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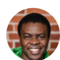

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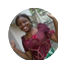

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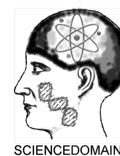

Antimicrobial Activity of Selected Medicinal Plants of South-Eastern Nigeria on *Pseudomonas* species Expressing Extended Spectrum Beta Lactamase (ESBL)

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Authors' contributions

This work was carried out in collaboration between all authors. Author ECO designed the study, and wrote the protocol. Authors IMN and ARA carried out the laboratory work. Authors IMN and NCD managed the literature searches. Author ALO wrote the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aim: To evaluate the antibacterial activity of selected medicinal plants from South-Eastern Nigeria against ESBL producing *Pseudomonas* species.
Study Design: Agar well diffusion assay for determination of sensitivity and Agar dilution method for determination of MIC were used.
Place and Duration of Study: Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra State, an Igbo speaking South-Eastern State in Nigeria, between February 2010 and october 2010.
Methods: The antibiograms of ten (10) ESBL producing *Pseudomonas* species to selected antibiotics as well as the antibiograms of these isolates against methanol leaf extracts of *Anthocleista djalonensis* A Chev. (Loganiaceae) (MLEA) (*Igbo* name- Uvuru or Ayuu), *Zapoteca portoricensis* H. M. Hem. (Fabeceae/mimosidea) (MLEZ) (*Igbo* name-

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Ayuu), *Gongrenema latifolium* Benth. (Asclepiadaceae) (MLEG) (*Igbo* name- Utazi) and *Psidium guajava* Linn. (Myrtaceae) (MLEP) (*Igbo* name- Gova), using doses of 3000.00 to 21.87 mg/ml using agar disk diffusion and agar well diffusion assays respectively were determined. The MIC of the plant extracts in comparison with that of gentamicin were also evaluated using the agar dilution method.
Results: All the ESBL producing *Pseudomonas* spp. were multi-drug resistant (IZD = 0) but, were all sensitive to imipenem. Only two strains of *Pseudomonas monteilli* were sensitive to MLEG with sensitivity decreasing with increasing concentrations of the MLEG. All the ESBL producing *Pseudomonas* spp were sensitive to MLEZ and MLEP. Also, sensitivity decreased with increasing concentration of the MLEZ and MLEP. MLEA showed no antimicrobial activity against the tested ESBL producing *Pseudomonas* spp. Gentamicin, with an MIC of 0.00015 µg/ml, was more active than the plant extracts. The MLEP was the more active with an MIC of 1 - 4.37 mg/ml, than MLEZ and MLEG which had MICs of 150 and 75 mg/ml respectively. Active constituents of these plant extracts especially that of *Psidium guajava,* may thwart the emerging resistance to carbapenems.
Conclusion: Development of a complex mixture of the active constituents or single active constituent(s) of *Psidium guajava* as antimicrobial agent(s) that will be effective against ESBL producing *Pseudomonas* species.

Keywords: Medicinal plants; active constituents; drug resistance; carbapenems; post-antibioticera.

1. INTRODUCTION

Members of *Pseudomonadaceae* are able to acquire drug resistance by various mechanisms more than Members of *Enterobacteriaceae* and this includes acquiring plasmids that encodes extended spectrum β- lactamase (ESBL) production. These resistance plasmids can be transferred between *Pseudomonas* species via transduction and conjugation. *Pseudomonades* also have innate resistance to many antibiotics [1-5]. An important member of this group is the opportunistic human pathogen, *Pseudomonas aeruginosa*. For the last two decades, this organism has become increasingly recognized as the causal agent of a variety of serious nosocomial infections, especially in immunocompomised patients [1]. There have been reports of other *Pseudomonas* spp causing human infections which rarely occur [6-8]. *Pseudomonas spp* are commonly found in the soil and water, but can sometimes be found on the surfaces of plants and animals.

ESBLs are known as extended-spectrum because they are able to hydrolyze a broader spectrum of β-lactam antibiotics than the simple parent β-lactamases from which they are derived. They have ability to even inactivate β-lactam antibiotics containing an oxyimino-group such as oxyimino-cephalosporins (e.g., ceftazidime, ceftriaxone, cefotaxime) as well as oxyimino- monobactam (aztreonam) [9,10]. They are not active against cephamycins and carbapenems. Generally, they are inhibited by β-lactamase-inhibitors such as clavulanate and tazobactam. ESBLs have been found in a wide range of gram-negative rods. However, the vast majority of species expressing these enzymes belong to the family *Enterobacteriaceae* [9]. *Klebsiella pneumoniae* remains the major ESBL producer. *Non-Enterobacteriaceae* ESBL producers are relatively rare with *Pseudomonas aeruginosa* being the most important organism [11,12]. ESBL enzyme-encoding genes SHV-2a and TEM-42 have been found in *Ps. aeruginosa* [2,3].

The antimicrobial activity of extracts of various plants has been proven scientifically. These extracts contain multiple active ingredients which could deter the development of resistance. For example; the methanol extracts of *Anthocleista djalonensis* has anti-bacterial and wound healing property [13]. Recently, the antimicrobial activity of the leaf extract of *Zapoteca portoricensis* have been authenticated in experimental models [14] while the methanol leaf extract of *Psidium guajava have* been known to posses antimicrobial activity [15]. Furthermore, the methanol leaf extracts of *Gongrenema latifolium* has an MIC of 1 mg against *Ps. aeruginosa* [16].

Currently, there is no medicinal plant that has been established to have antimicrobial activity against ESBL producing organisms, hence the need for the present study. In this research, extracts from selected medicinal plants from South-Eastern Nigeria known to possess antibacterial activity are evaluated against some ESBL producing *Pseudomonas species* and also to compare the MIC's of the said plants with that of Gentamicin. This will enable development of lead compounds for the control of these resistant pathogens.

2. MATERIALS AND METHODS

2.1 The Test Microorganisms

The test organisms used here are 10 ESBL producing *Pseudomonas* isolates gotten from swab samples of the intestine of animals killed on the spot. Pure cultures were obtained by streaking on MacConkey agar and Nutrient agar subsequently. The various isolates gotten were screened for the ESBL enzyme via the Double Disc Synergy Test (DDST). The ESBL were then characterized in order to determine which were *Pseudomonas* spp. by using MALDI –TOF. The isolation, screening for ESBL producers and characterization are well-detailed in our previous study [17]. Prepared pure cultures of the isolates were stored in nutrient agar slants at 2 - 4ºC and subsequently standardized to bring the cell population to McFarland's 0.5 nephelometer prior to any microbiological assay.

2.2 Herbal Extract

The plants used in this study which included *Anthocleista djalonensis*, *Zapoteca portoricensis, Gongrenema latifolium* and *Psidium guajava* were collected from Nsukka, Enugu State, South-East of Nigeria. They were identified and authenticated by a taxonomist at the International Centre for Ethnomedicine and Drug Development (INTERCEDD), Nigeria, formally Bio-resources Development Centre, BDC, and voucher specimens were deposited there. The leafy parts were air-dried at room temperature and reduced into fine powders using a mechanical blender. About 206 g of the powdered materials were extracted using 1000 ml analytical grade methanol (BHD laboratory, England) via cold maceration for forty-eight hours. The resulting mixture was filtered and the filtrate was concentrated using a Rotary evaporator (Model RE 300, Barloworld Scientific Ltd., UK) and kept at room temperature for the methanol to completely evaporate for 24 hrs. The resulting residue which was the methanol leaf extract of the various plants was stored in air-tight containers until they were used.

2.3 Standard Stock Solutions

Standard stock solutions of 350mg/ml, 1000 mg/ml, 2000mg/ml, 3000mg/ml, of the MLEG, MLEA, MLEG and MLEZ, respectively, were prepared in dimethylsulphoxide (DMSO, BDH-LABORATORY, ENGLAND). Two- fold serial dilutions of the stock solutions were prepared

while carrying out the various tests. A 100 µg/ml stock solution of gentamicin (a generous gift from Juhel Pharmaceutical Industry Ltd., Nigeria) was diluted to obtain concentrations of 1.2 µg/ml, 0.6 µg/ml, 0.3 µg/ml, 0.15 µg/ml and 0.075 µg/ml, which were used in our study.

2.4 Sensitivity of ESBL Producing *Pseudomonas* spp. to Plant Extracts and Standard Antibiotics

This was determined by using the agar well diffusion method for the plant extract and the disk diffusion method for the standard antibiotics [18]. Briefly, a Petri dish was divided into five sections; one section for the stock solution and each dilution of a plant extract in DMSO. 0.1ml of the standardized suspension of a *Pseudomonas* isolate was put into the empty sterile Petri dish. Bijou bottles containing 20 ml of sterile molten Muller-Hinton agar at 45℃ was poured into each of the plates containing the suspension of the isolate. These were gently rotated thoroughly and were allowed to set for 20 minutes. Six millimeter (6mm) cork borer dimension was used to bore holes into each section in the plates and each section was labeled properly. About 40 µl of the various concentrations of the extract placed into the wells and left for one hour at room temperature. The plates were incubated at 37ºC for 18-24 hours. The test was carried out in triplicates for each isolates. The experiment was carried out with all the plant extracts. The experiment was repeated using the standard antibiotics, but here instead of boring hole, the antibiotic disc (Oxiod); Ceftriaxone (30µg), Chloramphenicol (30µg), Ciprofloxacin (30µg), Cefuroxime (30µg), Amoxicillin/clavulanate (30µg), Gentamicin (30µg), Pefloxacin (30µg), Imipenem (30µg), Cefotaxime (30µg), Ceftazidime (30µg), Tetracycline (30µg) were placed on each section of the plate, up to a maximum of five sections. After the incubation period, the plates were observed and inhibition zone diameters (IZD) were measured.

2.5 Evaluation of the Minimum Inhibitory Concentration (MIC) of the Plant Extract

The minimum inhibitory concentration of the extracts against *Pseudomonas* species expressing ESBL were performed using the agar dilution method [13]. A 19 ml volume of sterilized molten nutrient agar was aseptically poured into sterile petri dishes containing 1 ml of the graded concentrations of the extracts. The plates were rotated to ensure even distribution of the plant extracts and allowed to set. The plates were swabed with 0.05 ml of standard suspension of an ESBL producing Pseudomonas isolate. This was repeated for all the other isolates and was performed in duplicates. The plates were incubated at 37ºC for 18-24 hours. Presence of growth was observed after incubation and the MIC recorded as the minimum concentration where no visible growth was observed on the plate.

3. RESULTS AND DISCUSSION

3.1 Antibiograms of ESBL Producing *Pseudomonas* spp.

The antibiogram of the ESBL producing *Pseudomonas* spp. are shown below in Table [1]. All the isolates of ESBL producing *Pseudomonas spp.* are multi-drug. Multiple-drug resistant organisms are resistant to at least one agent in three or more antimicrobial categories [19]. All the ESBL producing *Pseudomonas* spp. used in this study were multi-drug resistant. They were resistant to amoxicillin and augmentin but sensitive to imipenem. Their sensitivity to imipenem is in accordance with previous studies [20,21]. The resistance of the isolates to none-β-lactam antibiotics, including gentamicin (an aminoglycoside), tetracycline, and

erythromycin (a macrolide) is due to the coexistence of genes encoding drug resistance to other antibiotics on the plasmids which encode ESBLs [22].

Table 1. Antibiogram of ESBL producing *Pseudomonas* spp. before treatment with methanol leaf extract

	Inhibition Zone Diameter of Antibiotics (mm)								
Isolate	**CHL**	**AMC**	**AMX**	**CXC**	**GEN**	**COT**	**ERY**	**TET**	**IMP**
Pseudomonas putida (I1)	19	0	0	0	0	15	10	0	31
Pseudomonas monteilli (I1)	16	0	0	0	0	0	0	0	25
Pseudomonas monteilli (I2)	19	0	0	0	0	22	0	0	30
Pseudomonas putida (I2)	13	0	0	0	19	0	0	0	31
Pseudomonas monteilli (I3)	13	0	0	0	0	0	11	0	23
Pseudomonas fulva (F1)	15	0	17	0	0	9	13	0	25
Pseudomonas putida (I3)	13	0	0	0	20	18	0	0	30
Pseudomonas monteilli (F2)	20	0	0	0	12	0	0	0	27
Pseudomonas fulva (I2)	17	0	0	0	19	20	0	0	25
Pseudomonas mendocina (I2)	16	0	0	0	0	18	9	0	23

KEYS CHL-Chloramphenicol, AUG- Augmentin, AMX- Amoxicillin, CXC- Cloxacillin, CEN-Gentamicin, COT- Co-Trimoxazole, ERY-Erythromycin, TET- Tetracycline, IMP- Imipenem, F-Formites, I- Intestine of Cow

3.2 Sensitivity of ESBL Producing *Pseudomonas* spp. to Plant Extracts

The Methanol leaf extract of *Anthocleista djalonensis* was not active against any of the *Pseudomonas* species expressing ESBL at the dose levels (1000 – 65.5 mg/ml) used in this study. Antimicrobial activity studies on this plant have been done mainly on the root extract. Previous work on *Anthocleista djalonensis* showed that it had significant activities on bacteria isolates such as *Staphylococccus auerus, Pseudomonas aeruginosa, Shigella* spp, *Escherichia coli* [13]. The antimicrobial activity of methanol leaf extract of *Zapoteca portoricensis* against *Pseudomonas* species expressing ESBL is shown in Table [2] below. It was only active against 5 isolates, viz; *Pseudomonas monteilli*, *Ps. beteli*, *Ps. mendocina, Ps. monteilli* strain 6 and *Ps. monteilli* strain 7. The antimicrobial activity of the extract of *Zapoteca portoricensis* at lower doses (750 mg/ml and below) is variable as it does not inhibit some strains of the same *Pseudomonas specie*. Its activity though tends to increase with decreasing concentration with no activity at doses higher than 750 mg/ml. This may indicate that the constituent of the extract responsible for this activity may have a diffusion problem. The potency of *Zapoteca portoricensis* has been demonstrated previously where it was formulated into lozenges for the possible treatment of mouth and throat infections [23] and thus could be a potential source of drug for the treatment of ESBL infections.

Table 2. Antimicrobial activity of ESBL producing *Pseudomonas* species to the methanol leaf extract of *Zapoteca portoricensis*

Conc. (mg/ml)	Zones of Inhibition (mm)*									
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
3000	0	0	0	0	0	0	0	0	0	0
1500	0	0	0	0	0	0	0	0	0	0
750	6	0	6	7	0	9	8	0	0	0
375	7	0	6	7	0	7	7	0	0	0
187.5	9	0	10	8	0	11	10	0	0	0

**Each value represents the mean value of two determinants. P1= Pseudomonas monteilli, P2=Ps. putida, P3=Ps. beteli, P4= Ps. mendocina, P5= Ps. putida, P6= Ps. monteilli, P7= Ps. monteilli, P8= Ps. Putida, P9= Ps. fulva, P10= Ps. mendocina*

Table 3 shows the antimicrobial activity of methanol leaf extract of *Gongrenema latifolium* against *Pseudomonas* species expressing ESBL. Only *Pseudomonas monteilli* strains B1 and B6 were sensitive to the extract. Probably, strain B1 & B6 of *Pseudomonas monteilli* have a different type of ESBL which can be differentiated through characterization with PCR as they were the only isolates that were sensitive to the methanol leaf extract of *Gongrenema latifolium*. Methanol leaf extract of *Gongrenema latifolium* had a strong antimicrobial activity against non-ESBL producing *Pseudomonas aeruginosa* as mentioned elsewhere, but it is not active against ESBL producing *Pseudomonas aeruginosa* except for *Pseudomonas monteilli.* A previous work have shown that the methanol extracts of *G. latifolium* show activity against *S. aureus*, *L. monocytogenes, S. enteritidis, Ps. aeruginosa* and *Sal. Cholerasius ser typhimurium*. The highest activity observed was against *S. enteritidis, Sal. Cholerasius ser typhimurium* and *Ps. aeruginosa,* but when water was used to extract the plant active components, the solution show no activity against *S. aureus* [24].

Table 3. Antimicrobial Activity of the methanol extract of *Gongrenema latifolium* against *Pseudomonas species* expressing ESBL

Conc. (mg/ml)	Zones of inhibition (mm)*									
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
2000	0	0	0	0	0	0	0	0	0	0
1000	0	0	0	0	0	0	0	0	0	0
500	6	0	0	0	0	7	0	0	0	0
250	7	0	0	0	0	8	0	0	0	0
125	10	0	0	0	0	11	0	0	0	0

**Each value represents the mean value of two determinants. P1= Pseudomonas monteilli, P2=Ps. putida, P3=Ps. beteli, P4= Ps. mendocina, P5= Ps. putida, P6= Ps. monteilli, P7= Ps. monteilli, P8= Ps. Putida, P9= Ps. fulva, P10= Ps. mendocina*

The antimicrobial activity of the methanol extract of *Psidium guajava* against ESBL producing *Pseudomonas species* is variable as shown in Table 4 below. It was active against all the isolates except *Ps. mendocina* strain P10. The activity of the methanol leaf extract of *Psidium guajava* also decreased as the doses increased. This probably indicates that the active constituents in the extract were not able to diffuse through the pores of the agar. Previous work by Chah et al. showed that *Psidium guajava* had antibacterial properties against *E. coli, Proteus spp, Staphylococcus aureus* and *Pseudomonas species* [13].

Table 4. Antimicrobial Activity of the methanol extract of *Psidium guajava* against *Pseudomonas species* expressing ESBL

Conc. (mg/ml)	Zones of Inhibition (mm)*									
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
350	0	0	0	0	0	0	0	0	0	0
175	0	0	0	0	0	0	0	0	0	0
85.5	7	6	0	6	6	7	0	5	5	0
43.75	8	7	7	7	7	8	7	6	6	0
21.87	15	12	14	13	13	13	14	14	11	0

**Each value represents the mean value of two determinants. P1= Pseudomonas monteilli, P2=Ps. putida, P3=Ps. beteli, P4= Ps. mendocina, P5= Ps. putida, P6= Ps. monteilli, P7= Ps. monteilli, P8= Ps. Putida, P9= Ps. fulva, P10= Ps. mendocina*

The MIC of the plant extracts and that of the standard antibiotic, gentamicin are shown in Table 5. The MIC of Methanol leaf extract of *Anthocleista djalonensis* was not determined because it had no activity against any of the strains used in this study. Gentamicin is more active than the plant extracts. Its MIC is 0.00015 mg/ml across the strain. The methanol extract of *Psidium guajava* is the most active of all the extract with an MIC of between 1 - 4.37 mg/ml, followed by *Gongrenema latifolium* and then *Zapoteca portorinensis*. Previous study discovered the MIC of methanol leaf extract of *Psidium guajava* to be 2.5 mg/ml against typed strains and clinical isolates of *Pseudomonas aeruginosa* [25]. ESBL producing *Pseudomonas* spp may have destroyed the active ingredient in this extract and this is why its MIC is higher for most strains and on average (3.17 mg/ml). ESBL probably destroyed most of the antibacterial ingredients found in the methanol extract of *Gongrenema latifolium* as its MIC against *Pseudomonas aeruginosa* greatly increased [16] because according to Eleyinmi A F, 2007, the MIC of its methanol extract was 1 mg/ml. Some other study showed a much greater MIC for the methanol extract, up to 25 mg/ml against *Pseudomonas aeruginosa* [26].

Table 5. Minimum Inhibitory Concentrations of the plant extracts against the *Pseudomonas* species expressing ESBL

Isolates	Minimum inhibitory concentration			
	ZP (mg/ml)	PG (mg/ml)	GL (mg/ml)	GEN (mg/ml)
P1	150	1.09	75	0.15
P2	150	1.09	75	0.15
P3	150	2.18	75	0.15
P4	150	4.37	75	0.15
P5	150	4.37	75	0.15
P6	150	4.37	75	0.15
P7	150	4.37	75	0.15
P8	150	1.09	75	0.15
P9	150	4.37	75	0.15
P10	150	4.37	75	0.15

**Each value represents the mean value of two determinants. P1= Pseudomonas monteilli, P2=Ps. putida, P3=Ps. beteli, P4= Ps. mendocina, P5= Ps. putida, P6= Ps. monteilli, P7= Ps. monteilli, P8= Ps. Putida, P9= Ps. fulva, P10= Ps. mendocina ZP = Zapoteca portorinensis, PG = Psidium guajava, GL = Gongrenema latifolium*

The misuse and increasing use of antibiotics in human and veterinary medicine, and in agriculture have contributed to the development of antibiotic resistance. Agriculture accounts

for over 60% of antibiotic usage in the U. S, for disease prevention and growth promotion [27]. This high rate of misuse of antibiotics may be comparable elsewhere. Consequently, there will be a large pool of resistant organisms residing in these animals and poor hygiene may provide opportunities for vertical and horizontal transfer of resistant traits or genes to human pathogens. Thus there is need to maintain a constant pool of effective antibiotics. Plants remain an important source of medicines for both traditional and orthodox health care practices.

Therefore, these plants could join the other three quarters of plants worldwide, which provide active ingredients for prescription drugs. Among the 120 active compounds currently isolated from the higher plants and widely used in modern medicine today, 75 percent show a positive correlation between their modern therapeutic use and the traditional use of the plants from which they are derived [28]. The medicinal potential of four of such plant is evaluated here especially in the light of emerging resistance to cabapenems [29]. Immunocompromised patients like those with HIV and cancer are most susceptible to nosocomial infections caused by *Ps. aeruginosa* and other *spp*. More so, if they consume improperly cooked meat which may harbour other *Ps. spp*., which are ESBL producers. These ESBL producers can transfer their resistant plasmids to non-ESBL producing *Ps. aeruginosa,* making infections caused by such strain difficult to treat.

4. CONCLUSION

This study evaluates the antibacterial activity of the methanol leaf extract of four plants, known to have antibacterial activity, against ESBL producing *Pseudomonas* spp., found in the gut of animals in the abattoir. The methanol leaf extract of these plants, except for *Anthocleista djalonensis,* have antibacterial activity against these organisms at the dose level used in this study but their activity is hindered by diffusion as they had higher activities at lower doses. This is evidenced by their low MIC. It is worthy of note that the leaves of *Psidium guajava,* although eaten by ruminants, contained the most active extract with an MIC ranging from 1-4.37. It means that the organisms have not yet developed resistance to the complex active ingredients of its extract since time immemorial. If ruminant are fed mainly with these leaves instead of antibiotics supplemented feeds, this could curb the spread of ESBL genes. We recommend that the antibacterial activity of the leaf extract of these plants against ESBL producing *Pseudomonas* spp be evaluated at lower doses than those used in this study especially for *Anthocleista djalonensis.* Furthermore, fractions of these extracts could produce a higher antibacterial activity at lower doses which are comparable to that of standard antibiotics especially imipenem. The use and effect of plant extracts are however very safe at higher doses than is obtainable for pure compounds.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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Special appreciation goes to God, our friends, relatives and siblings

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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