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Research Article

In Vitro Antimicrobial Activity and Bactericidal Kinetics of the Leaf Extracts and Fractions of *Gnetum africanum* on Clinical Wound Isolates

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ABSTRACT

Replicating microorganisms at wound sites pose great health risks to humans especially due to increasing evolution of antimicrobial resistance to several antibiotics. *Gnetum africanum* has been used in ethnomedicine for successful management of wound infections and facilitation of the wound healing process. This study aimed at investigating the antimicrobial activity of leaves of *G. africanum* on bacterial isolates from wounds of diabetic patients. Antibiogram of twenty-six wound isolates was determined using disc diffusion method. Qualitative and quantitative phytochemical analysis of dried pulverized leaves was carried out. Successive gradient extraction of dried pulverized leaves was done with hexane, ethyl acetate and methanol. Bioassay-guided fractionation of methanol extract was carried out using vacuum liquid chromatography (VLC). Antimicrobial activities of the leaf extracts and fractions of *G. africanum* were screened by agar-well diffusion. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by agar-dilution. Bactericidal kinetics of the methanol extract was investigated using the viable count technique. Phytochemical screening detected saponins, alkaloids, tannins, phenols, anthraquinones and flavonoids, with minimal steroidal content. At 100 mg/ml, zones of inhibition ranged from 12-23 mm for all the extracts. MIC values ranged from <0.156->10 mg/mL for the crude extracts while the MIC values for the fractions ranged from 3.125-50 mg/mL. The kill kinetics showed a potent bactericidal activity which was concentration dependent with methanol extract. The study has shown that *G. africanum* extracts possess appreciable bactericidal activity and can be used in the management and treatment of wound infections.

Keywords: Ethnomedicine, Wound infections, MIC, Antibiogram, Phytochemical

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INTRODUCTION

Intact skin serves as a protective barrier against surface skin microbes as well as a protective covering for underlying tissues. The skin is however continuously exposed to different physical agents, wears and tears, as well as different pathogens; eventually leading to inflammation, infections and wounds. A wound is defined as breakage in the continuity of the skin and a resultant loss of continuous epithelium lining (Mummed *et al*., 2018). There are organisms that are resident in the skin and wounds without causing harm. However, when a certain threshold of these organisms is reached, coupled with the formation of biofilms, wound healing might be impeded (Bowler *et al*., 2001, Metcalf and Bowler, 2013, Negut *et al*., 2018). A moist, warm and nutritious environment established due to the breakage in the skin increases microbial colonization and proliferation. Serious bacterial wound infections such as tetanus and gas gangrene can result which may lead to chronic wound infections, long term disabilities and even death (WHO 2013).

Patients with diabetes are beset with several health challenges including but not limited to chronic renal failure, stroke, cardiovascular disease, skin wounds or ulceration. Diabetic patients especially the elderly are frequently faced with painful skin ulcerations which come with concomitant disintegration of the epidermis, dermis and most times subcutaneous tissues (Okonkwo and DiPietro, 2017). Although white blood cells are indispensable for proper functioning of the immune system, their functions are drastically reduced when the blood glucose levels are permanently high. At this stage, the body becomes unable to fight bacteria at wound sites and close up wounds (Endara *et al*., 2013).

Common pathogens implicated in wound infections include *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter* spp, *Proteus mirabilis, Acinetobacter* spp, *Bacteroides* spp, *Clostridium* spp, *Candida* spp, *Peptostreptococcus* spp, *Fusobacterium* spp, and *Aeromonas* spp (Manikandan and Amsath, 2013). Small

inocula of these infecting organisms may increase the risk of serious infections.

Drug resistance has contributed to the severity and duration of wound infections. Inappropriate use of antibiotics and several other factors over time have led to the emergence of resistant microorganisms which contribute to morbidity and mortality. Drug resistance can prolong treatment procedures, increase cost of treatment, reduce treatment options, as well as increase complications in wound infections (Sani *et al.*, 2012, Manikandan and Amsath, 2013).

Wound infections have drawn attention globally because of their significance in morbidity and mortality cases. Traditionally, medicinal plants are used in the treatment of wound infections of various types including external wounds (open, deep, chronic or suppurative). In the fight against antimicrobial resistance, evaluating the effect of plants can be a great boost in providing an alternative for wound infection therapy (Mummed *et al*., 2018).

Over the years, certain plants have been used to treat wound infections because they have specific secondary metabolites which confer medicinal relevance on them. Examples are plants that contain flavonoids such as *Moringa oleifera*, *Rosmarinus officinalis* and *Artemisia absinthium*. Also alkaloids in *Alstonia boonei* and polysaccharides in *Alstonia boonei* and *Parquetina nigrescens* have same effect (Odukoya *et al.*, 2012).

Vegetables are naturally herbaceous and have parts that may be eaten as a main or supporting meal (Edem, 2010). *Gnetum africanum is a* delicious leafy vegetable used in West African countries, especially Nigeria. The leaves of *G. africanum are edible and used in the preparation of* ukazi and afang soups by the Ibo and Ibibio tribes respectively (Nigeria) and Ero (Cameroun). Ethnomedicinally, it is used in the treatment of snake poisoning, diabetes mellitus, enlarged spleen, boils, nausea, sore throat. When cooked, there is significant increase in its nutritional constituents such as magnesium and phosphorus as well as its antioxidant capacity. There is also no significant loss in vitamins, carbohydrates, lipids and available secondary metabolites (Ogbonnaya and Chinedum 2013).

The numerous nutritional beneficial effects of vegetables to humans make them safer alternatives to processed foods and orthodox medicines. This study was undertaken to evaluate the antimicrobial activity of leaf extracts and fractions of *Gnetum africanum* on bacterial isolates from wounds of diabetic patients.

MATERIALS AND METHODS

Collection and Preparation of Plant Samples: The leaves of *Gnetum africanum* were freshly collected from Ikot Okodum in Eket LGA (Akwa Ibom State) and authenticated at the Department of Botany, University of Ibadan with voucher number UIH-222557. The leaves were air-dried, pulverized and weighed.

Phytochemical analysis: Qualitative screening for secondary metabolites of *G. africanum* was done using previously described procedures (Vinoth *et al*., 2012).

Plant extraction: Using the soxhlet apparatus, successive gradient extraction was carried out using solvents of increasing polarities; *n*-hexane, ethyl acetate, and methanol sequentially. Crude extracts were concentrated on a shaker water bath (Lab Tech shaker, model; LSI-3016R, Korea) set at 60 ^{o}C to obtain dried sample extracts. The extracts were weighed and then stored in the refrigerator at 4 °C for subsequent use.

Microorganisms used: Different clinical strains of bacteria obtained from wound ulcers of diabetic patients were collected from the Medical Microbiology Department, University College Hospital (UCH) and Adeoyo State Hospital, Ibadan, Nigeria. The isolates were subcultured on nutrient agar slants and screened for purity. Further biochemical tests were carried out to confirm the identity of the organisms prior to use for the antimicrobial assay. Organisms used for the study were; *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis,* and *Klebsiella pneumoniae.*

Antibiotic susceptibility testing of microbial isolates: Dilutions corresponding to 0.5 McFarland equivalent standard (1.5 x 10^8 CFU/mL) of each isolate were prepared from overnight cultures of the wound isolates. Sterile glass spreader was used to spread inoculum from each isolate on the surface of a separate Mueller Hinton agar plate. A pair of sterile forceps was used to place an antibiotic multi-disc gently but firmly on the surface of the inoculated plates. The Gram negative antibiotic multidisc (Abtek UK) contained the following; ceftazidime (30 µg), cefuroxime (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), augmentin (300 µg), nitrofurantoin (300 µg), ampicillin (10 µg) while the Gram positive antibiotic multidisc (Abtek UK) contained the following; ceftazidime (30 µg), cefuroxime (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), augmentin (300 µg), nitrofurantoin (300 µg), erythromycin (15 µg).

The plates were incubated at 37 °C for 24 h. Zones of growth inhibition were measured and interpreted according to the standards of Clinical Laboratory Standards Institute (CLSI) 2016.

***In vitro* antimicrobial screening of plant extracts:** The antibacterial activity of the plant extracts was determined by agar-well diffusion method. A 0.5 McFarland standard equivalent suspension of each isolate was made in 0.85 % saline and 0.1 mL of resulting isolate suspension was used to inoculate the plates of Mueller Hinton Agar. Wells of equal distances were bored with the aid of a standard sterile 8mm cork borer and 100 µL of different concentrations of extracts and control were placed into the corresponding wells. Gentamicin (10 µg) was used as the standard drug control. Diffusion of the extract was allowed by leaving the plates for about one hour at room temperature. The plates were then incubated at 37 °C for 24 h.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts: Double-fold serial dilutions of the plant leaf extracts (0.156-50 mg/mL) were made from which 1 mL was

taken and added to 19 mL of pre-sterilized molten Mueller Hinton agar at temperature of 40 °C. The media was poured into sterile petri dishes and allowed to solidify. The surfaces of the media were allowed to dry. A 0.5 McFarland standard equivalent suspension of each isolate was made in 0.85 % normal saline from which 0.1 mL was used to inoculate the plates. The plates were incubated at 37 °C for 24 h and later examined for the presence or absence of growth. The MIC was taken as the lowest concentration that prevented bacterial growth. For the MBC, sterile inoculating loop was swabbed on the MIC plates that did not show growth and used to inoculate freshly prepared nutrient broth. The inoculated broth suspensions were incubated at 37 °C for 24 h. The lowest concentration in which extracts did not allow growth of the test organisms was recorded as MBC (Adeniyi *et al*., 2016).

Vacuum Layer Chromatography (VLC): The methanol leaf extract was subjected to fractionation using VLC with solvents of varying polarities. Fifteen grams of the extract was adsorbed with silica gel (60-200 mesh size) and 100 mL of different ratios of solvents (hexane 100; hexane/chloroform 75:25, 50:50, 25:75, chloroform 100, chloroform/ethyl acetate 75:25, 50:50, 25:75, ethyl acetate 100, ethyl acetate/methanol 75:25, 50:50, 25:75 and methanol 100) was used for elution. The eluents were collected in clean tubes. Fractions were spotted on thin layer chromatographic (TLC) plates and developed with mobile phases of hexane/ethyl acetate/methanol (3:5:2), hexane/ethyl acetate (4:6). The fractions were pooled together based on their TLC profiles. The pooled fractions were concentrated to dryness and weighed. The antimicrobial assay was carried out using agar-well diffusion technique as previously described for the crude extracts.

Kill-kinetics Assay: One millilitre of overnight broth culture was inoculated into 4 mL of freshly prepared nutrient broth for each isolate. For the organism to reach an exponential phase, the broth culture was incubated for 18 h at 37°C. One millilitre of the crude leaf extract at a concentration equal to the MIC was put into 2.9 mL of nutrient broth and 0.1 mL of inoculum was added. Serial dilutions were made from this mixture and 0.1 mL each from 10^{-3} and 10^{-5} dilutions were used to inoculate freshly prepared nutrient agar at 30 minutes, 1 h, 1 h 30 minutes, 2 h, 4 h and 6 h (Lajubutu *et al*., 1995). After the incubation, microbial colony count was done and a graph of log of CFU/mL was plotted against time.

RESULTS

Phytochemicals detected were saponins, alkaloids, tannins, coumarins, anthocyanins, terpenoids, phenols, anthraquinones, flavonoids, and steroids (Table 1). Antibiogram results revealed that most of the test isolates employed in this study were Multi-Drug Resistant (MDR), being resistant to at least one antibiotic in three different classes of antibiotics (Magiorakos *et al*., 2012) as shown in Table 2. Most of the clinical isolates were sensitive to gentamicin and nitrofurantoin. The highest resistance to different classes of antibiotics was observed in *Klebsiella pneumoniae* followed by *Escherichia coli*.

Table 1:
Results of phytochemical analysis of *Gnetum africanum*

Parameters	Qualitative	Quantitative
Saponin	+	6.6%
Alkaloid	+	3.4%
Flavonoid	+	1.8%
Tannin	+	0.6%
Coumarin	+	ND
Steroid	+	ND
Terpenoid	+	ND
Cardiac Glycosides	-	ND
Anthocyanin	+	ND
Quinones	-	ND
Anthraquinone	+	ND
Phlobatanin	-	ND
Phenols	+	1.8%

Key: - : Absence of phytochemical, +: presence of phytochemical, ND: not done

The zone of growth inhibition results in Table 3 showed that *n*- hexane extract and ethyl acetate extract of *G. africanum* had better activity in comparison to the methanol extract. The crude extracts had remarkable antimicrobial activities on all the Gram negative organisms used while showing resistance to gentamicin, the standard drug control used for the study. MIC ranged from < 0.156 to 2.5mg/mL for *n*-hexane and ethyl acetate extracts while the MIC of methanol extract ranged from < 0.156-> 10 mg/mL as shown in Table 4. The Time-Kill analysis for *G. africanum* as shown in Figures 1 and 2 showed gradual reduction of surviving organisms per hour. The rate of kill for *S. aureus* was faster than that of *E. coli*. Complete kill was achieved for *S. aureus* at 1 hour when the concentration corresponding to the MIC was used while a total kill was achieved in 30 minutes when the concentration corresponding to 4MIC was used. For *E. coli*, complete bactericidal action was achieved at 4 hours for all the three concentrations used with 4MIC concentration having the fastest rate of kill.

Fractionation of the crude methanol extract yielded six fractions (G1-G6) with the physical characteristics of fractions and percentage yield shown in Table 5. Fractions of *Gnetum africanum* had lesser antimicrobial activity on the test isolates than the crude extracts as seen in the zones of growth inhibition (Table 6) with higher MIC and MBC values which ranged from 3.125-50 mg/mL and 25-100 mg/mL respectively (Table 7).

DISCUSSION

Gnetum africanum is an edible plant used in ethnomedicine for the treatment of wounds. This study investigated the activity of extracts and fractions of the plant on clinical wound pathogens from diabetic patients. Phytochemical screening revealed the presence of secondary metabolites such as saponins, alkaloids, flavonoids, tannins, coumarins, steroids, terpenoids, anthocyanins, anthraquinones and phenols.

Table 2:
Antibiogram profile of *Staphylococcus aureus* isolates

Isolate	CAZ (30µg)	CRX (30µg)	GEN (10µg)	CPR (5µg)	OFL (5µg)	AUG (30µg)	NIT (300µg)	ERY (15µg)	AMP (10µg)
S.a 1	R	R	S	S	S	R	R	R	NT
S.a 2	R	R	S	S	S	R	R	R	NT
S.a 3	R	R	S	S	S	R	R	R	NT
S.a 4	R	S	S	S	S	R	S	R	NT
S.a 5	R	S	S	S	S	R	S	R	NT
Ec 1	R	R	S	R	R	R	S	NT	R
Ec 2	R	R	S	R	R	R	S	NT	R
Ec 3	R	R	S	R	R	R	S	NT	R
Ec 4	R	R	S	I	S	R	S	NT	R
Ec 5	R	R	S	I	S	R	S	NT	R
Ec 6	R	R	S	I	S	R	S	NT	R
Kp 1	R	R	S	R	R	R	S	NT	R
Kp 2	R	R	R	R	R	R	S	NT	R
Kp 3	R	R	R	R	R	R	I	NT	R
Kp 4	R	R	R	R	R	R	I	NT	R
Kp 5	R	R	S	R	R	R	S	NT	R
Kp 6	R	R	R	R	R	R	R	NT	R
Kp 7	R	R	S	R	R	R	S	NT	R
Pm 1	I	I	I	I	I	R	I	NT	R
Pm 2	I	S	S	S	I	S	R	NT	R
Pm 3	I	S	S	S	I	R	R	NT	R
Pm 4	R	S	S	I	S	R	S	NT	R
Pm 5	R	I	R	I	I	R	I	NT	R
Pm 6	R	R	S	S	S	I	R	NT	R
Pa 1	R	R	R	R	R	R	R	NT	R
Pa 2	R	S	S	S	S	R	R	NT	R

S.a = Staphylococcus aureus, Ec = Escherichia coli, Kp = Klebsiella pneumoniae, Pm = Proteus mirabilis, Pa = Pseudomonas aeruginosa, CAZ= Ceftazidime, CRX= Cefuroxime, GEN= Gentamicin, CPR= Ciprofloxacin, OFL=Ofloxacin, AUG= Augmentin, NIT= Nitrofurantoin, ERY= Erythromycin, AMP=Ampicillin, NT=Not tested. R= Resistance, I= Intermediate, S= Susceptible

Table 3:
Antimicrobial screening of *G. africanum* extracts against clinical isolates

Isolates	*n*-Hexane extract				Ethyl acetate extract				Methanol extract				Gentamycin
	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	10µg
	Zones of inhibition(in mm)												
S. aureus$_1$	12	11	10	10	12	13	10	11	13	13	12	12	NZ
S. aureus$_2$	21	21	20	18	23	19	20	19	19	17	16	14	NZ
S. aureus$_3$	15	14	14	12	23	20	10	18	20	18	18	16	NZ
S. aureus$_4$	14	13	12	10	19	17	16	15	19	17	15	13	13
P. aeruginosa$_1$	20	20	15	10	20	18	16	14	18	16	13	15	NZ
P. aeruginosa$_2$	20	20	16	12	22	15	16	16	20	18	13	12	15
P. aeruginosa$_3$	16	15	15	12	18	18	18	16	22	17	16	15	20
P. aeruginosa$_4$	18	18	15	13	20	18	15	15	18	17	15	13	13
P. mirabilis$_1$	20	20	17	15	16	14	12	NZ	NZ	NZ	NZ	NZ	NZ
P. mirabilis$_2$	28	25	23	20	18	17	16	15	22	20	20	16	25
P. mirabilis$_3$	20	19	17	13	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
P. mirabilis$_4$	22	20	18	15	19	13	13	NZ	NZ	NZ	NZ	NZ	NZ
K. pneumoniae$_1$	20	17	15	15	15	15	13	13	20	17	15	13	NZ
K. pneumoniae$_2$	16	12	12	10	17	14	13	12	NZ	NZ	NZ	NZ	NZ
E. coli$_1$	14	12	11	11	15	15	12	11	14	13	12	12	NZ
E. coli$_2$	15	15	15	12	21	18	18	19	22	20	20	20	NZ

NZ = No Zone of growth inhibition, Ga = *Gnetum africanum*, *S. aureus = Staphylococcus aureus, E. coli = Escherichia coli, K. pneumoniae = Klebsiella pneumoniae, P. mirabilis = Proteus mirabilis, P. aeruginosa = Pseudomonas aeruginosa*

Table 4:
MIC and MBC of *n*-hexane, ethyl acetate and methanol extracts of *Gnetum africanum* on clinical isolates

Organism	*n*- Hexane extract (mg/mL)		Ethylacetate Extract (mg/mL)		Methanol Extract (mg/mL)	
	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus$_1$	0.625	>10	0.625	0.625	1.25	1.25
S. aureus$_2$	<0.156	>10	<0.156	0.625	< 0.156	0.625
K.pneumoniae$_1$	1.25	1.25	0.625	>10	2.5	>10
K.pneumoniae$_2$	2.5	1.25	2.5	>10	1.25>	>10
P.mirabilis$_1$	0.625	1.25	0.3125	0.625	1.25	1.25
P.mirabilis$_2$	0.625	1.25	0.625	0.625	0.625	0.625
E.coli$_1$	1.25	1.25	2.5	2.5	1.25	1.25
E.coli$_2$	< 0.156	<0.15625	< 0.156	<0.156	1.25	1.25
P.aeruginosa$_1$	0.625	0.625	< 0.156	< 0.156	< 0.156	1.25
P.aeruginosa$_2$	< 0.156	0.625	< 0.156	< 0.156	< 0.156	1.25

MIC= Minimum inhibitory concentration, MBC= Minimum bactericidal concentration, *S. aureus = Staphylococcus aureus*, *E. coli = Escherichia coli*, *K. pneumoniae = Klebsiella pneumoniae*, *P. mirabilis = Proteus mirabilis*, *P. aeruginosa = Pseudomonas aeruginosa*

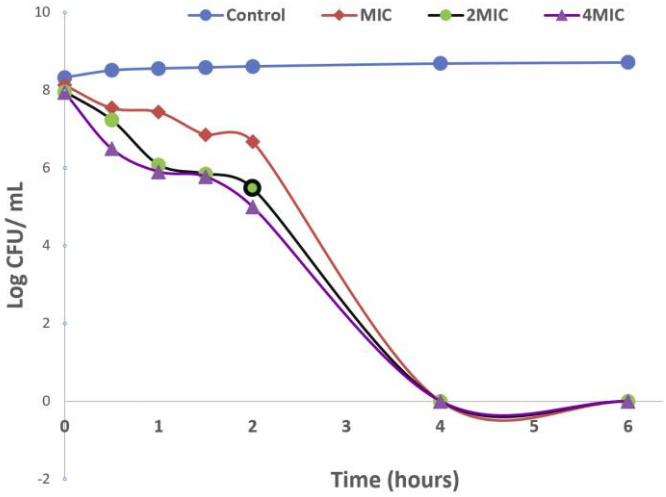

Figure 1
Time Kill kinetics of ethyl acetate extract of *Gnetum africanum* on *Escherichia coli*

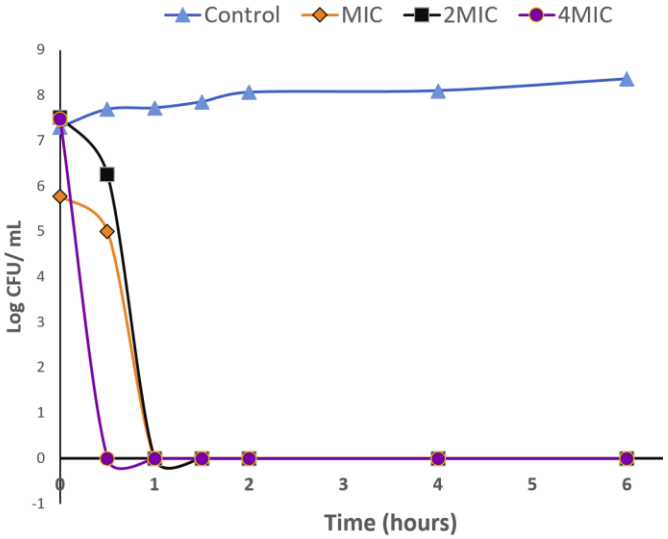

Figure 2
Time Kill kinetics of ethyl acetate extract of *Gnetum africanum* on *Staphylococcus aureus*

Table 5:
Physical characteristics, weight and percentage yield of fractions of crude methanol extract of *Gnetum africanum*

Fractions	Physical Characteristics	Weight(Grams)	Yield (%)
GA1	Light green	0.49	3.25
GA2	Dark green	0.42	2.8
GA3	Dark green	0.06	0.4
GA4	Dark green	0.79	5.3
GA5	Golden yellow	6.41	42.7
GA6	Dark green	2.79	18.65

Key: GA = Gnetum africanum fraction

Plants antimicrobial activity is ascribed to various secondary metabolites possessed. These secondary metabolites have the ability to simultaneously target different sites thus making it difficult for antimicrobial resistance to evolve (Padmanabhan and Jangle, 2012). Saponins, for example, are potent membrane permeabilizing agents which additionally have immunostimulatory, hypocholestrelomic, anti-carcinogenic, anti-microbial, anti-protozoan and antioxidant actions (Francis *et al.*, 2002; Sparg *et al.*, 2004). Flavonoids disrupt cytoplasmic membrane functions and also affect bacterial DNA gyrase. Alkaloids are responsible for intercalating with DNA thereby disrupting the bacterial DNA structure. Tannins form complexes with cell wall polysaccharides, leading to inhibition of microbial growth. They also inactivate microbial cell envelope (Raaman 2006, Usman *et al*., 2009). The observed antimicrobial activity in this study is most likely due to the different phytochemicals acting on different cell sites. The result is also consistent with what was reported by Eneh *et al*., (2017) where he reported antimicrobial activity of aqueous and methanol extract against various bacterial isolates.

The challenge organisms employed in this study were mostly multi-drug resistant pathogens (CLSI, 2016). The sensitivity of most of the clinical isolates used in the study to gentamicin and nitrofurantoin is an indication that these drugs may be useful in treating wound infections.

Table 6:
Antimicrobial activity of fractions obtained from VLC fractionation of crude methanol extract of *Gnetum Africanum* on selected isolates.

Fractions	**GA1**			**GA2**			**GA3**			**GA4**			**GA5**			**GA6**			**GN**	
Conc. (mg/mL)	100	50	25	100	50	25	100	50	25	100	50	25	100	50	25	100	50	25	10	100
Isolates	Zones of inhibition(mm)																			
S3	13	NZ	NZ	12	NZ	NZ	13	NZ	NZ	13	NZ	NZ	14	NZ	NZ	13	NZ	NZ	20	
S4	15	12	NZ	13	12	NZ	14	12	NZ	15	12	NZ	14	NZ	NZ	14	12	NZ	25	
E2	15	NZ	NZ	14	NZ	NZ	15	NZ	NZ	13	NZ	NZ	13	NZ	NZ	12	NZ	NZ	20	
P5	15	12	NZ	14	12	NZ	14	11	NZ	14	12	NZ	15	11	NZ	14	11	NZ	25	
K7	12	10	NZ	13	11	NZ	11	10	NZ	12	11	NZ	13	11	NZ	13	11	NZ	21	
PSI	13	11	NZ	13	11	NZ	14	12	NZ	14	11	NZ	13	11	NZ	12	10	NZ	20	

Key: GA = *Gnetum africanum* fraction, GN = Gentamycin, Conc. = Concentration, S = *Staphylococcus aureus,* E = *Escherichia coli,* P = *Proteus mirabilis,* K = *Klebsiella pneumoniae,* PS = *Pseudomonas aeruginosa,* NZ = No zone of growth inhibition.

Table 7:
Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the most active fraction of *Gnetum africanum.*

Isolate	**MIC (mg/ml)**	**MBC (mg/ml)**
E1	3.125	100
E2	50	50
E4	6.25	50
S2	12.5	25
S3	3.125	100
S5	3.125	50
K2	25	50
K6	25	50
P3	25	100
P6	50	100

The high antimicrobial resistance to different classes of antibiotics observed in Gram negative organisms in the study is not new, as there have been reports of high rates of resistance in Gram negative organisms (Okon, et al., 2014). Yishak and Biruk (2009) reported that about 51% of Gram negative organisms isolated from wounds are multidrug resistant, while in this study we found out that about 60% of the Gram-negative organisms were multidrug resistant. The ability of these organisms to exhibit this characteristic might be related to the different antimicrobial resistance strategies explored by the organisms such as modification of the antimicrobial target within the bacteria, expulsion of the antimicrobial agent from the bacterial cell, degradation of the active component of the antimicrobial agent or reduced access of the antimicrobial into the bacterial cell (Walsh 2000, Chellat *et al*., 2016). The resistant nature of these organisms to current antibiotics suggests an urgent need to explore other ways/sources of their elimination.

Gnetum africanum extracts as revealed by this study possess potent anti-bacterial activity with all the three extracts (n-hexane, ethyl acetate and methanol) showing anti-bacterial activity against the test organisms used. This is consistent with the results observed by Ayuk *et al.* (2017) where it was reported that extracts of *Gnetum africanum* had activity against *Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa.* Previous study by Ilodibia *et al.* (2015) showed minimal antimicrobial activity with ethanol extract of *G. africanum* but an appreciable activity with the aqueous extract against *S. aureus* and *E. coli*. However, this study has shown the constituents of the plant contained in the *n*-hexane, ethyl acetate and methanol extracts of *G. africanum* to be more active. The diameters for the zones of inhibition showed that hexane and ethyl acetate extracts of *G. africanum* had remarkable antimicrobial activity. Gentamicin, the standard drug control used for the study was not effective on most of the test isolates which were susceptible to the plant extracts.

Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial agent required to inhibit the growth of an organism. It is determined to evaluate the effectiveness of antimicrobials and extracts in inhibiting the growth of tested organisms. The observed diameter of the inhibition zone is inversely related to the MIC (Flanagan and Steck (2017). This means that the more susceptible a microorganism is to an antimicrobial agent, the larger the inhibition zone diameter and vice versa (Doughari *et al*., 2007). The MIC values of the extracts agreed with the corresponding antibacterial activities. The ethyl acetate extract which produced the widest inhibition zones and lowest MICs is considered as the most active extract. This indicated that the active principles of the plants are more concentrated in the mid-polarity spectrum. As the MICs were very low in a good number of cases, the appropriateness for the extracts as therapeutic products is possible.

The susceptibility tests of the fractions of methanol extract of *Gnetum africanum* revealed that the fractions had antimicrobial activity albeit less than the crude methanol extract. This indicates that the active metabolites responsible for antimicrobial activity in the plant have a synergistic and or additive effect and when separated the activity reduces. The MIC result of the fractions gave credence to the activity of the plant against *S. aureus*, suggesting its broad spectrum antimicrobial activity. Similar reports demonstrating the activity of *G. africanum* against *S. aureus* was reported by Obiukwu and Nwanekwu (2010).

The Time-Kill analysis for *G. africanum* showed a concentration dependent pattern with gradual reduction in the number of surviving organisms per hour. The kill-kinetics suggested that the action of the plant extracts may be cidal rather than inhibitory or static as there was no surviving organism at the end of 4 hours of incubation.

From the earlier discourse, extracts of *G. africanum* are effective against the bacteria isolated from wound sites of diabetic patients, this implies that active pure compounds of the plants can be harnessed and developed into drug compounds with the sole aim of augmenting the treatment regimen for patients with infected wounds. The findings of

this study highlighted that *G. africanum* has activities against bacteria isolated from wound infections. Subsequently, it will be useful in the treatment and management of wound infections. Furthermore, the positive findings from this study provide a scientific basis for the traditional use of the plant in the treatment of wounds.

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