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In-vitro Antimicrobial Activity 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 present study was conducted to investigate antimicrobial (bacteria and fungi) and cytotoxicity (MTT assay) of ethanol extract of *A. nilotica* (leaves). The ethanol extracts of *A. nilotica* (leaves) was screened of antimicrobial activity against four standard bacteria, two Gram-positive bacterial strains (*Bacillus subtilis* and *Staphylococcus aureus*), two Gram-negative bacterial strains (*Escherichia coli* and *Pseudomonas aeruginosa*) and two fungal strains (*Apergillus niger* and *Candida albicans*) using the cup plate agar diffusion method. And screened for the cytotoxicity using 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by using Vero cell line. The ethanol extract of *A. nilotica* (leaves). exhibited inhibitory effects against most of the tested organisms with zone of inhibition ranging from (21-28 mm). And MTT assay verified the safety of the examined extract. In conclusion: These studies conducted for both *A. nilotica* in the treatment of several bacterial and fungal.

Keywords: *In vitro*, antimicrobial activity and cytotoxicity, *Acacia nilotica* (leaves), Sudan.

1. Introduction
Acacia is the most significant genus of family: Leguminosae, first of all described by Linnaeus in 1773. It is estimated that there are roughly 1380 species of *Acacia* worldwide, about two-third of them native to Australia and rest of spread around tropical and subtropical regions of the world (Maslin *et al*, 2003: Orchard *et al*, 2003) [14]. *Acacia nilotica* (L) commonly called Acacia belongs to the family *Mimosaceae*. It is known as "Bagaruwa" among the "Hausa" speaking people of northern Nigeria. 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 between November and March. The powdered bark of the plant with little salt is used for treating acute diarrhea (Banso, 2009) [3]. The antibacterial activity of aqueous extract, different solvent extracts and isolated constituents of leaves of *A. nilotica* were evaluated and found to have high antibacterial activity (Raghavendra *et al.*, 2006) [16]. Extracts of *A. nilotica* were found effective against gram positive cocci and less effective against gram-negative bacilli, and antifungal activity against *Candida albicans* was found in the *n*hexane extract only (Mustafa *et al*., 1999) [13] and anti-*Plasmodium falciparum* activity (El-Tahir *et al*., 1999) [5]. The methanol extracts of *A. nilotica* showed significant inhibition against Gram-positive and Gram-negative bacteria (Abd el Nabi *et al*., 1992; Kambizi *et al*., 2001) [1, 10] including a moderate antimicrobial activity against multi-drug resistant *Salmonel typhi*. The decoction of the leaves used for astringent to the bowels, cure bronchitis, heal fracture, good for eye diseases. Bruised leaves applied to sore eyes in children. Paste of burnt leaves effective ointment in itch. Tender leaves beaten into a pulp are used as a gargle in spongy gums, sore throat and as wash in haemorrhagic ulcers and wound. Leaves extract is an astringent and injected to allay irritation in acute gonorrhoea and leucorrhoea. Further, bruised leaves formed into a poultice and applied to ulcer act as a stimulant and astringent. The tender leaves growing tops rubbed into a paste with sugar and water and given morning and evening act as a demulcent useful in coughs. The tender leaves

beaten into a pulp are given in diarrhoea as astringent (Farzana *et al*, 2014) [6]. The present study was conducted to investigate the antimicrobial and cytotoxicity of *A. nilotica* (leaves) in Sudan.

2. 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.

Tables (1) showed the yield % of ethanolic *A. nilotica* (leaves) antimicrobial and cytotoxicity investigated in this study.

Table (1): *Acacia nilotica* (leaves) investigated of antimicrobial and cytotoxicity

Scientific Name of Plants	Family name	Part Used	Yield %	Traditional medicine
Acacia nilotica	Mimosaceae	leaves	7.50 %	Antimalarial patients as a tonic, antimicrobial, diarrhoea and dysentery.

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

Preparation of crude extracts

Extraction was carried out for the fruits of *A. nilotica* plant by using overnight maceration techniques according to the method described by Harbone (1984). 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.

Test microorganisms

The ethanolic exracts solution of *A. nilotica* was tested against four bacterial species: two Gram-positive organisms, *Bacillus subtilis* (NCTC 8236) and *Staphylococcus aureus* (ATCC 25923), two Gram-negative bacterial strains *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) and two fungal strains *Apergillus niger* (ATCC 9763) and *Candida albicans* (ATCC 7596). The tested organisms were obtained from the Department of Microbiology, of Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI) and National Health Laboratory, Khartoum, Sudan.

The bacterial cultures were maintained on nutrient agar and inoculated at 37 °C for 18 h and then used for tests.

In vitro testing of extracts for antimicrobial activity

Testing for antibacterial activity

The cup-plate agar diffusion method (Kavanagh, 1972) was adopted with some minor modifications to assess the antibacterial activity of the prepared extracts. One ml of the standardized bacterial stock suspension 10^8 to 10^9 CFU/ml were thoroughly mixed with 100 ml of molten sterile nutrient agar which was maintained at 45 °C. 20 ml aliquots of the inoculated nutrient agar were distributed into sterile Petri-dishes. The agar was left to set and all of these plates 4 cups (10 mm in diameter) were cut using a sterile cork borer (No. 4) and agar discs were removed. The cups were filled with 0.1 ml sample of each extracts using automatic microlitre pipette, and allowed to diffuse a room temperature for two hours. The plates were then incubated in the upright position at 37 °C for 18 h. Two replicates were carried out for each extract against each of the test organisms. After incubation the diameters of the resultant growth inhibition zones were measured, averaged and the mean values were tabulated.

Testing for antifungal activity

The same method as for bacteria was adopted. Instead of nutrient agar, Sabouraud dextrose agar was used. The inoculated medium was incubated at 25 °C for two days for the *Candida albicans* and three days for *Aspergillus niger.*

Cytotoxicity Screening

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

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 (Patel *et al*, 2009).

Preparation of 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 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:

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

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. Student t test was used to determine significant difference between control and plant extracts at level of $P<0.05$.

3. Results and discussion

The ethanol extracts of the leaves of *A. nilotica* family (Mimosoideae) were screened for their antimicrobial activity against six standard microorganisms. Two Gram positive bacteria (*B. subtilis* and *S. aureus*), two Gram negative bacteria (*E. coli* and *P. aeruginosa*) and two fungi namely (*A. niger* and *C. albicans*) using the cup plate agar diffusion method. And cytotoxicity using 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT).

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

The ethanol extracts of the leaves of *A. nilotica* family (Mimosoideae) were screened for their antimicrobial activity against six standard microorganisms. Two Gram positive bacteria (*B. subtilis* and *S. aureus*), two Gram negative bacteria (*E. coli* and *P. aeruginosa*) and two fungi namely (*A. niger* and *C. albicans*) using the cup plate agar diffusion method.

As can be seen from Table (2) the extracts obtained from the leaves of *Acacia nilotica* exerted pronounced activity against all bacteria and fungi strains tested as indicated by diameter of growth inhibition zones that varied from (21-28 mm).The ethanol extracts of *Acacia nilotica* high activity against bacteria and fungi organism tested. This result was similar to that reported by El-Kamali *et al;* (2009) [4] but using different part of plant. Also similar to that reported by Rahman *et al*; (2012) [17] except that in their study *B. subtilis* was moderate. Howlader *et al*; (2012) [8] evaluation of antinociceptive antimicrobial and cytotoxic activities of ethanolic extract of *A. nilotica* (leaves) from Bangladesh by using disc mothed against gram negative bacteria (*Staphylococcus saprophyticus*, *Proteus spp, Escherichia coli, Staphylococcus aureus, Shigella Flexneri, Shigella Sonnei* and *Shigella boydii*) and gram positive (*Enterococcus faecalis, Streptococcus pyogenes, Streptococcus epidermidis* and *Streptococcus agalactiate*). Alavijeh *et al*; (2012) [2] the study of antimicrobial activity of few medicinal herbs by using different solvent (methanol) antibacterial activity against (*Bacillus subtilis, Escherchia coli, stphaylocuccus aureus and pseudomonas fluorescence*) and antifungal activity against (*Aspergillus flavus, Dreschlera turcica* and *Fusarium verticillioides*). The other study by Mahesh *et al*; (2008) [11] have reported *in vitro* activities of the *A. nilotica* (leaves) and methanol extract against other microorganisms by using disc mothed.

The result of minimum inhibition concentration from Table (2) showed that 12.5 µg/ml was the lowest concentration to inhibit the growth of all organisms tested. Comparison of observation given in Tables (3 and 4). Showed that the leaves extracts of *Acacia nilotica* dissolved in (DMSO) inhibited in ethanol inhibited all bacteria higher than 40 µg/ml Ampicillin and less Gentamicin. The leaves extracts of *Acacia nilotica* inhibited *P.s aeruginosa* higher than 40 µg/ml Gentamicin. The leaves extracts of *Acacia nilotica* inhibited *A.niger* similar to 20 µg/ml Clotrimazole and *C.albicans* higher than 50 µg/ml Nystatin. It is clear from Table 1 that the ethanolic extract of *Acacia nilotica* (leaves) showed high activity all bacteria and fungi and no toxicity.

Table (2): The antimicrobial activity of *A. nilotica* (leaves) against the standard bacterial and fungal:

Concentration (mg/ml)	Tested Bacteria				Tested Fungi	
	Gram (+ve)		Gram (- ve)			
	B.s	S.a	E.c	Ps.a	As.n	C.a
100	25	27	25	28	22	21
50	20	25	23	25	21	20
25	18	22	20	22	19	18
12.5	18	20	18	20	18	16

Key: Standard bacteria used: (*E.c = Escherichia coli, Ps.a = Pseudomonas aeruginosa, B.s = Bacillus subtilis, S.a = Staphylococcus aureus*, *C.a = Candida albicans* and *Asp.n = Apergillus niger*). MDIZ (mm) = Mean diameter of growth inhibition zone in mm. Interpretation of results: MDIZ (mm): >18 mm: Sensitive, 14 to 18 mm: Intermediate: <14 mm: Resistant. (-): No inhibition.

Table (3): Antimicrobial activity of *A. nilotica* (leaves) against the standard bacterial and fungal

Family/botanical/vernacular names	Part Extracted	Solvent system	MIZD* (mm)					
			Bacteria*				Fungi**	
			Gram (+ve)		Gram (- ve)			
			B.s	S.a	E.c	Ps.a	As.n	C.a
Mimosaceae (Gard)	leaves	Ethanol	25	27	25	28	22	21

***: Bacteria**: *B.s = Bacillus subtilis, S.a = Staphylococcus aureus, E.c = Escherichia coli, Ps.a = Pseudomonas aeruginosa.*
****: Fungi**: *A.n = Aspergillus niger* and *C.a = Candida albicans.*
MIZD*: Mean of Inhibition Zone Diameter in mm. Interpretation of results: MIZD*: 14 mm = Resistant; 18 mm = Sensitive; 14 to 18 mm = Moderate.

Table (4): Antibacterial and antifungal activity of reference antibiotics against standard microorganisms.

S. No	Drugs	Concentrations (µg/ml)	Standard microorganisms used MDIZ* (mm)			
			Tested bacteria used (M.D.I.Zmm)			
			Gram (+ve)		**Gram (-ve)**	
			B.s	**S.a**	**E.c**	**Ps.a**
1	**Ampicillin**	**40**	***15***	***25***	-	***16***
		20	14	***20***	-	***13***
		10	13	***18***	-	***12***
		5	12	***15***	-	-
2	**Gentamicin**	**40**	29	***35***	32	***23***
		20	22	***33***	30	***22***
		10	20	***30***	17	***21***
		5	17	***28***	-	***19***
			Tested fungi used(M.D.I.Zmm)			
			A.n		***C.a***	
3	**Clotrimazole**	**40**	30		42	
		20	22		40	
		10	***19***		3***3***	
		5	***16***		***30***	
4	**Nystatin**	**50**	28		17	
		25	26		14	
		12.5	23		-	

Key: Standard bacteria used: (*E.c = Escherichia coli, Ps.a = Pseudomonas aeruginosa, B.s = Bacillus subtilis, S.a = Staphylococcus aureus*, *C.a = Candida albicans* and *Asp.n = Apergillus niger*). MDIZ (mm) = Mean diameter of growth inhibition zone in mm. Interpretation of results: MDIZ (mm): >18 mm: Sensitive, 14 to 18 mm: Intermediate: <14 mm: Resistant. (-): No inhibition.

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

Howlader *et al*; (2012) [8] evaluation of antinociceptive antimicrobial and cytotoxic activities of ethanolic extract of *A. nilotica* (leaves) from Bangladesh by using brine Shrimp. Interestingly, the cytotoxicity assays were conducted in this study to evaluate the ethanolic extract of *A. nilotica* (leaves) their cytoxtoxicty effects by using MTT-assay include (vero cell line). The result of MTT assay verified the safety of the examined extract. Table (5).

Table (5): 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	***A. nilotica* (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 IC_{50} cannot be calculated.

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.

4. Conclusion

The leaves extracts of *A. nilotica* showed the various degree of inhibitory activity against the microorganisms tested. The obtained results may justify the use of the Sudanese leaves of *A. nilotica* as antimicrobial therapy in traditional medicine in Sudan and the neighboring countries. 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.

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