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Sensitivity of *Pseudomonas Species* Expressing Extended Spectrum Beta Lactamase to Different Solvent Fractions of *Milletia Aboensis*

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Abstract

The presence of Extended Spectrum Beta Lactamase (ESBL) producing organisms in abattoirs, a non-hospital community was investigated. A total of ten (10) isolates of *Pseudomonas* species out of twenty-six bacteria isolates expressing ESBL was obtained. The anti-pseudomonal activities of various solvent fractions of *Milletia aboensis* against the ESBL positive isolates of *Pseudomonas* species showed varying sensitivity. These results have suggested that *Milletia aboensis* possess potent anti-pseudomonal agents that could be used to treat infections due to *Pseudomonas* species expressing ESBL. These anti-pseudomonal metabolites are located in the ethanol, chloroform and methanol fractions but are absent in the ethyl acetate fractions.

Keywords: Extended Spectrum Beta Lactamase, *Milletia aboensis*, *Pseudomonas* species

1.0 Introduction

The emergence of antibiotic resistance among prevalent pathogens is a serious threat to the management of infectious disease. Beta-lactam (β-lactam) antibiotics are commonly used for the treatment of bacterial infections [1]. However the development of resistance to this class of antibiotics by bacteria through production of β-lactamases is the main mechanism of bacterial resistance to these antibiotics [2]. The extended-spectrum β-lactams became widely used in the treatment of serious infection due to Gram-negative bacteria [3] after failure of the Beta-lactams. However, resistance to these extended spectrum β-lactams due to β-lactamases emerged quickly [3, 4]. The first report of plasmid-encoded β-lactamases capable of hydrolyzing the extended-Spectrum cephalosporins was published in 1983[5]. Many Gram-negative bacteria possess naturally occurring, chromosomally mediated-lactamases, (e.g. AmpC cephalosporinases of *Enterobacteriaceae*) as well as plasmid-mediated β-lactamase e.g. TEM-1 and SHV-1[2] that inactivate these antibiotics. Extended Spectrum Beta Lactamases (ESBLs) are so called because they are able to hydrolyze extended spectrum β-lactam antibiotics. They have ability to inactivate β-lactam antibiotics containing an oxyimino-group such as oxyimino-cephalosporins (e.g., ceftazidime, ceftriaxone, cefotaxime) as well as oxyimino-monobactam (e.g. aztreonam) [3, 6]. The enzymes are not active against cephamycins and carbapenems. Generally, they are inhibited by β-lactamase-inhibitors such as clavulanate and tazobactam. ESBLs have been isolated in food producing animals, Community and Hospital settings and among paediatrics [7-9].

Milletia aboensis is widely distributed in tropical Africa and found abundantly in South East of Nigeria, Cameroon and Equatorial Guinea. The leaves are used for general healing and as laxatives while the root is used for treatment of venereal diseases [7]. Traditionally, the leaves decoction has been widely used in the treatment of pains and inflammatory disease conditions.

In this research therefore, the crude extracts and some solvent fractions of the crude extracts are evaluated against ESBL producing *Pseudomonas* species. The findings will enhance the search for new active ingredient(s) for the control of the ESBL producing pseudomonas species in particular and bacteria in general.

1.1 Materials and Methods

1.1.1 Isolation of ESBL producing *Pseudomonas* species using Double Disc Synergy Test (DDST)

A total of twenty-nine bacteria showing ESBL positivity isolated from One hundred and forty nine (149) samples collected from different Cow abattoirs at Awka metropolis Anambra State Nigeria were used for the study.

A 20 ml volume of Mueller Hinton agar was prepared and dispensed aseptically into each of the Petri dishes. A 0.1 ml suspension of each of the isolates equivalent to 0.5 ml MacFarland standard was aseptically seeded into the Petri dishes together with Mueller Hinton agar. This was allowed to stand for 1 hour to solidify. A combination disc

(Amoxicillin 20 µg and Clavulanic acid 10 µg) was placed at the centre of the Petri dish and antibiotics (Ceftazidime 30 µg and Cefuroxime 30 µg) were placed 15 mm apart centre to centre on both sides of the plates.
The set up was done in triplicate and it was left for 30 minutes for diffusion. It was incubated at 37 ^{o}C for 24 hours after which the various inhibition zone diameters were measured.

1.1.2 Extraction and fractionation of the plant materials

The fresh plant leaves were washed with clean water, then sliced into small fragments and sun-dried. The dried material was reduced to coarse powder with mortar and pestle and then reduced to a fine powder with a mechanical grinder.
About 500 g of the pulverized leaves were cold-macerated in aqueous ethanol (70%) for forty-eight hours. The ethanol extract was concentrated in vacuum. The extract was adsorbed on silica gel and eluted in succession with Chloroform, Ethyl acetate and Methanol. All the extracts and fractions were stored in refrigerator between 0-4 ^{o}C until used.

1.1.3 Sensitivity of test microorganisms to plant extracts

This was determined by the agar well diffusion method. A 20 ml quantity of sterile molten Mueller- Hinton nutrient agar was poured aseptically into Petri dishes containing 0.1 ml of the McFarland's standard of the test micro-organisms. The plates were gently rotated to ensure even distribution of the microorganisms and finally allowed to set. Using sterile cork borer, wells of 6 mm diameter were bored on the solidified seeded Mueller-Hinton nutrient agar plates. About 40 µl of the different concentrations of herbal extract and standard antibiotics were placed in the wells and left for one hour at room temperature after which the plates were incubated at 37 ^{o}C for 24 hours. The test was carried out in triplicate for each of the ten preparations. After the incubation period, the plates were observed for inhibition zone diameter (IZD).

1.1.4 Evaluation of the Minimum Inhibitory Concentration (MIC) of the plant extract

The minimum inhibitory concentration of the extracts and standard antibiotics against *Pseudomonas species* expressing ESBL were performed using the agar dilution method. A 19 ml volume of sterilized Mueller-Hinton nutrient agar was aseptically poured into a sterile Petri dish containing 1 ml of the graded concentration of the eight different preparations of the extracts. The plates were rotated to ensure an even distribution of the microorganisms and allowed to set. The plates were inoculated with a loopful of 0.5 McFarland standards of the test microorganisms. This was repeated for all the test microorganisms and each done in duplicates. The plates were incubated at 37 ^{o}C for 24 hours for all species of the ESBL producing *Pseudomonas*. Presence of growth was observed after incubation and the MIC recorded as the minimum concentration where no visible growth was observed on the plate.

1.2 Results and Discussions

The ethanol extract of *Milletia aboensis* has a yield of 86.74 g. The chloroform fraction had a yield of 8.46 g, the ethyl acetate fraction gave a yield of 5.48 g and that of methanol fraction was 35.46 g.
Phenotypic methods of detection of ESBL activity is based upon the resistance that ESBLs confer to oxyimino- β-lactams (e.g ceftriaxone, cefotaxime, ceftazidime and aztreonam) and the ability of a β-lactamase inhibitor, usually clavulanate, to block this resistance. A clear extension of the edge of the antibiotic's inhibition zone toward the disk containing clavulanate is interpreted as synergy, indicating the presence of an ESBL.
Table 1 thus shows high ESBL production with the organisms depicting the synergistic symbol that is characteristic of ESBL production appearing to be *Pseudomonas species*. ESBLs have been found in a wide range of gram-negative rods. However, the vast majority of strains expressing these enzymes belong to the family *Enterobacteriaceae* [3]. *Klebsiella pneumonia* seems to remain the major ESBL producers. Another very important organism is *Escherichia coli*. It is important to note the growing incidence of ESBLs in *Salmonella sp* [8]. ESBLs have become more prevalent among species with inducible AmpC β- lactamases [9].
Non *Enterobacteriaceae* ESBL producers are relatively rare with *Pseudomonas aeruginosa* being the most important organism [10]. ESBL has also been reported in *Acinetobacter spp*, *Burkholderia cepacia* and *Alcaligenes fecalis* [3].
Some solvent fraction of *Milletia aboensis* showed an appreciable level of antibacterial activity on the *Pseudomonas spp* producing ESBL (Table 2). Both the crude ethanol extract and methanol fraction showed appreciable activity against ESBL *Pseudomonas* isolates at the concentration of 25mg/ml and 12.5mg/ml respectively (Table 2). It was also observed that the activity increases as the concentration decreases which could be attributed to diffusion problems. Also, table 2 revealed that the crude extract of *Milletia aboensis* had no effect against two species of *Pseudomonas* (*putida* and *fulva*) while there are observable zones of inhibition when evaluated against *P.monteilli*, *P.beteli* and *P.mendocina.* There are zones of inhibition across the entire organisms challenged with the methanol extract of *Milletia aboensis* at the concentration ranges of 50mg/ml-12.5mg/ml.

Chloroform fraction exhibited antibacterial activity at the highest concentration (Table 2) while ethyl acetate showed no significant effect against the test ESBL isolates.

From table 3, the MIC of the Crude (ethanol) leaf extracts as well as the chloroform and methanol fractions of the extracts of *Milletia aboensis* against the positive test ESBL isolates of *Pseudomonas species* were shown. The ethanol extract had the lowest MIC value of 10 mg/ml. This could imply that the metabolites with highest activity may lie within the ethanol extract or that group of metabolites act synergistically to give a better performance than either the chloroform or methanol fractions.

Conclusion

The ESBL positive *Pseudomonas species* isolated are sensitive to metabolites found in the ethanol, chloroform and methanol fractions of *Milletia aboensis*.

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Table 1: Phenotypic confirmation of ESBL producing *Pseudomonas spp*

	CAZ	CAZ+AMC	CTX	CTX+AMC
P. *monteilli*	0	29	0	30
P. *putida*	21	28	0	22
P. *beteli*	17	28	0	28
P. *mendocina*	11	20	13	27
P. *putida*	15	28	0	21
P. *monteilli*	0	31	0	23
P. *monteilli*	15	33	0	22
P. *putida*	19	29	11	25
P. *monteilli*	0	30	13	29
P. *fulva*	21	34	0	24
P. *putida*	24	28	11	30
P. *monteilli*	0	31	0	24
P. *fulva*	21	29	14	29
P. *mendocina*	0	22	0	22

CAZ = Ceftazidime; CTX = Cefotaxime; AMC = Amoxicillin+ Clavulanic acid

Table 2: Sensitivity of solvent fractions of *Milletia aboensis* against *Pseudomonas species* expressing ESBL

Extract	Conc (mg/ml)	Zone of Inhibition (mm)									
		B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
Crude	200	0	0	0	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0	0	0	0
	50	0	0	6	0	0	0	0	0	0	0
	25	7	9	7	10	0	0	6	0	0	6
	12.5	8	10	10	12	0	0	7	0	0	6
Methanol	200	0	0	0	0	0	0	0	0	0	0
	100	0	7	0	0	0	0	0	0	0	0
	50	0	8	0	0	0	9	0	0	0	0
	25	7	8	4	6	8	14	8	7	0	6
	12.5	12	15	10	11	10	16	13	15	8	11
Chloroform	1000	8	8	8	6	0	9	10	0	6	11
	500	0	0	0	0	0	0	0	0	0	0
	250	0	0	0	0	0	0	0	0	0	0
	125	0	0	0	0	0	0	0	0	0	0
	65.5	0	0	0	0	0	0	0	0	0	0
Ethylacetate	250	0	0	0	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0	0	0	0
	25	0	0	0	0	0	0	0	0	0	0
	12.5	0	0	0	0	0	0	0	0	0	0

*Each value represents the mean value of two determinants. B_1= *Pseudomonas monteilli*, B_2=*P. putida*,B_3=*P.beteli*, B_4= *P. mendocina*, B_5= *P. putida*, B_6= *P. monteilli*, B_7= *P. monteilli*, B_8= *P. Putida*, B_9= *P. fulva*, B_{10}= *P. mendocina*

Table 3: Minimum inhibitory concentrations of solvent fractions of *Milletia aboensis* against *Pseudomonas species* expressing extended spectrum beta lactamase

Isolates	Minimum Inhibitory Concentration (mg/ml)			
	CEMA	CFMA	MFMA	GEN (µg/ml)
B1	10	50	50	0.15
B2	10	50	50	0.15
B3	10	50	50	0.15
B4	10	50	50	0.15
B5	10	50	50	0.15
B6	10	50	50	0.15
B7	10	50	50	0.15
B8	10	50	50	0.15
B9	10	50	50	0.15
B10	10	50	50	0.15

*Each value represents the mean value of two determinants. B1= *Pseudomonas monteilli*, B2=*P. putida*, B3=*P.beteli*, B4= *P. mendocina*, B5= *P. putida*, B6= *P. monteilli*, B7= *P. monteilli*, B8= *P. Putida*, B9= *P. fulva*, B10= *P. mendocina*. CEMA= Crude Extract of *Milletia aboensis*, CFMA= Chloroform Fraction of *Milletia aboensis*, MFMA= Methanolic Fraction of *Milletia aboensis*, GEN = Gentamycin