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In Vitro Antibacterial Activity and Phytochemical Screening of *Garcinia Kola* Extracts against Methicillin Resistant *Staphylococcus Aureus* (MRSA)

Abubakar U. Zage[1], Isma'il Ahmed[1], Lawan D. Fagwalawa[2], Muhammad Ali[1*]

[1]Microbiology Department, Kano University of Science and Technology Wudil Kano
[2]Biological Science Department, Kano University of Science and Technology Wudil Kano

Abstract:

Modern medicines have always depended on herbal extracts from plants as fundamental source of therapeutic ingredients. The aim of the study was to determine the phytochemical constituents and antibacterial activity of *Garcinia kola* seed extracts against clinical isolates of Methicillin Resistant *Staphylococcus Aureus* (MRSA). Total of 107 Staphylococcus isolates from infected wound and urine and were collected from Abubakar Imam Urology Center in Kano State, Nigeria over a period of eight months (October 2015 to May 2016). A disc diffusion method was used for characterization of MRSA. The phytochemical screening of the plant materials was done using conventional laboratory method while the antibacterial activity of the plant extracts was determined using agar well diffusion method. The result of bacterial characterization showed that eight isolates found were to be Methicillin Resistant *Staphylococcus aureus* (MRSA). Phytochemical screening of the seeds, leaves and stem bark extracts indicated the presence of Alkaloid, Tannin, Saponin, and Cardiac glycoside, Flavonoid, Terpenoid, Phenols Anthraquinone and Steroid. However, reducing sugar is absent. The antibacterial activity of the plant showed that the plant part extracts demonstrated antimicrobial effect against the test isolates with higher activity in seeds compared to leaves and stem. Statistical analysis of the result revealed that the overall average zone of inhibition shown by the extracts is 11.68 mm with methanolic seeds extract exerting the highest antibacterial effects on the test isolates with average zone of inhibition of 12.78 mm, followed by methanolic leaf extract 12.37 mm then aqueous seed extract with zone of inhibition of 11.87 mm. Least zones of inhibition were recorded in aqueous leaf extract and methanolic stem back extract with zones of inhibition of 11.28 and 10.43 mm respectively. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extracts range from 12.5 – 100 mg/ml of the extracts. Findings from this work support the use of seed extracts from *G. kola* as medicinal plant.

Keywords: Antibacterial activity; *Garcinia kola*; Methicillin Resistant; *Staphylococcus aureus*; phytochemical

Introduction

Plants are generally known to produce certain bioactive compounds used to protect them from microbial invasion. However, several researches conducted on such plants demonstrated that the bioactive components of the plants can also play an important role in protecting human diseases. The plants based compounds can be derived from leaves, stem bark, roots, fruits, seeds and flowers. As result of their versatile application, medicinal plants have become the richest source of drugs in traditional and modern medicine[1]. Several leaves and leaves extracts have been found to have antimicrobial activity against microorganisms. Several hundred genera of plants were utilized traditionally for medicinal purposes[2]. The antibacterial activity of plant is due to present of phytochemical constituents in them. Phytochemicals are secondary metabolites present in medicinal plants which include terpenoid, flavonoids, steroid, Alkaloids and phenolic compounds. The phytochemicals have impressive pharmaceutical properties such as analgesics, aesthetic, antibiotics, antiparasitic, anti-inflammatory, oral contraceptive, hormones and ulcer therapeutic laxative.

Garcinia kola is forest tree indigenous to sub-Saharan Africa and has been referred to as a 'wonder plant' because almost every part of it has been found to be of medicinal importance[3]. It occurs naturally from Sierra Lone to Southern Nigeria and on into Zaire and Angola, but is further distributed by man and is often found

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***Corresponding author:** Muhammad Ali, Biological Science Department, Kano University of Science and Technology, Wudil Kano, Email: alimuhd4real@gmail.com

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cultivated around villages. *Garcinia kola* belongs to a family of tropical plants known as Guttifera. It is an evergreen tree grown in the tropical rainforest of West Africa[4].

The parts used in this plant are the stem bark, seeds and root. The active ingredients are flavonoids, apigenin, kolaviron, biflavonoid - ametoflavone, saponins, tannins, resin[5]. Medicinally, the stem bark and seeds are used for acute fever, cough, and liver disorders and as an anti-vomiting agent[6]. It is also used as a remedy for inflammations of respiratory tract, bronchitis, throat troubles, stomach ache and gastritis[7]. The seed extract is very efficacious for hepatitis, antiseptic and is active against Gram-positive and negative bacteria[8]. The decoction of the root is used as aphrodisiac, evacuant, anticancer and is also recommended for dysentery, headache, malignant tumours and respiratory ailments[6]. The root is chewed for cleaning teeth and toothache. The phytochemical compounds isolated from *G. kola* include oleoresin, tannins, saponins, alkaloids, cardiac glycosides[9].

Staphylococcus aureus is a bacterium of significant importance because of its ability to cause a wide range of diseases and capacity to adapt to diverse environmental forms. The organism colonizes skin, skin glands and mucous membrane, causing infections both in human and animals such as rashes, inflammations of bones and the meninges as well as septicaemia[10]. In addition, *S. aureus* causes inflammation of the mammary gland in bovine and the lower part of the foot in poultry[11]. Penicillin and its derivatives, including methicillin have been used for the treatments of infections caused by *S. aureus*[12]. However, certain strains of *S. aureus* developed resistance known as methicillin resistant *Staphylococcus aureus* (MRSA). At present, less than 90 % of *S. aureus* strains are resistant to most penicillin derivatives[13] and ordinary antimicrobial agents like drugs from the family of amino glycosides, macrolides, chloramphenicols, tetracycline's and fluoroquinolones[14].

Materials and Methods

Ethical approval

Ethical approval for this research was obtained from the Hospital Service Management Board (HSMB), Kano based on the consent of Murtala Muhammad Specialist Hospital, Sir Muhammad Sunusi Specialist Hospital and Abubakar Imam Urology Centre Ethical Committees

Study area

Clinical isolates of *Staphylococcus aureus* were obtained from three hospitals within Kano metropolis which include Murtala Mohammed Specialists' Hospital, Sir Muhammad Sunusi Specialists' Hospital and Abubakar Imam Urology centre (MMSH, MSSH and AIUC). Characterization and identification of *Staphylococcus aureus* and Methicillin Resistance *Staphylococcus Aureus* (MRSA) was conducted in the Departments of Biological sciences and Microbiology of Kano University of Science and Technology Wudil. Determination of antimicrobial activity of the extracts against MRSA was done at Microbiology laboratory of Kano University of Science and Technology Wudil. Phytochemical screening of the extract was done at Biochemistry laboratory of Bayero University, Kano.

Sample collection

One hundred and seven (107) suspected *Staphylococcus* isolates were collected from three different hospitals within Kano State metropolis over a period of eight months (October, 2015 to May, 2016). The isolation of *Staphylococcal* isolates was done by culturing various clinical samples of wounds and pus (n = 37), High vaginal swab (HVS) (n = 29) and urine (n = 41) on a surface of Nutrient agar (Lifesave Biotech, USA). The plates were incubated at 37ºC for 24 h for colony formation. Each colony was isolated in a pure form by sub culturing for further studies and identification. Discrete colonies of each isolate were kept in peptone water. The bacterial strains were then stored at 4ºC for further experiments.

Identification of *staphylococcus aureus*

The isolates were confirmed as *Staphylococcus aureus* by conventional microbiological methods: Gram staining, Biochemical test (Coagulase test and Catalase test) and Mannitol fermentation test. Gram staining was done according to the methods described by Chessbrough[15]. Coagulase and Catalase tests were done according to the method described by Holt *et al*[16]. Mannitol fermentation test was done by inoculating the isolates onto the surface of Mannitol Salt agar and incubated at 37 for 24 hours[16].

Identification of methicillin resistant *staphylococcus aureus* (MRSA)

Eighty three (83); isolates confirmed as *Staphylococcus aureus* were later subjected to antimicrobial sensitivity testing for methicillin resistant using modified Kirby Bauer diffusion method on Muller Hinton agar and incubated for 24 hours at 37 . The methicillin resistant were tested using commercially prepared antibiotics discs containing Oxacillin (1 µg), Ampicillin (10 µg), Doxycycline (10 µg), Ciprofloxacin (30 µg), Cefoxitin (10 µg), and Ceftriaxone (30 µg) discs. The inoculums suspension was adjusted to match 0.5 McFarland turbidity standards. A sterile cotton swab was dipped into the adjusted suspension and excess inoculums was removed by pressing the swab firmly on the inside wall of the tube. The solidified surface of Mueller Hinton agar plate was inoculated by streaking the swab over the entire surface. The antimicrobial discs were placed firmly on the surface of the inoculated agar plate using sterile forceps. The plates were incubated at 37 for 24 hours. The plates were examined and the diameters of the zones of inhibition were measured. Results were classified as susceptible, intermediate, or resistant, according to the approved guidelines of the Clinical and Laboratory Standards Institute[17].

Plant Material

Collection, identification and processing

The plant materials used in the research work including seeds stem bark, and leaves of *Garcinia kola* were obtained from Kofar Mazugal kolanuts market in Kano municipal Local Government Area, Kano. Identification and authentication of the plant materials were done at Herbarium unit in the Department of Plant Science, Bayero University Kano with the following Herbarium accession number DPB/BUK/HIF/0667 and voucher specimens were deposited there for future references. The seeds of *Garcinia kola* were removed from the seed coat washed

and air dried for two weeks. The leaves and stem were also cut into bits for fast drying. They were air dried for two weeks and crushed to fine powder using a sterile mortar and pestle. The powdered form of the seed, stem bark and leaves of *Garcinia kola* were placed in different air tight containers, properly labeled and stored for further use.

Preparation of extracts

Aqueous and ethanol extracts of *Garcinia kola* leaves were prepared separately. Fifty grams (50 g) powder of the plant material was mixed with 500 ml each of distilled water and methanol respectively. The flasks were kept for 72 hours with intermittent shaking. Thereafter, filtration was done using Whatman filter paper. The methanol extracts was evaporated at 50˚C using rotary evaporator while the aqueous extract was evaporated at 40˚C in water bath until dried extract samples were obtained. The dried filtrates were reconstituted in 10 % DMSO thereby making a final concentration of 200 mg/ml as a stock concentration. The extract solutions were kept at 4˚C before use[18].

Qualitative phytochemical screening

The screening of the phytochemical constituents of the plant materials for various phytochemical constituents such as terpenoids, flavonoids, alkaloids, reducing sugars, steroid, glycoside, phenol, Anthraquinones, saponin and tannin was conducted using standard methods as described by Sofowora[19] and Trease and Evans[20].

Antibacterial activity of the extracts

The sensitivity of each extracts was determined using the agar well diffusion[21]. The prepared bacterial suspension equivalent to 0.5 McFarland Standard (1.5 x 10^6 CFU) was inoculated onto sterile surface of Mueller - Hinton agar medium (MHA) in a 13mm Petri - dish. A cork borer of 6 mm diameter size was utilized to produce 5 holes at equidistance on the medium. The holes were supplied with approximately 0.1ml of the prepared extracts at a various concentration of 25, 50, 75 and 100 mg / ml. The plates were allowed to diffuse on the laboratory bench for 1 hour. The inoculated plates were incubated at 37^{0}C for 24 hours. Zones of inhibition produced by the extracts against the test isolates were observed and measured. The experiment was conducted in triplicate and the average values were recorded. Clindamycin 125 mg / ml (Micro Lab limited) was served as a control (positive) for the experiment.

Determination of mic of the extracts

Broth dilution technique was employed to determine the minimum inhibitory concentration MIC of the extracts. Double fold dilutions were prepared by adding 2 ml of 100 mg / ml of the extract into a test tube containing 2 ml of Nutrient broth, thus producing solution containing 50 mg / ml of the extract. The process continue serially up to test tube No. 5, hence producing the following concentrations; 50, 25, 12.5, 6.25 3.125 mg/ml. Test tube No. 6 do not contain extracts and serve as negative control. Exactly 0.5 ml of 0.5 McFarland equivalent standards of test organisms were introduced into the test tubes and incubated at 37^0 C for 24 hours. After incubation the test tubes were observed for growth by checking for turbidity[21].

Determination of mic of the extracts

From each tube that did not show visible growth in the MIC, Briefly, 0.1 ml bacterial culture was pipetted from MIC tubes which did not show any growth and sub cultured onto the surface of Mueller Hilton agar plates. The inoculated plates were incubated at 37^{0}C for the period of 24 hours. The MBC was recorded as the lowest concentration of extract without single colony of bacteria on Mueller Hilton agar plates[21].

Statistical analysis

The data obtained on antibacterial activity of the extracts against the test isolates was analyzed using One - way analysis of variance (ANOVA). All the data were computed as means ± standard deviation using statistical program SPSS 21.0 (Statistical Package for the Social Sciences). Probability value of 0.05 was set to determine significant differences level on the activity of the extracts against the tested isolates.

Results

Identification of methicillin resistant *staphylococcus aureus* (MRSA)

Antibiotic susceptibility test was carried out for 83 *S. aureus* isolates, and the zones of inhibition obtained were classified based on Clinical and Laboratory Standards Institute in Tables 1 below. The antibiotic susceptibility test of the *S. aureus* isolates from wounds, HVS and urine showed that 8(15 %) isolates were resistant to the beta – lactam antibiotics used with equal resistance to Oxacillin, Ampicillin and Cefoxitin. Comparing the three isolates from the sample sites (wounds, HVS and urine): the sensitivity test of the *S. aureus* isolates showed that wounds isolates have the highest resistance to the antibiotics used 5(13.5 %), followed by urine isolates 2(9.61 %) and the least is HVS 1(5.76 %).

Table 1: Zone of inhibition of recovered MRSA strains against selected antibiotics.

Antibiotics / zones of inhibition (mm)							
Isolate code	**Oxa (1 µg)**	**Cef (30 µg)**	**Amp (10 µg)**	**Cip (30 µg)**	**Cex (10 µg)**	**Dox (10 µg)**	**Status**
W_4	09	06	09	10	09	17	MRSA
W_{11}	08	10	06	17	06	19	MRSA
W_{17}	06	06	10	10	10	21	MRSA
W_{18}	06	10	06	18	06	18	MRSA
W_{23}	09	10	10	10	10	20	MRSA
H_9	10	11	09	10	06	19	MRSA
U_5	06	06	06	15	08	19	MRSA
U_{19}	06	06	06	08	06	21	MRSA

Key W = Wounds isolates, H = HVS isolates, U = Urine isolates, Oxa = Oxacillin, Cef = Ceftriaxone, Amp = Ampicillin, Cex = Cefoxitin, Cip = Ciprofloxacin, Dox = Doxycycline

Phytochemical Screening

Phytochemical screening of leaves, seeds and stem back extracts in Table 2 indicates the presence of alkaloid, tannin, saponin, and cardiac glycoside, flavonoid, terpenoid, phenols anthraquinone and steroid. However, reducing sugar, flavonoid and steroid were absent in the leaves while reducing sugar, anthraquinone and saponin were absent in the stem back extract.

Table 2: Phytochemical screening of aqueous leaves, seeds and stem bark extracts of *G. kola.*

Phytochemical	Seeds extract	Leaves extract	Stem bark extract
Alkaloids	+	+	+
Anthraquinone	+	+	+-
Phenols	+	+	+
Cardiac Glycosides	+	+	+
Flavonoid	+	-	+
Terpenoid	+	+	+
Saponin	+	+	-
Steroids	+	-	+
Tannins	+	+	+
Reducing sugar	-	-	-

Key + = Presence of Phytochemical, - = Absent of Phytochemical

Antibacterial activity of the extracts

The antibacterial activity of the plant methanol seed extract against test isolates is presented in Table 3. The result shows that average zone of inhibition shown by the extracts is 11.68 mm with methanolic seeds extract exerting the highest antibacterial effects on the test isolates with average zone of inhibition of 12.78 mm, followed by methanolic leaf extract 12.37 mm then aqueous seed extract with zone of inhibition of 11.87 mm. Least zones of inhibition were recorded in aqueous leaf extract and methanolic stem back extract with zones of inhibition of 11.28 and 10.43 mm respectively. Zone of inhibition shown by control (125 mg/ml Clindamycin), ranges from 17.00 – 22.00mm.

Table 3: Zone of inhibition of different concentration of the *Garcinia kola* extracts against MRSA.

Extracts	Conc. (mg / ml)	Isolates / zones of inhibition (mm)							
		Is1	**Is2**	**Is3**	**Is4**	**Is5**	**Is6**	**Is7**	**Is8**
SME	50	12	10	06	06	11	13	09	11
	100	15	11	10	06	12	15	11	14
	150	14	15	11	09	15	15	12	13
	200	18	15	19	10	19	20	15	17
SAE	50	10	06	10	06	12	10	11	10
	100	13	11	10	06	15	12	13	12
	150	14	11	12	06	16	13	14	13
	200	15	13	16	10	18	17	16	10
LME	50	10	10	10	06	10	12	06	12
	100	09	11	13	10	12	15	10	14
	150	12	13	15	09	16	14	12	14
	200	17	12	15	11	17	18	16	17
LAE	50	10	06	10	06	11	13	06	11
	100	11	06	12	06	12	15	09	15
	150	10	09	14	06	14	14	12	17
	200	12	09	14	06	17	16	14	18
SBME	50	06	06	06	06	10	11	09	10
	100	10	09	10	06	12	12	11	12
	150	10	10	11	06	12	15	10	11
	200	10	12	15	06	15	16	14	15
SBAE	50	06	10	06	06	10	12	10	11
	100	11	11	09	06	12	14	11	14
	150	12	10	11	06	13	16	12	15
	200	11	13	1	06	15	18	14	16
Contrl	125	21	19	19	17	22	21	19	20

Key: SME= Seed Methanol Extract, SAE= Seed Aqueous Extract, LME= Leaves Methanol Extract, LAE= Leaves Aqueous Extract, SBME= Stem Bark Methanol Extract, SBAE= Stem Bark Aqueous Extract, Is = Isolates, Control = Clindamycin = 125 mg / ml

Minimum inhibitory concentration (mic) and mbc of the extracts

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts is represented in table 4 and 5. The result showed that the MIC and MBC of the extracts range from 12.5 – 100 mg/ml of the extracts.

Table 4: Minimum inhibitory concentration (MIC) of *Garcinia kola* extracts against MRSA.

	Isolates/ MIC (mg/ml)							
Extracts	**Is1**	**Is2**	**Is3**	**Is4**	**Is5**	**Is6**	**Is7**	**Is8**
SME	12.5	25.0	100	100	25	25	50	50
SAE	25	100	25	100	12.5	25	25	25
LME	25	25	25	100	50	12.5	100	12.5
LAE	50	NF	25	NF	12.5	12.5	100	12.5
SBME	100	100	100	NF	25	25	50	25
SBAE	100	25	100	NF	12.5	12.5	25	12.5

Table 5: Minimum bactericidal concentration (MBC) of *Garcinia kola* extracts against MRSA.

	Isolates / MIC (mg / ml)							
Extracts	**Is1**	**Is2**	**Is3**	**Is4**	**Is5**	**Is6**	**Is7**	**Is8**
SME	25	25	100	NF	50	50	100	50
SAE	25	NF	50	NF	25	50	50	100
LME	50	100	50	NF	100	50	NF	100
LAE	100	NF	50	NF	50	50	NF	100
SBME	NF	NF	NF	NF	50	100	100	100
SBAE	NF	100	100	NF	50	50	100	50

Key: SME= Seed Methanol Extract, SAE= Seed Aqueous Extract, LME= Leaves Methanol Extract, LAE= Leaves Aqueous Extract, SBME= Stem Bark Methanol Extract, SBAE= Stem Bark Aqueous Extract, Is = Isolates, NF = Not found

Discussion

The findings of this study demonstrated that MRSA isolates are resistant to beta lactam drugs such as oxacillin, ampicillin, ceftriaxone and cefoxitin (Table 1). The wide spread use of antibiotic resulted in the development of resistance to antibiotics through acquisition of the mobile cassette chromosome carrying the methicillin - resistant gene mecA[22] and mecC[23]. The resistance to methicillin was due to a penicillin-binding protein coded for by a mobile genetic element termed the methicillin - resistance gene –mecA[24]. In recent years, the gene has continued to evolve so that many MRSA strains are currently re-

sistant to several different antibiotics such as penicillin, oxacillin and amoxicillin[25].

The Phytochemical screening of the plant parts revealed the present of Alkaloid, Tannin, Saponin, and Cardiac glycoside, Flavonoid, Terpenoid, Phenols Anthraquinone and Steroid. However, Reducing sugar, Flavonoid and steroid were absent in the leaves while Reducing sugar, Anthraquinone and Saponin were absent in the stem back extract (Table 2). The finding of this study was inconformity with that of Adegboye *et al*.[26] on Phytochemical screening of *Garcinia kola,* in which the results shows that *Garcinia kola* seeds contain steroids, cardiac glycosides, flavonoids, tannins, saponins and reducing sugars. The presence of the above phytochemicals in the plant parts was responsible for its antibacterial activity. *Garcinia kola* seeds flavonoids have been shown to possess anti-inflammatory, anti - hepatotoxic and antimicrobial activities[27]. Saponins are known to possess antibacterial activities[28,29] whilst tannins play an important role in wound healing and also possess some antimicrobial activities. Complex mixtures of steroid compounds from plants are known to exhibit some bioactivity[30]. Phytochemical and biochemical studies of *Garcinia kola* showed the presence of sterols, terpenoids, flavonoids, glycosides, pseudo-tannins, saponin, proteins and starch[31]. This result was also inconformity with the present study.

The present study showed that the leaves, seeds and stem back of *G. kola* possess antimicrobial potential against MRSA. In line with the present finding, several other studies have reported antimicrobial potentials. The plant is used for the treatment of liver disorders and has been shown to possess anti-inflammatory, antimicrobial, antioxidant, antiviral, antidiabetic and anti-hepatotoxic activities[32]. The seeds are used in the treatment of bronchitis, throat infections, colics, headaches, chest colds, coughs, diarrhoea, hepatitis, asthma and dysmenorrheal/menstrual cramps[32]. The seed has also shown broad spectrum antibacterial activities[33]. The result of this finding was also in consistency with the result reported by Ogbulie *et al*.[34] on the antimicrobial efficacy of cold, hot water and ethanol extract of *G. kola* which revealed that cold and hot water extract of *G. kola* moderately inhibited the growth of *Staphlococcus aureus* and *Streptococcus pyogenes* with zone of inhibition of between 9-15mm. Also, found the cold and hot ethanol *G. kola* extracts profoundly inhibited the growth of *S. aureus, S. pyogenes, E. coli* and *S. typhi* to about 13 to 21mm. The ethanol extracts of the *G. kola,* also profoundly inhibited the growth of *S. aureus, S. pyogenes, E. coli* and *S. typhi* with zones of inhibitions ranging from 13 - 22mm.

Conclusion

The Phytochemical screening of the plant parts revealed the present of Alkaloid, Tannin, Saponin, and Cardiac glycoside, Flavonoid, Terpenoid, Phenols Anthraquinone and Steroid. The antibacterial activity of the *Garcinia kola* showed that the plant part extracts demonstrated antimicrobial effect against the test MRSA with higher activity in seeds compared to leaves and stem. Higher antibacterial activity was recorded in methanol extract when compared to aqueous extract. Findings from this work support the use of extracts from *G. kola* as medicinal plant.

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