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Mohammed I. Garbi. *et al.* / *International Journal of Biological & Pharmaceutical Research.* 2015; 6(10): 785-789.

e- ISSN 0976 - 3651
Print ISSN 2229 - 7480

International Journal of Biological & Pharmaceutical Research
Journal homepage: www.ijbpr.com

IJBPR

ANTIAMOEBIC AND CYTOTOXIC ACTIVITY OF *BAUHINIA RUFESCENS* (LAM) LEAF EXTRACTS

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ABSTRACT

The World Health Organization estimates that the protozoan is a major cause of morbidity worldwide, causing approximately 50 million cases of dysentery and 100,000 deaths annually. Intestinal amoebiasis due to the infection of E. histolytica is ranked third on the list of parasitic protozoan infections leading to death behind malaria and schistosomiasis. Metronidazole is used as a drug of choice against giardiasis. However, like a lot of other chemical agents, this drug has its own side effects. The present study was carried out to evaluate antiamoebic activites (*Entamoeba histolyica*) and cytotoxicity (MTT assay) of *Bauhinia rufescens.* Variety supreme court leaf petroleum ether and methanolic extracts. The highest activity against *Entamoeba histolytica*, with respect to time, was obtained from petroleum ether extract which exhibited 78.33% mortality within 72 h in 1000 ppm concentration, followed by the same extract which exhibited 75.12% mortality within 72 h with concentration of 500 ppm. On the other hand the lowest antiamoebic activity was recorded by methanol ether extract 60.20% mortality with 125 ppm concentration in 72 hours. The cytotoxicity of petroleum ether and methanol extracts had varying degrees of toxicity to Vero cell lines with IC_{50} 391.39µg/ml for the petroleum ether extract and 488.32µg/ml for the methanol extract. These studies conducted for *Bauhinia rufescens* leafs was proved to have potent activities against *Entamoeba histolyica* trophozoites *in vitro*. And MTT assay verified the safety.

Key Words: *Bauhinia rufescens*, *Entamoeba histolyica*, Cytotoxicity, Antiamoebic.

INTRODUCTION

The plant *Bauhinia rufescens* Lam is a scandent shrub or small tree belonging to the giant family Leguminosae, subfamily Leguminosae- caesalpiniodeae; usually 1-3 m high, sometimes reaching 8 m; often scraggy, stunted and multi-stemmed. Bark ash-grey, smooth, very fibrous and scaly when old, slash pink, twigs arranged in 1 plane like a fishbone, with thornlike, lignified, lateral shoots, 10 cm long. The leafs are very small, bilobate almost to base, with semi-circular lobes, glaborous, with long petioles, greyish-green, less than 3 cm long. Flowers are greenish-yellow to white and pale pink, petals 5, spathulate, 15-20 mm long; stamens 10, filaments

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hairy at the base. Fruits aggregated, long, narrow pods, twisted, up to 10 cm long, glaborous, obliquely constricted, shining dark red-brown, with 4-10 seeds each (Burkill, 1995).

The plant is deciduous in the drier area and evergreen in the wetter area, often found in the dry Savannah region, especially near streams or river banks; occuring throughout West Africa and extends across Africa up to Sudan. It has wide array of medicinal and socio-cultural uses. Several *Bauhinia* species are utilized as folk medicines worldwide, including Africa, Asia, South America and Central America, An extract of the root is used as an astringent or antipyretic in local medicine. Leaves and fruit are applied for the treatment of diarrhea, dysentery and ophthalmic diseases. The bark of the roots and trunk is used to cure chest complaints, syphilis and other venereal diseases, leprosy, diarrhea and dysentery and to reduce fever (Ayensu, 1978).

Medicinal plants are still invaluable source of safe, less toxic, lower price, available and reliable natural resources of drugs all over the world. People in Sudan and in other developing countries have relied on traditional herbal preparations to treat themselves. Therefore, it is useful to investigate the potential of local plants against these disabling diseases (Amaral *et al.,* 2006; Koko *et al.,* 2008).The infection of intestinal parasite is common in developing countries and hs negative effects on the feed and human health (WHO, 1984). Some of the intestinal parasites cause sudden and acute diarrhea which continues for many days as in the cases of amoebiasis (Abu-zeid *et al.,* 1989). The intestinal parasites may be cause anemia and a malnutrition (Aust *et al.,* 1974).

Intestinal amoebiasis caused by *E. histolytica* is ranked third after malaria and schistosomiaison on the list of parasitic protozoan infections leading to death (Farthing *et al.,* 1996). Amoebiasis is the infection of human gastrointestinal tract by *E. histolytica*; a protozoan parasite capable of invading the intestinal mucosa and that may spread to other organs, mainly the liver which usually leads to amoebic liver abscess. This infection remains a significant cause of morbidity and mortality world-wide (Stanley and Reed 2001). Amoebiasis is a rare occurrence in developed countries of the world, but only found in travelers, immigrants, homosexuals and institutionalized persons.

Bauhinia rufescens was selected to evaluate the activity of petroleum ether and methanol crude extracts against *Entamoeba histolytica* trophozoites and also the cytotoxicity against Vero cell line was evaluated.

MATERIALS AND METHODS

Plant materials

Bauhinia rufescens study was collected from West Sudan, on February 2012. The taxonomic identification of the plant was carried out at Medicinal and Aromatic Plants and Traditional Medicine Research Institute, National Center for Research by Dr. Hider Abdelgadir. A voucher specimen was deposited at the herbarium of the institute. The (Leafs) were air-dried at room temperature (28-30^{o}C) for three weeks and coarsely ground to powder by a mechanical grinder.

Preparation of crude extracts

30 grams of the coarsely ground material of the leaf were successively extracted for by soxhlet apparatus using petroleum ether and methanol. The extracts were then filtered and evaporated under reduced pressure using rotatory evaporator apparatus.

Parasite isolate

E. histolytica used in all experiments were taken from patients of Ibrahim Malik Hospital (Khartoum). All positive samples were examined by wet mount preparation. The positive sample was transported to the laboratory in Roswell Park Memorial Institute (RPMI 1640) medium. Trophozoites of *E. histolytica* were maintained in RPMI 1640 medium containing 5% bovine serum at 37 ±1^{o}C. The trophozoites were maintained for the assays and were employed in the log phase of growth.

In vitro susceptibility assays

In vitro susceptibility assays used the sub- culture method of (Cedillo *et al.*,2002), which is being described as a highly stringent and sensitive method for assessing the anti-protozoal effects (gold standard) particularly in *Entamoba histolytica*, *Gairdia intestinalis* and *Trichomonas vaginalis* (Arguello *et al.,* 2004).

5 mg from each extract was dissolved in 50 µl of dimethyl sulfoxzide (DMSO) at Eppendorf tube containing 950 µl distilled water in order to reach concentration of 5 mg/ml (5000 ppm). The concentrates were stored at -20°C for further analysis.

Sterile 96-well microtite plate was used for different plant extracts, positive control (metronidazole) and negative control (culture medium plus trophozoites).

Three out of 8 columns of microtitre plate wells (8 columns × 12 rows) were chosen for each extract, 40 µl of an extract solution (5 mg/ml) were added to the first column wells C-1: On the other hand, 20 µl of complete RPMI medium was added to the other wells the second column and third column (C-2 and C-3) . Serial dilutions of the extract were obtained by taking 20 µl of extract to the second column wells and taking 20 µl out of the complete solution in C-2 wells to C-3 wells and discarding 20 µl from the total solution of C-3 to the remaining 20 µl serial solutions in the successive columns. 80 µl of culture medium was complemented with parasite and added to all wells. The final volume in the wells was 100 µl. In each test metronidazole pure compound [(1-(2-hydroxyethl)-2-methyl-5 nitroimidazole], was used as positive control in concentration 312.5 ppm, whereas untreated cells were used as a negative controls (culture medium plus

trophozoites). For counting, the samples were mixed with Trypan blue in equal volume. The final number of parasites was determined with haemocytometer three times for counting after 0, 24, 48, and 72 h. The mortality % of parasite for each extracts activity was carried out according to the following formula:

Mortality of parasite (%) =

$$\frac{\text{(Control negative – tested sample with extract)}}{\text{Control negative}} \times 100\%$$

Cytotoxicity Screening

Microculture tetrazolium MTT assay was utilized to evaluate the cytotoxicity of the studied plants.

Microculture Tetrazolium (MTT) Assay

Principle of MTT assay

This Colorimetric assay is based on the capacity of Mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble, blue colored formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells (Patel *et al*,. 2009).

Preparation of Bauhinia rufescens extracts for MTT assay

Using a sensitive balance 5 mg of each extracts were weighed and put in eppendorf tubes. 50 µl of DMSO were added to the extract and the volume was completed to 1 ml with distilled water obtaining a concentration of 5 mg/ml. The mixture was vortexed and stirred by magnetic stirrer to obtain a homogenous solution.

Cell Line and Culturing Medium

Vero (Normal, African green monkey kidney) cells were cultured in a culturing flask containing a complete medium consisting of 10% fetal bovine serum and 90% minimal essential medium (MEM) and then incubated at 37^{o}C. The cells were subcultured twice a week.

Cell counting

Cell counts were done using the improved Neubauer chamber. The cover slip and chamber were cleaned with detergent, rinsed thoroughly with distilled water and swapped with 70% ethanol, then dried. An aliquot of cell suspension was mixed with equal volume of 0.4% trypan blue in a small tube. The chamber was charged with cell suspension. After cells had settled, the chamber was placed under light microscope. Using 40 X objective, cells in the 4 large corner squares (each containing 16 small squares) were counted. The following formula was used for calculating cells:

$$\text{(Cells/ml) } N = \frac{\text{Number of cells counted X Dilution factor X } 10^4}{4}$$

MTT procedure

The monolayer cell culture formed in the culturing flasks was trypsinized and the cells were put in centrifuging tube and centrifuged for 5 minutes separating the cells from the supernatant that flicked out. 1 ml complete medium was added to the cells and all the cell suspension was contained in a basin. In a 96- well microtitre plate, serial dilutions of each extracts were prepared. 3 duplicated concentrations for each extracts i.e. 6 wells for each of 8 extracts. All wells in rows A, B and C were used in addition to first 4 wells from each rows D, E and F. The first 2 wells of row G were used for the negative control and the first 2 wells of row H were used for the positive control Triton X. 20 µl complete medium pipetted in all wells in rows B, C and mentioned wells of rows E and F. Then 20 µl from each extracts were pipetted in rows A and B and first 4 wells of rows E and F. 20 µl taken from row B were pipetted and mixed well in row C from which 20 µl were taken and flicked out. The same was done from E to F. After that 80 µl complete medium were added to all used wells. Then adjusting the cell account to 3000 cell/well, 100 µl of cell suspension were added completing all wells to the volume 200 µl. Now, we have duplicated three concentrations 500, 250, 125 µg/ml for each extract. Then the plate was covered and incubated at 37^{o}C for 96 hours.

On the fourth day, the supernatant was removed from each well without detaching the cells. MTT suspension stock (5 mg/ml) prepared earlier in 100 ml phosphate buffer solution (PBS) was diluted (1:3.5) in a culture medium. To each well of the 96-well plate, 50 µl of diluted MTT were added. The plate was incubated for further 4 hours at 37^{o}C. MTT was removed carefully without detaching cells, and 100 µl of DMSO were added to each well. The plate was agitated at room temperature for 10 minutes then read at 540 nm using microplate reader. The percentage growth inhibition was calculated using the formula below:

$$\% \text{ cell inhibition} = 100-\{(Ac-At)/Ac\} \times 100$$

Where, **At** = Absorbance value of test compound; **Ac** =Absorbance value of control.

Statistical analysis

All data were presented as means ± S.D. Statistical analysis for all the assays results were done using Microsoft Excel program 2007.

RESULTS

The yield % of *Bauhinia rufescens* leaf petroleum ether, methanol extract was 3.8, 23.9 respectively. The leafs of *Bauhinia rufescens* family (Leguminosae) was screened for antiamoebic activity

against (*Entamoeba histolytica*) trophozoites *in vitro*. And cytotoxicity using 3- (4, 5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) agents Vero cell line.

Antiamoebic activity of *Bauhinia rufescens* extract: The antiamoebic potential of the petroleum ether and methanol extract of *Bauhinia rufescens*, with different concentrations (500, 250 and 125 ppm) and Mertronidazole (the reference control) with compared with Metronidazole which gave 96% inhibition at concentration 312.5 µg/ml at the same time against *Entamoeba histolytica* (Figure 1-2).

The maximum concentration used was 500 µg/mL. When this concentration produced less than 50% inhibition, the IC_{50} cannot be calculated.

This table indicates the % inhibition of Vero cell line growth *in vitro* by methanolic extract and Petroleum ether extract of the *Bauhinia rufescens.* MTT colorimetric assay was used. Reading in triplicate for different concentrations 500-125 µg/mL. The result of MTT assay verified the safety of the examined extract of petroleum ether and methanol extract.

Table 1. Inhibition percentage and IC_{50} of *Bauhinia rufescens* MTT assay against Vero cell line

Name of plant	Concentration (µg/ml)	Petroleum ether		Methanol	
		Inhibition (%) ± SD	IC_{50} (µg/ml)	Inhibition (%) ± SD	IC_{50} (µg/ml)
Bauhinia rufescens	500	55.31 ± 0.21	>100	56.20 ± 0.31	>100
	250	40.12 ± 0.65		38.31 ± 0.15	
	125	32.08 ± 0.16		30.71 ± 0.12	
*Control		95.3 ± 0.01		95.3 ± 0.01	

Key: *Control = Triton-x100 was used as the control positive at 0.2 µg/mL.

Fig 1. *In vitro* activity of *Bauhinia rufescens* petroleum ether extract against *E.histolytica*

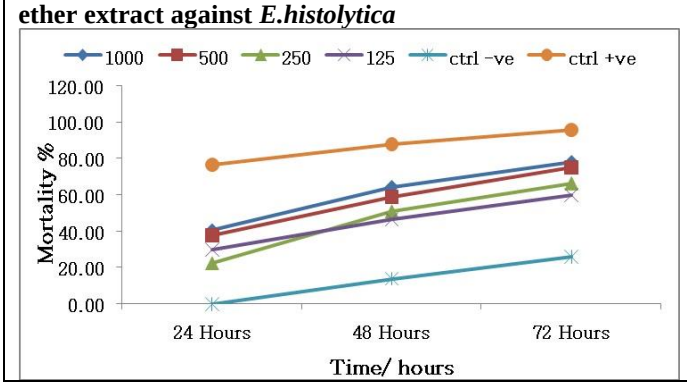
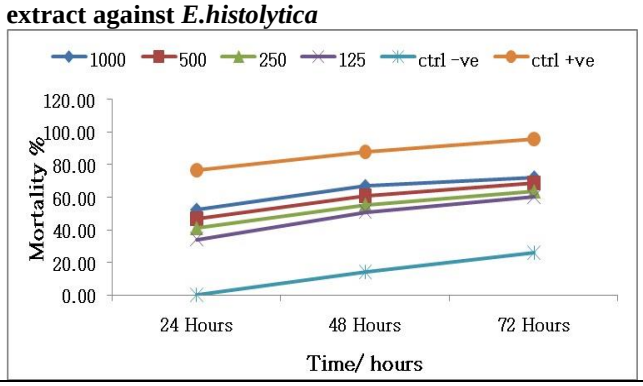


Fig 2. *In vitro* activity of *Bauhinia rufescens* methanol extract against *E.histolytica*



DISCUSSION

In the present study, methanol extract of leaf were found to have a wide range of percentage yield in comparison to their respective petroleum ether extracts. This might be associated with the high content of polar constituents in the matrices of the plant materials. The leaf extract were found greenish in colour with the exception of the petroleum ether and methanol extracts. The problems of treating of gastrointestinal diseases by chemotherapy are well known. Therefore, new, safer, and more effective drugs are necessary. In this context medicinal plants have made and are continuing to make important contributions to this area of therapeutics.

In the present work, petroleum ether extracts of *Bauhinia Rufescens* leaf studies have more mortality effect than methanol extract against *Entameba hostaletica*. This may be due to the ability of the petroleum ether to elute a wide range of chemical constituents from the plant leaf while the methanol might have extracted less numbers of the ingredients. (Maydell and Von, 1986), they said that *Bauhinia Rufescens* root and fruit for treatment of diarrhea, dysentery and ophthalmic or as tonic .The bark of the root is used to cure chest complaints, syphilis and other venereal diseases, leprosy and reduce fever, the fruit against dysentery and venereal diseases -Some of these diseases such as, dysentery and venereal diseases -this study did not confirm which type of venereal disease are protozoal diseases and may possibly confirm the antiprotozoa activity of the plant. Indeed, it could explain the antiprotozoal activity of *Bauhinia Rufescens* on different classes of protozoa such as *Giardia lamblia* and *Entameba hostaletica.* (Maydell and Von, 1986).

This is the first report of antiamoebic activities of the *Bauhinia Rufescens* extracts was investigated using *in vitro* bioassays that included the standard drug, metronidazole as positive control. The *Bauhinia Rufescens* extracts against *Entamoeba histolytica* given in Fig (1-2). Both petroleum ether and methanol extracts of *Bauhinia Rufescens* were highly effective against *Entamoeba*

histolytica and were less efficient than metrondizole. In other studies the extracts of *Bauhinia Rufescens* stem bark was tested for gram-positive and gram-negative bacteria and showed moderate inhibiting activity. Other study on *B. rufescens* had a antibacterial substances compared to root of *B. racemosa* and bark of *B. variegate* (Jain *et al.*, 2008).

Apparently, the introduction of the *Bauhinia Rufescens* extracts Showed effect against the aforementioned parasites compared with the standard drug metronidazole. In addition, the *Bauhinia Rufescens* extracts were non-toxic to the Vero cell lines this result agree with (Aminu and Hasnah 2013), who studied the acute toxicity of the extract combination of *Bauhinia Rufescens* extract in brine shrimp larvae and the (LC_{50}, 2.52 mg/mL) value showed that the highest dose can be administered without lethal effect, indicating that the extract has became safe.

CONCLUSION

Our results revealed a moderate pharmacological activity against *Entamoeba histolytica* we suggested that the extracts have the potential of being used in parasitic infection. The results presented here providing motivation for further exploration of isolation active compounds, particularly as antiamoebic agents from *Bauhinia Rufescens* extracts with important advantages for the development of new anti-parasitic agents.

ACKNOWLEDGEMENT: None.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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