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Detection of Carbapenemase Producing Enterobacteriaceae from Clinical Samples and their Susceptibility to Conventional Antibiotics and Medicinal Plant Extracts

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Abstract

Carbapenamases are enzymes that renders inactive, the carbapenems antibiotics which are often considered as the last treatment option for all serious infections. Carbapenemase-producing Enterobacteriaceae (CPE) has already been identified all over the world. In Kano and other part of Northern Nigeria, there are several reports that CPE is in circulation at a rate researchers have never imagined and is rapidly spreading to far rural communities. This study aims to detect *Carbapenemase* producing *Enterobacteriaceae* from clinical sample and determine their susceptibility to conventional antibiotics and *Ficus ovata* and *Guiera senegalensis* plant extracts. Isolates of *Pseudomonas aeruginosa*, *E. coli* and *Klebsiella pneumoniae* were collected from Murtala Muhammad Specialist Hospital, reconfirmed and screened for *Carbapenemase* production using the Modified Hodges Test. The highest occurrence of *carbapenemase* was observed in E. coli (14.3%), while the least was observed in *K. pneumoniae* 3(12%) and *P. aeruginosa* 2(6.7%). Ficus ovata and *Guiera senegalensis* Plants were extracted using ethanol, N-butanol and N-hexane as separate solvents. Of all the three plants extracts, highest yield was observed in N-Butanol extracts having 32.7% in Ficus ovata. Susceptibility test using antibiotics revealed a decreased sensitivity of the test isolates to some commonly used antibiotics; Cefotaxime (25µg), Cefixime (5µg), Levofloxacin (5µg), Amoxicillin (30µg) and Cefuroxime (30µg). Agar well diffusion test was employed at a concentration of 20, 10 and 5mg/ml. All the six extracts were observed to have the highest activity against *P. aeruginosa* with *Guiera senegalensis* having more inhibitory activity. This demonstrates the extracts potentials for extraction of chemotherapeutic agents.

Keywords: Carbapenemase-Producing Enterobacteriaceae; *Escherichia Coli; Klebsiella Pneumonia; Pseudomonas Aeruginosa*; Ficus Ovata, Guiera Senegalensis

Introduction

Carbapenemase enzymes are regarded as a serious threat affecting the global healthcare system. These enzymes are diverse and are in rapid circulation throughout the world [1]. Their health burden is coupled with the fact that the antibiotics which it renders inactive, are the ones regarded as the last resort treatment option against all serious bacterial infections caused by both Gram positive and Gram negative organisms [2]. To date, these enzymes are known to be

produced by only Gram negative Enterobacteriaceae such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Acinetobacter baumannii* among others [2,3].

In Northern Nigeria, there are several reports including that of Yusuf T, et al. [4] that the rate of spread of these enzymes is moving at an alarming rate which if nothing is been done, there is the fear that it will continue to spread to far rural areas which have weak health care system. In Kano, Kaduna, Jigawa and other Northwestern states, different classes of Carbapenemase enzymes producing isolates have been reported to be in circulation. These enzymes are responsible for drug and surgical failures, prolong hospitalization, as well a high number of deaths [2].

For many years, the treatment of many infectious diseases using plant concoctions continue to yield promising outcomes. The activity recorded by these plants are mainly due to the reservoirs of bioactive constituents, and these constituents are reported to be in different proportion in different plants. However certain bioactive compounds were reported to be present only among given species of plants. The antibacterial activity of many medicinal plants and plant preparations has not been examined in Nigeria [5,6]. Ficus ovata known as "Gamji dankwarai" in Hausa and *Guiera senegalensis* known as "Sabara" in Hausa, are known to be very active in managing different ailments such as stomach pain, wound and infertility in Kano and other part of Hausa land [7-9].

As newer classes and groups of carbapenemase enzymes continue to be discovered in Nigeria and Kano state in particular, and the high resistance profile coupled with fatal public health consequences exhibited by bacterial harboring Carbapenamases genes, it become very important to test their susceptibility to different classes of other antibiotics as well as *Ficus ovata* and *Guiera senegalensis* leaf extracts which were known to be effective in the treatment of various form of bacterial infections (Appendix 1 & 2).

Materials and Method

Sampling Site

The sampling site is Urology Center, Murtala Muhammad Specialist Hospital, located in Kano Municipal City, Kano state Nigeria.

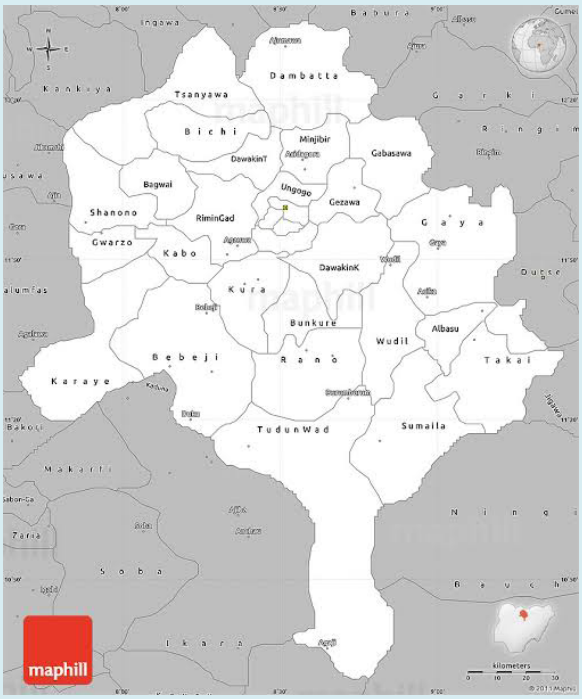

Figure 1: Map of Kano State Showing Kano Municipal City Local Gov't, where the sampling site is located [10].

Ethical Clearance

Ethical approval for the study was obtained from Kano State Health Hospitals Management Board.

Isolate Collection and Reconfirmation

A total of three clinical isolates (E. coli, K. pneumoniae and P. aeruginosa) obtained from urine culture were

collected from Murtala Muhammad Specialist Hospital Urology Centre, located at Kano Municipal City Local Gov't. Kano, over a period of two weeks. For every round of sample collection, the specimens were transported without delay, to the Microbiology Laboratory, Skyline University Nigeria, for re-confirmation as described by Cheesbrough M, et al. [11].

Modified Hodge Test for the Detection of Carbapenemase Production

Modified Hodge test (MHT) was employed for the phenotypic detection of Carbapenamases by the isolates. This was carried out as described by CLSI C.L.S.I [12]. McFarland 0.5 suspension of *E. coli* ATCC 25922 was prepared in peptone water to arrive at a dilution of 1:10. Using Mueller Hinton Agar, a lawn culture was prepared were *E. coli, K. pneumoniae* and *P. aeruginosa* were tested on the same plate using a single 10 µg ertapenem disc. The plate was incubated at 37°C for 18-24hrs. A positive test was observed following the appearance of a clover leaf-like indentation of *E. coli* ATCC 25922 along the test organism. The absence of growth was regarded as negative test as described by Nordmann PM, et al. [3].

Extraction of Plant Materials

The plant samples (Guiera senegalensis and Ficus ovata) were air dried, grounded and extracted by percolation using 3 different solvents (N-butanol, N-hexane and ethanol) to arrive at a total of six different extracts.

Antibacterial Susceptibility Test of the Extracts

The plants extracts were re-calculated to arrive at a concentration of 20mg/ml, 10mg/ml and 5mg/ml. Agar well diffusion test was used in testing their activity against the 3 different isolates.

Result and Discussion

Following the reconfirmation of the isolates using morphological appearance, motility, Gram reaction and biochemical test. All the isolates were observed to be Gram negative rod, motile, catalase positive with special ability to produce gas (Table 1). These characteristics as exhibited by all the test isolates (*E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) are in agreement with the report of Cheesbrough M, et al. [11,13].

isolate	Gram reaction	Morphology	Motility test	Catalase test	Gas production
E. coli	_	Rod	+	+	+
Klebsiella pneumoniae	_	Rod	+	+	+
Pseudomonas aeruginosa	+	Rod	+	+	+

Keys: +:Positive, -:Negative
Table 1: Microscopy and Biochemical test.

In all the two plants, the highest yield was observed in N-Butanol having 32.7% in *Ficus ovata* and 26.3% in *Guiera senegalensis*. The lowest yield was observed in N-Hexane extract having 5.6% for *Ficus ovata* and 6.7% in *Guiera senegalensis* (Table 2). This may be due to the variation in the Phytoconstituents present in each of the plant. More so, the place of sample collection as well as time of sample collection was reported to directly affect the presence and or level of Phytoconstituents presents in plant extracts [1,14,15]. As reported by Yusha'u M, et al. [16] the varying extracts colour may be due to the level of abundance of some given Phytoconstituents which gives a distinct colour to each extracts.

Plant	Solvent of extraction	Solvent polarity	Amount Weighed	Amount obtained	(%) yield	Colour	Smell
Ficus ovata	NH	0.009	47	2.63	5.6	Greenish black	Pleasant smell
	NB	0.586	47	15.37	32.7	Greenish brown	Choky smell
	ET	0.654	44	10.75	24.4	Greenish brown	Pleasant smell
Guiera senegalensis	NH	0.009	50	3.37	6.7	Dark green	Pleasant smell
	NB	0.586	50	13.16	26.3	Dark green	Pleasant smell
	ET	0.654	50	5.06	10.1	Brownish green	Pleasant smell

Keys: NH: N-Hexane, NB: N-butanol, ET: Ethanol.
Table 2: Physical properties of *Ficus ovata* and *Guiera senegalensis* plants extracts.

The Modified Hodge Test was carried out for the Detection of carbapenemase production (Table 3 & Figure 2), where the highest occurrence of carbapenemase was observed in *E. coli* (14.3%), while the least was observed in *K. pneumoniae* and *P. aeruginosa* having 3(12%) and 2(6.7%) respectively. These variations in the occurrence of this enzyme may be because not all strains or species of these isolates are capable of producing Carbapenamases. More so, it may be because the test isolates are clinical isolates and not all patients harbored these categories of isolates that produce Carbapenamases [2]. The work of Yusuf T, et al. [4], which report the first cases of the circulation of Carbapenemase isolates in Kano also revealed that carbapenemase-producing isolates are in circulation in different parts of Kano, at varying levels and locations.

Isolates	Number of Isolates screened	Positive isolates (%)	Negative isolates (%)
E. coli	35	5 (14.3)	30 (85.7)
K. pneumoniae	25	3 (12)	22 (88)
P. aeruginosa	30	2 (6.7)	28 (93.3)

Table 3: Modified Hodge Test for the Detection of carbapenemase production among isolates collected from Murtala Muhammed Specialist Hospital.

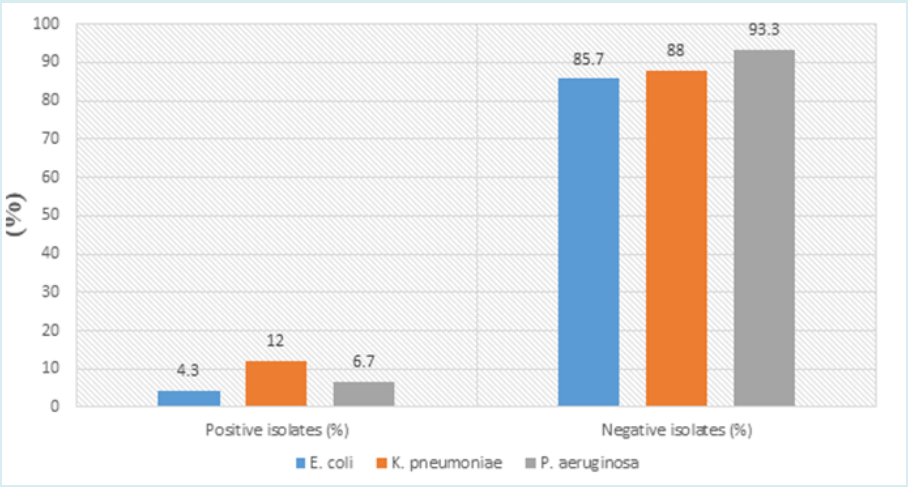

Figure 2: Occurrence of Carbapenamase Produsing Isolates among clinical isolates collected from murtala muhammad specialist hosipital.

The Antibacterial susceptibility test of the test isolates (E. coli, Klebsiella pneumoniae and Pseudomonas aeruginosa) revealed a decreased sensitivity of the test isolates to some commonly used antibiotics such as Cefotaxime (25 µg}, Cefixime {5µg}, Levofloxacin {5 µg}, Amoxicillin{30µg} and Cefuroxime{30µg} (Table 4). This shows that the resistant isolates exhibit resistance to other different classes of antibiotics which is a clear indication of their public health implications. These isolates have the capability in transferring resistance among the same and or different species making their existence a continuing threat [17,18].

Zone of inhibition (mm)				
Antibiotics	**Concentration {µg}**	***E. coli***	***Klebsiella pneumoniae***	***Pseudomonas aeruginosa***
Amoxicillin	30	9	6	9
Cefotaxime	25	4	4	4
Ceftriaxone	45	13	11	12
Cefixime	5	6	0	0
Levofloxacin	5	12	0	9

Ciprofloxacin	5	9	15	8
Imipenem	10	11	13	9
Cefuroxime	30	4	9	8
Ofloxacin	5	9	14	12
Erythromycin	15	8	14	13
Gentamycin	10	6	14	6
Azithromycin	15	12	14	11

Table 4: Antibacterial susceptibility test of *E. coli, Klebsiella pneumoniae* sand *Pseudomonas aeruginosa* to some commonly used antibiotic.

The result of the antibacterial activity of Ficus ovata and Guiera senegalensis extracts against some clinical isolate of Enterobacteriaceae (Table 5 and 6), shows that the activity of the extracts is quite dependent on the level of concentration. The activity decreases with a decrease in concentration from 200 mg to 100 mg and then 50 mg. This is in agreement with the work of Yaro MN, et al. [7] and Yusha'u M, et al. 16]. All the extracts of the two plants were observed to have the highest activity against *P. aeruginosa*, followed by *E. coli* while the least activity was recorded in K. pneumoniae isolate. Considering the very high resistance profile exhibited by Pseudomonas aeruginosa, as it remains one of the few isolates which have found to contain almost all the resistant genes that has been reported to exist among the diverse groups of Enterobacteriaceae [19-21]. The extracts activity can serve as a source of hope for the extraction of novel therapeutic agents that can be used to curtail the health problems posed by this bacterium.

Extracts	Concentration (mg)	E. coli	Klebsiella pneumoniae	Pseudomonas aeruginosa
FO-NB	20	8	12	9
	10	4	8	6
	5	0	6	0
FO-NH	20	11	13	8
	10	10	9	6
	5	4	0	6
FO-ET	20	14	8	11
	10	9	6	6
	5	6	4	0

Keys: FO: *Ficus ovata*, NB: N-Butanol, NH: N-Hexane, ET: Ethanol.
Table 5: Antibacterial activity of *Ficus ovata* Extracts against some clinical isolate of Enterobacteriaceae.

Extracts	Concentration {mg}	E. coli	Klebsiella	Pseudomonas
GS-NB	20	14	14	12
	10	12	11	8
	5	8	6	7
GS-NH	20	11	6	14
	10	6	4	9
	5	4	4	7
GS-ET	20	8	11	12
	10	6	9	8
	5	4	4	4

Keys: GS: *Guiera senegalensis*, NB: N-Butanol, NH: N-Hexane, ET: Ethanol
Table 6: Antibacterial activity of *Guiera senegalensis* Extracts against some clinical isolate of Enterobacteriaceae.

The activity recorded by *Ficus ovata* extracts as recorded in this study is in agreement with the findings of Oyeleke SB, et al. [5] and Kuetea V, et al. [9] who reported the presence of Alkaloids, flavonoids and tannins in the plants with inhibitory activity against E. coli, Shigella and Salmonella spp. The work of Onwuliri FC, et al. [22], Ogbeba J, et al. [6] conducted in Bauchi State and that of [8] conducted in Borno state, (North east) Nigeria report the aqueous and ethanol extracts of Guiera senegalensis to contain Tannins, flavonoids, saponins and alkaloids, with a significant inhibitory activity against both Gram negative (E. coli, Salmonella) and Gram positive (S. aureus) bacterial pathogens. Yaro MN, et al. [7] also report a significant activity by the extracts of *Guiera senegalensis.*

Conclusion

The highest occurrence of carbapenemase was observed in *E. coli* (14.3%), while the least was observed in K. pneumoniae and P. aeruginosa having 3(12%) and 2(6.7%) respectively. Of all the three plants extracts, highest yield was observed in N-Butanol extracts having 32.7% in *Ficus ovata*. The test isolates were observed to have a decreased sensitivity to some commonly used antibiotics. All the extracts of the two plants were observed to have the highest activity against P. aeruginosa, followed by *E. coli* while the least activity was recorded in K. pneumoniae isolate. The inhibitory activity exhibited by the extracts against the test isolates could justify their use locally in traditional medicine. The extracts have also demonstrated the potentials for the extraction of novel chemotherapeutic agents therefore future research should focus on identifying the individual components of each of the extracts with a view to identifying the specific active compounds. On the spread of CPE, the populace needs to be enlightened on the dangers of over-the-counter drugs, reckless use of antibiotics, and the need for adequate personal and community hygiene and dose completion therapy among others.

Recommendation

- The use of plants locally to treat infections should be backed by scientific evidences, else they can be toxic to humans. This also necessitate the need for toxicity studies on the extracts.
- Carbapenemase producing isolates can be spread easily as such extra care must be taken in ensuring an all-round environmental and personal hygiene as this has been noted as a means of dissemination of infectious agents with CPE inclusive.
- The co-selection of antibiotics, use of over the counter medication and over consumption of carbapenems antibiotics has been identified as the major causes and persistence of carbapenems resistance, therefore extra caution need to be applied when handling antibiotics.
- People with urinary tract infection should be fully diagnosed for confirmation before treatment using carbapenems antibiotics.
- Future research should focus on identifying the individual components of each of the extracts via Gas chromatography Mass spectroscopy (GC-MS), Thin layer chromatography and or phytochemical test with a view to identifying the specific active compounds.
- A larger sample size should be tested to cover a wider range of isolates, and appropriate bodies should take necessary measures to avoid the risk of occurrence of these enzymes of medical importance

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- **Conflict of Interest**
  The authors declare no conflict of interest and have read and agree to the content of the manuscript.

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