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Susceptibility of Multi-Drug Resistant Wound Pathogens to Extracts and Fractions of *Ficus Vogelii* (Miq) and *Telfairia occidentalis* (Hook F.) and Bactericidal kinetics

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: Wound infection is the third most common nosocomial infection worldwide, with resultant high mortality and morbidity rates in developing countries. Many pathogenic microrganisms implicated in wound infections have evolved antimicrobial resistance, necessitating a focused search for important therapeutic bioactive compounds from natural sources.

Objective: To investigate the antimicrobial potential of leaf extracts and fractions of *Ficus vogelii* and *Telfairia occidentalis* against wound clinical isolates.

Materials and Methods: Pulverized plant leaves were screened for the presence of phytochemicals. Extraction of ground leaves was by cold maceration with methanol, and fractions were obtained by partitioning into hexane, ethyl acetate and methanol. Antibiogram of clinical isolates was determined via disc diffusion method. Antimicrobial susceptibility of test isolates was determined by agar-well diffusion while minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by microbroth dilution assay. Time-kill assay of the methanol fraction of plants was carried out using the viable count technique.

Results: Alkaloids, anthraquinones, cardiac glycosides, flavonoids, saponins, steroids, tannins, and terpenoids were the phytochemicals detected. The plant extracts and fractions had appreciable inhibitory effects against the multi-drug resistant test organisms, with MIC and MBC values ranging from 3.125 to 12.5 mg/mL and 12.5 to > 50 mg/mL, respectively. A total kill of the organisms was achieved at 24 hours at 3.125mg/mL and 6.25 mg/mL of methanol fractions of both plants.

Conclusion: *Ficus vogelii* and *Telfairia occidentalis* contain bioactive compounds that can be developed into standard chemotherapeutics for the management and treatment of wound infections.

Keywords: *Ficus vogelii*, *Telfairia occidentalis*, Wound, Antimicrobial, Time-kill Assay

INTRODUCTION

Plants have long been utilized for therapeutic purposes in many countries, and they are sources of a number of efficacious medications. (Mahbubur-Rahman *et al.*, 2013). In the treatment of infections, some plants have been found effective due to the presence of biologically active chemical substances like tannins, essential oils, alkaloids, saponins, flavonoids, and other chemical compounds which are curative. Therefore, over time, the use of plants as medicine has been warmly embraced despite the advancement of pharmaceutical research (Mazid *et al.*, 2012).

Wound infection is the presence of replicating microorganisms within a wound and these organisms delay healing of wounds, cause the wound to break down, ultimately increasing the cost of treatment

(Ogba *et al.,* 2014). There are a number of pathogenic organisms in wound infections which include *Staphylococcus aureus, Acinetobacter spp.*, Beta-hemolytic Streptococci *(S. pyogenes, S. agalactiae), Escherichia coli, Stenotrophomonas* spp and *Proteus* spp. Antibiotics such as Amoxicillin-clavulanate, Cephalexin, Clindamycin, Dicloxacillin, Doxycycline, Gentamicin, and Trimethoprim-sulfamethoxazole are commonly used to treat infected wounds, however the emergence of multidrug-resistant wound pathogens pose a challenge to health care practitioners. Medicinal plants, on the other hand, show potential as a treatment option for wound infections.

Ficus vogelii belongs to the fig genus in the family Moraceae. It is mostly found in West and Central Africa. It is popularly called rubber tree and locally called 'kujung' by the Obudu people in Nigeria. Ethnomedicinally, it is used to treat cardiovascular diseases and diabetes. Extracts of the leaves have been reported to have local antimicrobial effects and is also useful for the treatment of wounds (Igile *et al.,* 2015). *Ficus vogelii* has been shown to be effective as an antiulcer, anti-anemia, and anti-diabetic.

Telfairia occidentalis is a vine in the family Cucurbitaceae. It is commonly called fluted pumpkin. Saponins, anthraquinones, tannins, alkaloids, flavonoids, steroids, and reducing sugars have been found in the leaves and stem. Cucurbitacins, a very bitter and toxic substance found in this plant, has been extensively studied as a phytochemical of great interest. (Ajuru and Nmom, 2017). Ethnobotanically, it is used for the treatment of malaria, sudden attack of convulsion, and anemia (Obinaju *et al.,* 2015). It also has the ability to regenerate testicular damage and increase spermatogenesis (Nwangwa *et al.,* 2007). Extracts of *Telfairia occidentalis* have been shown to be hepatoprotective, ameliorates the severity of BPH and inhibit pathogenic microorganisms (Nwakama *et al.;* 2014; Ajani and Akinyemi, 2015; Oladele *et al.,* 2017).

There is currently insufficient information on the antimicrobial activity of *Ficus vogelii* and *Telfairia occidentalis* on microorganisms implicated in wound infections, hence the antibacterial activity of *Ficus vogelii* and *Telfairia occidentalis* on clinical wound isolates was investigated in this study

METHODOLOGY

Plant collection

The leaves of *Ficus vogelii* and *Telfairia occidentalis* were collected freshly from the Botanical Garden of the University of Ibadan in Oyo state, Nigeria. The samples were authenticated at the Forestry Research Institute of Nigeria (FRIN), Ibadan with voucher numbers FHI 112110 for *Ficus vogelii* and FHI 112109 for *Telfairia occidentalis.* The leaves were air-dried under shade at about 29^{o}C, pulverized, weighed and stored in airtight containers.

Phytochemical Screening

Qualitative phytochemical screening was carried out on the pulverized leaves to detect the presence of secondary metabolites (Sofowora, 2012).

Plant Extraction and Fractionation

Dried pulverized leaves (1000 g of *Ficus vogelii* and 800 g of *Telfairia occidentalis*) were extracted by cold maceration in methanol. The plant sample was transferred into a glass container and five liters (5L) of methanol was added, stirred intermittently and left to stand for 72 hours. Then the solvent containing the extract was collected with a muslin bag. The above process was repeated by the addition of pure methanol solvent into the shaft collected. The crude extract obtained from each plant was then filtered with Whatmans filter paper (1mm diameter). The filtrate was concentrated using rotary evaporator set at 40°C. The crude methanolic extract was further concentrated using a vacuum oven set at 40°C with a pressure of 700mmHg. The extract was weighed and percentage yield computed. To obtain the methanol, ethyl acetate and hexane fractions of the crude extracts, the crude methanolic extract of *Ficus vogelli* (10.30g) and *Telfairia occidentalis* (5.85g) were mixed with 100 mL of methanol each and stirred. Distilled water of about 100 mL was added to the solution, stirred and transferred into a 500 mL separating funnel and 200 mL of pure hexane was added, shaken and allowed to stay for 15 minutes for proper partitioning of the hexane and methanol/water layer. The hexane layer was collected. Pure hexane (200mL) was added to the methanol / water layer again until a clear hexane layer was obtained. The above process was repeated using ethyl acetate solvent to obtain ethyl acetate fraction of the extract. The fractions were concentrated using rotary evaporator set at 40°C. With the use of a vacuum oven set at 40°C with a pressure of 700mmHg,

further concentration was achieved. The fractions were weighed and the percentage yield calculated.

Test organisms

Clinical isolates from wounds were obtained from the Department of Medical Microbiology, University College Hospital, Ibadan. The isolates which were previously identified by the source laboratory were screened for purity and confirmation of their identity was done by standard biochemical tests. The organisms included *Cronobacter sakazaki* (1), *Enterobacter cloacae* (1), *Escherichia coli* (2), *Klebsiella pneumoniae* (2), *Proteus vulgaris* (3), *Pseudomonas aeruginosa* (3) and *Staphylococcus aureus* (4). The microorganisms were inoculated on nutrient agar slants and stored at 4°C.

Antibiotic susceptibility testing

Antibiotic sensitivity pattern of the clinical isolates was determined by standard disc diffusion method (CLSI, 2018). Inoculum of overnight broth culture of the bacterial isolates was transferred into nutrient broth and dilutions corresponding to McFarland standards were used to inoculate the surface of sterile Mueller Hinton agar plates using sterile applicators. The seeded plates were dried for 30 minutes and the antibiotic multi-discs firmly placed on the surface of the agar using a pair of sterile forceps. The plates were incubated at 37ºC for 24 hours and the zones of inhibition were measured. Interpretation of results was done using CLSI (2018) standard. The antibiotic multi-discs for the Gram-negative organisms contained Ceftazidime (CAZ) 30µg, Cefuroxime (CRX) 30µg, Gentamicin (GEN) 10µg, Ceftriaxone (CTR) 30µg, Erythromycin (ERY) 5µg, Cloxacillin (CXC) 5µg, Ofloxacin (OFL) 5µg, Augmentin (AUG) 30µg (Abtek UK). The antibiotic multi-discs for Gram positive organisms contained Ceftazidime (CAZ) 30µg, Cefuroxime (CRX) 30µg, Gentamicin (GEN) 10µg, Cefixime (CXM) 5µg, Ofloxacin (OFL) 5µg, Augmentin (AUG) 30µg, Nitrofurantoin (NIT) 300µg, and Ciprofloxacin (CPR) 5µg (Abtek UK).

***In vitro* antimicrobial screening of plant extracts and fractions**

Antimicrobial screening was conducted using agar well diffusion as described by Coker and Onu (2019) with slight modification. The extracts were dissolved in 40% $DMSO_4$ to give concentrations which ranged from 3.125-100 mg/mL. Bacterial suspension adjusted to 0.5 McFarland standard was used to inoculate Mueller Hinton agar plates with the aid of sterile cotton tipped applicator. Equidistant wells of about 8 mm were bored with a cork borer in the set agar. The wells were filled with graded concentrations (3.125 mg/mL-100 mg/mL) of the prepared extracts and fractions. Gentamicin (10µg) was used as the standard drug control (positive control) while 40% $DMSO_4$ was used as negative control. The plates were left on the bench for an hour to allow the extracts diffuse into the agar before incubating at 37 ºC for 24 h. Zones of inhibition were measured after the incubation period. The experiment was done in duplicates.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC of the extracts were determined using a micro-broth dilution assay (Kuete *et al*. 2008). The crude extracts and fractions were dissolved in Mueller Hinton Broth (MHB) to obtain stock concentration of 50 mg/ml, after which they were serially diluted to obtain 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 3.125 mg/mL, 1.5625 mg/mL, and 0.78125 mg/mL concentrations. Serial two-fold dilutions of each sample were made with MHB and 100 µL of each concentration was put into the wells of the microtitre plates which contained 95 µL of MHB and 5 µL of inoculum. The wells which contained 195µL of MHB and 5 µL of inoculum served as the negative control while gentamicin served as the positive control. The plates were covered with sterile covers and mixture was agitated with a shaker. The plates were incubated at 37ºC for 24 hours. The MIC of the samples was obtained on addition of 40µL of 0.2mg/ml of p-iodonitrotetrazolium chloride to contents of the wells and incubated at 37ºC for 30 minutes. A color change to pink indicated the presence of viable bacteria cells. The lowest concentration in which there was no growth was taken as the MIC. A loop full of the inoculum from the wells showing no visible growth was inoculated on Muller Hinton Agar and incubated at 37ºC for 24 hours. The lowest concentration showing no growth was taken as MBC.

Kill kinetics

The concentrations used for the kill kinetics of plant extracts were the MIC, 2MIC, 4MIC values which are 3.125 mg/mL, 6.25 mg/mL and 12.5 mg/mL respectively. Five milliliters (mls) of Tryptone Soya

Broth (TSB) was prepared in a universal bottle and sterilized after which it was cooled and inoculated with the selected organism for 18 hours at 37ºC. One milliliter of the overnight broth culture was sub cultured in 4 mls of already prepared and sterilized TSB broth to maintain a sustained log phase of growth. Using aseptic procedures, 0.1 mL of the culture was transferred to 2.9 mls of TSB broth which already contained 1ml of the crude plant extract making a total of 4mls. 0.1 mL of the mixture was aseptically taken and serially diluted to obtain a 10^{-4} dilution and 0.1 mL of this dilution was seeded to an already prepared and sterilized Mueller Hinton agar in a bottle. The bottle was gently rotated between palms and poured into the sterile plates and allowed to set. This gave zero minute. Other volumes were withdrawn at selected intervals which were 30mins, 1 hour, 1.5 hours, 2 hours, 4 hours, 6 hours and 24 hours. The procedure was carried out for the concentrations of MIC, 2MIC, 4MIC, the positive control (Gentamicin) and the negative control which contained no crude plant extract. The plates were incubated for 24 hours and the numbers of colonies counted using Stuart Scientific Colony Counter. A graph of Log CFU/mL against exposure time was plotted.

RESULTS AND DISCUSSION

Qualitative phytochemical screening of the leaves of *Ficus vogelii* and *Telfairia occidentalis* revealed the presence of terpenoids, steroids, flavonoids, anthraquinones, alkaloids, tannins, and saponins. Cardiac glycosides were present in only *Telfairia occidentalis* (Table 1). This corroborates the result of the work done by Igile *et al.* (2015) and Inuwa *et al.* (2012), who reported the presence of these compounds in their study plant.

The presence of these compounds can be said to be responsible for their pharmacological activities. According to Arabski *et al.* (2012), saponins are detergent-like compounds capable of increasing the permeability of bacteria cell membranes. A similar work done by Maatalah *et al* (2012) revealed the antibacterial and antifungal properties of alkaloids. Terpenoids have been isolated from a variety of plants and have demonstrated antibacterial activity against Gram positive and Gram negative organisms.

Table 1: Qualitative phytochemical results of leaf extracts of *Ficus vogelii* and *Telfairia occidentalis*

Phytochemicals	*Ficus vogelii*	*Telfairia occidentalis*
Terpenoids	+	+
Steroids	+	+
Anthraquinones	++	+
Alkaloids	+	+
Tannins	+	+
Flavonoids	+	+
Saponin	+	+
Cardiac glycosides	-	+

Key: -; absence of phytochemical, +; presence of phytochemical, ++; abundance of phytochemical.

From the antibiogram profile, all the test organisms were multi-drug resistant being resistant to more than two classes of antibiotics The organisms were also resistant to the control drug used (Gentamicin). These organisms may pose a threat to mortality and morbidity of patients because treating infections caused by these organisms will be difficult due to their resistance to standard antibiotics.

Table 2: Antibiogram profile of test organisms

Organisms	CPR	NIT	GEN	CRX	OFL	CAZ	CXM	AUG	CTR	ERY	CXC
Cr.s	R	S	R	R	R	R	R	R	NA	NA	NA
En.c	R	S	R	R	R	R	R	R	NA	NA	NA
Es.c.1	R	S	R	R	R	R	R	R	NA	NA	NA
Es.c.2	R	R	R	R	R	R	R	R	NA	NA	NA
Kl.p.1	R	S	R	R	I	R	R	R	NA	NA	NA
Kl.p.2	R	S	R	R	R	R	R	R	NA	NA	NA
Pr.v.1	R	S	R	R	R	R	R	R	NA	NA	NA
Pr.v.2	R	S	R	R	R	R	R	R	NA	NA	NA
Pr.v.3	R	S	R	R	R	R	R	R	NA	NA	NA
Ps.a.1	S	R	R	R	S	R	R	R	NA	NA	NA
Ps.a.2	S	R	S	R	S	R	R	R	NA	NA	NA
Ps.a.3	S	R	S	R	S	R	R	R	NA	NA	NA
St.a.1	NA	NA	R	R	S	R	NA	R	R	R	R
St.a.2	NA	NA	R	R	R	R	NA	R	R	R	R
St.a.3	NA	NA	R	R	R	R	NA	R	R	R	R
St.a.4	NA	NA	R	R	S	R	NA	R	R	R	R

Key: I= Intermediate, R= Resistant, S= Sensitive, NA= not applicable
CAZ = Ceftazidime, CRX = Cefuroxime, GEN = Gentamicin, CXM = Cefixime, OFL = Ofloxacin, AUG= Augmentin, NIT= Nitrofurantoin, CPR = Ciprofloxacin, CTR = Ceftriaxone, ERY = Erythromycin, CXC = Cloxacillin
Cr.s= *Cronobacter sakazaki*, En.c= *Enterobacter cloacae*, Es.c = *Escherichia coli*, Kl.p = *Klebsiella pneumoniae,*, Pr.v = *Proteus vulgaris*, Ps.a = *Pseudomonas aeruginosa*, St.a = *Staphylococcus aureus.*

Despite exhibiting multi-drug resistance, the test organisms were susceptible to the methanolic crude extract and fractions of both plants with zones of inhibition ranging from 10 mm to 23 mm (Table 3). This implies that polar bioactive components are abundant in both plants. As a result, purifying and separating the methanol fraction can yield highly potent bioactive molecules. Asides from the peptidoglycan-based inner wall, Gram negative organisms have an extra outer membrane. Chemical agents such as antibiotics and even plant extracts find it difficult to permeate the cell's outer membrane yet the crude extract and fractions had appreciable activity on Gram negative organisms. Ayisa *et al.* (2020) reported appreciable activity of *Telfairia occidentalis* ethanolic extracts at 50mg/mL on *E. coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Stephen (2020) reported antibacterial activity of *Ficus vogelii* on *E. coli*, *S. typhimurium*, and *C. albicans*. The MICs recorded were relatively high with values ranging from 3.125mg/mL to 25mg/mL and MBCs ranging from 12.5mg/mL to >50 mg/mL (Table 4) in comparison to the MICs of *Telfairia occidentalis* reported by Ayisa *et al.* (2020).

Table 4: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of fractions of *Ficus vogelii* and *Telfairia occidentalis* on clinical test organisms

Isolates	Hexane fraction				Ethylacetate fraction				Methanol fraction			
	MIC (mg/mL)		MBC (mg/mL)		MIC (mg/mL)		MBC (mg/mL)		MIC (mg/mL)		MBC (mg/mL)	
	FV	TO	FV	TO	FV	TO	FV	TO	FV	TO	FV	TO
Cr.s	6.25	6.25	25	25	6.25	6.25	12.5	12.5	3.125	3.125	12.5	12.5
En.c	25	12.5	25	25	12.5	6.25	25	25	12.5	6.25	12.5	12.5
Es.c	12.5	12.5	25	12.5	12.5	12.5	12.5	12.5	12.5	6.25	12.5	12.5
Kl.p	12.5	12.5	25	25	12.5	12.5	25	25	12.5	12.5	12.5	12.5
Pr.v	25	25	>50	>50	25	25	>50	50	25	25	50	50
Ps.a	12.5	12.5	25	25	12.5	12.5	25	12.5	12.5	12.5	12.5	12.5
St.a	6.25	6.25	12.5	12.5	6.25	3.125	12.5	12.5	6.25	3.125	12.5	12.5

Key: TO=*Telfairia occidentalis,* FV = *Ficus vogelii*,Cr.s= *Cronobacter sakazaki*, En.c = *Enterobacter cloacae,* Es.c = *Escherichia coli*, Kl.p = *Klebsiella pneumoniae*, Pr.v = *Proteus vulgaris*, Ps.a = *Pseudomonas aeruginosa*, St.a = *Staphylococcus aureus*

Nonetheless, the antibacterial activity of both plants on the test organisms, as measured by zones of inhibition, MICs, and MBCs, suggests that their bioactive components have broad spectrum action, as they were active against both Gram positive and Gram negative bacteria.

Further purification of the bioactive components contained in the plants will yield pure bioactive compounds which may have stronger inhibitory properties. Bactericidal kinetics showed a concentration dependent activity of the methanol fractions of both plants against test isolates. There was a gradual reduction in number of the viable microorganisms with time, but at 6.25mg/mL and 12.5mg/mL, a total kill was observed at 24 hours (Fig 1 and2).

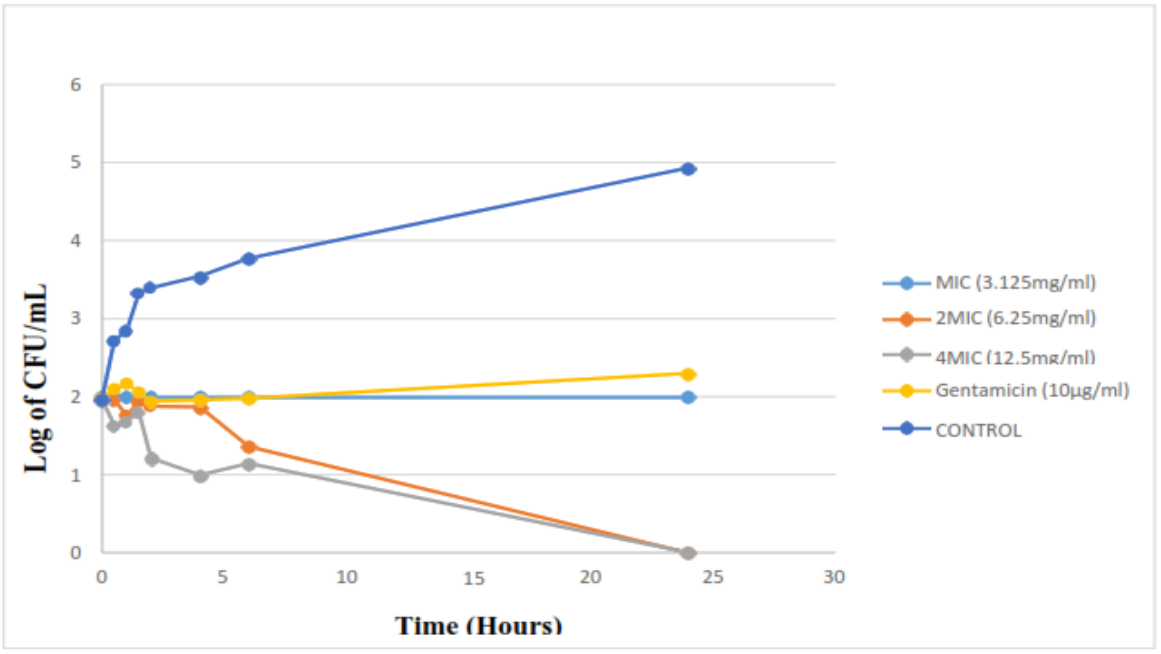

Figure 1. Bactericidal kinetics of the methanol fraction of *Telfairia occidentalis* on *Cronobacter sakazakii* showing gradual kill in response to the varying concentrations over 24 hours

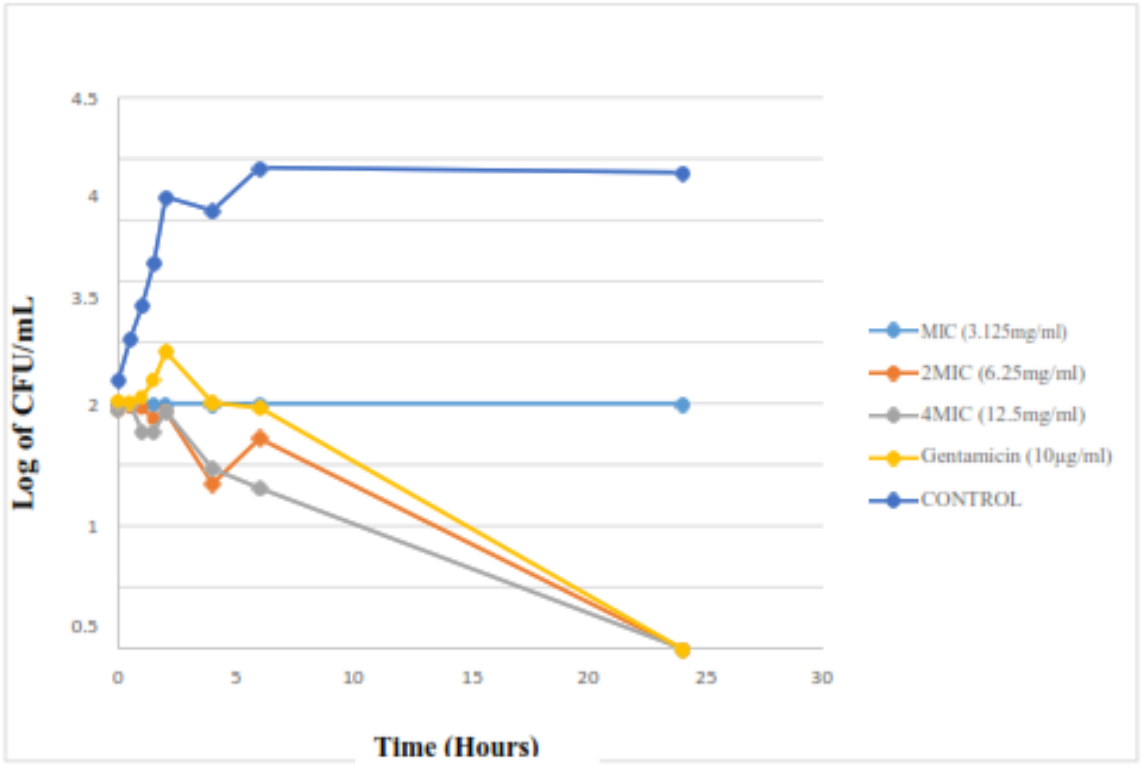

Figure. 2: Bactericidal kinetics of the methanol fraction of *Ficus vogelii* against *Staphylococcus aureus* showing gradual kill in response to varying concentrations over 24 hours

Table 3 Antimicrobial screening of the hexane, ethylacetate, and methanol fractions of *Telfairia occidentalis* and *Ficus vogelii*.

Isolates	Crude Methanolic extract						Hexane Fraction						Ethyl acetate Fraction						Methanol Fraction						Gen (10µg/mL)
	100 mg/mL		50 mg/mL		25 mg/mL		100 mg/mL		50 mg/mL		25 mg/mL		100 mg/mL		50 mg/mL		25 mg/mL		100 mg/mL		50 mg/mL		25 mg/mL		
	To	Fv	To	Fv	To	Fv	To	Fv	To	Fv	To	Fv	To	Fv	To	Fv	To	Fv	To	Fv	To	Fv	To	Fv	
Zones of Inhibition (mm)																									
Cr.s	17	17	16	15	14	13	15	14	14	11	13	11	13	14	12	11	10	11	19	18	19	16	16	13	13
En.c	19	15	18	14	16	11	14	12	15	12	13	11	12	12	12	12	10	10	22	17	21	16	18	11	11
Es.c.1	18	16	18	14	16	10	15	14	12	12	10	10	14	11	12	11	12	10	21	17	20	17	17	14	12
Es.c.2	17	15	16	12	14	10	14	12	12	12	10	10	12	12	11	12	11	10	18	18	18	16	15	13	12
Kl.p.1	15	14	15	13	13	12	14	12	13	12	12	10	12	12	12	12	10	10	17	15	17	13	16	12	12
Kl.p.2	20	15	19	13	15	12	13	12	12	12	12	11	13	12	12	12	12	11	23	17	22	14	18	12	11
Pr.v.1	16	15	16	14	15	12	12	12	12	12	10	11	12	12	12	12	10	11	19	18	18	17	18	17	13
Pr.v.2	17	13	15	13	13	12	12	12	12	12	12	10	12	12	10	12	9	10	19	17	17	15	17	12	11
Pr.v.3	17	14	17	13	15	11	12	11	11	10	10	10	13	11	13	10	10	10	20	16	18	14	18	10	13
Ps.a.1	15	14	14	12	13	10	14	13	13	11	10	10	14	13	13	11	10	10	18	16	17	15	14	12	13
Ps.a.2	19	16	16	14	14	12	14	14	14	12	12	12	14	14	14	12	12	12	22	19	18	15	15	13	12
Ps.a.3	18	15	17	13	15	11	15	12	14	12	13	10	12	12	12	12	10	10	20	17	19	13	16	12	12
St.a.1	21	18	19	16	18	13	14	14	14	12	12	12	14	14	14	12	12	12	23	19	20	15	20	13	16
St.a.2	21	18	20	15	17	12	14	13	12	12	12	12	14	13	12	12	12	12	22	20	22	17	18	14	18
St.a 3	20	19	18	15	18	14	15	14	14	13	13	13	15	14	14	13	11	13	22	18	21	16	19	13	18
St.a.4	20	18	18	14	17	12	15	12	13	12	12	10	14	12	13	12	12	10	22	18	20	15	19	14	20

Key: To=*Telfairia occidentalis*, Fv = *Ficus vogelii*, - = No Zone of Inhibition, Cr.s= *Cronobacter sakazaki*, En.c = *Enterobacter cloacae*, Es.c = *Escherichia coli*, Kl.p = *Klebsiella pneumoniae*, Pr.v = *Proteus vulgaris*, Ps.a = *Pseudomonas aeruginosa*, St.a = *Staphylococcus aureus*, Gen- Gentamicin

CONCLUSION

The antibacterial activity exhibited by extracts and fractions from the plants in this study may be due to the presence of secondary metabolites. Despite being resistant to multiple antibiotics, the test organisms were susceptible to the plants' extracts and fractions, indicating that the plants have the potential to be alternative options for treating wound infections. However, further tests could be carried out on the active extracts to determine their lethal dose, active principles as well as other parameters that would account for the eligibility of the plant in clinical use.

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