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CYTOTOXICITY AND *IN VITRO* ANTIAMOEBIC ACTIVITY OF *SENNA DIDYMOBOTRYA* CRUDE ROOT EXTRACTS IN COMPARISON WITH METRONIDAZOLE AGAINST *ENTAMOEBA HISTOLYTICA*

Lydia Bonareri Nyamwamu [a*], Moses Ngeiywa [a], Margaret Mulaa [a], Agola Eric Lelo [b], Johnstone Ingonga [b], Albert Kimutai [c], Michael Gicheru[d]

[a]University of Eldoret, Department of Biological Sciences, P.O. BOX 1125-30100, Eldoret, Kenya.
[b]Kenya Medical Research Institute, Centre for Biotechnology Research and Development, P.O. BOX 54840-00200, Nairobi, Kenya.
[c]University of Kabianga, Department of Biological Sciences, P.O. BOX 2030-20200, Kericho, Kenya.
[d]Kenyatta University, Department of Zoological Sciences, P.O. BOX 43844-00100, Nairobi, Kenya

***Corresponding author e-mail:** lydianyamwamu@yahoo.com

ABSTRACT

The cytotoxicity and an *in vitro* antiamoebic effect of *Senna didymobotrya* (Irwin Fresen) were studied. 300g of crushed *S. didymobotrya* was isolated in several solvents namely Dichloromethane, ethyl acetate, methanol, hexane and water extracts. The minimal inhibitory concentration (MIC) and IC_{50} of the extracts in comparison with metronidazole were then obtained on trophozoite of *E. histolytica*, HM-1: IMSS strain in LYI-S-2 medium. The MIC for *S. didymobotrya* dichloromethanolic, methanolic successive, hexanic and water extracts after 48 hours was 500mg/ml each while methanolic total had a MIC of 250mg/ml. MIC for metronidazole was 62.5mg/ml whereas ethyl acetate was inactive. The IC_{50} for *S. didymobotrya* dichloromethanolic, methanolic total, methanolic successive, hexanic and water extracts was 2.15, 3.63, 0.3, 0.46, 0.12 and 0.58 respectively. The IC_{50} for metronidazole was 0.03. *Senna didymobotrya* is effective on the trophozoites of *E. histolytica* species and the methanolic total extract exhibited the greatest antiamoebic activity, at the lowest MIC.

Keywords: Antiamoebic, *Entamoeba histolytica*, *Senna didymobotrya*

1. INTRODUCTION

Amoebiasis is a disease caused by *Entamoeba histolytica*, a parasitic protozoan that infects humans and is responsible for 40,000 to 110,000 deaths per year [1, 2]. Ten percent of infected persons exhibit clinical symptoms; 80% to 98% of these are intestinal, and 2% to 20% are extra intestinal [3]. *Entamoeba histolytica* is the pathogenic and the etiologic agent of amoebic colitis and liver abscess [4]. Differential diagnosis between *E. histolytica* and *Entamoeba dispar* species is essential both for treatment decision and public health knowledge [5]. The WHO suggested that *E. histolytica* should be specifically identified and, if present, treatment is crucial [6]. The incidence of amoebiasis has decreased significantly in recent years because of improved sanitation in many countries and the use of effective therapeutic agents. The World Health Organization and the Pan-American Health Organization recommend the treatment of all patients with confirmed *E. histolytica* infection, regardless of the presence of symptoms [3]. In spite of the effective therapeutic agents that are available for the treatment of amoebiasis, it still constitutes a global health problem [7]. The prevalence of amoebiasis varies from 1% in industrialized countries to 50%–80% in tropical countries [1].

Among parasitic infections, amoebiasis ranks third worldwide in lethal infection, after malaria and schistosomiasis [8, 9]. Although it is asymptomatic in 90% of cases, about 50 million people are estimated to suffer from the symptoms of amoebiasis [10]. These

infections result in 40 000–110 000 deaths annually [11]. Prevalence rates of amoebiasis are highest in developing countries in Asia, particularly the Indian subcontinent and Indonesia, the sub-Saharan and tropical regions of Africa, and areas of Central and South America [12]. The estimated number of infected cases may be much higher due to the lack of a sensitive and specific diagnostic test [13].

The current treatments of choice are one of a family of Nitroimidazole (usually metronidazole), nitrofurans, quinacrine or paromomycin [14].However, these drugs have been reported to cause mutagenicity in bacteria [15], and are carcinogenic in rodents [16]. It has been reported that metronidazole and also its hydroxy metabolites are potentially genotoxic and carcinogenic [17]. Moreover, it seems to act as an immunosuppressive agent in experimental rats, both in cell-mediated and humoral immune responses [18]. Therefore, there is need to develop a safe and effective alternative antiamoebic agent. For people in developing countries, medicinal plants are popular because their products are safe and widely available at low cost [19]. Some compounds extracted from medicinal plants already play an important role against infectious diseases for instance quinine from *Cinchona* species, and artemisinin from *Artemisia annua*; both are effective against malaria [20].

Senna didymobotrya (Fres.) Irwin & Barneby (syn *C. didymobotrya*; belonging to the family Fabaceae (Leguminosae) is a widely used medicinal plant in East Africa [21]. Worldwide, its potential as a medicinal plant are being utilized by traditional practitioners [21]. In Kenya, Kipsigis community has traditionally been using these plants to control malaria as well as diarrhea [21]. In addition, it has been used to treat skin conditions of humans and livestock infections as well [22]. In Congo, Rwanda, Burundi, Kenya, Uganda, and Tanzania, root decoction of these plants was used for the treatment of malaria, other fevers, jaundice and intestinal worm [21]. In addition, root or leaf mixed with water or decoction of fresh parts is used to treat abscess of the skeletal muscle and venereal diseases [23]. The plant is also useful for the treatment of fungal, bacterial infections, hypertension, hemorrhoids, sickle cell anemia, a range of women's diseases such as inflammation of fallopian tubes, fibroids and backache, to stimulate lactation and to induce uterine contraction and abortion [24]. In the present study, *S. didymobotrya* root extract was selected for investigation because it is routinely used to cure diarrhea in the Kipsigis traditional medical practice. It is, therefore, of interest to scientifically evaluate its cytotoxicity and its effect on amoebiasis for potential antiamoebic activity *in vitro* and *in vivo*. The effect of different solvents on the plant extract was also investigated.

2. MATERIALS AND METHODS

2.1 Entamoeba histolytica trophozoites culture

Entamoeba histolytica HM-1: IMSS strains were used in all experiments. The parasite and LYI-S-2 culture media were obtained from University of Boulevard, USA, France. The trophozoites were cultured axenically in screw-capped tubes at 35.5^0C on LYI-S-2 medium, supplemented with 10% (v/v) heat-inactivated bovine serum [25]. Subcultures were performed routinely at 48 hr intervals by replacing the medium without detaching the monolayer. Cells were harvested by replacing the medium with a fresh one, chilling on ice for 20 min, and inverting gently to detach the monolayer.

2.2 Collection of *Senna didymobotrya* roots

The roots of *S. didymobotrya* were collected randomly during the months of October-November, 2012 and were authenticated. The plant materials were taxonomically identified by a taxonomist and the voucher specimens were preserved at the Centre for Biotechnology Research and Development, Kenya Medical Research Institute (KEMRI) for future reference.

2.3 Preparation of extracts

The roots were washed, cut into small pieces and air-dried for three weeks under a shed. The dried specimens were shred using an electrical mill in readiness for extraction. The sample preparation and extraction procedure were carried out as described by Harbone, (1994) [26]. Briefly, Cold sequential extraction was carried out on plant material with analar grade organic solvents of increasing polarity, which included n-hexane, dichloromethane, ethyl acetate and methanol. Six hundred millilitres of n-hexane was added to 300 g of the shred specimen and flasks placed on a shaker and soaked for 48 h. The residue was filtered using a Buchner funnel under vacuum until the sample dried. The sample was soaked further with 600 ml of nhexane for 24 h until the filtrate remained clear. The filtrate was then concentrated under vacuum by rotary evaporation at 30 - 35°C [26]. The concentrate was transferred to a sample bottle and dried under vacuum; the weight of the dry extract was recorded and stored at -20°C until required for bioassay. The process was repeated sequentially for dichloromethane, ethyl acetate and methanol.

2.3.1 Solvent Extraction

300g of dried powder were taken in 600ml of hexane in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 190-220 rpm for 24 hrs. After 24hrs the supernatant was collected and the solvent evaporated. The residue obtained was collected and stored at 4 °C in airtight bottles. The process was repeated sequentially for ethyl acetate, Dichloromethane, methanol and hexane.

2.3.2 Aqueous Extraction

300g of dried powder were added to 600ml of distilled water in a conical flask and boiled on slow heat for 2 hrs. It was then filtered using No. 1 Whitman filter paper and centrifuged at 5000rpm for 10 min. After 6 hrs, the supernatant was collected at an interval of every 2 hrs pooled together and concentrated using a rotary evaporator. The residue obtained was collected and stored at 4 °C in airtight bottles.

2 .4 *In vitro* exposure and evaluation

A concentration range of 0-100mg/ml of plant extract were prepared in test tubes by serial dilution and air-dried under sterile conditions. To each of the test tubes LYI-S-2 medium was added. The contents of the test tubes were then turned into a suspension by using the ultrasound bath for 30 min and kept at rest for 24 h at 4° C. To each of the test tubes there was subsequent addition of 1.0×10^3 trophozoites of *E. histolytica* followed by incubation at 35.5° C for 24 h and 48 h [27]. The experimental controls were assigned in the following manner: I) LYI-S-2 medium+Trophozoites and II) LYI-S-2 medium + trophocytes+metronidazole.

After the 24 h and 48 h periods, the tubes were chilled for 20 min and the attached trophozoites detached by gentle inversion. The number of viable cells was determined by observation under the microscope using eosin 0.01% stain [28]. The inhibitory capacity of the extracts of *S. didymobotrya* were assayed using eosin, as a result of which viable trophozoites of *E. histolytica* remained clear whereas dead cells were light red in color [33]. The criteria for viability being motility and dye exclusion. The lowest concentration of each plant extract which completely inhibited the growth of trophozoites of *E. histolytica* was considered the minimum inhibition concentration (MIC) [27]. After determining the MIC, a concentration range of 125 -1000mg/ml were used. Growth rate (GR) was defined as the difference between the number of viable protozoa counted at 0h and after 24 h and 48 h. The percentage of growth inhibition (% GI) was calculated using the following formula [29]:

$$\%\ GI = 1 - \frac{GR\ Extract}{GR\ Control} \times 100$$

The experiments were performed in duplicate and repeated three times.

2.5 Cell toxicity

The cytotoxic concentration causing 50% cell lysis and death (CC_{50}) was determined for the extracts following the method described by Kurokawa *et al*. (2001) [30]. VERO cells are lineages of cells used in cell cultures and are used as host cells for eukaryotic parasites. Briefly, VERO cells were cultured and maintained in Minimum Essential Medium (MEM) supplemented with 10% FBS. The cells were cultured at 37^0C in 5% CO_2, harvested by trypsinization, pooled in a 50 ml vial and 100 µl cell suspension (1 x 10^5 cell/ml) put into 2 wells of rows A-H in a 96-well micro-titer plate for one sample. The cells were incubated at 37^0C in 5% CO_2 for 24 hours to attach, the medium aspirated off and 150µl of the highest concentration of each of the test samples serial diluted. The experimental plates were incubated further at 37^0C for 48 hours. The controls used were cells with no drugs, and medium alone (no drugs and no cells). Thiazolyl Blue Tetrazolium (MTT) reagent (10µl) was added into each well and the cells incubated for 2-4 hours until a purple precipitate was clearly visible under a microscope. The medium together with MTT was aspirated off from the wells, Dimethylsulfoxide (DMSO; 100µl) added and the plates shaken for 5 minutes. The absorbance was measured for each well at 562nm using a micro-titer plate reader [31]. Cell viability (%) was calculated at each concentration via an excel program as described by Mosmann (1983) [32] using the formula:

$$CV\,(\%) = \frac{\text{Average absorption of duplicate drug wells - Average absorption of blank wells}}{\text{Average absorption of control wells}}$$

3. RESULTS

3.1 Extraction of compounds of *Senna didymobotrya*

Three hundred grams of S. *didymobotrya* yielded 1.77g 3.85g, 7.35g, 6.23g and 1.52g and 7.5g of S. *didymobotrya* when extracted with dichloromethane, ethyl acetate, methanol total, methanol successive, hexane extract and water respectively. There was a significant difference ($p< 0.05$) in the yields obtained.

3.2 *In vitro* antiamoebic activity of crude extracts of *Senna didymobotrya* on *Entamoeba histolytica* trophozoites

Incubation of *E. histolytica* trophozoites at a concentration range of 250-1000 mg/ml of extracts caused growth inhibition. When the concentration range increased, the number of living trophozoites decreased significantly. However, population densities of the amoeba in the control tubes were increased on the average. The growth inhibition after 24 and 48 hours are shown in the Figures 1 and 2. Methanol total was the most active agent against the trophozoites of *E. histolytica* at 24hour period (Figure 1). The Minimum Inhibition Concentration (MIC) for methanol total after 48hr was 250mg/ml, whereas the MIC for metronidazole, the current drug of choice, was 62.5mg/ml after 48hour, respectively (Table 1).

There was a significant difference in antiamoebic activity between the different types of extracts with methanol total having the highest activity ($\rho<0.05$; $\rho= 5.94x10^{-6}$). There was also a significant difference between the different concentrations with 1000mg/ml being the most active concentration for all the extracts with the exception of ethyl acetate that demonstrated inactivity and with methanol total having the lowest concentration (250mg/ml; Figure 1; $\rho <0.05$; $\rho = 0.013$) after 24hour. There was also a significant difference in antiamoebic activity between the different types of extracts and concentrations ($\rho <0.05$; $\rho = 2.04x10^{-6}$, $\rho = 0.01$) respectively with 500mg/ml being the most active concentration after 48hour period with the exception of ethyl acetate that demonstrated inactivity and with methanol total having the lowest concentration (250mg/ml). However, there was no significant difference in antiamoebic activity between methanol total and the current drug of choice metronidazole (Figure 2; $\rho >0.05$; $\rho=0.39$) after the 48 hour period although at low concentration, (125ml) methanol total demonstrated antiamoebic inactivity.

When dissolved in Dichloromethane, Methanol successive, Hexane and Water extracts, *S. didymobotrya* was able to destroy the trophozoites of *E. histolytica*, but at higher concentrations (500 mg/ml 48 hr, respectively; Table 1). However, *S. didymobotrya* in ethyl acetate extract was inactive even at a higher concentration (Table 1). The inhibition appeared to be time and dose-dependent. Moreover, the results revealed that a longer period of exposure to *S. didymobotrya* at the same concentration decreases the number of viable trophozoites (Figures 1 and 2).

When the MICs activity of *S. didymobotrya* in the different extracts was compared with metronidazole, Methanol total exhibited the lowest MIC (250mg/ml; Table 1), whilst dichloromethane, methanol successive, hexane and water extracts exhibited an MIC of 500mg/ml (Table 1) each. However, ethyl acetate extract exhibited no antiamoebic activity even at a higher concentration. Metronidazole the current drug of choice had an MIC of 62.5mg/ml (Table 1).

3.4 Cytotoxicity of crude root extracts of *Senna didymobotrya*

The IC_{50} values of *S. didymobotrya* for dichloromethane, ethyl acetate, methanol, hexane and water extracts on trophozoites were significantly different ($p<0.01$; Table 2). The IC_{50} and MIC values *S. didymobotrya* in methanol total and hexane samples were significantly lower ($p<0.001$) but compared well with those of Metronidazole ($p>0.05$). The IC_{50} values of the cytotoxicity of the samples to VERO cells showed that the ethyl acetate extract was significantly less toxic ($p<0.05$) compared to metronidazole. However, *S. didymobotrya* in hexane and methanol total were significantly more toxic (had lower IC_{50}; $p<0.05$) although less toxic than metronidazole. The MIC values revealed that concentrations of below 500mg/ml were not toxic for all the *S. didymobotrya* in all extract solvents except ethyl acetate which did not produce tangible results.

4. DISCUSSION

In this study, we investigated the cytotoxicity and an *in vitro* antiamoebic effect of *S. didymobotrya* (Irwin Fresen), crude root extracts using different solvents. The aqueous extraction produced the highest yields compared to the other solvents. This is because water is more polar compared to the organic solvents hence its able to extract more compounds from a plant material [34]. This is in agreement to the study by Kigondu, [35], who obtained similar results. Water extracts produced the highest yields as compared to the organic solvents. When the organic solvents were compared, Methanol total produced the highest yields compared to the other organic solvents. This is in agreement with the study carried out by Korir *et al*., [34]. This activity could be explained by the ability of methanol to extract both polar and non polar compounds [34].

Parasite growth depends primarily on the number of active parasites, temperature and culture medium [36]. In this study, in similar experimental conditions, the results of the experiments in the study group were significantly different from those of the negative control group ($P<0.01$). In this study, methanol total

exhibited the greatest antiamoebic activity, at the lowest MIC (250mg/ml), which the methanolic total extract showed 100% lysis of trophozoites of *E. histolytica* at a concentration of 250mg/ml in comparison to the ethyl acetate extract which exhibited almost no antiamoebic activity. Behnia *et al*., [36], showed that Iranian *A. sativum* is effective on the trophozoites of *E. histolytica* species and the essential oil exhibited the greatest antiamoebic activity, at the lowest MIC.

The antiamoebic activity of *S. didymobotrya* crude root extracts of dichloromethane, methanol successive, hexane and water was lower than that of methanol total although better than that of the ethyl acetate extract which showed limited or no activity against *E. histolytica* trophozoites. This is in agreement with Rani [37], who obtained similar results, whereby the greatest activity was found in methanol extracts and even water extracts of the same plants displayed less activity. It is possible that the active components in *S. didymobotrya* have high polarity since the highly polar solvent (methanol) seems to extract compounds with activity against *E. histolytica* parasites [34].

The IC_{50} values of the cytotoxicity of *S. didymobotrya* extracts to VERO cells showed that ethyl acetate was significantly less toxic compared to metronidazole. However, hexane and methanol total were significantly more toxic but less toxic than metronidazole the current drug of choice for amoebiasis.

5. CONCLUSION

From the findings of this study concerning the antiamoebic property of *Senna didymobotrya*, it can be concluded that the methanolic total crude root extract seems to be a good antiamoebic candidate for amoebiasis treatment and inhibition of growth of *E. histolytica* is dose-dependent. However, some in vivo studies in regards to its effects on human need to be investigated.

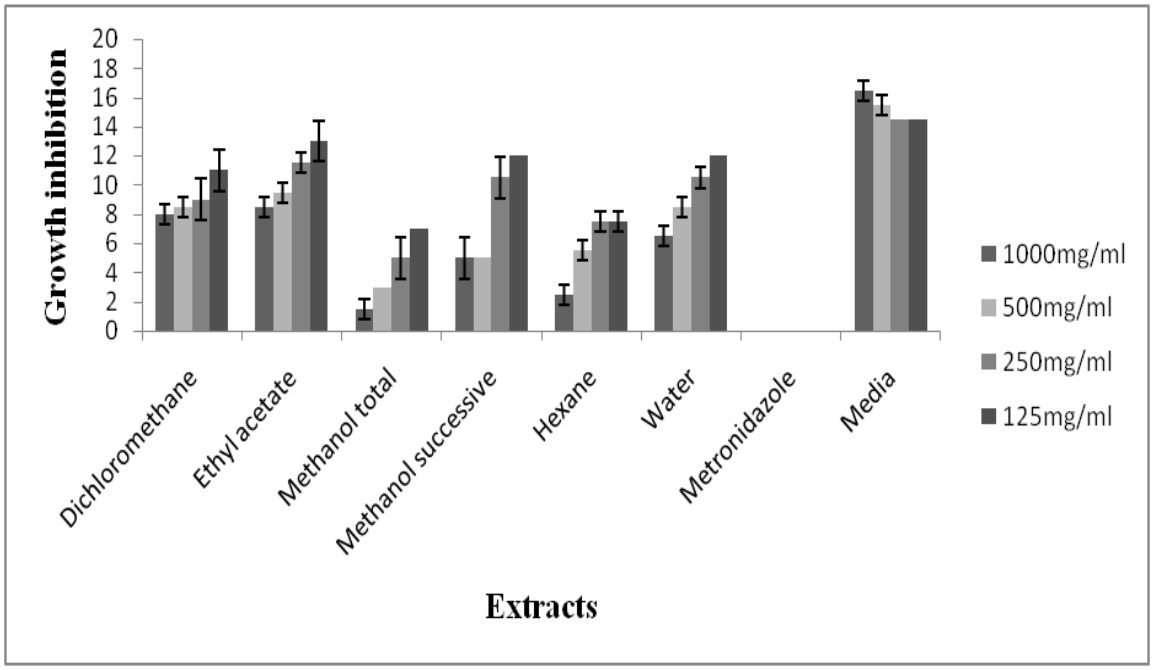

Figure 1: Antiamoebic activity of metronidazole and *S. didymobotrya* Crude root extracts at different concentrations on *E. histolytica* trophozoites at 24hour

Figure 2: Antiamoebic activity of metronidazole and *S. didymobotrya* Crude root extracts at different concentrations (mg/ml) on *E. histolytica* trophozoites at 48hr

Table 1. *In vitro* activity of *S. didymobotrya* root extracts against *E. histolytica* trophozoites

Extracts	MIC (mg/ml)
Dichloromethane	500
Ethyl acetate	Not active
Methanol total	250
Methanol successive	500
Hexane	500
Water	500
Metronidazole	62.5

Table 2. Cytotoxicity of extracts of *Senna didymobotrya* to VERO cells

Extract	IC_{50}
Dichloromethane	2.15
Ethyl acetate	3.63
Methanol total	0.3
Methanol successive	0.46
Hexane	0.12
Water	0.58
Metronidazole	0.03

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