



International Journal of
Experimental Pharmacology

www.ijepjournal.com

**ANTI GIARDIAL, ANTIOXIDANT AND CYTOTOXICITY OF
ETHANOLIC LEAVES EXTRACT OF ACACIA NILOTICA (L)**

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ABSTRACT

Acacia nilotica (L) related to (family Fabaceae-Mimosoideae). The division of *Acacia nilotica* is Magnolophyta and class is Magnolipsida. The genus is *Acacia* and species is *nilotica*. The ailments treated by this plant include colds, congestion, fever, gallbladder, hemorrhage, hemorrhoids, leucorrhoea, ophthalmic, sclerosis and small pox. Acacia bark is drunk for intestinal pains and used for treating acute diarrhea. Other preparations are used for gargle, toothache, ophthalmic and syphilitic ulcers. The roots of Acacia are used to treat Tuberculosis. This study was carried out to evaluate anti giardial activity (*Giardia lamblia*), antioxidant (DPPH assay) and cytotoxicity (MTT assay) of ethanol extract of *A. nilotica* (leaves). The ethanol extracts of *A. nilotica* (leaves) was screened for its anti giardial activities (*Giardia lamblia*), antioxidant screening for their free radical scavenging properties using 2,2-Di (4-tert-octylphenyl)-1-picryl-hydrazyl (DPPH), while propyl galate was used as standard antioxidant and screened for their cytotoxicity using 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), was obtained from *A. nilotica* (leaves) ethanol extract which exhibited 100% mortality within 96 h, at a concentration 500 µg/ml; this was compared with Metronidazole which gave 96% inhibition at concentration 312.5 µg/ml at the same time. The tested antioxidant activity gave (65 ± 0.04 RSA%) in comparison to the control of propylgalate levels (88 ± 0.07RSA%) And MTT assay verified the safety of the examined extract. In conclusion: These studies conducted for both *A. nilotica* (leaves) was proved to have potent activities against *Giardia lamblia* trophozoites *in vitro*.

Keywords: *In vitro*, Anti giardial activity (*Giardia lamblia*), Metronidazole, Antioxidant activity (DPPH), Cytotoxicity activity (MTT-assay), *Acacia nilotica* (leaves), Sudan.

INTRODUCTION

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[1, 2].

Acacia nilotica (L) commonly called Acacia belongs to the family *Mimosaceae*. It is known as “garad” among the speaking people in Sudan. The plant is a tree with yellow mimosa-like flowers and long grey pods constricted between seeds. The bark and branches are dark with fissures. The branches bear spikes about 2 cm long. The leaves are five and densely hairy with 3 - 6 pairs of pinnae consisting of 10 - 20 pairs of leaflets that are narrow with parallel margins that are rounded at the apex and with a central midrib closely crowded. The inflorescence consists of bright yellow flowers in auxillary head on stalks that are half way up. The flowering period of the plant is

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between November and March. The powdered bark of the plant with little salt is used for treating acute diarrhea [3].

Giardiasis is the most common cause of parasitic gastro-intestinal disease and it is estimated that up to two hundred million people are chronically infected with *giardia lamblia* globally, and 500,000 new cases reported annually [4]. *Giardia lamblia* is a major cause of diarrhoea in humans [5]. *Giardia* is a flagellate protozoan with worldwide distribution that causes significant gastrointestinal diseases in a wide variety of vertebrates including cats and human. Giardiasis is one of the intestinal protozoa that cause public health problems in most developing countries as well as some developed countries. *Giardia lamblia* is considered to be one of the leading causative agents of diarrhoea in both children [6, 7, 8] and adults [9, 10].

With the purpose of searching for new anti giardial agents, in the present work *A. nilotica* which are used traditionally for treatment of clinical signs associated with giardiasis were selected to evaluate the activity of their ethanolic crude extracts against *Giardia lamblia* trophozoites *in vitro*.

II. MATERIALS AND METHODS

Plant materials

The *A. nilotica* (leaves) were collected from central Sudan between January 2008 and February 2008. The plant was identified and authenticated by the taxonomists of Medicinal and Aromatic Plants Research Institute (MAPRI). All plant parts were air-dried, under the shadow with good ventilation and then ground finely in a mill until their uses for extracts preparation.

Preparation of crude extracts

Extraction was carried out for the leaves of *A. nilotica* by using overnight maceration techniques according to the method described by [11]. About 50 g were macerated in 250 ml of ethanol for 3 h at room temperature with occasional shaking for 24 h at room temperature, the supernatant was decanted and clarity field by filtration through a filter paper, after filtration, the solvent was then removed under reduced pressure by rotary evaporator at 55°C. Each residue was weighed and the yield percentage was calculated then stored at 4°C in tightly sealed glass vial ready for use. The remaining extracts which is not soluble by successively extracted by Ethanol using the previous technique. Extracts kept in deep freezer for 48 h, then induced in freeze dryer (Virtis, USA) until completely dried. The residue was weighed and the yield percentage was calculated. The extracts were kept in 4°C until the time of their use

Parasite isolate

G. lamblia used in all experiments were taken from patients from Ibrahim Malik Hospital (Khartoum). All taken samples were examined by wet amount preparation;

the positive samples were transported to the laboratory in nutrient broth medium. Trophozoites of *G. lamblia* were maintained in RPMI 1640 medium containing 5% bovine serum at 37 ± 1°C. The trophozoites were maintained for the assays and were employed in the log phase of growth.

Inoculum

G. lamblia was inoculated in the RPMI 1640 medium and incubated at 37 ± 1°C for 48 h. parasites were counted under the microscope by haemocytometer chamber.

In vitro susceptibility assays

In vitro susceptibility assays used the sub-culture method by [12], which is being described as a highly stringent and sensitive method for assessing the anti-protozoal effects (gold standard) particularly in *E. histolytica*, *Gairdia intestinalis* and *Trichomonas vaginalis* [13]. 5 mg from each extract and compound was dissolved in 50 µl of dimethyl sulfoxide (DMSO) at Eppendorf tube containing 950 µl D.W in order to reach concentration of 5 mg/ml (5000 ppm). The concentrates were stored at -20°C for further analysis. Sterile 96-well microtitre plate was used for different plant extracts, positive control and negative control. Three columns of a microtitre plate wells [8 columns (C) × 12 rows (R)] 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 were added to the other wells of 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 (a trichomonocide) pure compound [(1-(2-hydroxyethyl)-2-methyl-5 Nitroimidazole], a was used as positive control in concentration 312.5 µg/ml, 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 four times for counting after 24, 48, 72 and 96 h. The mortality % of parasite for each extracts activity was carried out according to the following formula:

$$\text{Mortality of cells (\%)} = \frac{(\text{Control negative} - \text{tested sample with extract}) \times 100\%}{\text{Control negative}}$$

Only 100% inhibition of the parasite was considered, when there was no motile parasite observed.

Antioxidant activity of plant extracts

DPPH radical scavenging assay: The DPPH radical scavenging was determined according to the method of [14] with some modification. In 96-wells plate, the test samples were allowed to react with 2,2-Di (4-tert-octylphenyl)-1-picryl-hydrazyl stable free radical (DPPH) for half an hour at 37°C. The concentration of DPPH was kept as 300 µM. The test samples were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517 nm using multiplate reader spectrophotometer. Percentage radical scavenging activity by samples was determined in comparison with a DMSO treated control group and Propyl Gallate (PG). All tests and analysis were run in triplicate.

Cytotoxicity Screening

Microculture tetrazolium MTT assay was utilized to evaluate the cytotoxicity of *A. nilotica* (leaves).

Microculture Tetrazolium (MTT) Assay:

Principle

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 [15].

Preparation of *A. nilotica* (leaves) Extracts, Solutions

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°C. The cells were subcultured twice a week.

Cell line used

Vero cells (Normal, African green monkey kidney).

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 10 X objective, cells in the 4 large corner squares (each containing 16 small squares) were counted. The following formula was used for calculating cells:

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

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°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°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:

$\% \text{ cell inhibition} = 100 - \left\{ \frac{\text{Ac}-\text{At}}{\text{Ac}} \right\} \times 100$ <p>Where, At = Absorbance value of test compound; Ac = Absorbance value of control</p>

Statistical analysis

All data were presented as means ± S.D. Statistical analysis for all the assays results were done using Microsoft Excel program. Student t test was used to determine

significant difference between control and plant extracts at level of $P < 0.05$.

RESULTS AND DISCUSSION

The leaves of *A. nilotica* (family: Mimosoideae) were screened for anti-giardial activity against (*giardia lamblia*) trophozoites *in vitro*, in comparison to the Mertronidazole (the reference control), antioxidant screening for their free radical scavenging properties using 2.2Di (4-tert-octylphenyl)-1-picryl-hydrazyl (DPPH), while propyl galate was used as standard antioxidant. And cytotoxicity using 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) Vero cell line, while triton-x was used as standard (control).

Antigiardial activity of *A. nilotica* (leaves) extract

Giardia lamblia is an important cause of acute and chronic gastrointestinal disease throughout the world and has been identified as the etiologic agent in numerous waterborne outbreaks of diarrheal disease. Although *G. lamblia* is among the most prevalent enteric protozoal infections in humans, it is relatively recently that improvements in the *in vitro* cultivation of this organism have allowed reliable, reproducible tests to assess the *in vitro* activity of therapeutic agents against *G. lamblia* [16]. Despite the previous comprehensive screening of Sudanese medicinal plants for their antiprotozoal activity [17 - 19].

The anti-giardial potential of the Ethanolic extract of the medicinal plant of *A. nilotica* (leaves) were extracted by ethanol, with different concentrations (500, 250 and 125

ppm) and Mertronidazole (the reference control) with concentration (312.5 µg/ml) to be investigated against *giardia lamblia* trophozoites *in vitro*. Ethanolic extracts of *A. nilotica* (leaves). showed 100% inhibition at a concentration 500 µg/ml after 96 h; this was compared with Metronidazole which gave 96% inhibition at concentration 312.5 µg/ml at the same time against *Giardia lamblia*. Fig (1).

This result proves the say; In Sudan garad had been used as anti-parasitic agent. The plant extracts were found to exhibit anti-diarrhoeal, antibacterial, antimalarial and inhibition of lipid peroxidation [19, 20, 21, 22 and 2].

Antioxidant activity of *A. nilotica* (leaves) extract

As shown in Table (2), the results of antioxidant activity *A. nilotica* (leaves) showed moderate antioxidant activity against the DPPH free radical (65 ± 0.04 RSA%) (Table 2).

Cytotoxicity assay of *A. nilotica* (leaves) extract

This table indicates the % inhibition of Vero cell line growth *in vitro* by ethanolic extract of *A. nilotica* (leaves). MTT colorimetric assay was used. Reading in triplicate for different concentrations 125-500 µg/mL.

Interestingly, the cytotoxicity assays were conducted in this study to evaluate the ethanolic extract of *A. nilotica* (leaves) their cytotoxicity effects by using MTT-assay include (Vero cell line). The result of MTT assay verified the safety of the examined extract.

Table 1. Preliminary quantitative data on the amount of *A. nilotica* (leaves) used in the anti-giardial activity, antioxidant activity and cytotoxicity study

Scientific Name of Plant	Family name	Part Used	Yield %	Traditional medicine
<i>Acacia nilotica</i>	Mimosaceae	leaves	7.50	skin diseases, diarrhea, dysentery, cough, diabetes, eczema, wound healing, burning sensation and as an astringent, demulcent, anti-asthmatic antimalarial patients as a tonic, antimicrobial, and dysentery.

This table indicates the scientific names, families, parts used, yield% of ethanol extract and traditional uses of *A. nilotica* (leaves).

Table 2. Antioxidant activity of *A. nilotica* (leaves)

No	Name of plant	Part	%RSA* ± SD (DPPH)
1	<i>A. nilotica</i>	leaves	65 ± 0.04
2	*Control	PG	88 ± 0.07

Key: RSA* = Radicals scavenging activity *Control = P.G = Propyl Gallate.

This table indicate the anti DPPH of ethanol extract of *A. nilotica* (leaves) the reading and propyl gallate was used as standard drug level. The tested antioxidant activity gave (65 ± 0.04 RSA %) in comparison to the control of propylgallate levels gave (88 ± 0.07 RSA %).

Table 3. Cytotoxicity of *A. nilotica* extracts on normal cell lines (Vero cell line) as measured by the MTT assay:

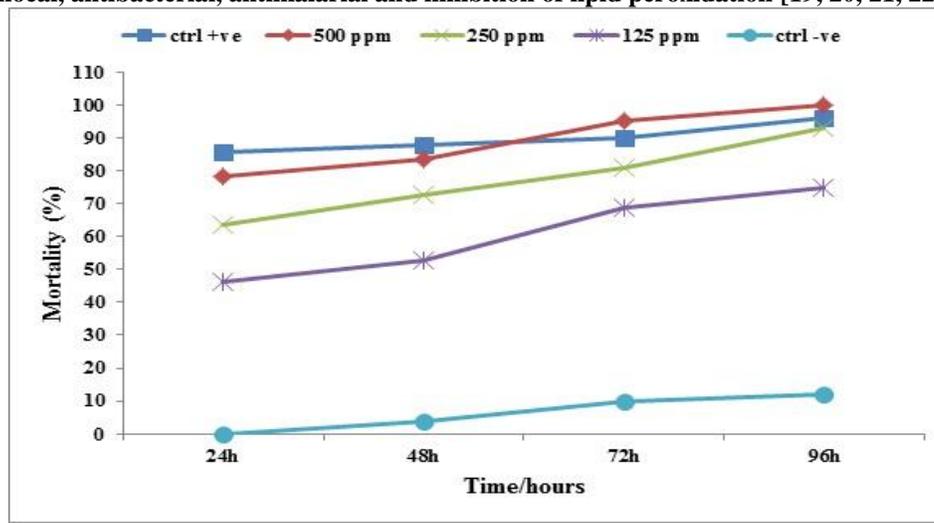
No.	Name of plant (part)	Concentration (µg/ml)	Absorbance	Inhibition (%) ± SD	IC ₅₀ (µg/ml)
1	<i>A. nilotica</i> (leaves)	500	1.43	50.9 ± 0.05	>100
		250	1.53	47.5 ± 0.03	
		125	2.07	29.1 ± 0.02	
2	*Control		0.14	95.3 ± 0.01	

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

The maximum concentration used was 500 µg/mL. When this concentration produced less than 50% inhibition, the IC₅₀ cannot be calculated.

Fig. 1. *In vitro* activity of *A. nilotica* (leaves) ethanolic extract against *Giardia lamblia*.

This result proves the say; In Sudan garad had been used as anti-parasitic agent. The plant extracts were found to exhibit antidiarrhoeal, antibacterial, antimalarial and inhibition of lipid peroxidation [19, 20, 21, 22 and 2].



CONCLUSION

This result enhances the ethno botanical uses of *A. nilotica* (leaves) as antidiarrheal in cases associated with Giardiasis in Sudan. Further investigations regarding the mode of action and other related pharmacological studies such as *in vivo* investigation, drug formulation and clinical trials are highly recommended.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Amel Mahmoud Abdrabo, Head department of Microbiology and Parsitology, Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI) Khartoum, Sudan.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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