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Phytochemical Screening and Antimicrobial Activity of Methanol Extracts of *Usteria guineensis* and *Sphaerocoryne gracilipes* on Pathogens and ESBL-Producing *Escherichia coli*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Antibiotic resistance of microbial pathogens has become a threat to public health, with observed increase in outbreak of infections, therapeutic failure, morbidity and mortality. Pathogenic *Escherichia coli* and other extended spectrum beta lactamase (ESBL) producing bacteria causes serious health challenges due to antimicrobial resistance. This has caused an increasing research on medicinal plants as a source of alternative potential therapeutic agents. In this study, antimicrobial activity of *Usteria guineensis* (UG) and *Sphaerocoryne gracilipes* (SG) was investigated on standard organisms and clinical isolates of ESBL-producing *E. coli.*

The leaves of *Usteria guineensis* and *Sphaerocoryne gracilipes* were extracted using methanol. Phytochemical analysis was carried out on the medicinal plants according to standard procedure. The clinical isolates of *E. coli* were screened for the production of ESBL using double disc synergy test, with *E. coli* ATCC 25922 as standard. Agar well diffusion method was used to determine antibacterial activity of the extracts at 100 and 25 mg/mL while Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were assessed using broth micro-dilution method. Statistical analysis was done using ANNOVA.

The two plants contain cardiac glycoside, alkaloids and saponin and 11/15 (80%) of the *E. coli isolates* show the production of the ESBL. The extracts of the two plants showed antibacterial activity against ESBL *E. coli* with zone of inhibition ranging between 11 to 18 mm for *Usteria guineensis* and 12 to 15 mm for *Sphaerocoryne gracilipes*. The MIC of the extracts ranged between 0.78 to 50 mg/mL and MBC from 12.5 to >50 mg/mL for the ESBL producers and the standard strains.

Therefore, the two plants, especially *Usteria guineensis* have potentials to be developed as alternative therapeutic agents for the treatment of infections caused by ESBL-producing pathogens like *Escherichia coli.*

Keywords: Usteria guineensis; Sphaerocoryne gracilipes; phytochemical screening; resistance; ESBL-producing Escherichia coli.

1. INTRODUCTION

The increased usage of beta-lactam antibiotics like penicillins and cephalosporins has produced an increase in resistance of bacteria in the family Enterobacteriaceae to many antibiotics of therapeutic importance [1,2]. Pathogenic *Escherichia coli* and other extended spectrum beta lactamase (ESBL) producing bacteria causes serious health challenges due to antimicrobial resistance [2,3]. The primary mechanism of resistance to beta-lactam antibiotics is the formation of extended-spectrum beta-lactamases (ESBLs), which inactivate beta-lactam antibiotics. This is the primary cause of β-lactam antibiotic resistance among Enterobacteriaceae worldwide. ESBL-producing enterobacteria are common among the antibiotic-resistant bacteria that cause nosocomial and community-acquired illnesses [4,5].

Bacteria produce an enzyme called ESBLs that break down β-lactam drugs (except for cephamycins and carbapenems) [4], thus allowing them to become resistant to extended-spectrum penicillin, cephalosporins, and monobactams. Beta-lactamase inhibitors, such as clavulanic acid, also act to prevent enzymatic hydrolysis of β-lactam drugs. Report on the development of resistance to extended spectrum cephalosporin by ESBL-producing Enterobacteriaceae has been on the rise, which requires serious attention. ESBLs have been found in Enterobacteriaceae, especially *Klebsiella* species and *E. coli* [6,7], and also among the non-lactose fermenters like *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. The gene responsible for the expression of antibiotic resistance is usually mediated by the chromosome or plasmid. Though ESBL is plasmid-mediated, it is easily spread across Enterobacteriaceae members; not just to beta-lactams, but also to other regularly used antibiotics such fluoroquinones, aminoglycosides, and sulphonamides. As a result, many patients require antibiotics of last resort, such as carbapenems [2]. Hence, there is need for routine surveillance for ESBL-production and also the rational choice of antibiotics for the treatment of infections and reduction in the burden of antimicrobial resistance in clinical settings [8].

In Africa and Asia, a majority of the populace still depends on traditional medicine for their primary healthcare needs [9] due to the growth in antibiotic resistance and the scarcity of conventional antibiotics. Plants have been used over time as a source of natural products for human health. Therefore, many medicinal plants have been investigated for their antimicrobial properties and some have been utilized as therapeutic alternatives [10-12,7]. Secondary metabolites in plants such as saponins, phenolic compounds, flavonoids and alkaloids have been identified as responsible for this antimicrobial and therapeutic properties [13, 14, 12]. The use of traditional medicine, commonly referred to as complementary and alternative medicine is expanding quickly in developed nations including United States, UK, Germany where plant portion's active ingredients are used to treat diseases, prevent illness and manage cancer [15]. Moreover, many local medicinal plants of Ethiopia e.g. *Centella asiatica* and *Silybum marianum* have shown good antibacterial activity against multidrug resistant (MDR) pathogens [16, 12, 17, 18]. The onus rests on natural product scientists to search and discover more medicinal plant products or phytochemicals that can be used as alternative medicines in the treatment of diseases. Therefore, two Nigerian medicinal plants, with little or no report on phytochemical and pharmacological activities are hereby investigated for antibacterial activity against ESBL producing microbes represented by Escherichia coli of the family Enterobacteriaceae. This will also provide useful scientific information on the 'new' plants, especially phytochemical and antimicrobial.

Usteria guineensis Willd. (Loganiaceae) is a native plant to the tropical region of Africa and the only *Usteria* species in West Africa. The plant is found in the Southern Nigeria where it identified under the names 'Ukala' (Edo), ' Oporo' and 'Esinsin-ile' (Yoruba), where they use it for medicine and as fibres [19]. According to Iwu, [20] the roots and leaves of *U. guineensis* are used to treat cough, parasitic skin infections and bronchitis. The Mende people of Sierra Leone use the roots to treat malaria by rubbing them on their foreheads, necks, and joints; but in Togo, the root decoction is used to cure gonorrhoea [21].

Sphaerocoryne gracilipes (Benth.) X.Guo & R.M.K Saunders (Annonaceae). Former name is *Oxymitra longipedicellata* (Baker f.) Sprague & Hutch. (Annonaceae). The native range of this species is Nigeria to West Central Tropical Africa. It is a climbing shrub. Petals are yellowish on the outside and purple on the interior at the base and it has scarlet fruits. The genus *Sphaerocoryne* Scheff. ex Ridl. (Annonaceae) is native to: Nigeria to Kenya, South Tropical Africa, Indo-China to Malesia. Two species of Sphaerocoryne are known from Africa, *S. gracilipes* in Nigeria and Central Africa *S. gracilis* in Zimbabwe and East Africa. The plant, *S. gracilipes* is 'novel' with no much work done on it and no information is available on its ethno-medicinal use [22]. Therefore, our reports on its phytochemical constituents and antimicrobial activity will contribute to scientific knowledge on the plant.

Fig. 1. *Sphaerocoryne gracilipes* (Annonaceae)
Source: https://km.wikipedia.org/wiki:Rumdoul.jpg

Fig. 2. *Usteria guineensis* (Loganiaceae)
Source: https://images.app.goo.gl/GbB8

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

The leaves of *Sphaerocoryne gracilipes* and *Usteria guineensis* were obtained from Ikire in Irewole Local Government of Osun State (latitude and longitude — 7.3699703, 4.1872178) and was authenticated at Forestry Research Institute of Nigeria (FRIN), with deposited voucher specimen number FHI-114074 and FHI-114073, respectively.

2.2 Extraction

The powdered leaves of *Sphaerocoryne gracilipes* (150 g) and *Usteria guineensis* (100 g) were weighed into a clean apparatus and soaked with enough methanol (extracting solvent). The soaked plant samples were shaken vigorously at intervals of 4 hours for 72 hours. The extracted matter was filtered using fresh cotton plug and then using filter paper (Whatman No. 1). The filtrate was concentrated using a rotary evaporator. The resulting crude extract of each solvent was weighed and stored in the refrigerator below 4^{o}C [23], until used for microbial assay.

2.3 Phytochemical Screening for Secondary Metabolites

The extracts were screened for secondary metabolites using the powdered samples of the plants' leaves. The tests were carried out in accordance with standard procedures [14].

2.3.1 Alkaloids

About 1 g each of the powdered leaf sample of *U. guineensis and S. gracilipes* was extracted with 10 ml HCl on water bath. The extracts were filtered and the pH was adjusted to 6.5 using Sodium Hydroxide solution. Drangendorff′s reagent, Meyer′s reagent and Wagner′s reagent was added to 3 ml of the filterate drop by drops. The test tubes were shaken and colour change was observed as follows: Drangendorff′s reagent (reddish brown), Wagner′s reagent (reddish brown) and Mayer′s reagent (creamy precipitate).

2.3.2 Anthraquinone Glycosides

About 0.2 g each of the powdered leaf samples of the plant extracts was placed in a dry clean test tube and 5 mL diluted sulphuric acid was added. The test tube was heated for 5 minutes and cooled. The contents were partitioned against the same volume of chloroform and the layers were allowed to separate. The chloroform layer was then carefully transferred to a clean test tube and then shaken together with 5 mL of 10% ammonium solution. A pink color observed in the aqueous layer indicate presence of anthraquinones.

2.3.3 Saponin Glycosides

10 ml of distilled water was added to about 1g of each powdered samples of the plant extracts in test tubes and heated for approximately 10 minutes. This was filtered while hot and the aqueous extract was used to demonstrate

frothing by diluting 2 ml of the filterate to 10 ml with water and it was shaken. Formation of a persistent froth indicated presence of saponins.

2.3.4 Cardiac Glycosides

2.3.4.1 Keller-Killiani test

Exactly 0.3 ml of 10% of ferric chloride in 50% glacial acetic acid was added to a portion of the dried extract residue of the plants in clean test tubes. Then 2ml of conc. sulphuric acid was added to the side of the test tube. This produces another layer below the acetic acid layer. A brown ring formation at the interphase indicates the presence of deoxy-sugar.

2.3.4.2 Kedde test

The dried residue was also mixed with 1 ml of 2% 3,5 dinitrobenzoic acid in ethanol. Then 5% sodium hydroxide solution was added to the solution. It was mixed thoroughly and the formation of brown purple color shows the presence of an unsaturated lactone ring.

2.3.4.3 Tannin

1g each of powdered plant samples was boiled for 5 minutes. Filteration was done and each filterate was made up to 10 ml volume; 5% ferric chloride solution was then added. A blue-black precipitate shows presence of tannin.

2.4 Test Organisms

Fifteen (15) *Escherichia coli* isolates from urine samples were collected from Molecular Lab, Faculty of Pharmacy, University of Ibadan. *Escherichia coli* ATCC 11175, *Bacillus subtilis* ATCC 6633*, Salmonella typhimurium* ATCC 14028*, Staphylococcus aureus* ATCC 29813 and *Pseudomonas aeruginosa* ATCC 27853 used as reference were collected from Department of Pharmaceutical Microbiology Laboratory, University of Ibadan. They were all maintained on agar slants at 4˚C prior to use.

2.5 Detection of ESBL-Producing Bacteria Using Double Disc Synergy Test (DDST)

The detection of the ESBL production among the clinical isolates was determined by double disc synergy test using two third generation cephalosporin discs; ceftazidime and cefotaxime [8,7]. A fresh pure culture of *E. coli* was prepared and used to prepare a suspension of the test organism at the standard of 0.5 McFarland standard (1.0 x 10^8 cfu/mL). The prepared suspension of the test organisms was applied on the Mueller Hinton agar surface using sterile cotton. Next, the discs were put on the plates that had been infected. To enable accurate assessment of the diameter of the zone of inhibition, an augmentin disc was positioned in the center, and ceftazidime and cefotaxime discs were positioned 20 mm away from the clavulanic acid disc. After that, it was incubated for 18 to 24 hours at 37˚C. The tests were carried out in duplicates.

2.6 Antimicrobial Activity of Extracts

The procedures of agar well diffusion method [12] were followed. The Mueller Hinton agar was seeded with a standardized bacteria suspension (based on a turbidity of 0.5 McFarland standard, or 1 ×10^8 cfu/mL) using the agar well diffusion method. On the agar surface, consistent wells were punched using a sterile cork borer with a 9mm diameter. Using a sterile Pasteur pipette, the crude extracts (25 and 100 mg/mL) were then added to the wells and left to diffuse. The positive and negative controls were represented by control wells that contained 10% DMSO4 and 10µg/mL of gentamycin, respectively. The tests were carried out in duplicates. The zones of inhibition were measured 24 hours after the bacterium plates were incubated at 37˚C.

2.7 Determination of Minimum Inhibitory Concentration (MIC) of Plant Extracts

The MIC was determined using broth microdilution technique following CLSI guideline [24] using broth dilution in 96-well micro-titer plate. The bacteria suspension having turbidity equivalent to that of 0.5 McFarland Standard (1.5 x 10^8 cfu/mL) were inoculated into broth microdilution plates already containing different dilutions of the extracts (50 to 0.097 mg/mL) and control antibiotics (gentamycin) into 100 µL of Mueller Hinton broth. The plates were incubated at 37 ^{0}C for 24 hours. The plates were examined for growth by the addition of tetrazolium salt. A change in colour to red or pink is indicative of growth of test organism. The lowest concentration of the antibiotics showing absence of growth is taken as the MIC. Two columns of the titre plate containing broth and antibiotics and broth alone were used as positive and negative control, respectively.

2.8 Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration was determined by inoculating freshly prepared Mueller Hinton broth in test tubes with inoculum from the wells showing no visible growth as indicated by no change in colour (in the MIC determination above). The tubes were then incubated at 37^0C for 24 hours. The lowest concentration of the extract showing absence of the growth is recorded as the MBC [25].

2.9 Statistical Analysis

The results were statistically analyzed, and compared with standard drug (positive control) using ANOVA multiple comparisons test.

3. RESULTS

3.1 Extraction Yield

The percentage yield of the plants was calculated with the formula: dry weight of extract/ dry weight of plant samples × 100. The yields obtained were 4.3% for *Usteria guineensis* which was lower than 5.9% for *Sphaerocoryne gracilipes* (Table 1).

The results of phytochemical screening, as shown in Table 2 revealed that the leaves of the two plants, though belonging to different family, have similar secondary metabolites, viz: saponin, alkaloid and cardiac glycoside. However, while *Usteria guineensis* was found to possess tannins, *Sphaerocoryne gracilipes* showed absence of tannins.

Table 1. Yield of plants' samples on extraction with methanol

Plants/Leaves	Wt of Sample (g)	Wt of Extract (g)	Yield (%)
U. guineensis	100.0	4.3	4.3
S. gracilipes	150.0	8.9	5.9

Table 2. Phytochemical results for *U. guineensis* and *S. gracilipes*

Phytochemicals	U. guineensis	S. gracilipes
Alkaloids	+	+
Cardiac glycoside	+	+
Anthraquinone	-	-
Tannins	+	-
Saponins	+	+

Keys: + = present, - = absent

Table 3. Detection of synergy between Amoxicillin/ Clavulanic acid disc (30/10µg) and two third generation cephalosporin disc

Isolate	Ceftazidime(30µg)			Cefotaxime(30µg)		
	M	Mc	≥5mm	M	Mc	≥5mm
E1	14	28	14	16	30	14
E2	14	30	16	20	31	11
E3	10	22	12	16	28	12
E4	20	30	10	24	38	14
E5	-	-	-	-	-	-
E6	12	30	18	20	29	9
E7	15	24	9	16	15	1
E8	5	9	4	5	20	15
E9	-	-	-	-	-	-
E10	12	39	27	16	31	15
E11	12	24	12	20	32	12
E12	12	32	20	24	32	8
E13	30	32	2	42	42	0
E14	-	-	-	-	-	-
E15	18	30	12	20	30	10

Keys: M= Diameter of Zone of inhibtion without Clavulanic acid disc, Mc= Diameter of Zone of inhibtion with Clavulanic acid disc, - = No inhibition

Table 4. Antimicrobial activity of extracts on clinical isolates and common pathogens

	Zones of Inhibition of Extracts and Controls (mm)					
Extracts	***Usteria guineensis***		***Sphaerocoryne gracilipes***		**Gentamycin**	**DMSO**
Isolates	**25mg/ml**	**100mg/ml**	**25mg/ml**	**100mg/ml**	**10µg/ml**	**10%**
E1	-	-	-	-	18	-
E2	-	-	-	-	17	-
E3	12	14	-	-	-	-
E4	-	-	-	-	18	-
E5	12	-	-	-	-	-
E6	-	-	-	-	-	-
E7	-	-	-	-	16	-
E8	12	-	-	-	-	-
E9	-	-	-	-	14	-
E10	-	-	-	-	-	-
E11	14	14	12	15	15	-
E12	11	11	11	12	13	-
E13	11	11	-	12	11	-
E14	14	14	12	15	-	-
E15	-	-	-	13	20	-
Ec	-	-	-	-	18	-
Sa	14	15	18	19	13	-
Pa	11	11	-	11	14	-
Bs	-	-	-	-	10	-
Stm	-	14	-	14	16	-

Keys: - = Not active, Ec= Escherichia coli ATCC 11175, Bs= Bacillus subtilis ATCC 6633, Stm = Salmonella typhimium ATCC 14028, Sa = Staphylococcus aureus ATCC 29813 and Pa = Pseudomonas aeruginosa ATCC 27853

Table 5. MIC and MBC of plants' extracts on selected isolates and standard strains

	MIC (mg/ml)		MBC (mg/ml)	
ISOLATES	**UG**	**SG**	**UG**	**SG**
E2	6.25	50.0	12.5	50.0
E3	6.25	25.0	25.0	50.0
E4	12.5	12.5	25.0	25.0
E5	6.25	50.0	12.5	50.0
E6	12.5	12.5	25.0	50.0
E8	6.25	12.5	25.0	25.0
E9	25.0	12.5	12.5	25.0
E14	12.5	ND	25.0	ND
Ec	6.25	50.0	50.0	>50.0
Sa	0.78	1.56	50.0	>50.0
Pa	3.13	12.5	12.5	100.0
Bs	12.5	12.5	25.0	>50.0
Stm	12.5	12.5	50.0	>50.0

Keys: E: Escherichia coli Isolates, Ec: Escherichia coli ATCC 11175, Bs: Bacillus subtilis ATCC 6633, Stm: Salmonella typhimium ATCC 14028, Sa: Staphylococcus aureus ATCC 29813, and Pa: Pseudomonas aeruginosa ATCC 27853, UG: Usteria guineensis and SG: Sphaerocoryne gracilipes, ND: Not determined

The results of ESBL production of the clinical isolates using double disc synergy test showed that specifically 73.33% of the *Escherichia coli* isolates were ESBL producers. Three of the *E. coli* were resistant to cefotaxime and ceftazidime, while the remaining *E. coli* did not show a ≥5 mm increase in inhibition zone of cephalosporin with Clavulanic acid (according to [24]) (Table 3).

Antibacterial screening of the methanol extracts demonstrated a poor antibacterial activity on the gastrointestinal pathogens and standard isolates with the exception of *Staphylococcus aureus*.

The concentration of the plant extracts at 25mg/mL and 100mg/mL showed little or no zones of inhibition compared to the gentamycin used as positive control. The diameters of zone of inhibition were between 11-15mm and 8-18mm at 25mg/mL and 100mg/mL of *U. guineensis* while the diameters obtained from *Sphaerocoryne gracilipes* was between 11-18mm and 12-19mm at concentrations 25 and 100mg/mL, respectively (Table 4).

The MIC value of both the methanolic leaf extract of *U. guineensis* and *S.* gracilipes were found to range between 0.78-12.5 mg/mL and 1.56-50.0 mg/mL, respectively while the MBC ranged from 6.25 - 50mg/mL and 12.5- >50 mg/mL for *U. guineensis* and *S. gracilipes* on the common pathogens and clinical isolates. This result is presented in Table 5.

4. DISCUSSION

Bacteria pathogens are responsible for a large number of gastrointestinal tract infections with young children, the elderly and immunocompromised individuals at greater risk. Infections of the gastrointestinal tract have a significant impact on morbidity and mortality with success of treatment being threatened by the production of Beta Lactamases which inactivates most antibiotics [26]. Enterotoxigenic *Escherichia coli* in its pathophysiology colonises the upper GIT where they produce an enterotoxin that stimulates the mucosa cells to secrete fluid via an increase in intracellular cAMP which leads to diarrhoea [2,3]. Invasive bacteria such as *Salmonella* also colonises the lower ileum, which may lead to the production of painful stools with blood [27]. This infection is usually prominent in areas with poor sanitation practices. The gastrointestinal pathogens used in this study were found to include ESBL producers as presented by double disk synergy test [7]. Among the 15 *E. coli*, eleven showed positive results as ESBL which may have posed a serious threat to the use of antibiotics, rendering them ineffective. The three *E. coli* strains that showed no sensitivity to cefotaxime and ceftazidime may be Multidrug resistant (MDR) and most likely will not respond to conventional treatment with the antibiotics. The emergence of this antibiotic resistance has been attributed to misuse of antibiotics [28].

However, methanolic leaf extract of *U. guineensis* showed zones of inhibition (ZOI) as wide as 11-14mm and 11-15 mm on most of the organisms at 25mg/mL and 100mg/mL, respectively. The MIC obtained for *S. aureus* strain was found to be 0.78mg/mL which is lower to the MIC value obtained for the ESBL-producing *E. coli, Klebsiella, Pseudomonas*, *Salmonella and Bacillus* species used in this study which range from 3.125- 25mg/mL. It was noted that the tested S. aureus was more susceptible to the plant extracts with ZOI of 14-19mm. This is not strange as the typed strain a sensitive Gram-positive organism. But the organism Stm was more or less non-susceptible, though a typed strain, is an MDR *Salmonella typhimurium*. In comparism, the methanolic leaf extract of *S. gracilipes* had diameters of ZOI ranging from 11-12 and 11-15mm at concentrations 25mg/ml and 100mg/mL, respectively. The MIC ranged from 12.5-50mg/mL on most strains, except for *S. aureus* which showed sensitivity to the crude extract at MIC of 1.56mg/mL. The two plant extracts can thus be used as a therapeutic alternative in the treatment of infections caused by *S. aureus.* Also*, U. guineensis* had higher antibacterial activity (considering the recorded ZOI and MIC) than *S. gracilipes,* therefore may be more effective in the treatment of ESBL-producing *E. coli* infections. The bactericidal activity of the plant extracts was obtained by calculating the MIC index (MBC/MIC), which was found to be 2 for *U*. *guineensis* (greater and more consistent than for *S. gracilipes*) for most of the tested organisms. The implication of this is that *U. guineensis* is bactericidal in action, unlike *S. gracilipes* with lower MIC index which implies that it is more of bacteriostatic in action [16] at least at the tested concentrations.

The two plant extracts were found to have alkaloids, saponins and cardiac glycosides as parts of their secondary metabolites, which may be responsible for their antibacterial activity on the tested organisms [12,18, 29]. The absence of tannins in *S. gracilipes* might have contributed to less activity of the extracts on the ESBL *E. coli* as tannins have been reported to possess antimicrobial activity [30, 29], [31-34].

5. CONCLUSION

The extracts used in this study had fair activity on ESBL *E. coli,* the activity recorded on *U. guineensis* showed it has a fair potential in the treatment of ESBL *E. coli* infections. The result of this study supports the traditional application of *U. guineensis* and *S. gracilipes* to treat infections caused by *Staphylococcus aureus*. This proves

that the plant can be used as an alternative therapeutic agent in the presence of alarming antimicrobial resistance.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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