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Antimicrobial and anti-diarrheal activity of some Sudanese medicinal plants from anacardiaceae family

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Abstract
In the present study, methanolic extract of Rhus abyssinica, Heeria insignis and Lannea schimperi leaves, stem and roots were evaluated by employing *in vitro* antimicrobial and anti- diarrheal activity. Standard methods were adopted for testing the bioactivity of the components towards four bacterial species (Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus), two fungal species (Aspergillus niger and Candida albicans), two protozons (Entamoeba histolytica and Giardia lamblia) Methanol extract of Lannea schimperi stem showed high antibacterial activity against Bacillus subtilis (27 mm) as compared with the standard antibiotics ampicillin (20 mm) and gentamicin (25 mm). %). Extract from Lannea schimperi leaves had potent antigiardial and antiamoebic activities (IC50 ≤ 100 µg/ml) against Giardia lamblia and Entamoeba histolytica, 80.82 and 92.21 µg/ml respectively.

Keywords: antimicrobial, anti- diarrheal, anacardiaceae family

Introduction
Medicinal plants provide a subsidy from nature that can be of great value to rural people and may be of value to global society as a source of new drugs. More than 50% of all the drugs in clinical use currently have a natural product origin [1]. The cultivation of plants as sources of drugs is the best and the most promising way to satisfy market's expanding demand for these raw materials. This is certainly the only applicable way in which we can stop their decline and secure their long-term survival. In addition, there will be an accelerated poverty reduction through creation of rural income [2].

African traditional medicine is the oldest and perhaps the most diverse of all medicine systems. Africa is considered to be the cradle or mankind with a rich biological and cultural diversity marked regional difference in healing practices. Unfortunately, the systems of medicines are poorly recorded and remain so to date [3]. Yet the documentation of medicinal uses of African plants is becoming increasingly urgent because of the rapid loss of the natural habitats of some of these plants because of anthropogenic activities. The African continent is reported to have one of the highest rates of deforestation in the world.

The oldest written information in the Arabic traditions comes from the Sumerians and Akkadians of Mesopotamia, thus originating from the same areas as the archaeological records [3].

Traditional medical practices play an important role in Sudan, and 90% of population particularly those who are living in frontiers and rural areas depend mainly on herbal medicine for the treatment of various types of diseases [4]. Sudan folklore-medicine represents a unique blend of indigenous cultures with Egyptian, Indian, Arabian, East and West African cultures. This in view of a number of factors, such as draught, desertification, expansion of Agricultural schemes and the introduction of health services to primitive areas; which initiated astonishingly rapid changes, leading to the least use of native medicines, which would eventually disappear. As in many developing countries, herbal drugs are of major importance in Sudanese folk medicine [5].

Literature Review
Reference strains of bacteria and fungi, Mueller Hinton agar Saboraud dextrose agar, Ampicillin, Ciprofloxacin, Fulcin and Gentamicin, dimethyl sulfoxide (DMSO), [(1-(2-hydroxyethyl)-2-methyl-5- nitroimidazole].

Collection and preparation
The roots, stem-bark and leaves of L. schimperi, R abyssinica and H. insignis were obtained from Erkowit 700 km east of Khartoum. The plant materials were dried under shade for 14 days except the roots of L. schimperi which was dried for 28 days. They were ground into fine powder in preparation for extraction. The finely powdered materials were weighted separately and stored at room temperature.

General Procedure of Extraction
Methanol crude A hundred g of the finely ground material was soaked in methanol solvent for 48 hours with occasional swirling to ensure through extraction. The soaked material was filtered and the crude extract collected in clean containers. The extract of each sample was weighed, transferred to small container and stored at room temperature until tested.

Antimicrobial tests
Four bacterial strains and two fungi were used as test organisms in this study.

Reference strains of bacteria

Table 1

Bacillus subtilis	**NCTC 8239**	**(Gram positive rods)**
Echerichia coli	ATCC 25922	(Gram negative rods)

Table 2

Staphylococcus aureus	**ATCC 25923**	**(Gram positive rods)**
Pseudomonas aeruginosa	ATCC 27853	(Gram negative rods)

Reference strains of fungi

Table 3

Candida albicans	**ATCC 7596**	**(Yeast fungi)**
Aspergillus Niger	ATCC 9763	(Filamentous fungi)

ATCC is the American Type Culture Collection, Rockville, Maryland, U. S. A

Standard antibiotic
Ampicillin, Ciprofloxacin, Fulcin and Gentamicin were used as a positive control for bacteria.

Determination of antimicrobial activity
Culture media
Nutrient broth

This medium contains peptone, yeast extract and sodium chloride. It was prepared according to Barrow and Feltham [6] by dissolving 13 grams of the medium in one liter of distilled water. The pH of the medium was adjusted to 7.4 and the medium was then distributed into screw capped bottles, 5ml of each was taken and sterilized by autoclaving at 121˚C for 15 minutes.

Nutrient agar
The medium contained lab-lemco powder (1.0 g), yeast extract (2.0 g), peptone (5.0 g) and agar No.3 (15.0 g). 28 grams of dehydrated medium were dissolved in one liter of distilled water and the pH was adjusted to 7.4. The dissolved medium was sterilized by autoclaving at 121˚C for 15 minutes.

Mueller Hinton agar
Mueller Hinton dehydrated media (38 g) was dissolved in liter of purified water and heated with frequent agitation. Media was sterilized at 121˚C for 15 minutes and cooled to 45 - 50˚C and dispensed into sterile Petri dishes.

Saboraud dextrose agar
The medium contained mycological peptone (10.0 g), dextrose (D-glucose) (40.0 g) and agar (15.0 g). 65 grams of dehydrated medium were suspended in a liter of distilled water, dissolved by heating and the pH was adjusted to 5.6. The medium was sterilized by autoclaving at 121˚C for 15 minutes.

Preparation of reference strains of bacteria
One ml aliquots of 24 hours' broth culture of tested organisms were aseptically added to nutrient agar slopes and incubated (Griffin and George Ltd, England) at 37˚ C for 24 hours. The bacterial growth was harvested and washed off by addition of sterile normal saline. The harvested bacteria were suspended in a suitable volume of normal saline to produce a suspension containing about 10^{18}- 10^{9} colony forming units per ml (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 [7].

Preparation of reference strains of fungi
The fungal culture was grown on saboraud dextrose agar, incubated at 25˚C for 4 days. The suspended fungal growth was harvested in 100 ml of sterile normal saline and the suspension was stored in refrigerator till used.

Testing for antibacterial activity
The antibacterial activity test was performed using the disc Diffusion assay [8]. Sterile filter paper discs (Whatman No. 1, 0.5 mm in diameter) were impregnated with one of each extract (20mg/ml) and left to dry to remove residual solvent, which might interfere with the determination. A bacterial suspension was prepared and added to the sterilized medium before solidification. The media with bacteria was poured into sterilized Petri dishes under aseptic condition. Extract discs were then placed on seeded agar plates. Each extract was tested in triplicate.
Muller Hinton Agar plates were incubated with 0.2 ml bacterial suspension of overnight culture of each bacterium and uniformly spread out. Duplicate were maintained. The plates were incubated at room temperature for 24 hours [9]. After the incubation period, the inhibition zone around the discs were measured and recorded, each test was carried out in triplicates.

Testing for antifungal activity
The same method as for bacteria was adopted. Instead of nutrient agar, Saporaud dextrose agar was used. The molten medium was incubated with the specific organism and the medium was incubated at 25˚C for one day.

Dilution method
The aim of broth and agar dilution methods is to determine the lowest concentration of the assayed antimicrobial agent (minimal inhibitory concentration, MIC) that, under defined test conditions, inhibits the visible growth of the bacterium being investigated. MIC values are used to determine susceptibilities of bacteria to drugs and to evaluate the activity of new antimicrobial agents.
The most commonly used method in the dilution techniques is the broth dilution technique, described by Rahman *et al*. [10]

Anti-Diarrhea
Method of Entamoeba histolytica and Giardia lamblia
Parasite isolation

Entamoeba histolytica and Giardia lamblia used in all experiments were taken from patients of Ibrahim Malik Hospital (Khartoum). All positive samples were examined by wet mount preparation. Then the positive sample was transported to the laboratory in nutrient broth medium.
Trophozoites of Entamoeba histolytica were maintained in RPMI 1640 medium containing 50% bovine serum at 37 ± 1˚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 [11] which is being described as a highly stringent and sensitive method for assessing the anti-protozoal effects (gold standard) particularly in Entamoeba histolytica, Gairdia intestinalis and Trichomonas vaginalis [12]. 5mg from each extract was dissolved in 50µl of dimethyl sulfoxide (DMSO) at Eppendorf tube containing 950µl D.W to reach concentration of 5mg/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 and negative control.

Three out of eight columns of microtite plates wells (8 columns x 12 rows) were chosen for each extract, 40µl of an extract solution (5mg/ml) were added to the first column wells C-1: On the other hand, 20µl of complete RPMI medium were added to the other wells the second column and the third one (C-2 and C-3). Serial dilutions of the extract were obtained by taking 20µl of extract to the second column wells and taking 20µl out of complete solution in C-2 wells to C-3 wells and discarding 20µl from the total solution of C-3 to the remaining 20µl serial solutions in the successive columns. 80µl of culture medium was complemented with parasite and added to all wells. The final volume in the wells was 100µl.

In each test metronidazole (a trichomonocide) pure compound [(1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole], was used as positive control in concentration 312.5µg/ml, whereas untreated cells were used as 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:

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

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

All data were presented as mortality (%). Statistical analysis for all the assays results were done using Excel

Results and Discussion

Antibacterial and antifungal activities:

All the crude extracts obtained from leaves, stems and roots of R. abyssinca, H. insignis and L. schimperi were screened for their antimicrobial activity against four standard bacterial species (Bacillus subtilis, Echerichia coli, Pseudmonas aeroginosa, Staphylococus aureus) and two fungal species (Aspergillus niger and Candida albicans). The antibacterial and antifungal assays were conducted by using the disc diffusion method. The tests were carried out at a concentration of 20 mg/ml. The zones of inhibition of bacterial and fungal growth were measured after 24 hours and the measurements were done (in mm) from the end of the growth of one side of the disc to the beginning of growth of the other side including the diameter of the disc. Minimum inhibition concentration (MIC) was done only for test organisms that showed inhibition zones greater than 18 mm. The means of diameter of the growth inhibition zones obtained in the experiment are shown in Tables (1, 2 and 3). The results were interpreted in terms of commonly used terms: sensitive, intermediate and resistant. On this basis, plant extracts resulting in greater than 18 mm growth inhibition zones are considered to possess relatively high antibacterial activity, and those resulting in 14-18 mm inhibition zone are of intermediate, and those resulting in zones below 14 mm are low or inactive [13]. Susceptibility of standard bacteria to different plants extracts were arranged according to their activity. The results clearly indicated variation in the activity against different bacterial and fungal species ranging between relatively high to resistant. L. schimperi stem extract showed the highest activity specially towards B. subtilis (27 mm) and S. aureus (21 mm) while R. abyssinca stem extract showed the lowest activity specially towards P. aeroginosa (11 mm) and A. niger (10 mm). B. subtilis was resistant to leaves and stem extracts of R. abyssinca, leaves extract of H. insignis and roots extract of L. schimperi, while they were intermediate to stems extract of H. insignis and highly active towards leaves and stems extracts of L. schimperi. H. insignis stem extract showed resistant to the two fungal species while leaves extract showed resistant towards A. Niger only. The calculated MIC values of methanol extracts of R. abyssinca leaves, L. schimperi leaves and L. schimperi stem are shown in Table (3). R. abyssinca leaves and L. schimperi stem extracts had MIC values of 3.125 mg/ml towards C. albicans and S. aureus while the MIC value of L. schimperi leaves and stems extracts towards Bacillus subtilis was 12.5 mg/ml The antimicrobial activity of methanolic extract of the all tested samples were compared with some selected standard antibiotics, Ampicillin at a concentration of 20 mcg/disc, Ciprofloxacin at concentration of 5 mcg/disc, Gentamicin at 10 mcg/disc and Fulcin at 1 mcg/disc. Ampicillin did not display any inhibition zone against E.coli while all tested samples showed activity except leaves of L. schimperi. Ciprofloxacin showed the highest inhibition zones comparing with other tested antibiotics against all bacterial species except with P. aeroginosa, it exerted inhibition zone of 10 mm which is lower than inhibition zones of all tested samples against the same species. Although Gentamicin had moderate activity (17 mm) against P. aeroginosa and S. aureus but it was higher than the activities of all tested sample against these two strains except the stem extact of L. schimperi which showed high activity with inhibition zone of 21 mm. When comparing antifungal activity of studied samples with antibiotic Fulcin only R. abyssinica leaves extracts showed inhibition zone similar to Fulcin (20 mm) against C. albican, others samples had lower activity than Fulcin. In a previous study on one of Rhus genus (R. typhina) [14], the antimicrobial activity of fruit extract was tested against twelve strains including Gram-positive and Gram-negative bacteria as well as yeasts. The extract showed a strong antimicrobial activity with a concentration-dependence and a broad antimicrobial spectrum for all tested bacteria species, also in this study methanol extract of leaves and stem of R. Abyssinica showed antimicrobial activity against all tested strains except B. subtilis. Crude methanol and water extract of the roots and stem as well as the leaves of L. schimperi exhibited antifungal activity [15]. This finding was agreed with the finding of this study when using L. schimperi roots, stem and leaves extracts which showed inhibition zones ranged between 10 mm to 17mm.

Table 4: Anti-microbial activity of methanol Extracts from Rhus abyssinica, Heeria insignisand Lannea schimperi against four bacterial and two fungal species

Plant part	Inhibition zone (mm)					
	Bacterial strains				Fungal strains	
	B. s	E. c	P. a	S. a	A. n	C. a.
RL	0	14± 0.2	13 ± 0.7	15±0.2	10 ± 0.3	20 ± 0.1
RS	0	12 ± 1.1	11±0.2	13±0.5	10±0.2	13 ± 0.2
HL	0	12± 1.2	12 ± 0.3	11± 0.5	12±0.5	0
HS	15 ± 0.2	12±0.7	14±0.2	12 ± 0.2	0	0
LL	21 ± 0.4	0	11 ± 0.7	14 ± 0.2	10	15 ± 0.2
LS	27 ± 0.2	15 ± 0.2	11 ± 0.2	21±1.6	10 ± 0.3	15 ± 0.3
LR	0	15 ± 0.2	11 ± 0.2	14 ± 0.5	15 ± 0.2	17±1.1

Key: B.s.: Bacillus subtilis, E.c.:Echerichia coli, P.a.:Pseudmonas aeroginosa, S.a.:Staphylococus aureus, A.n.: Aspergillus niger,C.a:Candida albicans.
RL: Rhus abyssinica leaves, RS: Rhus abyssinica stem, HL: Heeria insignis leaves, HS: Heeria insignis
stem, LL: Lannea schimperi leaves, LS: Lannea schimperi stem, LR: Lannea schimperi roots.

Table 5: MIC values for antimicrobial activity of methanolic extract from Rhus abyssinica, Heeria insignis and Lannea schimperi

Plant sample	Microbial strains MIC values (mg/ml)
R. abyssinca leaves extract Candida albicans	3.125
L. schimperi leaves extract Bacillus subtilis	12.5
L. schimperi stem extract Bacillus subtilis	12.5
L. schimperi stem extract Staphylococus aureus	3.125

Table 6: Antimicrobial activity of the standard antibiotics against the tested bacterial and fungal species

Antibiotic	Concentration (mcg/ disc)	E. c	S. a	P. a	S.t	C.a	A.n
Ampicillin	20	0	20	11	20	0	0
Ciprofloxacin	5	35	33	10	20	0	0
Gentamicin	10	8	17	17	25	0	0
Fulcin	1	0	0	0	0	20	25
Control 1	DMSO	0	0	0	0	0	0
Control 2	Normal saline	0	0	0	0	0	0

Key: B.s.: *Bacillus subtilis, E.c.:Echerichia coli, P.a.:Pseudmonas aeroginosa, S.a.:Staphylococus aureus*, A.n.: *Aspergillus niger,C.a:Candida albicans.*

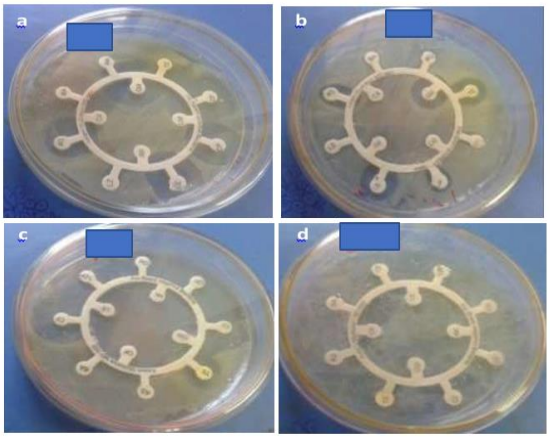

Fig 1: Antibacterial activity of standard antibiotics; ampicillin (20 mcg/disc), ciprofloxacin (5 mcg/disc) and gentamicin (10 mcg/disc) a, against; Bacillus subtilis b, against Staphyllococcus aureus; c, against Pseudomonas aeruginosa and d, against Escherichia coli

Fig 2: Anti-fungal activity of standard Fulcin (1mcg/disc), against Candida albicans.

Fig 3: Anti-bacterial activity of methanol extracts of the Heeria stem against Staphylococus aureus showing the inhibition zones

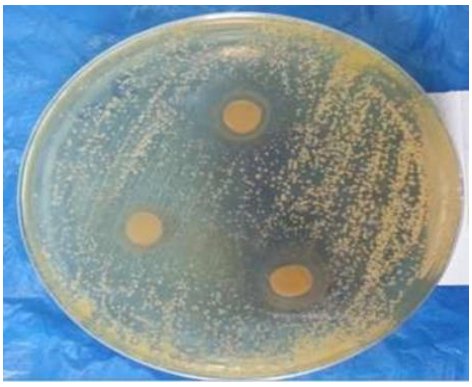

Fig 4: Anti-fungal activity of petroleum ether and chloroform extracts of the leaves against

Candida albicans showing the inhibition zones

Anti-diarrhea

In this experiment trials were made to test the anti-diarrheal effect of extracted components from H. insignis, L. schimperi and R. abyssinica against Giardia lamblia and Entamoeba histolytica trophozoites. Metronidazole drug (antiprotozoal medication) was used as positive control and dimethyl sulfoxide (DMSO) was used as negative control. The percentage of trophozoites growth inhibition was calculated by comparison with controls. The 50% inhibitory concentration (IC50) was defined as the concentration of the extract that inhibited growth by 50%.

Antiamoebic activity

The crude extracts were tested in triplicate on Entamoeba

histolytica culture, the activity of the extracts of R. abyssinica, H. insignis and L. schimperi were investigated using three different concentrations and the recorded results are presented in Table (4) and Figure (1). Extract is considered active with mortality value ≥ 50%. The methanol crude extracts of all samples were effective against G. lambilia especially at high concentrations (all extracts were concentration dependant). Lannea schimperi roots showed the highest activity against at all level of concentrations, it gave 88.4% for the highest concentration 500 ppm compared with the positive control which gave 92.21% mortality, while the other concentrations gave 69.9 and 51.3% mortalities at 250 and 125 ppm concentration, respectively. Leaves and stem extracts of H. insignis showed the lowest anti amoeba activity as compared with the other tested plant samples. According to the criteria of Amaral *et al*. [16], who established extracts with IC 50 ≤ 100 µg/ml as highly actives; extract of L. schimperi leaves (IC50 = 92.21 µg/ml) was very active towards the protozoan, being as an outstanding when compared with the other extract samples.

3.7.4.2 Anti- giardial activity

The activity of different s extracts from R. abyssinica, H. insignis and L. schimperi were investigated using three different concentrations (500, 250 and 125 µg/ml). The experiment was carried out on a pure culture of Giardia lamblia protozoan. The obtained results are presented in Table (5) and Figure (2). The results of this experiment revealed that the methanolic crude extracts of all samples were effective against G. lambilia especially at high concentrations (all extracts were concentration dependant). L. schimperi roots showed the highest activity against at all level of concentrations; it gave 86.7% for the highest concentration 500 pmm compared with the positive control which gave 92.21% mortality, while the other concentrations gave 68.1 and 54.2% mortalities at 250 and 125 pmm, respectively. The calculated IC50 values ranging from 80.82 to 278.90 µg/ml. The strongest level of inhibition was recorded by extract from L. schimperi leaves with an IC50 = 80.82 µg/ml. Referring to Amaral *et al*. [16] criteria, this finding suggested that L. schimperi leaves methanol extract could have ability for killing G. lambilia.
However, antigiardial activity has been described for pentacyclic triterpenes, such as qunonamethides [17]. It is speculated that mechanism of action of triterpenes is due to a distribution on microorganism′s cellular membrane [18]. Thus, the interesting antigiardial activity displayed by all extracts of R. abyssinica, H. insignis and L. schimperi could be attributed to their richness in triterpenes.

Table 7: Anti-giardial activity of methanolic extracts from Rhus abyssinica, Heeria insignis and Lannea schimperi

Extract	Concentration (µg/ml)			IC50 (µg/m)
	Mortality (%)			
	500	250	125	
R. abyssinica leaves	74.3	58.1	38.6	190.64
R. abyssinica stems	76.5	58.7	44.8	161.79
H. insignis Leaves	65.7	50.9	40.1	221.74
H. insignis stems	63.7	40.4	38.5	278.90
L. schimperi leaves	73.0	60.5	58.3	80.82
L. schimperi stems	68.9	57.2	40.4	191.86
L. schimperi roots	86.7	68.1	54.2	108.68
Control	95.2			

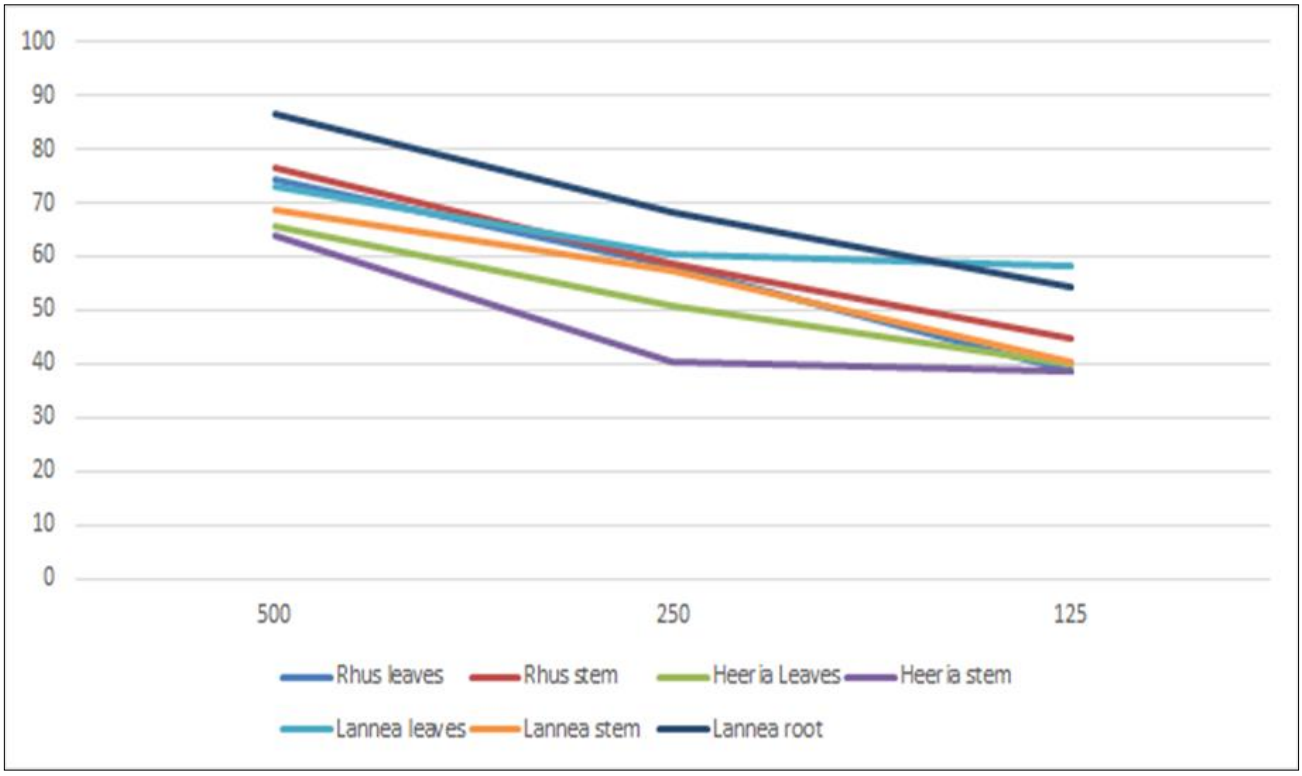

Fig 5: Comparison for anti-giardial activity of R. abyssinica, H. insignis and L. schimperi extracts at different levels of concentrations

Table 8: Anti-amoebic activity of methanolic extracts from Rhus abyssinica, Heeria insignisand Lannea schimperi

Concentration (µg/ml)				
Extract	**Mortality (%)**			**IC50 (µg/m)**
	500	250	125	
R. abyssinica leaves	72.0	50.6	42.2	201.47
R. abyssinica stems	71.0	55.2	40.0	196.37
H. insignis Leaves	60.1	51.0	41.0	237.62
H. insignis stem	60.1	41.3	30.5	329.62
L. schimperi leaves	75.1	63.2	55.2	92.21
L. schimperi stems	67.3	55.0	35.2	224.85
L. schimperi roots	88.4	69.9	51.3	118.99
Control	95.2			

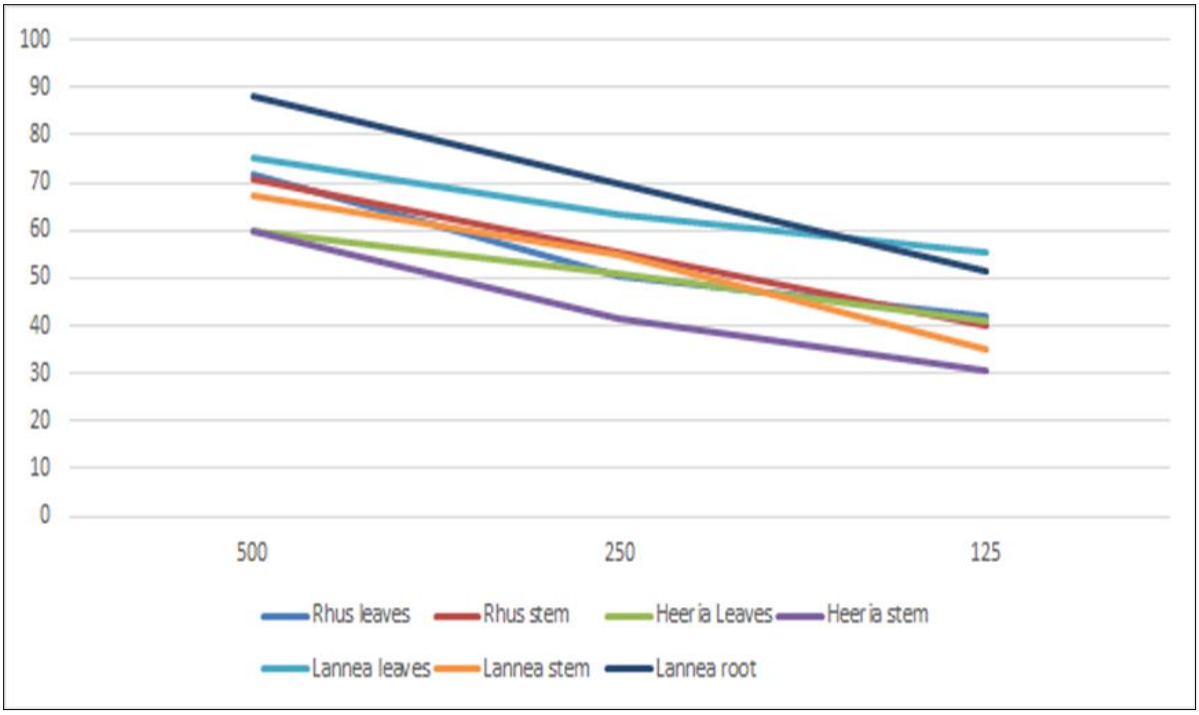

Fig 6: Comparison for anti-amoebic activity of R. abyssinica, H. insignis and L. schimperi extracts at different levels of concentrations

Conclusions

Rhus abyssinica, Heeria insignis and Lannea schimperi are small trees or shrubs belong to Anacardiaceae family. The three plants and preparations derived from them are widely used by the tribal communities at eastern Sudan for medicinal purposes.

The methanolic extract of L. schimperi stem showed the highest antimicrobial activity especially towards B. subtilis and S. aureus. L. schimperi leaves extract showed remarkable antigiardial and antiamoebic activities against G. lamblia and E. histolytica trophozoites.

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