Advancement in Medicinal Plant Research
Vol. 2(3), pp. 50-57, September 2014
ISSN: 2354-2152
Full Length Research Paper

In vitro amoebicidal, antimicrobial and antioxidant activities of the plants *Adansonia digitata* and *Cucurbit maxima*

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Accepted 15 September, 2014

ABSTRACT

The present study was conducted to investigate the amoebicidal activities of two medicinal plants used in Sudan. The World Health Organization (WHO) estimates that the protozoan *Entamoeba histolytica* is a major cause of morbidity worldwide, causing approximately 50 million cases of dysentery and 100,000 deaths annually. Amoebiasis is due to infection with the protozoan parasite *E. histolytica*. The patients infected with *E. histolytica* must be treated right after definite diagnosis and no need to treat infected individuals with *E*. *histolytica* dispar isolates. Metronidazole is used as a drug of choice against amoebiasis. However, like a lot of other chemical agents, this drug has its own side effects. Whole plants of *Adansonia digitatata* (leave) and *Cucurbita maxima* (seed) were extracted by ethanol, with different concentrations (500, 250 and 125 µg/ml) and Metronidazole (312.5 µg/ml) to be investigated against *E. histolytica* trophozoites *in vitro*. Ethanolic extracts of *A. digitatata* (leave) exhibit 100% inhibition against *E. histolytica* at concentration 500 µg/ml after 72 h, this was compared with Metronidazole powder which gave 75% inhibition at concentration 312.5 µg/ml at the same time. Ethanolic extracts of *Cucurbita maxima* (seed) exhibit 100% inhibition at concentration 500 µg/ml after 96 h; this was compared with Metronidazole powder which gave 80% inhibition at concentration 312.5 µg/ml at the same time. The tested plants showed high antimicrobial activities to the tested bacterial and fungal strains. Moreover, *C. maxima* (seed) extract showed a moderate antioxidant activity via DPPH assay free radical test, however *Adansonia digitatata* (Leave) extract showed low antioxidant activity through the same test. On the other hand, no activity was presented in iron chelating assay. In conclusion, these studies conducted for both *A. digitatata* (leave) and *C. maxima* (Seed) were proved to have potent activities against *E. histolytica.*

Keywords: *In vitro*, amoebicidal, *Entamoeba histolytica,* Metronidazole, *Adansonia digitatata* (leave), *Cucurbita maxima* (seed).

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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 (Amaral et al., 2006; Koko et al., 2008).

The leaves of *Adansonia digitata* are important protein sources in complementing the amino acid profile and thereby improving the protein quality of the diet. Young leaves are commonly used as a vegetable in soups or cooked and eaten as spinach. Dried green leaves are used throughout the year, mostly in soups served with the staple dish of millet. Flowers can be eaten raw or used as flavour in drinks. In the folk medicine *A. digitata* is used in the treatment of fevers, diarrhea, malaria, haemoptysis and scorbutic complaints (vitamin C deficiency) and dysentery. Pulp extract is applied as eye-drops in cases of measles. In many medicinal uses, stem bark is used. When prepared it is made into a decoction for internal use and functions due to its soluble and insoluble tannin, and gummy and albuminious constituents beta-sitosterol has been studied and this occurs in the bark and also the seed oil. Root bark is also used in traditional medicine. This contains beta-sitosterol and two glycosides. The leaves form a component or herbal remedies and a mash prepared from the dried powdered roots is given to malarial patients as a tonic. A semi-fluid gum obtained from *A. digitata* bark is used to treat sores (Yusha'u et al., 2010).

The pumpkin, *Cucurbita maxima* Duchesne belongs to the family Cucurbitaceae. It is a large climbing herb, annual or perennial. Its aerial part consists of flexible succulent stem with trifoliate leaves. *C. maximum* is widely cultivated throughout India and in most warm regions of the world. Both of its fruits and the aerial parts are commonly consumed as vegetable. However, the plant has been used traditionally in many countries such as China, India, Yugoslavia, Brazil and America as antidiabetic, antitumor, antihypertensive, anti-inflammatory, immunomodulatory and antibacterial agents (Saha et al., 2011; Caili et al., 2006).

The treatment of Amoebiasis consists of the use of one or more drugs, with metronidazole being the first choice. Other Ntroimidazolic derivatives (secnidazole, Tinidazole and Ornidazole), Benzimidazoles (Albendazole and Mebendazole), Furazolin, Quinacrine and Paromomycin have also been employed in therapeutic regimens. However, these drugs have adverse effects including gastrointestinal disturbances, nausea, headache, leucopoenia, myopia, neuralgia, and allergic dermatitis and an unpleasant taste in the mouth. Furthermore, they can lead to neurotoxic effects, ataxia, convulsions and vertigo, bringing about the interruption of treatment. In addition, mutagenic and carcinogenic effects have been described in laboratory animals (Campanati and Monteiro-Leal, 2002; Upcrof et al., 2006).

Thus the need of alternative drugs to reduce their burden of purchasing the synthetic drugs especially after the problem of getting resistant to many clinical patients against Metronidazole (Iran and Kezaeian Mal Izaddoost, 2006; Tiwari et al., 2008) and thus new antiamoebical drugs are probably required. The World Health Organization (WHO) estimates that the protozoan *Entamoeba histolytica* is a major cause of morbidity worldwide, causing approximately 50 million cases of dysentery and 100,000 deaths annually (World Health Organization, 1997; Ravdin and Stauffer, 2005). 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 (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 Jr and Reed, 2001). Amoebiasis is a rare occurrence in developed countries of the world, but only found in travelers, immigrants, homosexuals and institutionalized persons. *E*. *histolytica*-associated dysentery is a common occurrence in the less developed and developing countries of the world, but is more common in areas of low socio-economic status, poor sanitation and nutrition especially in the tropics (Ravdin and Stauffer, 2005). Thus the majority of *E*. *histolytica* infections, morbidity and mortality occur in Africa, Central and South America and the Indian sub-continent (Haque et al., 2000).

Metronidazole is the drug now widely used and recommended in the treatment of amoebiasis (Townson et al., 1994). But it is less effective in the tissue than in the gut lumen (Bhopale et al., 1995). In addition, it can eradicate only up to 50% of laminae infections (Tierney et al., 1999). Metronidazole sometimes causes adverse effects, example, myoplasia, neuralgia, and allergic dermatitis (Upcrof et al., 2006). The present study was conducted to investigate the amoebicidal, antimicrobial and antioxidant activities of *A. digitatata* (leave) and *Cucurbit maxima* (seed) in Sudan.

MATERIALS AND METHODS

Plant materials

The leaves *A. digitata* and seeds of *C. maxima* 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 barks and fruits of selected plants 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.

Parasite isolate

E. histolytica used in all experiments were taken from patients of Ibrahim Malik Hospital (Khartoum). All taken samples were examined by wet mount preparation; the positive samples were transported to the laboratory in nutrient broth medium. Trophozoites of *E. histolytica* 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.

Inoculums

E. histolytica 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 of Cedillo-Rivera et al. (2002), 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* (Arguello-Garcia et al., 2004). 5 mg from each extract and compound was dissolved in 50 µl of dimethyl sulfoxzide (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-hydroxyethl)-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 three times for counting after 0, 24, 48, 72, 96, 120, 144 and 168 h. The mortality % of parasite for each extracts activity was carried out according to the following formula:

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

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

Antimicrobial activity of plant extracts

Microbial strains

The antibacterial activity of plants extract was assessed against four bacterial species: Gram positive (+ve) bacteria *Staphylococcus aureus* (ATCC 25923 American Type Culture Collection, Rockville, Maryland, USA) and *Bacillus subtilis* (NCTC 8236 National Collection of Type Culture, Colindale, England) and two Gram negative (-ve) bacteria: *Pseudomonas aeruginosa* (ATCC 27853 American Type Culture Collection, Rockville, Maryland, USA) and *Escherichia coli* (ATCC 25922), in addition to two fungal species: *Candida albicans* (ATCC 7596 American Type Culture Collection, Rockville, Maryland, USA) and *Aspergillus niger* (ATCC 9763 American Type Culture Collection, Rockville, Maryland, USA).

Preparation of bacterial suspensions

One ml aliquots of a 24 h broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37°C for 24 h. The bacterial growth was harvested and washed off with 100 ml sterile normal saline, to produce a suspension containing about 10^8 to 10^9 CFU/ml. The suspension was stored in the refrigerator at 4°C till used.

The average number of viable organisms per ml of the stock suspension was determined by means of the surface viable counting technique (Miles and Misra, 1938). Serial dilutions of the stock suspension were made in sterile normal saline solution and 0.02 ml volumes of the appropriate dilution were transferred by micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drops to dry and then incubated at 37°C for 24 h. After incubation, the number of developed colonies in each drop was counted. The average number of colonies per drop (0.02 ml) was multiplied by 50 and by the dilution factor to give the viable count of the stock suspension, expressed as the number of colony forming units per ml suspension.

Each time a fresh stock suspension was prepared. All the above experimental conditions were maintained constant so that suspensions with very close viable counts would be obtained.

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 at

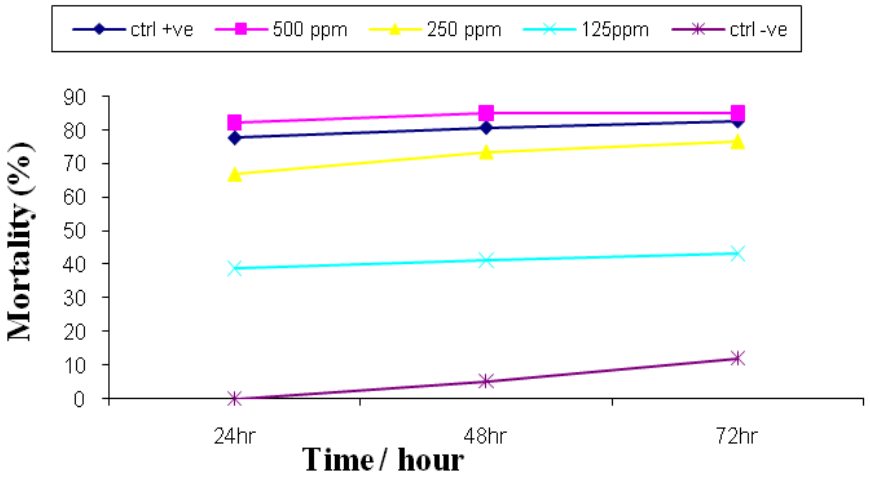

Figure 1. *In vitro* activity of *Adansonia digitata* (leave) ethanolic extract against *Entamoeba histolytica.*

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.*

Determination of minimum inhibitory concentration (MIC) by agar plate dilution method: The principle of the agar plate dilution is the inhibition of growth on the surface of the agar by the plant extracts incorporated into the medium. Plates were prepared in the series of increasing concentrations of the plant extract. The bottom of each plate was marked off into 6 segments. The organisms tested were grown in broth over night to contain 10^8 CFU/ml. A loop-full of diluted culture was spotted with a standard loop that delivers 0.001 ml on the surface of segment. The end point (MIC) is the least concentration of antimicrobial agent that completely inhibits the growth. Results were reported as the MIC in mg/ml.

Antioxidant activity of plant extracts

DPPH radical scavenging assay: The DPPH radical scavenging was determined according to the method of Shimada et al. (1992) with some modification. In 96-wells plate, the test samples were allowed to react with 2.2Di (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.

Iron chelating assay: The iron chelating ability was determined according to the modified method of Dinis et al. (1994). The Fe^{2+} were monitored by measuring the formation of ferrous ion-ferrozine complex. The experiment was carried out in 96 microtitre plate. The plant extracts was mixed with $FeSO_4$. The reaction was initiated by adding 5 mM ferrozine. The mixture was shaken and left at room temperature for 10 min. the absorbance was measured at 562 nm. EDTA was used as standard, and DMSO as control. All tests and analysis were run in triplicate.

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

Amoebicidal activity of plant extracts

The activity of ethanolic extract of *A. digitata* (leave) gave inhibition 100% after 96 h in concentrations of 500 and 250 ppm after 96 h, while in Mertronidazole (the reference control) 80% mortality was shown at the same time (Figure 1).

The activity of ethanolic extract of *C. maxima* (seed) gave inhibition 100% after 96 h in concentration of 500 ppm after 96 h, while in Mertronidazole (the reference control) 80% mortality was shown at the same time (Figure 2).

Antimicrobial activity of plant extracts

The antimicrobial potential of the Ethanolic extract of the medicinal plants *A. digitata* (leave) and *C. maxima* (seed) were evaluated against four standard bacterial strains and two fungal species which include: *Escherichia coli* (ATCC 25922) *Pseudomonas aeruginosa* (ATCC 27853), (ATCC 6380) *Bacillus subtilis* (NCTC 8236),

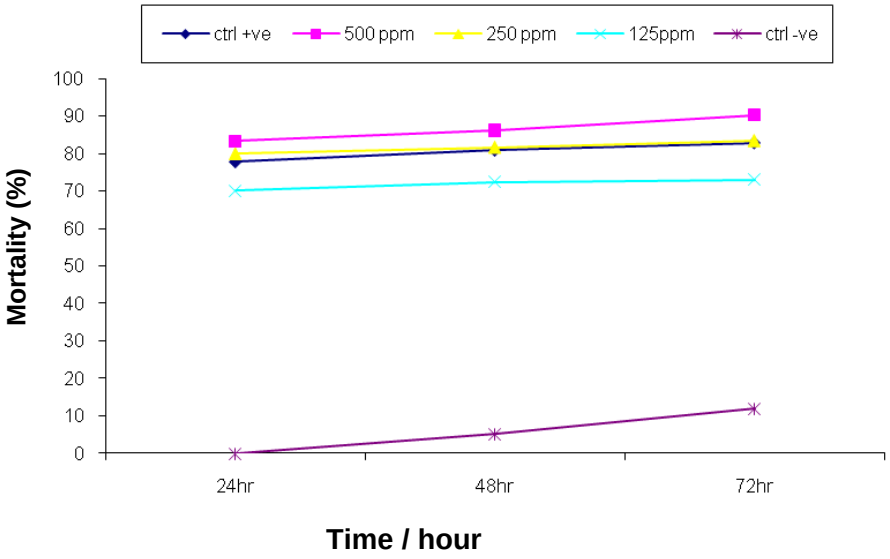

Figure 2. *In vitro* activity of *Cucurbita maxima* (seed) ethanolic extract against *Entamoeba histolytica.*

Table 1. Minimum inhibitory concentrations (MIC) of *Adansonia digitata* (leave) Ethanolic extract against the standard microorganisms.

Standard microorganisms	Concentration (mg/ml) Clear zone diameter (mm)			
	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml
Tested bacteria				
E.c	17	16	15	14
Ps.a	18	17	16	15
B.s	15	14	13	12
S.a	15	14	13	12
Tested fungi				
C.a	17	16	15	13
Asp.n	18	16	15	14

Key: Standard microorganisms (*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*).

Staphylococcus aureus (ATCC 25923), *Candida albicans* (ATCC 7596) and *Aspergillus niger* (ATCC 9763) (Tables 1, 2 and 3).

Antioxidant activity of plant extracts (DPPH)

As shown in Table 4, the results of antioxidant activity *C. maxima* (seed) extract showed moderate antioxidant activity against the DPPH free radical (38 ± 0.02 RSA%) however, *A. digitatata* (leave) extract detected to have a low antioxidant activity for the same test (13 ± 0.03 RSA %). On the other hand, no activity was presented in iron chelating assay (inactive).

DISCUSSION

Amoebicidal activity of plant extracts

The present study, ethanolic extracts of *A. digitata* (leave) exhibit 100% inhibition at concentration 500 and 250 µg/ml after 96 h, this was compared with Metronidazole (the reference control) powder which gave 80% inhibition at concentration 312.5 µg/ml at the same time (Figure 1).

In the present study, ethanolic extracts of *C. maxima*

Table 2. Minimum inhibitory concentrations (MIC) of *Cucurbita maxima* (seed) (leave) ethanolic extract against the standard microorganisms.

Standard microorganisms	Concentration mg/ml			
	Clear zone diameter (mm)			
	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml
Tested bacteria				
E.c	23	18	16	15
Ps.a	23	20	18	16
B.s	21	17	16	14
S.a	22	19	17	14
.				
Tested fungi				
C.a	20	18	16	15
Asp.n	22	19	18	16

Key: Standard microorganisms (*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*).

Table 3. Antibacterial and antifungal activity of reference antibiotics against standard microorganisms.

Antibiotic	Concentration (µg/ml)	*Standard microorganisms used **MDIZ (mm)			
		Tested bacteria			
		E.c	***Ps.a***	***B.s***	***S.a***
Ampicillin	40	18	-	15	23
	20	16	-	14	20
	10	13	-	13	18
	5	-	-	12	15
Tetracyclin	40	-	24	16	23
	20	-	19	13	21
	10	-	-	12	20
	5	-	-	-	18
		Tested fungi			
		E.c		***Ps.a***	
Nystatin	40	32		27	
	20	28		17	
	10	26		14	
	5	23		-	
Clotrimazole	40	42		30	
	20	40		22	
	10	31		20	
	5	28		-	

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.

(seed) exhibit 100% inhibition at concentration 500 and 250 µg/ml after 120 h, this was compared with Metronidazole (the reference control) powder which gave 95% inhibition at concentration 312.5 µg/ml at the same

Table 4. Antioxidant activity of plant extracts.

Material	%RSA* ± SD (DPPH)	%Iron chelating ± SD
Extracts of *Adansonia digitatata* (leaves)	13 ± 0.03	Inactive
Extracts of *Cucurbita maxima* (seed)	38 ± 0.02	Inactive
PG/EDTA	91 ± 0.01	98 ± 0.02

RSA* = Radicals scavenging activity.

time (Figure 2).

Antimicrobial activity of plant extracts

The mean diameter of inhibition zone produced by *A. digitata* (leave) and *C. maxima* (seed) extracts on the tested standard microorganism are presented. On the other hand, Table 1, 2 and 3 showed antimicrobial activity of the reference chemotherapeutic drugs against the test microorganisms. The results were interpreted as sensitive, intermediate and resistant. Based on the results of Table 2 and 3, plants extracts resulting in ≤15 mm mean diameter inhibition zone are considered to be active and those resulting in >15 mm are inactive [30]. The minimum inhibitory concentration (MIC) of the ethanolic extract of *A. digitata* leaves against standard microorganism, are shown in Tables 1, 2 and 3.

It is clear from Table 1 that the ethanolic extract *A. digitata* leaves showed high activity only against *Pseudomonas aeruginosa* and *Aspergillus niger*, whereas it was an intermediately active against *E. coli, Bacillus subtilis* and *Candida albicans*.

It is clear from Table 1 that the ethanolic extract of *C. maxima* seed showed high activity only against *P. aeruginosa* and *A. niger*, whereas it was an intermediately active against *E. coli, B. subtilis* and *C. albicans.*

It is clear from Table 1 that the ethanolic extract of *C. maxima* (seed) showed high activity all bacteria and fungi.

Antioxidant activity of plant extracts

To explain the health benefits attributed to both plants focused in this present work, antioxidant activity tests was carried for ethanolic extract of *A. digitata* leaves and ethanolic extract of *C. maxima* seed through DPPH and iron chelating assays. Our study confirm and verify previous study by Oloyede et al. (2010) exploring the antioxidant activity of the plant *A. digitata* in which the author predict moderate to low antioxidant activity using hexane, ethylacetate and water where prediction of high activity was obtained by using buanol (Oloyede et al., 2010). However, in this study, a different solvent was used which is ethanol. On the other hand, many studies were carried out on nutrient values of *C. maxima* specially the fruit part where been proven to have a potentially good antioxidant activity for their procession of ascorbic acid and some phenols (Yadav et al., 2010). Moreover, the antioxidant activity proven for the ethanolic *C. maxima seeds* extract through DPPH assay may add new scientific value for the plant with future recommendations for using throughout techniques and different solvents and exploration of active ingredients.

CONCLUSION

This result enhances the ethno botanical uses of the plant as antidiarrheal in cases associated with Amoebiasis in central 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 Mr. Mohammed Gharby, laboratory of International University of Africa. Mr. Mohammed Omer Medical Biochemistry Unit and laboratory of Microbiology, Medicinal and Aromatic Plants Research Institute (MAPRI), for their strong assistance with this study.

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Citation: Kabbashi AS, Koko WS, Mohammed SEA, Musa N, Osman EE, Dahab MM, Fadul Allah EF, Mohammed AKh, 2014. *In vitro* amoebicidal, antimicrobial and antioxidant activities of the plants *Adansonia digitata* and *Cucurbit maxima*. Adv Med Plant Res, 2(3), pp. 50-57.