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Antimicrobial Efficacy of Methanolic and Aqueous Extracts of Partially Purified Protein from Young and Matured Root of *Guiera senegalensis* (Moshi Medicine)

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Authors' contributions

This work was carried out in collaboration among all authors. Author MKJ designed the study, performed the statistical analysis. Author OAO wrote the protocol. Author MIS managed the analyses. Author MS managed the literature of the study. Author CEM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: Antibiotic resistance among pathogenic bacteria is increasing at an alarming rate leading to the need for traditional medicine as an alternative.
Aim of the Study: The study aimed to evaluate the antimicrobial activity of methanolic and, aqueous extracts of partial purified protein of young and matured roots of *Guiera senegalensis*.
Methodology: Antimicrobial activity was determined by disc diffusion and broth dilution techniques, Quantitative phytochemical analysis was carried out by standard procedure, the gel chromatography technique was used to fractionate the crude protein. The test isolates were Bacillus subtilis, *Staphylococcus aureus*, *E. coli*, *Salmonella typhimurium* and *Candida albicans*.
Results: The antimicrobial activity showed all the extracts were quite effective against most of the test isolates except *Candida albicans* (fungus). The crude and partially purified proteins were active

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against Gram-positive bacteria. The maximum zone of inhibition (37.33±5.03b mm) was observed in methanolic extracts of young root against *Staphylococcus aureus* at 100 mg/ml. Most extracts of methanolic exhibited minimum inhibitory concentration (MIC) at the range of 6.25 mg/ml and 12.5 mg/ml and minimum bactericidal concentration (MBC) at 12.5 mg/ml and 25 mg/ml. The young root was more active than the matured root. Quantitative phytochemicals showed a high amount of saponins (9.98% and 6.42%) in matured and young roots respectively.

Conclusion: *Guiera senegalensis* has broad-spectrum antimicrobial activity and a potential source of new antibiotics that could be useful for the treatment of infectious diseases.

Keywords: Guiere senegalensis; minimum inhibitory concentration; minimum bactericidal concentration; phytochemistry; microbial.

1. INTRODUCTION

Plant materials remain an important resource to combat serious diseases in the world. According to WHO [1]. 80% of the world's population is dependent on traditional medicine and a major part of the traditional therapies involve the use of plant extracts or their active constituents. Antibiotic resistance among pathogenic bacteria is increasing at an alarming rate, and at the same time, few new antibiotics reach the market. Today, resistant and multi-drug resistant (MDR) pathogenic strains are widespread causing problems with the treatment of common bacterial infections [2]. Infectious diseases due to a variety of bacterial pathogens and drug resistance are the world's leading cause of premature death commonly reported all over the world [3]. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases. One approach is to screen local medicinal plants for their antimicrobial properties.

Antimicrobial peptides (AMPs) are important part of the innate immune system, made up of small molecules that may present antibacterial, antifungal, ant parasitic, and antiviral activity [4]. Usually, these molecules are composed of 10–50 amino-acid residues and arranged in different groups depending on the amino-acid composition, size and conformation [5]. AMPs have promising therapeutic properties, they kill microbes rapidly, have broad activity-spectra and there are few reports of emerging bacterial resistance and therefore much effort is focused on finding potential novel antibacterial drugs among AMPs.

Amino acids such as lysine, arginine, histidine, glycine, phenylalanine, glutamic and aspartic have antimicrobial activity against some pathogens either in a pure state or combined with other elements or compounds [6].

Guiera senegalensis is being used in traditional medicine for the remedy of many ailments/diseases. The leaves are widely used for pulmonary and respiratory diseases like cough as a febrifuge, colic and diarrhea, syphilis, beriberi, leprosy, impotence, rheumatism, as diuretic. Also use for dysentery and gastrointestinal pain. In addition, partially purified anthocyanin fraction from leaf extract of *G. senegalensis* has been shown to possess antioxidant property against CCl_4 induced oxidative stress in rats [7].

In view of this, the antimicrobial activity of the methanolic and aqueous extracts and partially purified protein of the *G. senegalensis* will be determined.

2. MATERIALS AND METHODS

2.1 Sample Collection

Fresh leaves and roots of *Guiera senegalensis* were collected from Giwa, Zaria Local Government Area of Kaduna State, Nigeria. They were authenticated at the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria, Kaduna State. Voucher Number of *Guiera senegalensis*: 1823.

2.2 Preparation of Extracts

Fresh young and matured roots of *G. senegalensis* were washed with distilled water and dried at room temperature. The roots were pulverized using a mechanical grinder and 50 g of the pulverized roots was extracted in 300 ml methanol and 300 ml cold water as detailed below according to the method [8].

2.2.1 Methanol extraction of dried root of *G. senegalensis*

Fifty gram of young root powder was extracted in a soxhlet apparatus with 300 ml methanol. The solution of the methanol extract was gently

evaporated to dryness in a water bath at 40°C in fume cupboard. The resultant extract was transferred into airtight sample bottles and kept at 4°C until required. This was repeated for matured root.

2.2.2 Cold water extraction of dried leaf *G. senegalensis*

Fifty gram of young root powder was soaked in 300 ml distilled water in a 500 ml sterile conical flask with a constant stirring using magnetic stirrer. The mixture was allowed to stand at room temperature for 48 hours, after which it was filtered using No. 1 Whatman filter paper. The filtrate was gently evaporated to dryness in a water bath at 40°C and stored in a refrigerator at 4°C until required. The same was repeated for matured root.

2.3 Quantitative Phytochemical Constituents of *Guiera senegalensis*

Total alkaloids, flavonoids, Tannins, cyanogenic glycosides, oxalate, phytates, and saponins were determined from the dried leaves and roots samples using the method described by Mir et al. [9].

2.4 Preparation of Stock Solution of Extract

The stock solutions of the four extracts, methanol extracts of matured root and young root aqueous extracts were prepared by dissolving 1.0 g of each extract in 10 ml of sterile distilled water to give a concentration of 100 mg/ml. The stock solutions were reconstituted to graded concentrations of 50 mg/ml, 25 mg/ml and 12.5 mg/ml using two-fold dilution. They were well labeled and stored at 4°C until required.

2.5 Preparation of Test Organisms

The stock bacterial and fungal isolates used were obtained from Ahmadu Bello University Teaching Hospital Zaria, Kaduna State. Fresh pure plates of the test organisms were made from the isolate cultures obtained on agar slants.

The isolates were sub-cultured on selective and differential solid media and re-identified using colony morphology, gram reaction, motility test, haemolytic activity and biochemical tests namely catalase, bile solubility, litmus milk, citrate, oxidase and fermentation of sugars- mannitol, lactose and sorbitol [10]. With the aid of sterile wire loop, colonies of fresh cultures of the different bacterial isolates were picked and suspended in 5ml nutrient broth in a well-labeled sterile 10 ml bijou bottles. They were incubated at 37°C for 24 hours.

2.5.1 Determination of preliminary antimicrobial activity of extract

The antimicrobial activity of the extracts were determined using agar well diffusion test and broth dilution technique [11]. The antimicrobial activity of the plant extracts was tested on four standard bacteria species and fungus namely; *Bacillus subtilis, Staphylococcus aureus, Eschericia coli*, *Salmonella typhimurium* and *Candida albican* in the Microbiology Laboratory, Faculty of Sciences Ahmadu Bello University Zaria. These were standard laboratory cultures whose susceptibility on commonly used antibiotics was already established.

Staphylococcus aureus and *Bacillus subtilis* represented gram-positive bacteria while *Eschericia coli* and *Salmonella typhimurium* represented gram-negative bacteria.

2.5.2 Agar well diffusion assay

The agar well diffusion technique as modified by Mohamed et al. [12] was the standard method used to determine the antibacterial activity of the bioactive compounds. Briefly in the method, the media of Mueller hinton agar (Becton Dicknson M.D USA) and the potatoes dextrose agar was prepared and treated according to manufacturer's guidelines, where 38 g of mueller hinton agar was mixed with one litre of distilled water and enclosed in a container and autoclaved at 121°C for 15 minutes. The media was later dispensed into 90 mm sterile agar plates (Oxoid, UK) and left to set for bacterial assay while 19g of potatoes dextrose agar was mixed with one litre of distilled water and autoclaved at 121°C for 15 minutes for fungus assay. The agar plates were incubated for 24 hours at 37°C to confirm their sterility. Absence of growth after 24 hours showed that the plates were sterile. The sterile agar plates were inoculated with the test culture by surface spreading using sterile wire loops and each organism evenly spread on the entire surface of the plate to obtain uniformity of the inoculum. The culture plate had at most 4 wells of 6 mm diameter and 5mm depth made into it using a sterile agar glass borer. Ciprofloxacin was used

as a positive control for bacteria and ketoconazole for fungus. Approximately 0.2 ml of the bioactive test compound of concentration 100 mg/ml, 50 mg/ml, 25 mg/ml and 12.5 mg/ml was suspended in the wells and thereafter inoculated plates/culture were incubated for 24 hours at 37°C. The plates/cultures were examined for the presence of inhibition zones around each well.

Antimicrobial activity was determined from the zone of inhibition around the wells. Single readings were carried out. Non-active compounds did not show any inhibition zone. The zones of inhibition were measured using a ruler and a pair of divider and results were reported in millimeters (mm).

2.5.3 Determination of Minimum Inhibitory Concentration (MIC)

The MIC was evaluated on plant extracts which showed activity on any bacteria organism. The method used was the tube dilution method.The plant extracts were serially diluted from the solutions of 50 mg/ml to obtain varying concentration. The concentration were; 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml and 3.125 mg/ml Double dilutions of the extract were incorporated in Muller Hinton broth (Oxoid, UK) and then inoculated with 0.1 ml each of standardized suspension of the test organisms into the various test tube containing varying concentrations and another set of test tubes containing only Mueller Hinton broth were used as negative control, another test tube containing Mueller Hinton broth and test organisms was used as positive control. All the test tubes and controls were then incubated at 37°C for 24hrs. After incubation period, the presence or absence of growth in each tube was rated using the following scale: - no growth, + = scanty growth, ++ =moderate growth, +++ =heavy growth. A loop full from each tube was further sub cultured on nutrient agar to comfirm bacterial growth was inhibited. The growth of bacteria on solid media indicated that a particular concentration of the extract was unable to inhibit the bacterial growth. The lowest concentration of extract showing no growth indicated the amount of extract in grams per millilitre to which the organism is susceptible.

This was the minimum inhibitory concentration (MIC).

2.5.4 Determination of Minimum Bactericidal Concentration (MBC)

The MBC was determined by collecting 1 ml of broth culture from the tubes used for the MIC determination and subculturing into fresh solid nutrient agar plates. The plates were incubated at 37°C for 24 h. The least concentration that did not show any growth after incubation was regarded as the MBC [13].

2.6 Partial Purification of Antimicrobial Proteins/Peptides of Young and Matured Roots of *G. senegalensis*

Antimicrobial proteins and peptides were analyzed using the method of Bibiana et al. [14] with slight modification in buffer concentration. The fresh roots samples of 50 g each were homogenized using 0.1 M phosphate buffer, pH 7.4 and then filtered. The crude sample was centrifuged at 10,000 rpm for 30 mins. The crude extracts were saturated with 80% ammonium sulphate. The saturated extract was subjected to dialysis. After dialysis, these samples were subjected to spectrophotometric analysis to determine the concentration of the protein. The supernatant was subjected to gel filtration chromatography using Sephadex G-15 Approximately 40 fractions (3.0 ml each) were collected at the flow rate of 1 ml/21 seconds with potassium phosphate as eluting buffer and absorbance (OD) was measured at 280 nm.

3. RESULTS

The antimicrobial activity of the following test isolates *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Salmonella typhimurium* and *Candida albicans* showed that both methanolic and aqueous extracts of the young and mature roots were effective against most of the test isolates compared to purified protein which was effective only on Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*). All extracts were ineffective against the fungus *(Candida albicans).* The zone of inhibition for methanolic extracts of the young root at the concentration of 100 mg/ml was 37.33±5.03 mm which was more than that of the standard drug ciprofloxacin, 35.00±1.00 mm, against *Staphylococcus aureus* of the same concentration.

The crude protein of the root showed activity against Gram-positive bacteria only. The fractions were active against Gram-positive bacteria (*Bacillus subtilis and Staphylococcus aureus*).

The extracts MIC against *Bacillus subtilis*, *Staphylococcus aureus and Escherichia coli*, *Salmonella typhimurium* was 6.25 mg/ml to 12.5 mg/ml and 25 mg/ml respectively.

Phytochemical analysis of *G. senegalensis* showed the presence of alkaloids, saponins, tannins, cyanogenic glycosides, phytic acids, oxalates and flavonoids. Least percentages were observed in oxalates and cyanogenic glycosides of all the roots.

4. DISCUSSION

The antimicrobial activity of the following test isolates "*Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Salmonella typhimurium* and *Candida albicans* shows that both methanol and aqueous extracts were effective against the test isolates except *Candida albicans* whereas purified protein fraction was effective only on ~~to~~ Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*). All extracts were ineffective against the fungus; *Candida albicans.* According to chemical and laboratory standard institute CLSI [15] any plant material should be considered an effective therapeutic agent if its extract produces zones of inhibition ≤ 15.00 mm on the target pathogenic organism. Activity of plant extracts to test bacteria is normally expressed invitro as zone of inhibition in millimeter (≤10.00 mm) is regarded as effective [16].

Generally, both extracts showed a wide range of antimicrobial activity when compared to the positive control but there was a slight difference between the extracts. Methanol extract of young root showed activity against three (3) isolates except *Salmonella typhimurium* while Methanol and aqueous extracts of matured roots showed activity against *Bacillus subtilis* and *Staphylococcus aureus* which agreed with similar reports documented by Falmata et al. [17]. The observed antimicrobial effects on the isolates are believed to be due to the presence of tannins, flavonoids and saponins which have shown to possess antimicrobial properties [18].

The zone of inhibition for methanolic extracts of the young root was more than that of the standard drug ciprofloxacin against *Staphylococcus aureus* of the same concentration. This result is in accordance with the experiment carried out by Townsend et al. [19].

The crude protein of the l root showed activity against Gram-positive bacteria only. The fractions were active against Gram positive bacteria (*Bacillus subtilis and Staphylococcus aureus*).

The extracts exhibited MBC at a low concentration against all isolates except *E. coli* whose MBC was at higher concentration. The low MIC and MBC exhibited by the extracts against *Staphylococcus aureus, E. coli, Salmonella typhimurium* and *Bacillus subtilis* are of great significance in the health care delivery system, since it could be used as an alternative to orthodox antibiotic in the treatment of infections caused by these microbial pathogens, especially as they frequently developed resistance to known antibiotics.

The root extracts of the plant showed Phytochemical constituents such as alkaloids, saponins, tannins, cyanogenic glycosides, phytic acids, oxalates, and flavonoids. saponins and several other aromatic compounds are secondary metabolites of plants that serve a defense mechanism against invasion by many microorganisms, insects and other herbivores [20,21]. Flavonoids are hydroxylated phenolic substance known to be synthesized by plants in response to microbial infection [22]. Antimicrobial property of saponin is due to its ability to cause leakage of proteins and certain enzymes from the cell [23]. Tannins bind to proline-rich proteins and interfere with protein synthesis [24]. The medicinal properties and pharmacological actions of *G. senegalensis* are well-known to

Table 1. Quantitative phytochemical of young and matured roots of *G. senegalensis*

Phytochemicals compounds (mg/100 g)	Matured root	Young root
Tannins	1.06±0.02	1.05±0.02
Flavonoids	4.16±0.01	3.95±0.10
Saponins	9.98±0.02	6.42±0.07
Cyanogenic glycosides	0.55±0.06	1.51±0.05
Phytic acids	0.58±0.25	2.55±0.02
Oxalates	0.08±0.06	0.14±0.01

Table 2. Antimicrobial screening of different concentrations of methanol extracts of matured root of *Guiera senegalensis*

Concentration of extract/Standard drugs (mg/ml)	*Bacillus subtilis*	Zones of inhibition of micro organisms (mm)			
		Staphylococcus aureus	*Escharichia coli*	*Salmonella typhimurium*	*Candida albican*
100	18.67± 1.15 [b]	19.33± 1.53 [b]	NI	NI	NI
50	16.67 ±0.58[b]	15.33 ±1.15[c]	NI	NI	NI
25	15.33 ±0.58[bc]	12.67± 1.53[bc]	NI	NI	NI
12.5	12.00± 1.00[bc]	NI	NI	NI	NI
Cipr 100	45.33± 1.53[a]	35.00± 1.00[a]	22.33 ±1.53[a]	36.33±2.08[a]	NI
Keto.100					40.00 ±1.00[a]

([a,b,c]) = Means in the same column with different superscripts letter indicates statistically significant differences (P< 0.05). Values are mean± standard deviation of triplicate NI = No inhibition, Cipr = Ciprofloxacin (Antibacterial drug), keto = Ketokonazole (Antifugal drug)

Table 3. Antimicrobial screening of different concentrations of methanol extracts of young root of *Guiera senegalensis*

Concentration of extract/Standard drugs (mg/ml)	Zones of inhibition of micro organisms (mm)				
	Bacillus subtilis	*Staphylococcus aureus*	*Escharichia coli*	*Salmonella typhimurium*	*Candida albican*
100	16.00± 1.00[b]	37.33 ±5.03 [b]	13.33± 1.15 [b]	NI	NI
50	15.33± 0.58 [b]	29.67± 2.08[bc]	12.33± 0.58[bc]	NI	NI
25	12.00± 1.00 [b]	26.67 ±5.13[bc]	11.33± 0.58[bc]	NI	NI
12.5	11.33 ±0.58 [b]	25.33 ±3.06[bc]	10.67± 0.58[bc]	NI	NI
Cipr 100	45.33 ±1.53[a]	35.00 ±1.00 [a]	22.33± 1.53 [a]	36.33± 2.08 [a]	NI
Keto.100					40.00 ±1.00 [a]

([a,b,c]) = Means in the same column with different superscripts letter indicates statistically significant differences (P< 0.05). Values are mean± standard deviation of triplicate NI = No inhibition. Standard drugs: Cipr = Ciprofloxacin (Antibacterial drug), keto = Ketokonazole (Antifugal drug)

Table 4. Antimicrobial screening of different concentrations of aqueous extracts of matured root of *Guiera senegalensis*

Concentration of extract/Standard drugs (mg/ml)	Zones of inhibition of micro organisms (mm)				
	Bacillus subtilis	***Staphylococcus aureus***	***Escharichia coli***	***Salmonella typhimurium***	***Candida albican***
100	17.00± 1.00[b]	18.33± 0.58 [b]	NI	NI	NI
50	16.00± 1.00 [b]	16.33 ±1.00 [b]	NI	NI	NI
25	13.00± 1.73[c]	15.67± 0.58 [c]	NI	NI	NI
12.5	11.33± 0.58 [c]	12.00± 1.00 [c]	NI	NI	NI
Cipr 100	45.33± 1.53[a]	35.00± 1.00 [a]	22.33± 1.53 [za]	36.33± 2.08 [a]	NI
Keto.100					40.00± 1.00 [a]

(a,b,c) = Means in the same column with different superscripts letter indicates statistically significant differences (P< 0.05). Values are mean± standard deviation of triplicate NI = No inhibition. Standard drugs: Cipr = Ciprofloxacin (Antibacterial drug), keto = Ketokonazole (Antifugal drug)

Table 5. Antimicrobial screening of different concentrations of aqueous extracts of young root of *Guiera senegalensis*

Concentration of extract/ Standard drugs (mg/ml)	Zones of inhibition of micro organisms (mm)				
	Bacillus subtilis	***Staphylococcus aureus***	***Escharichia coli***	***Salmonella typhimurium***	***Candida albican***
100	18.67± 1.53 [b]	17.33 ± 1.53 [b]	NI	NI	NI
50	15.67± 0.58[c]	16.00± 1.00 [b]	NI	NI	NI
25	12.00 ± 1.00 [C]	12.67± 1.53[bc]	NI	NI	NI
12.5	11.33± 0.58[b]	11.67 ± 0.58[bc]	NI	NI	NI
Cipr 100	45.33± 1.53[a]	35.00± 1.00 [a]	22.33 ± 1.53 [a]	36.33 ± 2.08 [a]	NI
Keto.100					40.00± 1.00 [a]

([a,b,c]) = Means in the same column with different superscripts letter indicates statistically significant differences (P< 0.05). Values are mean± standard deviation of triplicate NI = No inhibition. Standard drugs: Cipr = Ciprofloxacin (Antibacterial drug), keto = Keto Conazole (Antifugal drug)

Table 6. Mininmum Inhibitory Concentration (MIC) of methanol and aqueous extracts of roots of *Guiera senegalensis* (mg/ml)

Bacteria isolates	**MT matured roots (mg/ml)**	**MT young roots (mg/ml)**	**AQ matured roots (mg/ml)**	**AQ young roots (mg/ml)**
Bacilus subtilis	12.5	25	6.25	12.5
Staphylococcus aureus	12.5	6.25	6.25	12.5
Escherichia coli	–	12.5	–	–
Salmonella typhimurium	–	25	–	–

Key: AQ = Aqueous extract, MT = Methanol extract, - = No Minimum Inhibitory Concentration (MIC)

Table 7. Minimum Bactericidal Concentration (MBC) of Methanolic and Aqueous extracts of the root of *Guiera senegalensis*

Bacteria isolates	AQ matured roots	AQ young roots	MT matured roots	MT young roots
Bacilus subtilis	12.5	25	25	50
Staphylococcus aureus	12.5	25	25	12.5
Escherichia coli	_	_	_	12.5
Salmonella typhimurium	_	_	_	50

Key: AQ = Aqueous extract, MT = Methanol extract, - = No Minimum Bactericidal Concentration (MBC)

Table 8. Purification of bioactive protein of young and matured roots of *Guiera senegalensis*

Purification steps	Total protein (mg/ml)	Total activity (AU)	Specific activity (AU/mg)	Recovery (%)	Purification fold
Matured roots					
Crude protein	9.04	2500	276.55	100	1
80% ammonium sulfate precipitation	6.5	2300	352.85	92	1.28
Gel filtration Sephadax	2	2100	1050	84	3.80
Young roots					
Crude protein	11.22	2800	249.55	100	1
80% ammonium sulfate precipitation	6.74	2600	385.76	92.86	1.55
Gel filtration Sephadax	1.1	2000	1818.18	71.43	7.29

Table 9. Antimicrobial activity of crude and partial purified protein fractions of *Guiera senegalensis*

Samples	*Bacillus subtilis*	*Staphylococcus aureus*	*Escherichia coli*	*Salmonella typhimurium*	*Candida albicans*
Crude extract of matured roots	+	+	-	-	-
Crude extract of young root	+	+	-	-	-
Peak of 20th fraction of gel filtration of matured root	-	+	-	-	-
Peak of 24th fraction of gel filtration of young root	-	+	-	-	--

Key: +++ = very strong activity, ++ = strong activity, + = activity present, −= activity absent

Nigeria's traditional medicine. This plant is known to contain various active principles of therapeutic value and possess biological activity against a number of diseases [25].

The present study reveals the existence of antimicrobial substances in *Guiera senegalensis* and further studies are required to find out the active components of medicinal properties in this valuable plant root.

5. CONCLUSION

The results obtained in this research imply that the root extract of *Guiera senegalensis* possesses antimicrobial activity against pathogenic bacteria and may be used in susceptibility studies and treatment of infections caused by *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli.*

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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