Silva et al., 2008; Esteves et al., 2008; Bou et al., 2013). However, the analyzed essential oil from the state of Rio de Janeiro showed α-humulene (α-caryophyllene) as the major compound.

There are few studies related to cytotoxic activity of essential oils from Salicaceae in tumor cell lines (Silva et al., 2008; Bou et al., 2013; Nikolic et al., 2014; Hayan et al., 2016). This data suggested that sesquiterpenes have cytotoxic activity, and indicated that further chemical and biological studies with other species from *Casearia* genus should be undertaken.

In recent years, the clonogenic assay is frequently used in cancer research laboratories to determine the survival and proliferation of tumor cells after experimental treatments (Hoffman, 1991; Tian et al., 2016). In these experiments, treatment with essential oil of *C. sylvestris* at 4.0 µg/ml reduced the colony-forming ability of A549 cells to 6% of the value obtained with the control. This effect could be due to interference of essential oil in the membrane integrity, DNA fragmentation or mitochondrial depolarization (Li et al., 2014; Ruan et al., 2015).

According to literature, the clonogenic assay is the method of choice to determine cell reproductive death after treatment with cytotoxic agents (Franken et al., 2006). The comet assay is widely used to evaluate the genotoxic potential in animal tissues, especially in studies on cancer therapy (Fairbairn et al., 1995; Dantas et al., 2002). According to other studies, the essential oils cause damages on tumor cell lines. For instance it has also been detected that the MCF-7 cell line is not able to repair the lesions on DNA, possibly due to the production of epoxides which induces DNA damage caused by essential oils, as well as by lipid peroxidation of essential oil rich in sesquiterpenes on V79 cells (Péres et al., 2009; Ortiz et al., 2016). There is evidence that DNA damages can be caused due to the epoxide formation by the induction of epoxide hydrolase 1 (EPHX1) responsible for the detoxification of exogenous chemicals (Ortiz et al., 2016).

In addition, this assay is extremely recommended when the toxicity of the agent tested is unknown, especially when there is no available data on cytotoxicity or genotoxicity (Tice et al., 2000). To the best of our knowledge, this is the first DNA damage study of the essential oil of *C. sylvestris* in a tumor cell line using two assays to detect potential harmful effects.

Conclusion

The essential oil from leaves of *C. sylvestris* exhibited potent DNA damage on A549 tumor cell line.

Conflict of interests

The authors have not declared any conflicts of interests.

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Full Length Research Paper

Phytolacca octandra (L.), *Phytolacca dodecandra* (L'Herit) and *Balanites aegypiaca* (L.) extracts as potential molluscicides of schistosomiasis transmitting snails

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Schistosomiasis is a widespread parasitic infection whose intermediate host is aquatic snails and affects more than 250 million people worldwide. Although control of the snails with synthetic molluscicides is possible, it is not greatly preferred due to concerns of environmental toxicity and the relatively high cost of the chemicals. Conversely, organic plant-derived molluscicides are a better alternative that can be used to reduce the incidence of the disease. The objective of this study was to evaluate the molluscicidal activity of the plants *Phytolacca octandra*, *P. dodecandra* and *Balanites aegypiaca.* The major parts of the whole plant (berries, leaves, stems and roots) were collected, air dried to constant weight, macerated to a fine powder and extracted separately using methanol in soxhlet apparatus. The extracts were screened for activity using brine shrimp lethality test and thereafter tested for molluscicidal activity. There was no significant difference observed in the activity of the plant parts studied and of the three plant species in the brine shrimp lethality test. Similarly, no significant difference in molluscicidal activity of the plant parts studied and in the three plants against *bulinus* snails was detected. It was concluded that that the three plants can be used in the control of schistosomiasis transmitting snails.

Key words: Schistosomiasis, molluscicidal, *Bulinus* snails, brine shrimp, *Phytolacca octandra*, *Phytolacca dodecandra*, *Balanites aegypiaca*

INTRODUCTION

Globally, schistosomiasis is the most devastating tropical disease after malaria and intestinal helminthiasis (Jenkins-Holick and Kaul, 2013) with Steinmann et al. (2006) stating that in terms of number of people at risk and those infected, it is second to malaria. The disease is parasitic and caused by any of five species of

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Schistosoma trematodes, giving rise to acute and chronic diseases with clinical manifestations due to urinary (*S. haematobium*), intestinal (*S. mansoni*), or hepatosplenic involvements (Oliveira-Filho and Paumgartten, 2000). Steinmann et al. (2006) reported that more than 700 million people are at risk of infection whereas 207 million people are infected with the disease. According to World Health Organisation (WHO) (2016), the disease has been reported in 78 countries, whereby preventative chemotherapy is required in 52 endemic countries with moderate to high transmission. It is estimated that 90% of those requiring treatment live in Africa (WHO, 2016) and it is responsible for at least 200,000 deaths annually in sub-Saharan Africa (Jenkins-Holick and Kaul, 2013).

Schistosomiasis is regarded as a disease of poverty, occurring in areas that are remote and poverty-stricken with little or no safe water or sanitation and limited health care (Bruun and Aagaard-Hansen, 2008). Bruun and Aagaard-Hansen further stated that it is a disease classified by WHO as neglected by local, national and global actors. The lifecycle of the flatworms that cause human schistosomiasis involves a sexual stage in the human and an asexual stage in the freshwater snail host (Jenkins-Holick and Kaul, 2013). There are four main strategies to destroy the parasites: killing the worms in humans by anthelminthic drugs, killing the intermediate host snails by chemical (molluscicides) or biological control agents, stopping people infecting snails by preventing contamination of freshwater bodies with human waste and finally stopping infection of man by keeping people out of infested water bodies (Knopp et al., 2012).

Niclosamide is so far the only WHO recommended molluscicide but has disadvantages of requiring multiple applications for total elimination of the snails, making it time consuming and less cost effective (Inobaya et al., 2014). Oliveira-Filho and Paumgartten (2000) observed that although niclosamide is effective and less hazardous to environment and human health when compared with other molluscicides, it is costly for developing countries and is toxic to some non-target species like bacteria, algae, and first instar larvae of mosquitoes. Various researches have evaluated the effectiveness of plant-derived molluscicides for instance, Yadav and Singh (2011) noted that when the latex powder of *Euphorbia hirta* is used together with other substances (rutin, betulin, taraxerol and ellagic acid) it has potent molluscicidal activity on *Lymnaea acuminata* and *Indoplanorbis exustus* snails possibly due to neurotoxicity. *Euphorbia milii* latex was found to be a rather potent and selective plant molluscicide that is nontoxic to bacteria, algae, and first instar larvae of mosquitoes (Oliveira-Filho and Paumgartten, 2000).

Yadav and Singh (2014) found that sub-lethal exposure of *Jatropha gossypifolia* latex, leaf and stem bark and their different combinations with betulin, ellagic acid, rutin and taraxerol caused a significant reduction in survival and hatchability of snail *Lymnaea acuminata*, killing the snails and rendering them sterile. The objective of this study was to evaluate the molluscicidal activity of the plants *Phytolacca octandra*, *P. dodecandra* and *Balanites aegypiaca.*

MATERIALS AND METHODS

Description of plants

Balanites aegyptiaca (Fam. Balanitaceae) is an indigenous a tree or shrub up to 12 m high with yellowish-grey to dark brown vertical cracked bark. Shoots have strong green spines up to 9 cm long, usually alternate and opposite to petioles of the leaves. Leaves are bifoliate with obovate folioles, pubescent underneath in young and glabrous in mature leaves. Flowers are green-yellowish, a little over 1 cm in diameter, solitary or fasciculate with cymes born at the base of leaves or spines. The fruit is an ellipsoid drupe, 3 to 4 cm long, turning from green to yellow when mature with a brown, ellipsoid hard stone surrounded by yellowish edible pulp (Food and Agriculture Organisation. 2016). *Phytolacca dodecandra* (Fam. Phytolaccaceae) is an indigenous climbing or scrambling dioecious, semi-succulent shrub or liana with glabrous stem. Leaves are petiolate, alternate, simple, entire and exstipulate with ovate blade, rounded base and acute glabrous apex. Inflorescence is axillary or terminal raceme. Flowers are unisexual, 5-merous; male flowers with narrowly oblong sepals, apetalous, 10 to 20 stamens in 2 whorls and a rudimentary ovary; female flowers have oblong to ovate sepals, apetalous, rudimentary stamens, with superior ovary of 4 to 5 carpels, curved styles 1 to 2 mm long and linear stigmas. Fruits are in clusters of 4 to 5, 1-seeded berries with kidney-shaped shiny black seeds. (Prota, 2016).

Phytolacca octandra (Fam. Phytolaccaceae) is a bushy perennial, up to 1 m high believed to have been introduced from South America but now naturalised in Kenya. Leaves are alternate, narrowly elliptic-lanceolate, hairless, margin entire and petiole to 2 cm long. Inflorescences are in terminal or lateral, leaf-opposed racemes, 4 to 14 cm long. Flowers are bisexual, white to yellowish-green with 8 stamens in one whorl. Fruits are depressed globose, mostly 6-8-lobed, 5-8 mm in diameter, hairless, dark purple to black when ripe (Flora of Zimbabwe, 2016).

Collection of plants and snails

The plants *P. dodecandra* and *P. octandra* were collected in Njoro area of Nakuru County whereas the plant *Balanities aegyptiacca* samples were collected in Kitui County. The plants were identified at Egerton University. Snails were collected in Timboni dam in Kilifi County. The dam was chosen as it had a sandy beach, established vegetation such as water lilies and other floating vegetation which offer an ideal environment for the *Bulinus* spp. snails. The infection caused by *S. heamatobium* or urinary schistomiasis is very common in this area especially among school going children who swim and play in the infected waters of the dam. After collection, the snails were transported in a beaker, containing wet cotton wool to prevent dehydration, to the institute of Primate Research Malacogy Laboratory at Fort Jesus, Mombasa, where an indoor culture was established. Each aquarium measuring 45 by 32 cm and 16 cm deep, made of polycarbonate material was used whose inside surfaces were rough to provide a gripping surface for the snails. To each aquarium was put approximately 5 litres of dechlorinated water. The bottom was lined with about 2 cm deep sand substrate. Aeration was by a whisper number 100 air pump, which was operated at low pressure to create minimal turbulence in the aquaria. The room temperature was maintained between 26

and 27°C. The collected snails were fed with lettuce and kept in these aquaria for 24 h for acclimatisation process before they could be used for the bioassay procedure.

Preparation of plant materials

The plant materials were separated into berries, leaves, stem and roots and each part was packed in a clear labelled plastic bag. The parts were air dried for a period of six weeks after which the dried materials were ground using a hammer mill and the powdered samples were packed in clear labelled plastic bags ready for extraction.

Extraction of the plant material

A total of 250g of each grounded sample was extracted using methanol in a soxhlet apparatus for a period of eight hours. The solvent was removed in vacuo to give the concentrated methanol extract, which was used for bioactivity test, and the rest dried in an evacuated dessicator.

Bioassay

The World Health Organisation guidelines were followed in the bioassay tests. Each extract was tested at 200, 100 and 50 mg per litre of dechlorinated water. Three plastic containers plus one control were prepared for each extract concentration. Five acclimatised snails were placed into each of these containers with a total volume of 200 ml of the test extract solution. The snails were exposed to the test solution for 24 h at room temperature and normal diurnal lighting. The extract was decanted after 24 h and the snails rinsed twice with a stream of dechlorinated water. The snails were then fed with lettuce and left in clean water for a further 24 h in order to recover. Snails were considered dead if they remained motionless, not feeding, no heartbeat when observed under a microscope or shell looked discoloured at the end on the period. Control snails were not exposed to the test extract solution.

Isolation and purification of active compounds

The crude extracts of the different parts of the different plants were isolated and purified as follows. They were dissolved to give an aqueous solution. The solvents used were distilled water, *n*-butanol, methanol and diethyl ether which were used in the case of *P. octandra* and *P. dodecandra* berries and cold methanol was used with all other different plants' parts. Filtering gave a brown precipitate for *P. octandra* stem and white crystals for the other plant parts after washing with cold methanol, but also with pet-ether and ethyl acetate for *P. octandra* stem. The melting point was determined using a Sanyo melting point apparatus. Thin layer chromatography (TLC) analysis of the crystals was done with methanol and silica gel. Antimony chloride in concentrated hydrochloric acid (HCL) was used as the spraying reagent. Various chemical tests done included: test for triterpenoids using Liebermann-Buchard reagent (acetic anhydride in concentrated sulphuric acid), alkaloids using Dragendorff reagent (Bismuth nitrate and Potassium iodide in water) and test for saponins. Elemental analysis was carried out using the CE 440 Elemental Analyser Exeter Analytical instrument.

Screening the plants' extracts by use of brine shrimp lethality test

The extracts were tested at 1000, 100 and 10 ppm. Five test tubes and one control were prepared at each concentration for a total of 18 test tubes per extract. Fifty milligrams of the dried extract were weighed and dissolved in 5 ml of methanol. From this solution 500, 50, and 5 µl were transferred into test tubes corresponding to 1000, 100 and 10 ppm, respectively using a micro-pipette syringe. The solvent was evaporated using a blow drier. Extracts that were insoluble in methanol were dissolved in dimethylsalfoxide (DMSO)-AR and up to 50 µl per 5 ml of brine were used to avoid DMSO toxicity affecting the results. The prepared extracts were stored in a dry place.

A saline solution was prepared by dissolving 33 g of sea salt in one litre of water. Ten millilitres of this solution was transferred into a petri-dish on which two milligrams of brine shrimp eggs were sprinkled. The brine shrimps eggs were placed in an incubator and allowed to hatch for 24 h. After hatching ten brine shrimps larvae were transferred into each prepared extract tube using a Pasteur pipette. Five millilitres of the saline solution were then added into each of the test tubes containing the larvae and the extracts. The number of surviving larvae were then counted and recorded. From the data the % mortality was calculated using Abbott's formula after correction for control. The formula is given below:

$$\% \text{ Mortality} = \frac{(\text{sample mortality} - \text{control\% mortality})}{100 - \text{control\% mortality}} \times 100$$

Statistical analyses

Statistical analysis was done to determine LD_{50} using Probit procedure. These values were subjected to analysis of variance (ANOVA) using general linear model (GLM) procedure and the means separated by using Duncan's Multiple Range Test (DMRT). SAS proprietory software release 8.1 (1999 to 2000) was used in these analyses. Correlation was done on the mean LD_{50} and the percent mortality for the three plants and their parts.

RESULTS

Percent yield of the plant materials using Soxhlet apparatus

Soxhlet extraction method gave the highest per cent yield for the berries of *P. octandra* and *P. dodecandra*, and the lowest for *B. aegyptiaca* berries (Table 1). However, analysis of variance showed no significant ($P > 0.05$) difference in mean percent yields of the three plants.

Brine shrimp assay

Mean percent mortality for the berries, leaves, stem and roots of the plants

Calculation for the mean percent mortality after correction for control for the different plants parts showed that the leaves of *P. octandra* had the highest percent mortality when compared to other plants *(P. dodecandra and. B. aegyptiaca)* parts, whereas the lowest mean percent mortality was recorded in the stem of *B. aegyptiaca* (Table 2). The berries with the highest percent mortality were those of *P. octandra*. The stem of *P*. *octandra* was

Table 1. Percent Yield of the plant materials of the three plants.

Plant	Plant part	% yield	Mean % yield
P. octandra	Berries	30.95	15.27[a]
	Leaves	18.56	
	Stem	8.56	
	Roots	8.24	
P. dodecandra	Berries	30.89	14.04[a]
	Leaves	7.92	
	Stem	11.53	
	Roots	5.82	
B. aegyptiaca	Berries	2.75	9.76[a]
	Leaves	14.76	
	Stem	11.08	
	Roots	10.45	

[a]Means with the same superscript are not significantly different at 5 % level

Table 2. Mean percent mortality for the berries, leaves, stem and roots of each of the three plant species.

Plant part	Mean % mortality ± *SEM**		
	P. octandra	***P. dodecandra***	***B. aegyptiaca***
Berries	27.00±5.42	22.00±9.37	24.89±7.35
Leaves	37.67±2.83	18.00±2.34	35.11±8.57
Stem	32.5±6.21	21.44±6.59	11.23±2.89
Roots	32.19±4.49	32.89±3.02	36.02±2.20

*standard error of the mean

Table 3. Mean LD_{50} for the berries, leaves, stem and roots for the three plants species.

Plant part	Mean LD_{50} ± SEM		
	P. octandra	***P. dodecandra***	***B. aegyptiaca***
Berries	2.2627±0.7097	2.9865±0.0243	2.6839+0.1837
Leaves	2.4181+0.1923	2.5397+0.1466	2.6770±0.4604
Stem	2.4197+0.3446	2.8193+0.2517	2.8523±0.0954
Roots	2.5672+0.1815	2.5332+0.1089	2.0931±0.00

found to have the highest mean percent mortality whereas the roots of *B. aegyptiaca* were found to have the highest mean percent mortality. LD_{50} for the berries, leaves, stem and roots for the three plants species LD_{50} is the dose that kills 50% of the organisms exposed to the test substance within 24 h of exposure. Potent substances therefore, give lower LD_{50} values. Analysis of variance showed no significant difference ($P > 0.05$) in the mean LD_{50} values for the different plant parts that is the berries, leaves, stem and roots of the three plants (Table 3). The best mean LD_{50} value for the berries, leaves and stem were recorded for the plant *P. octandra* extracts. However, these were not significantly different ($P > 0.05$) from the LD_{50} values recorded for berries, leaves, stem of *P. dodecandra* and *B. aegyptiaca.* The plant *B. aegyptiaca* recorded the best mean LD_{50} value for the roots extract although it is not significantly different ($P > 0.05$) from the LD_{50} value of roots recorded for *P.*

Table 4. LD_{50} for the berries, leaves, stems and roots of the plants *P. octandra, P. dodecandra* and *Balanites. Aegyptiaca.*

Plant part	Mean LD_{50} ± SEM		
	P. octandra	***P. dodecandra***	***B. aegyptiaca***
Berries	1.4520±0.1011	1.1427±0.1356	0.9609±0.0020
Leaves	0.8846+0.0742	0.8749±0.0356	0.7975±0.0719
Stem	1.0438±0.0093	0.7305±0.1349	0.9415±0.0062
Roots	1.0310±0.1205	0.8885±0.0742	1.2482±0.3551

octandra and *P. dodecandra*.

Comparison of mean LD_{50} and mean percent mortality

Correlation analysis performed on the mean LD_{50} and the percent mortality for the three plants species and their parts showed significant negative correlation (P = 0.0345, r = -0.61178). This means that an increase in the LD_{50} value may be associated to a greater extent with a decrease in the percent mortality for the plants and plant parts studied.

Muluscicide test: LD_{50} values for the plant parts of the three plant species

Analysis of variance showed no significant difference (P > 0.05) in the mean LD_{50} values for the different plant parts of the three plants (Table 4). The best mean LD_{50} value for the berries and leaves were recorded in *B. aegyptiaca,* whereas *P. dodecandra* recorded the best mean LD_{50} for the stem and roots. However, these were not significantly different (P > 0.05) from the mean LD_{50} values recorded for the berries, leaves, stem and roots of *P. octandra,* the berries and leaves of *P. dodecandra* and the stem and roots of *B. aegyptiaca*.

DISCUSSION

From the experimental data, the regenerative parts of the plants appear to be equally active as any other part of the plants since no significant difference (P > 0.05) exists among the parts. This makes the regenerative parts suitable for development of new molluscicides since their exploitation would not endanger the plants which grow in many parts of the country. In the experiment, the extracts were tested within the range of between 1000 to 10 mg/L instead of the recommended 100 to 10 mg/L (WHO, 1961). This is due to the low activity recorded in the recommended range which could probably be as a result of very low concentration of saponins in the extracts as reported by Treyvaud et al. (2000). The mode of action of the extracts is probably haemolysis since the colourless extract solution turned reddish in colour six hours after exposure of the snails to the extract. These findings are consistent with those of Harborne (1984). In Andwa, Ethiopia a five year study of the effect of the application of *P. dodecandra* berries in local streams lead to the reduction of snail population which resulted to reduced prevalence of *S. mansoni* in children from 50% at the start of the project to 7% at the end and a reduction of incidence in the entire population of the area from 63 to 34% (Lemma, 1983). Similarly, Molla (2011) found that different plant parts of *Balanites aegyptiaca* were effective in reducing the population of schistosomiasis-transmitting snails. The use of these plants is supported by Lemma (1983) who reported that application of P. dodecandra berries in the control of the schistosomiasis-transmitting snails had no obvious adverse effects on other microflora and fauna of treated streams. From the results of molluscicidal activity, no significant (P > 0.05) difference exist between the plant parts (the berries, leaves, stem and roots) and the three plants studied. These results are consistent with those obtained in the Brine shrimp lethality in that no significant difference in activity was recorded between the plant parts and between the plants studied. These results are supported by earlier findings of the World Health Organization that the effect of a potential molluscicide on fish and shrimp can be used to determine the effectiveness of a compound (WHO, 1961).

CONCLUSIONS AND RECOMMENDATIONS

Since the regenerative parts are equally active, communities would be encouraged to use these parts which would lead to conservation of the plants in the country. Similarly, as there is no significant difference in the molluscicidal activity of the three plants studied, communities should be advised to conserve and utilize any of these plants that can grow well their locality for controlling schistosomiasis-transmitting snails.

It is possible to monitor active extracts for molluscicidal activity using Brine shrimp lethality test because it has the advantages of being rapid (24 h following introduction of the shrimps), inexpensive, and simple (for example no aseptic technic required). It also utilizes a large number of organisms for statistical consideration and requires no

special equipment and a relatively small amount of sample (20 mg for screening initial extracts at 1000 ppm, lesser amounts for active fractions). From this study, the following are recommended as areas of further research:

1. Testing the extracts and compounds against genus *Biomphalaria* snails which are intermediate host of fecal schistosomiasis.
2. Evaluating the miracidiacidal and cercariacidal properties of the extracts and compounds.
3. Spectroscopic (x-ray crystallography, NMR, Mass) analysis on the isolated compounds to determine their structure.

Conflict of interest

The authors have not declared any conflict of interests.

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