

In Vitro Evaluation of the Antimicrobial and Antioxidant Activity of Hibiscus Sabdariffa L

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Abstract: Prevalence Of Resistant Microbial Strains Is Increasing With Time; Due To The Extensive And Indiscriminate Use Of The Broad Spectrum Antimicrobials That Rendered The Current Used Antimicrobial Agents Insufficient To Control Some Microbial Infections. The Present Study Was Aimed To Investigate The Antimicrobial And Antioxidant Potential Of Methanolic Extract Of The Medicinal Plant Hibiscus Sabdariffa L. The Agar Well Diffusion Technique Was Used To Determine The Antimicrobial Activity Of Methanolic Extract Of Hibiscus Sabdariffa L And The Antioxidant Activity Was Determined Through The DPPH Radical Scavenging Assay. The Methanolic Extract Of Hibiscus Sabdariffa L Was Examined Against Six Reference Bacterial Strains, One Fungal, And One Parasitic Clinical Isolate Which Include : Escherichia Coli (ATCC 25922) Pseudomonas Aeruginosa (ATCC27853), Klebsiella Pneumoniae (ATCC 15380), Salmonella Typhi (ATCC), Bacillus Subtilis (NCTC 8236), Staphylococcus Aureus (ATCC 25923), Candida Albicans (ATCC 7596) And Giardia Lamblia. The Result Of The Preliminary Screening Extract Showed Activity Against All Microorganisms Tested The Inhibition Zones Ranged From 20 To 25 Mm. The Minimum Inhibitory Concentrations Were Determined For The Extract Against The Selected Reference Strains. The Methanolic Extract Of The Hibiscus Sabdariffa L Screened For Antigiardial Activity Against (G. Lamblia) Trophozoites In Vitro Showed Antigiardial Activity With Inhibition Concentrations (IC50) More Than 180µg/ML. The Methanolic Extract Of Hibiscus Sabdariffa L Revealed That The Selected Plant Had A Significant Potential Effect Inhibiting The Growth Of Bacterial, Fungal, Parasitic Strains In Addition To Its Radical Scavenging Activity.

Key Words: Hibiscus Sabdariffa L, Methanolic Extract, Giardia Lamblia, Antimicrobial Activity, DPPH

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I. Introduction

Infectious Diseases Pose A Constant Threat To Human Beings¹. Every Individual On The Earth Could Be Affected With A Disease². The Emergence And Re-Emergence Of Infectious Diseases Have Become A Significant Worldwide Problem³. An Infectious Disease Is Caused By Various Microbes Or Pathogens, Most Of Them Are Usually Microorganisms And Few Of Them Are Visible By Naked Eyes⁴. The Most Common Pathogens Are Different Types Of Viruses, Bacteria, Fungi And Protozoa³. Humans Have Struggled Throughout The Years To Control Infectious Diseases Through Reducing Contact With Infected People, Prophylaxis, Vaccination And Antibiotics⁵.

Antibiotics Are One Of The Most Important Weapons In Fighting Infections And Have Greatly Benefited The Health-Related Quality Of Human Life Since Their Introduction⁶. However, Over The Past Few Decades These Health Benefits Are Becoming Less Significant Since Many Commonly Used Antibiotics Have Become Less And Less Effective Against Certain Illnesses Not Only Because Many Of Them Produce Toxic Reactions But Also Due To Emergence Of Drug Resistant Bacteria⁷. Resistance Development Is An Even Bigger Problem Since The Bacterial Resistance Is Often Not Restricted To The Specific Antibiotic Prescribed, But Generally Extends To Other Compounds Of The Same Class⁸. Bacterial Resistance And Its Rapid Increase Is A Major Concern Of Global Public Health And Are Emerging As One Of The Most Significant Challenges To Human Health^{6,8}.

The Global Emergence Of Antibiotics Resistance Is Fueled By The Wide Spread Use Of Broad-Spectrum Antimicrobial Agents, Creating Continuous Selective Pressure, And By Lapses In Infection Control, Which Facilitate Transmission Of Resistant Pathogenic Microorganisms^{6,9}. The Dynamics Of Antibiotic

Resistance Within Hospital Settings Are Determined By Introduction Of Resistance, Cross-Transmission And Induction Of Resistant Strains During Antibiotic Therapy¹⁰. Nature Has Been A Huge Source Of Antimicrobial And Other Medicinal Products Since Pre -Historic Times, The Importance Of Using Herbal Products In Treatment Of Various Human Diseases Are Not Limited¹¹. It Is Obvious That The Plant Kingdom Harbours Inexhaustible Sources Of Active Ingredients That Are Valuable In The Management Of Many Serious And Complicated Diseases⁹. Therefore, Medicinal Plants Are Significant For The Study Of Their Conventional Uses Through The Confirmation Of Their Pharmacological Effects^{11,12}. Treating Bacterial Infections By Antibiotics Is Beneficial But Their Indiscriminate Use Has Led To An Alarming Resistance Among Microorganisms And Led To The Re-Emergence Of Old Infectious Diseases^{6,13}. One Approach To Treat Infectious Diseases Is The Use Of Plant Extracts Individually And /Or As An Alternative Approach Is The Use Of Combination Of Antibiotics With Plant Extracts^{9, 14}. This Latter Approach I.E. Combination Therapy Or Synergistic Therapy; Against Resistant Microorganisms May Could Pave The Path For The Development Of Novel Alternatives Effective In Combating Various Infectious Diseases And Probably Representing Potential Area For Further Future Investigations⁶.

II. Materials And Methods

Study Design

Its Descriptive Laboratory Based Study.

Study Area

This Research Was Conducted In Medicinal Aromatic Plants And Traditional Medicine Research Institute, National Center For Research, Khartoum, Sudan.

Study Population

Selected Reference Strains Includings. Aureus (ATCC 25923), E.Coli (ATCC 25922), Ps. Aeruginosa (ATCC 27853), B. Subtilis (NCTC 8236), K. Pneumoniae (ATCC 53657), S. Typhi (ATCC14028)C. Albicans (ATCC 7596), And G. Lamblia Clinical Isolate.

Data Collection

Data Was Collected By Reading Diameter Of Inhibition Zone Of The Plants Extract And Selected Antibiotics.

Data Analysis

Data Was Analyzed Statistically Through The Program Of SSPS Version 16.0. American Type Culture Collection (ATCC) Rockville, Maryland, USA.

III. Methods

Collection Of The Plant Materials

Calyxes Of Hibiscus Sabdariffa L Werebought Fromsupermarket, Omdurman, Sudan In May 2016. They Were Authenticated By The Herbarium Of Medicinal Aromatic Plants And Traditional Medicine Research Institute (MAPTMRI), Khartoum, Sudan.

Preparation Of Crude Extracts

Hundred Gram Of Hibiscus Sabdariffa L Was Grounded Using Mortar And Pestle And Successively Extracted By Soaking 80 % Methanol For About 72 Hours With Daily Filtration And Evaporation, The Solvent Was Evaporated Under Reduced Pressure To Dryness Using Rotary Evaporator Apparatus, The Extract Was Allowed To Dry In The Air Till Complete Dryness Then The Residue Was Weighed And The Yield Percentage Was Determined¹⁵.

IV. Preparation Of The Test Organisms

Preparation Of Bacterial Suspensions

One MI Aliquots Of A 24 Hours Broth Culture Of The Test Organisms Were Aseptically Distributed Onto Nutrient Agar Slopes And Were Incubated At 37°C For 24 Hours. The Bacterial Growth Was Harvested And Washed Off With 100 MI Sterile Normal Saline, To Produce A Suspension Containing About 108- 109 C.F.U/ ML. The Suspension Was Stored In The Refrigerator At 4°C Till Used¹⁶.

Preparation Of Fungal Suspension

The Fungal Cultures Were Inoculated Onto Sabouraud Dextrose Agar Andincubated At 25 °C For 4 Days. The Fungal Growth Was Harvested And Washed With Sterile Normal Saline And Finally Suspension In 100ml Of Sterile Normal Saline Were Stored In The Refrigerator Until Used^{17,18}.

In Vitro Testing Of Extracts For Antimicrobial Activity By Agar Well Diffusion Method

The Antimicrobial Activity Of The Plant Extract Was Determined Using The Agar Well Diffusion Method. The Extract (Hibiscus Sabdariffa L) Was Dissolved In Methanol To Prepare 100 (W/V) % Concentration. The Individual Reference Strains Were Standardised By Adjusting The Absorbance Of The Inoculum To (0.08–0.13) At OD 625 Nm¹⁶. Hundred Ml Of The Standardised Inoculums Were Spread On The Surface Of Nutrient Agar Using Disposable Sterile Glass Spreader, And The Surface Was Allowed To Dry. Wells (10 Mm In Diameter) Were Cut From The Inoculated Medium Using A Flame-Sterilized Cork Borer, And Then Were Filled With Plant Extract. The Plates Were Incubated At 37°C For 24 Hours; Then The Zones Of Inhibition Around Each Well Were Measured¹⁹.

In Vitro Testing Of Extract For Antigiardial Activity

In Vitro Susceptibility Assays Were Performed Following The Sub-Culture Method, Which Is A Highly Stringent And Sensitive Method For Assessing The Anti-Protozoal Effects (Gold Standard) Particularly In *E. Histolytica*, *Giardia Intestinalis* And *Trichomonas Vaginalis*, 5mg From Both Plant Extract And Compound Was Dissolved In 50 Ml Of Dimethylsulfoxide (DMSO) In An Eppendorf Tube Containing 950 Ml In Order To Reach The Concentration Of 5 Mg/Ml (5000 Ppm). The Mixture Was Vortexed And Stirred By Magnetic Stirrer To Obtain A Homogenous Solution. The Concentrates Were Stored At -20°C For Further Analysis. Sterile 96 Multi-Well Plate (8 Columns (C) × 12rows (R)) Was Used For The Plant Extract, Positive Control And Negative Control With Three Columns Were Used For Each Extract. 40 Ml Of The Plant Extract Solution (5 Mg/Ml) Were Added To The First Column Wells C-1: On The Other Hand, 20 Ml Of Complete Roswell Park Memorial Institute(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 Made By Taking 20 Ml Of Extract From C1 To The Second Column Wells And Were Mixed, Then 20 Ml Were Transferred From C-2 Wells To C-3 Wells And Then 20 Ml Were Discarded, From C-3 Wells. 80 Ml Of Culture Medium Were Matched With Trophozoites(1 X10³ Cell/Ml) And Were Added To All Wells. The Final Volume In The Wells Was 100 Ml. Metronidazole (A Trichomonocide) Pure Compound (1-2-Hydroxyethyl-2-Methyl-5 Nitroimidazole), Was Used As Positive Control At A Concentration Of 312.5 Mg/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 After 24, 48, 72 And 96 H From The Assay²⁰.

The Mortality % Of Parasite For Each Extract Was Calculated According To The Following Formula:

$$\text{Mortality \% Of Cells} = \frac{(\text{N}^\circ \text{ Of Cells In Negative Control} - \text{N}^\circ \text{ Of Cells In Tested Sample With Extract}) \times 100}{\text{N}^\circ \text{ Of Cells Negativecontrol}}$$

100% Inhibition Of The Parasite Was Considered, When There Was No Motile Parasite Were Observed.

DPPH Radical Scavenging Assay

The Antioxidant Activity Of H. Sabdariffa L Methanolic Extract Was Determined Through The DPPH Radical-Scavenging Assay²¹. One Milliliter Of Each Plant Extracts Was Added To 3 Ml Of The Methanolic DPPH Solution. The Mixture Was Then Shaken And Allowed To Stand At Room Temperature In The Dark For 30 Minutes Then The Decrease In Absorbance At 517 Nm Was Measured Against A Blank (Methanol Solution) Using A Jenway Spectrophotometer. A Mixture Consisting Of 1 Ml Of Methanol And 3 Ml Of DPPH Solution Was Used As The Control. The Radical-Scavenging Activity Of Sample Was Expressed As Percentage Inhibition Of DPPH, Which Was Calculated According To The Formula:

$$\text{Inhibition \%} = \frac{[(AB - AA)/AB] \times 100}{\text{N}^\circ \text{ Of Cells Negativecontrol}}$$

Where AB And AA Are The Absorbance Values Of The Control And Of The Test Sample, Respectively.

V. Results

The Antimicrobial Activity Of H. Sabdariffa L Showed Various Levels Of Microbial Growth Inhibition Against Gram Positive And Gram Negative Bacteria. This Indicates The Broad Spectrum Nature Of The Plant Extract. The Antimicrobial Potential Of The Methanolic Extract Of H. Sabdariffa L Was Evaluated Against *E. Coli* (ATCC 25922) *P. Aeruginosa* (ATCC27853), *K. Pneumoniae* (ATCC 15380), *S. Typhi* (ATCC14028), *B. Subtilis* (NCTC 8236), *S. Aureus* (ATCC 25923), *C. Albicans* (ATCC 7596) And *G. Lambli*a Clinical Isolate.

The Mean Mdzs Produced By H. Sabdariffa L Extract On The Tested Microorganisms (Table 1) And Mdzs Of The Extracts Plus The Selected Antibiotics (Figure 1) Reveals That The Screening Of This Medicinal Plant Could Verify It Is Use In Traditional Medicine. The Results Were Interpreted As Sensitive, Intermediate And Resistant For The Selected Antibiotics, While The Mdzs Of Plant Extract Was Interpreted Following The Method Adopted By Cruickshank Et Al., 1975²³, Where Plants Extracts Resulting In 15 Mm Or More With Mean

Diameter Inhibition Zone Were Considered To Be Active And Those Resulting In Less Than 15 Mm Were Inactive. The Minimum Inhibitory Concentrations (MIC) Of The Methanolic Extract Of H. Sabdariffa L Methanolic Extract Against Reference Strains Yielded Mics Between 6.25 And 12.5 Mg/MI Which Is Significantly Low Compared To Many Commercial Antibiotics(Table 2). The Extract Was Screened For Antigiardial Activity Against G. Lamblia Trophozoites In Vitro Showed In (Table 3).

The Results In (Figure 1) Illustrates That The Methanolic Extract Of H. Sabdariffa L Showed High Activity Against S. Aureus (25mm) Which Is Almost More Than 40 µg/MI Gentamicin And Similar To 5 µg /MI Ciprofloxacin. It Inhibits K. Pneumoniae (20mm) Which Is More Than Activity That Produced By 40µg/MI Gentamicin.The Methanolic Extract Of H. Sabdariffa L Exhibited Mdizs Of (25mm, 23mm, 20mm, 20mm, 20mm, 22mm And 21mm) Against S. Aureus , Ps. Aeruginosa , K. Pneumoniae , B. Subtilis , E. Coli, S. Typhi , And C. Albicans Respectively, Which Was Higher Than 40µg/MI Gentamicin. Table (4) Showed The Radical Scavenging Potential Of Methanolic Extract Of H. Sabdariffa L Compared To Propyl Gallate (Ascorbic Acid). The Antigiardial Potential Of The Methanolic Extract Of H. Sabdariffal With Different Concentrations (500, 250 And 125 Ppm) And Metronidazole (The Control) With Concentration (312.5 Mg/MI) Was Investigated Against G. Lamblia Trophozoites In Vitro. The Methanolic Extract Of The H. Sabdariffa L Showed 72% Inhibition At A Concentration Of 500µg/MI After 72hrs; Which Was Compared With Metronidazole Giving 95% Inhibition At Concentration 312.5 Mg/MI At The Same Time Against G. Lamblia (Table 3).

Table (1): Antimicrobial Activity Of Methanolic Extract Of Hibiscus Sabdariffa L Against Selected Reference Strains

Plant	Reference Strains */MDIZ Mm						
	Escherichia Coli	Pseudomonas Aeruginosa	Klebsiella Pneumoniae	Salmonella Typhi	Bacillus Subtilis	Staphylococcus Aureus	Candida Albicans
H.Sabdariffa L	20	23	20	22	20	25	21

MDIZ: Mean Diameter Inhibition Zone.

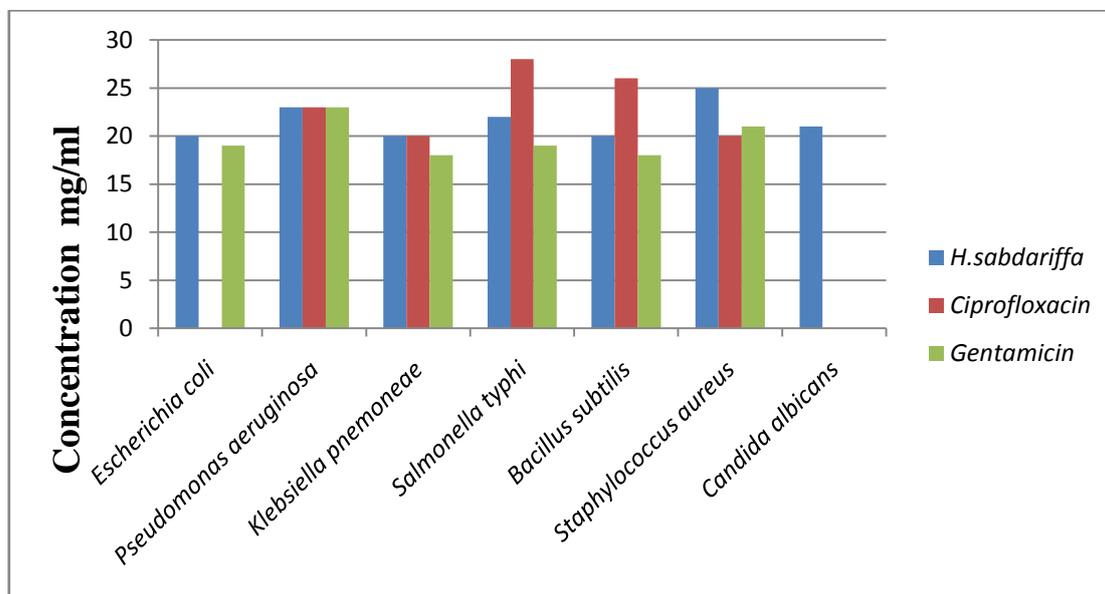


Figure (1): Antimicrobial Activity Of Plant Extract And Antibiotics Against Reference Strains

Table (2): Minimum Inhibitory Concentration (MIC) Of The Methanolic Extract Of Hibiscus Sabdariffa

Plant	MIC Of Reference Strains Mg/MI					
	Escherichia Coli	Pseudomonas Aeruginosa	Klebsiella Pneumoniae	Salmonella Typhi	Bacillus Subtilis	Staphylococcus Aureus
Hibiscus Sabdariffa L	12.5	12.5	12.5	12.5	6.25	6.25

Table (3): Anti-Giardial Activity Of The Methanolic Extract Of Hibiscus Sabdariffa L

	Mortality (µg/Ml)			IC50 (µg/Ml)
	Mortality (%) ± SD			
	500	250	125	
Hibiscus Sabdariffa L	72.57 ± 0.4	53.09 ± 0.9	44.02 ± 0.03	184.28
Control	95.03 ± 0.03			

Table (4): Antioxidant Activity Of The Methanolic Extract Of Hibiscus Sabdariffa L

No.	Sample	%RSA ±SD(DPPH)
1	Hibiscus Sabdariffa L	53± 0.09
2	Propyl Gallate	93± 0.01

VI. Discussion

The Methanolic Extract Of H. Sabdariffa L Revealed Broad Spectrum Activity Against The Tested Strains With References. Aureus Being The Most Sensitive Bacterium (25mm). The Findings Of This Study Were Similar To Those Reported By Abd-Ulgadir In Sudan Et Al. (2015)⁹, While These Results Differ From Those Reported In Ghana With Only Ps. Aeruginosa And S. Aureus Being Similar To Their Report Solaleye (2008) In Nigeria²². These Variations Could Be Due To The Difference In The Selected Strains Or Due To Variation In Phytochemical Content Of The Tested Extracts. The Methanolic Extract Of H. Sabdariffa L Exhibited Higher Activity Against The Gram Positive Organisms Than The Gram Negative Strains In This Study And This Could Be Due To The Fact That The Cell Wall Of Gram Positive Bacteria Is Less Complex And Lack The Natural Sieve Effect Against Large Molecules Due To The Small Pores In Their Cell Envelope. H. Sabdariffa L Methanolic Extract Screened For Antigiardial Activity Against G. Lamblia Trophozoites In Vitro Showed Antigiardial Activity With An Inhibition Concentration (IC50) More Than 180µg/Ml.

The 2, 2 Diphenyl-2-Picryl Hydrazyl (DPPH) Radical Widely Used For Screening The Scavenging Activities Of Natural Compounds Such As Phenolic Or Crude Extracts Of Plants. DPPH Is A Relatively Stable Radical At Room Temperature And Accepts An Electron Or Hydrogen Radical To Become Stable Diamagnetic Molecule. The Assay Is Based On The Measurement Of The Scavenging Ability Of Antioxidants Towards The Stable Radical DPPH Which Reacts With A Suitable Reducing Agent Mensor Et Al. (2001)²⁴. DPPH Radical Is Scavenged By Antioxidants Through The Donation Of Proton Forming The Reduced DPPH. The Colour Changes From Purple To Yellow After Reduction, Which Can Be Quantified By Its Decrease Of Absorbance At Wavelength 518 Nm. Radical Scavenging Activity Increase With The Increase In Percentage Of The Free Radical Inhibition. The Degree Of Discoloration Indicates The Free Radical Scavenging Potentials Of The Sample By Its Hydrogen Donating Ability.

The Antioxidant Properties Of The Methanolic Extract Of H. Sabdariffa L Tested Was Compared With The Activity Of The Well Known Antioxidants Ascorbic Acid, Tested Plant Showed (53%) Activity. The Effectiveness Of H. Sabdariffa L Extract In Scavenging Free Radicals Was Reported Numerously by Tolulope et Al. (2007) In Nigeria²⁵, Al-Hashimi (2012) In Iraq²⁶, Andyanget Al. (2012) In China²⁷; Majority Of These Studies Attributed The Antioxidant Activity Of H. Sabdariffa L Calyx To The Presence Of Phenolic Compounds. Microbiological Tests Revealed That, The H. Sabdariffa L Plant Extract Has Antimicrobial Properties That Verify Its Use In Folkloric Medicine In The Treatment Of Abscesses, Biliary Conditions, Cough, And Scurvy Reported By Ali Et Al. (2005) In Saudi Arabia²⁸.

In conclusion H. Sabdariffa L Calyxes Showed The Broad Spectrum Antimicrobial Activity Against Various Reference Strains And One Clinical Isolate. The Calyxes, Showed High Antimicrobial Activity Against Bacteria, Antimicrobial Activity Against G. Lamblia, And Antioxidant Activity. The Screening Of H. Sabdariffa L Proved To Have Significant Antimicrobial, Antigiardial And Radical Scavenging Activity That Could Be The Reason Behind It Is Effectiveness In Folkloric And Traditional Therapy.

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