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EVALUATION OF ANTIBACTERIAL ACTIVITY AND CYTOTOXICITY OF ROOTS OF *WALTHERIA INDICA*

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ABSTRACT

Background and Objective: The aim of this study was the determine *in vitro* antibacterial activity of different extracts of *Waltheria Indica* roots against standard bacteria, determine the Minimum Inhibitory Concentrations (MICs), study the cytotoxic effect of active extracts against different cell line and identify the major chemical components of the plants extracts. **Material and Method:** The cup agar plate diffusion method was used to screen the antibacterial activity of plant extracts and antibiotic. The Minimum Inhibitory Concentrations (MICs) of the most active extracts were determined using the agar plate dilution method. Micro-culture-tetrazolium MTT-assay was utilized to evaluate the cytotoxicity of extracts. All data were expressed as mean ± standard Deviation mean. Analysis of variance was performed by ANOVA using the SPSS software. Significant differences between means were determined by Least Significant Difference (L.S.D) and student t-test. A significant difference was considered at the level of $P < 0.05$. **Results:** The results indicated that most extracts exhibited inhibitory effect against stranded bacteria. The lowest minimum inhibitory concentration (MIC) values was <3.125 mg/ml. *Ps.aeruginosa*, *E.coli*, S.aureus and *B.subtilis* were sensitive to *Amoklan* at concentration 100µg/ml, while *S.aureus* was resistant. Most extracts inhibited organism more than drug used. Alkaloid, flavonoids, tannins, saponins, sterols and triterpenes were found in extracts. The results of Micro-culture-tetrazolium (MTT) assayrevealed that all extracts tested were non-toxic.

KEYWORDS: Antibacterial, Cytotoxicity, *Waltheria Indica*, MTT-assay, Roots, Fraction

INTRODUCTION

Antibiotics and the innovative antimicrobials, antibiotics and the chemotherapeutic agents have been of value in controlling many infections but they depend on judicious use to minimize the incidence of resistant forms.[1] In developing countries, due to the cost of efficient antimicrobials, a large proportion of the population utilizes medicinal plants for the treatment of infectious diseases.[2] Medicinal and aromatic plants and their derivatives represent an integral part of life in Sudan.[3] In Sudan, people have been tapping their herbal remedies for medication for time immemorial. For this purpose they use a vast variety of plants ranging from the rain forest vegetation in the south, to the desert vegetation of the north, and from the semi-Mediterranean climatic zone of the red sea, to the rich savanna of the west.[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.[5] *Waltheria indica* L belonging to the family Malvaceae. In traditional medicine, *Waltheriaindica* is used for the treatment of minor ailments and complicated ailments. In Sudan. The water extract of the roots is used for tooth and stomach pain, for rheumatism and as tonic.[6] It is used as an aspirin-likeanti-inflammatory drug. Various extracts are used in management of inflammation and inflammation conditions. *Waltheria indica* is used to treat diarrhea by traditional healers in Nigeria, Burkina Faso, Mexico and Panama. Also it is traditionally used to treat malaria, dysentery, hemorrhoids, cancers, syphilis, epilepsy, syphilis, infertility, erectile dysfunction and impotence. Infusion of roots were used to treat gingivitis, anemia and diarrhea.[7]

MATERIALS AND METHODS

Plant material: *Waltheria indic*a was obtained from the Northern Kordofan, Sudan at 2016 and authenticated by Mr. Yahia Suliman at herbarium of Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), National Center for Research, Khartoum, Sudan.

Preparation of the extracts: The plant sample was grounded using mortar and pestle and extracted by soaking 80% methanol for about five day with daily filtration and evaporation.

Solvent was evaporated under reduced pressure to dryness using rotary evaporator apparatus and the extract allowed to air till complete dryness.[8]

Fractionation of methanolic extract: Specific weight of each methanolic extract(crude extract 18g, 11.25%) was dissolved in 500 ml of distilled water and shacked, three times with 100 ml of petroleum ether each time using separatory funnel. Aqueous layer was then re-shacked three times with 100 ml of chloroform in each time using reparatory funnel. Chloroform layers were combined together and evaporated under reduced pressure using rotary evaporator. Aqueous layer was then re-shacked, three times with 100 ml of ethyl acetate in each time using reparatory funnel. Ethyl acetate layers were combined together and evaporated under reduced pressure using rotary evaporator apparatus. Aqueous layer was finally shackhed, three times with 100 ml of n-butanol in each time using separatory funnel. n-Butanol layers were combined together and evaporated under reduced pressure using rotary evaporator apparatus. Aqueous layer was lyophilized using freeze-drier machine till dryness and the yield percentage of each fraction was calculated.[9]

Organisms

The standard bacterial strain used *Bacillus subtilis*(NCTC 7596), *Staphylococcus aureus*(ATCC 25923), *Escherichia coli*, (ATCC 25922), and *Pseudomonas aeruginosa*(ATCC 27853) were obtained from the Department of Microbiology and Parasitology, Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), National Centre for Research, Sudan.

Determination of minimum inhibitory concentration (MIC)

The agar plate dilution method was used to determine the least concentration of plant extract that completely inhibits the growth of microorganism[7] at concentrations (50, 25, 12.5, 6.25 and 3.125mg/ml).[10]

Antimicrobial screening

The cup-plate agar diffusion method.[11] was adopted, to assess the antibacterial activity of the prepared extracts.[12]

Antibacterial activity of reference drug

In the present work, *Amoxicillin/clavulanic acid* the trade name (Amoklan) was used as reference drugs. Antibacterial activity of drug was tested at different concentrations obtained

by taking 375mg of each powdered drug and dissolved in 375 ml sterile distilled water to give a concentration of 1000 µg/ml followed by serial dilutions to give concentrations of 100, 40, 20 and 10 µg/ml.

Cytotoxicity screening

Micro-culture-tetrazolium MTT-assay was utilized to evaluate the cytotoxicity of plants. 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-diphenyltetrazolium bromide (MTT) into an insoluble, blue colored-form azan, a product 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.

Cell Line and Culture Medium

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

Statistical analysis

All data were expressed as mean ± standard Deviation mean. Analysis of variance was performed by ANOVA using the SPSS software. Significant differences between means were determined by Least Significant Difference (L.S.D) and student t-test. A significant difference was considered at the level of $P < 0.05$.

RESULTS

Methanolic crude extract, n-butanol extract, extract of ethyl acetate, and extract of ethyl acetate of *Waltheria indica* roots had high activity against all organisms at concentration 100mg/ml. The extract of water had high activity against all microorganisms except *P.aeruginosa*, Only *S.aureus* was sensitive to petroleum ether extract. Table (1) and Figure(1).

Table (1): Diameters (in mm) of inhibition zones obtained in the fraction of the antibacterial activity of *Waltheria indica* roots.

*Waltheriaindica*roots	Con (mg/ml)	Standard Bacteria use			
		Mean Diameter of growth Inhibition Zone in mm (MDIZ*)			
		B.s	S.a	E.c	P.s
Crude extract	100mg/ml	14.5 ± 7^{a}	25 ± 0^{a}	15 ± 0^{a}	22.5 ± 3.5^{a}
Pet. ether extract	100mg/ml	0.0^{a}	15 ± 0^{b}	0.0^{b}	11 ± 0^{b}
Chloroform extract	100mg/ml	15.5 ± 7^{a}	15.5 ± 7^{c}	14.5 ± 7^{a}	15 ± 0^{c}
Ethyl acetate extract	100mg/ml	25 ± 0^{b}	24.5 ± 7^{a}	21 ± 0^{c}	21 ± 1^{a}
n-Butanol extract	100mg/ml	25 ± 1^{b}	26.5 ± 7^{d}	23.5 ± 7^{d}	24.5 ± 7^{a}
Water extract	100mg/ml	20.5 ± 7^{c}	19.5 ± 7^{e}	14.5 ± 7^{a}	12.5 ± 7^{bc}
Sig		**	**	**	**

Data in table were given as mean ± standard Deviation mean (Std Deviation) of five species of pathogenic bacteria (triple replications for each). Statistical comparison between groups applied using Post hoc (LSD) test in columns and t-test in columns.

**= High significant differences with ($P\leq0.01$).

Means with different superscripts letters are significantly different at ($P<0.05$).

***MDIZ**= Mean diameter of growth inhibition zone in mm.

0.0 = No inhibition.

Interpretation of results: **MDIZ**(<18 mm: Sensitive, 14-18 mm, Intermediate: >14 mm: Resistant.

Where:-, ***B.s:*** *Bacilus subtilis* ***S.a:*** *Staphylococcus aureus.* **E.c:** *Escherichia coli* and **P.s:** *Pseudomonas aeruginosa.*

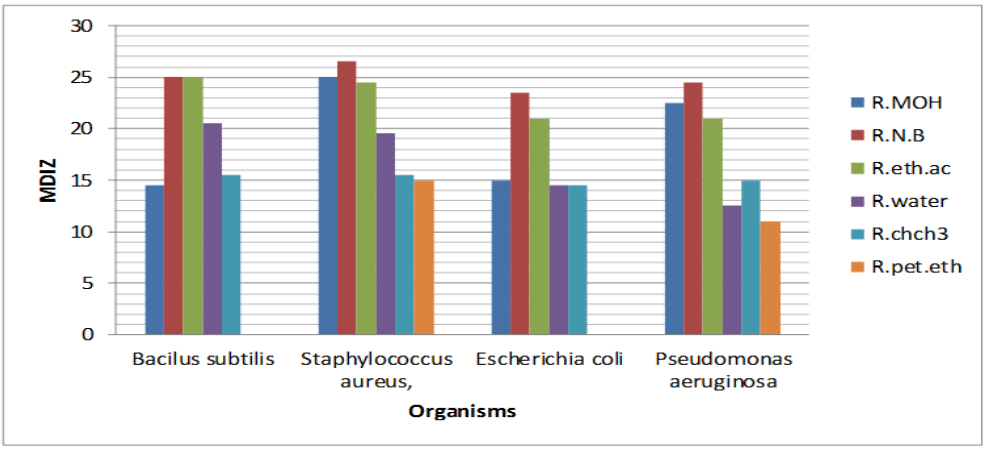

Figure (1): The Antimicrobial activity of different extracts of *Waltheria indica* roots against the Standard Organisms.

**** M.D.I.Z =: Mean diameter of growth inhibition zone in (mm).**

MOH=Methanol extract of roots, Pet. ether = Petroleum ether extract of roots, N.B= n-Butanol extract of roots, Eth.ac= Ethyl acetate extract of roots, Water = Water extract of roots, $Chcl_3$= Chloroform extract of roots.

The minimum inhibitory concentrations of four extracts(n-butanol, ethyl acetate and water) of roots were determined against four standard microorganisms(*B.subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa)* by agar dilution method at concentrations (50, 25, 12.5,6.25 and 3.125mg/ml). Table(2). The lowest minimum inhibitory concentration (MIC) values was obtained was<3.125 mg/ml for the extract of n-Butanol and extracts of ethyl acetate against *B.subtilis*. The range of MICs of n-Butanol extracts of roots between (25-100mg/ml, 3.125mg/ml) for all organisms. The ethyl acetate extract of roots showed low MICs for *B.subtilis* <3.125 mg/ml and 100mg/ml for *Escherichia coli.* The range of MICs of water extracts of roots between (6.25-100mg/ml, 3.125mg/ml) for organisms.

Table (2): Determination of the Minimum Inhibitory Concentrations (MICs).

Standard microorganisms	MICs		
	N-Butanol	ethyl acetate	Water
B. subtilis	**<3.125 mg/ml**	**<3.125 mg/ml**	**100 mg/ml**
S. aureus	**6.25 mg/ml**	**6.25 mg/ml**	**6.25 mg/ml**
E.coli	**25 mg/ml**	**100 mg/ml**	**50 mg/ml**
P. aeruginosa	**6.25 mg/ml**	**12.5 mg/ml**	**100 mg/ml**

The antibacterial activity of the selected drug *Amoxicillin/clavulanic acid* the trade name (Amoklan) against studied standard organisms were determined at four different concentrations(100,40,20 and 10µg/ml). *Ps. aeruginosa*, *E.coli* and *B.subtilis* were sensitive to *Amoklan* at concentration 100µg/ml, while *S.aureus* was resistant. all organisms were resistant to *Amoklan* at concentrations(40,20,10 µg/ml) Table (3).

Table (3): Zones of antibacterial inhibition of reference antibiotic Amoklan of different Concentrations.

Concentrations	Standard Bacteria use			
	Mean Diameter of growth Inhibition Zone in mm (MDIZ*)			
	B.s	S.a	E.c	P.s
100 µg /ml	**15**	**0**	**18**	**18**
40 µg /ml	**0**	**0**	**0**	**0**
20 µg /ml	**0**	**0**	**0**	**0**
10 µg /ml	**0**	**0**	**0**	**0**

The cytotoxicity of active plant extracts was evaluated using Micro-culture-tetrazolium (MTT) assay. Four extracts from *Waltheriaindic*a were evaluated for cytotoxic activity on (Normal, African green monkey kidney) cellas presented in Table (10) The results revealed that all extracts tested were non-cytotoxic, extracts tested had LC_{50} values greater than 100 µg/mL the cut-off point.

Table (10): Cytotoxicity of plant extracts on normal cell lines (Vero cell line) as measured by the MTT assay.

NO	Name of Extracts	Concentration (µg/ml) Inhibition (%) ± SD			IC_{50} (µg/ml)	IC_{50}
		500	250	125		
1	Water	70.2± 0.05	61.3± 0.06	45.1± 0.06	155.3	> 100
2	N-Butanol	71.8± 0.07	57.8± 0.04	40.4± 0.05	186.5	> 100
3	Ethyl acetate	77.7 ± 0.05	56.7± 0.06	40.9± 0.08	182.3	> 100
4	*Control	96.28± 0.01				< 30

Key: IC_{50}< 30 µg/ml: high toxic, > 100 µg/ml: no toxic *Control = Triton-x100 was used as the control positive at 0.2 µg/mL.

DISCUSSION

All extracts showed effect on types of standard bacteria at concentration 100mg/ ml, there was significant difference (P<0.05) between the zones of inhibition of different extracts. The crude(methanol) extract of roots showed high effect on all types of standard bacteria at concentration 100mg/ ml. Similar result reported Fatokun *et al*.,[13], the methanolic extract of roots of *Waltheria indic*a showed highest activity against *Staphylococcus aureus, Streptococus pneumonia* and *Klebsiella pneumonia*. Similarly (Mongalo *et al*.,[14] found that the 20 mg/ml of methanolic extract of *W. Indica* showed zone of inhibition 12.5 ± 1.50 mm against *S. aureus* and 12.1 ± 1.67 mm against *Pseudomonas aerogenosa*. Similarly (Olajuyigbe *et al*.,[15] reported that the ethanolic extract of *Waltheria indica* roots was active against *E coli* and S. *aureus*. Also Zailani *et al*.,[16] found that the roots of *Waltheria indica* had high activity against *Pseudomonas aerogenosa* and *E.coli*. The n-butanol extract of roots showed high effect on all types of standard bacteria at concentration 100mg/ml, the Gam positive bacteria were more affected than Gram negative bacteria. The strong activity suggested used of plant for treatment of infections caused by *P. aeruginosa* and S. *aureus*.

The ethyl acetate extract of roots showed high effect on all types of standard bacteria at concentration 100mg/ml.

The zones of inhibition of the antibacterial activity of the water extract of roots at concentration 100mg/ml recorded high activity for *Staphylococcus aureus* and *Bacillus subtilis*, while *Escherichia coli* and *Pseudomonas aeruginosa* were resistant, so the Gram positive bacteria are more affected than negative bacteria. Similarly (Osman,[1] found that the zone of inhibition of water extract of roots was 20 mm against *Bacillus subtilis* and 18mm against *S.aureus*. Similarly (Mongalo *et al*.,[14] found that water extracts of *Waltheria indica* showed least activity against the *S. aureus*.

The Chloroform extract and water extract of roots were effective on all types of standard bacteria at concentration 100 mg/ml. Similarly (Osman, 2009) found that the zone of inhibition of Chloroformextract18 mm against *Bacillus subtilis*, 14mmagainst *E.coli*, 17mm against *Pseudomonas aeruginosa* and *S. aureus*.

Petroleum ether extract of *Waltheria indica* roots was effective on *Bacillus subtilis*, while *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* were resistant at concentration 100 mg/ml.

On extract of *Waltheria indica* roots *E.coli* was higher significance ($p<0.05$) inhibition zone values had been observed on n-butanol extract (23.5 ± 7^{d}), The lower significance ($p<0.05$) inhibition zone value had been observed on petroleum ether extract (0.0^{b}). *Pseudomonas aeruginosa* was higher significance ($p<0.05$) inhibition zone values had been observed on n-butanol extract (24.5 ± 7^{a}), The lower significance ($p<0.05$) inhibition zone value had been observed on petroleum ether extract (11 ± 0^{b}). *Bacillus subtilis* was higher significance ($p<0.05$) inhibition zone values had been observed on ethyl acetate extract and n-butanol extract (25 ± 0^{b}, 25 ± 1^{b}) respectively, The lower significance ($p<0.05$) inhibition zone value had been observed on petroleum ether extract (0.0^{a}). *Staphylococcus aureus* was higher significance ($p<0.05$) inhibition zone values had been observed on n-butanol extract(26.5 ± 7^{d}), The lower significance ($p<0.05$) inhibition zone value had been observed on petroleum ether extract (15 ± 0^{b}).

The minimum inhibitory concentrations were determined for the three most active extracts against the four standard microorganisms. The MICs ranged between < (3.125-100mg/ml). The lowest MICs values showed by n-butanol extract of roots for *B.subtilis* (< 03.125 mg/ml). The MICs of water extract of roots ranged between (6.25-100mg/ml) for the four standard microorganisms.

P.aeruginosa, *E.coli* and *B.subtilis* were sensitive to Amoklan, while *S.aureus* was resistant at concentration 100µg/ml. On concentrations(40,20,10 µg/ml) all organisms were resistant to the drug.

The cytotoxicity study indicated that all extracts tested was non-toxic and hence support the traditional healers' claims who believe that the herbal medicines they use are safe.

CONCLUSION

These consequences suggest that *Waltheria indica* used, contain bioactive substances whose antibacterial potentials are highly comparable with that of antibiotic used in sensitivity test against microorganisms. The activity of plant against microorganisms may be indicative of the presence of broad-spectrum antibiotic compounds in that plant. All extracts of medicinal plant tested was non-toxic, these results may support the safety concern raised by traditional healers regarding administration of extracts from this plant. The *Waltheria indica* could become promising natural antimicrobial agents with potential applications in pharmaceutical industry for controlling the pathogenic bacteria.

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