ECRONICON OPEN ACCESS

EC MICROBIOLOGY
Research Article

Combined Activities of Some Selected Nigerian Medicinal Plants against ESBL Producing Strains of *Escherichia coli* and *Klebsiella pneumonia*

Ikegbunam Moses N[1]*, Achugbu Adaeze N[2] ,Nwachukwu Judith C[2], Okoye Adaora M[2] and Uba Chibuzo C[2]

[1]*Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University, Awka, Nigeria*

[2]*Department of Botany, Nnamdi Azikiwe University, Awka, Nigeria*

***Corresponding Author:** Ikegbunam Moses N, Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University, Awka, Nigeria.

Received: February 02, 2018; **Published:** June 27, 2018

Abstract

Background: Bacteria resistance to orthodox medicine is currently on the increase and is a threat globally.

Objectives: To evaluate the combined activities of leaf extracts of; *Ocimum gratissimum* and *Ocimum canum*; *Piper guineense* and *Xylopia aethiopica*; *Nauclea latifolia* Stem Bark extracts against ESBL producing strains of *Escherichia coli* and *Klebsiella pneumonia*.

Methods: Agar diffusion method was used in individual-plant evaluation for antimicrobial effects. while the checkerboard continuous variation method was adopted in the combination studies. The ESBL producing ability of the organisms was confirmed using double disk synergy test.

Results: *O. canum* and *O. gratissimum* methanol extracts had no activity against resistant strains of *E. coli* but recorded activity against *K. pneumonia* at 250 mg/ml. *X. aethiopica* inhibited the growth of *E. coli* and *K. pneumonia* across all concentrations while *P. guineense* recorded no activity. Methanol and chloroform extracts of *N. latifolia* showed activity against *E. coli* at 100 mg/ml and 50 mg/ml respectively but had no activity against *K. pneumonia.* All combinations of *O. canum* and *O. gratissimum* showed synergistic effect against resistant strains of *K. pneumonia.* Combinations of *P. guineense* and *X. aethiopica* showed no activity against *E. coli* but recorded antagonistic and synergistic effects against *K. pneumonia.* Combination of methanol and chloroform extracts of *N. latifolia* recorded synergistic and antagonistic effects against *E. coli* at different combination ratios. All combination ratios showed synergistic effect against *K. pneumonia.*

Conclusion: This work has established that combination of above named extracts could possibly improve ease and effectiveness of control of resistant bacteria especially *E. coli* and *K. pneumonia* responsible for infectious diseases.

Keywords: *Antimicrobial; Combined Activity; ESBL; Escherichia coli; Klebsiella pneumonia; Medicinal Plants*

Abbreviations

ESBL: Extended-Spectrum ß- Lactamases; MIC: Minimum Inhibitory Concentration; FIC: Fractional Inhibitory Concentration; ME: Methanol Extract; MCE: Methyl Chloroform Extract

Introduction

Infectious diseases are the world leading cause of premature death, killing almost 50,000 people every day [1]. Many of these infections are caused by bacteria such as *Escherichia coli, Salmonella spp., Vibrio cholera, Klebsiella spp., Staphylococcus spp.* and *Pseudomonas spp.* With the proliferation of pathogenic bacteria and consequent increase in mortality, use of antibacterial drugs has become widespread over several decades. However, continuous and extensive use of these drugs (which are mainly synthetic) has not only been known to present serious clinical implications such as increased susceptibility to subsequent diseases including diarrhoea and pathogenic infection as a result of alteration of microbial flora in the body [2] but also lead in selection and spread of resistant bacteria. Consequently, antibacterial drugs are becoming less effective or even ineffective giving rise to an accelerating global health security emergency that is rapidly outpacing available treatment options [3].

Extended-spectrum ß- lactamases (ESBLs) have emerged as a major source of antimicrobial resistance in gram-negative pathogens such as *Escherichia coli and Klebsiella pneumonia,* thereby impeding the antimicrobial treatment of infections caused by Enterobacteriaceae [4].

Plants have been known to provide the basis for traditional treatment for different types of diseases with vast potential as source of new chemotherapeutic agents [5]. Medicinal plants generally contain a number of bioactive constituents, mainly the secondary metabolites such as alkaloids, flavonoids, tannins and phenolic compounds. These phytochemicals have been proven to possess immunomodulatory and antioxidant properties and may be potential natural antibacterial for the treatment of common bacterial infections [6]. Furthermore, different plant parts, plant extracts or plant species can be used in combination to achieve the same goal with great efficacy. In fact, it is thought that herbal remedies have the advantage in combining their active components to obtain synergistic or additive effects which give to the plants an efficiency superior to some of their isolated components [7].

Ocimum canum (Lamiaceae), popularly called African basil or curry leaf, has been used in successful management of various disease conditions like bronchial asthma, chronic fever, cold, cough, malaria, dysentery, convulsions, diabetes, diarrhea, arthritis, emetic syndrome, skin diseases, insect bite etc. and in treatment of gastric, hepatic, cardiovascular and immunological disorders [8]. *Ocimum gratissimum* (Lamiaceae), popularly known as Scent leaf, fever plant or Nchanwu, in Igbo is used in the treatment of epilepsy and high fever [9]. It has also been reported to be used traditionally to treat bacterial infections such as diarrhoea, dysentery and other gastrointestinal infections; upper respiratory tract infections, urogenital infections, skin infections, wounds and ulcers, headache, and bacterial fevers such as typhoid fever [10]. Studies have shown this plant to possess antibacterial activity on *E. coli and S. aureus* [11], antioxidant properties [12] and anti-leishmanial activity [13]. *Piper guineense,* (Piperaceae) also known as African Black Pepper, Ashanti Pepper, Uziza (Igbo), Iyere (Yoruba) or Odusa (Ibibio) has been found to have preservative [14] as well as antimicrobial properties [11,15]. *Xylopia aethiopica* (Dunal) A. Rich (Annonaceae) a valuable medicinal plant widely distributed in the West African rainforest is used traditionally for treatment of ailments including skin infections, candidiasis and cough [16] and as an analgesic [17]. It has been shown to have good antimicrobial activity against commonly encountered food pathogens [18]. *N. latifolia*, also known as African peach or Pin cushion tree, a valuable medicinal plant is used traditionally in the treatment of diabetes mellitus [19], yellow fever [20], malaria and bacterial infections like dysentery and diarrhoea [17].

Although studies have been carried out on the antimicrobial activities of these plants, there is need for more combination studies to establish claims of greater efficacy of medicinal plants, prevent the emergence of resistance, and provide broader-spectrum of activity than monotherapy regimens against pathogenic bacteria as well as reduce toxicity [21].

Methods and Material

Sample Collection

Fresh and healthy leaves of African basil (*O. canum)* and clove basil (*O. gratissimum)* plant samples were collected from Agulu and Amawbia town respectively in Anambra state. Freshly harvested leaves of uziza (*Piper guineense*) was procured locally from a popular market in Nnewi, Anambra State while Uda (*Xylopia aethiopica*) leaves was gotten from Nsukka, Enugu State. The plant was identified by Mrs Aziagba Bibian, a plant Taxonomist in botany department in Nnamdi Azikiwe University, Awka.

The plant sample of *Nauclea latifolia* (stem bark) was collected at Midjivin village of Kaele in the Far North region (10° 10, 800°N, longitude 14° 20, 070°E and altitude 456masl), Cameroon and it was authenticated at the National Herbarium in Yaoundé, Cameroon, where a voucher sample was deposited. The plant samples were air dried at room temperature and pulverized into fine powder using a mechanical grinder. The powdered plant sample was then stored until further use.

Sample Preparation

Extraction of plant materials

Methanol extraction

150g of dried leaf powder of each of the sample (*O. canum, O. gratissimum, X. aethiopica* and *P. guineense*) was dissolved in 900 ml of methanol and extracted using a soxhlet extractor at a temperature of 80°C for 6 hours.

750g of the powdered, air dried *N. latifolia* was cold macerated in aqueous methanol for 3 days with intermittent shaking. The maceration process was then repeated the second time for maximal extraction. At the end of the extraction, the suspension was filtered using Whatman No. 1 filter paper (Whatman international Ltd, Maidstone, England) and the filtrate was concentrated almost to dryness under vacuum using rotary evaporator (Model RE 300) at 40°C and then stored at 4°C until further use.

Fractionation of *N. latifolia* Extract

The crude methanol extract of *N. latifolia* was first absorbed on silica gel and sequentially extracted in succession using, chloroform, petroleum ether, ethyl acetate and methanol in increasing order of polarity. All of the fractions so obtained were filtered using Whatman No 1 filter paper and a rotary evaporator, RE300 was used to concentrate the fractions at 45° ± 5°C. The fractions obtained were stored at 4°C until further use.

Percentage yield of the extracts was calculated thus:

$$\frac{crude\ extract\ weight}{initial\ dry\ weight} \times 100$$

Microbial Analysis

Isolation of the test organisms

Using a wire loop, a colony of test organisms, ESBL producing strains of *Escherichia coli* and *Klebsiella pneumonia* were collected from pure cultures at the department of Pharmaceutical Microbiology and Biotechnology laboratory, and placed in an agar slant. This was incubated at 37°C and stored in a refrigerator.

Screening and Detection of ESBL production

The antibiogram of the test isolates (*K. pneumonia* and *E. coli*) was performed. A representative strain of *Escherichia coli* and *Klebsiella pneumonia* resistant to any of the third generation cephalosporin's (including ceftazidime and cefotaxime) according to the breakpoints of the Clinical Laboratory Standard Institute (CLSI), were tested for ESBL production using phenotypic technique [22]. ESBL production in the *K. pneumonia* and *E. coli* isolates were detected phenotypically using the double disk synergy test (DDST) method as described in other works [23,24]. Augmentin disk (30 µg) was aseptically placed at the center of a Mueller-Hinton (MH) agar already inoculated with

the test bacterium. Ceftazidime (30 µg) and cefotaxime (30µg) single antibiotic disks were each placed adjacent to the central disk (Augmentin) at a distance of 15 mm. The plates were incubated at 37°C for 18 - 24 hrs. A ≥ 5 mm increase or difference in the inhibition zone diameter for either of the cephalosporins (ceftazidime and cefotaxime) tested in combination with Augmentin (a beta lactamase inhibitor) versus its zone when tested alone confirms ESBL production phenotypically.

Antimicrobial Test Procedures

Preparation of Stock Solution

500 mg/ml stock solution of the *O. canum, O. gratissimum, X. aethiopica* and *P. guineense* extracts was prepared by dissolving 2g of the plant extracts in 4 ml of DMSO. From the stock solution a double fold serial dilution was done to obtain 250 mg/ml, 125 mg/ml, 62.50 mg/ml and 31.25 mg/ml. 100 mg/ml stock solution of the *N. latifolia* extracts was prepared by dissolving 2g of the plant extracts in 2 ml of DMSO from which a double fold serial dilution was done to obtain 100 mg/ml, 50 mg/ml, 25 mg/ml and 12.5 mg/ml. The stock solutions were then labelled appropriately and stored at room temperature till required for use.

Antimicrobial Evaluation

Antimicrobial evaluation on each of the crude extract was done using agar diffusion method. A pure culture of resistant strains of *Escherichia coli* and *Klebsiella pneumonia* were exposed to different dilutions of the individual crude plant extract for antimicrobial evaluation. Mueller-Hinton agar was prepared according to manufacturer's instruction and sterilized using autoclave at 121°C at 15pa. The sterilized nutrient media were allowed to cool to warm touch before introducing it into sterile petri dishes. 100 µl of standardized microorganisms containing an approximate number of 1 x 10^5 cfu was introduced into the media and shaken for even distribution in the petri dish. This was allowed to cool and gel. Holes of 9 mm in diameter were bored in the petri dish using a cork borer. Different concentrations (500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml and 12.5 mg/ml) of the extract were introduced into the hole and incubated at 37°C for 18 hrs. The zone of inhibition was measured and recorded.

Determination of Minimum Inhibitory Concentration

The minimum concentration of the crude extract at which no visible growth was seen was determined by plotting the value of X^2 against the log concentrations of the double fold serial dilutions of the plant extract.

$$X^2 = \left[\frac{mean\ IZD - well\ diameter}{2}\right]^2$$

Where Well diameter is the diameter of the cork borer.

Interaction studies

Interaction study techniques, known as the Checkerboard method as employed by Afunwa., *et al.* 2011 [25], were used in this study but with a little modification. The interaction studies were between leaf extracts of *O. canum* and *O. gratissimum*; Leaf extracts of *P. guineense* and *X. aethiopica;* Methanol extract and Methyl chloroform extracts of *N. latifolia.*

Stock solutions of 469.32 mg/ml and 490 mg/ml were prepared for *O. gratissimum* and *O. canum* leaf extracts respectively by dissolving appropriate quantity of each of the plant in 60 ml volume of Dimethyl sulfoxide (DMSO). Stock solutions of 300 mg/ml each were prepared for *Xylopia aethiopica* and *Piper guineense* extracts by dissolving appropriate quantity of each of the plants in 60 ml of Dimethyl sulfoxide (DMSO). Stock solutions of 83.16 mg/ml and 62.64 mg/ml were prepared for Methanolic Extract and Methyl Chloroform Extracts of *N. latifolia* respectively by dissolving appropriate quantity in appropriate volume of Dimethyl sulfoxide (DMSO).

The stock solutions of the individual plants were combined in different ratios starting from A10:B0 to A0:10B for each interaction study between the selected plants. For each interaction study, A and B represents leaf extracts of *O. gratissimum* and *O. canum*; Leaf extracts of *P. guineense* and *X. aethiopica;* Methanol extract and Methyl chloroform extracts of *N. latifolia.*

A two-fold serial dilution was performed on each of the combinations using DMSO as the diluent. Agar diffusion technique was employed to determine the MICs of the individual plants and in combination ratios.

Interaction between the plant extracts were evaluated by determining their Fractional inhibitory concentration Index (FIC index) using the Equation below:

FIC index= FIC A + FIC B

Where

$$FIC\ A = \frac{Mic\ of\ Drug\ A\ in\ combination\ with\ Drug\ B}{Mic\ of\ Drug\ A\ alone}$$

$$FIC\ B = \frac{Mic\ of\ Drug\ B\ in\ combination\ with\ Drug\ A}{Mic\ of\ Drug\ B\ alone}$$

A and B are two antimicrobial agents being combined.

The effects of the combinations were classified as synergistic, additive, indifference and antagonistic, if the FIC Index < 1, = 1, > 1 ≤ 2 and > 2 respectively

Statistical Analysis

Mean IZD were calculated and presented with SPSS v22.

Results

Antimicrobial evaluation

The antimicrobial evaluation of the plant extracts used in the study revealed that both *O. canum, O. gratissimum* has no antibiotic activity against resistant strains of *E. coli* used at different concentrations ranging from 500 mg/ml to 31.25 mg/ml (Table 1). Antibiotic activity against the resistant strains of *K. pneumonia* was however recorded at 500 mg/ml and 250 mg/ml concentrations with IZD of 14.00 ± 1.41 and 10.50 ± 0.71 respectively for *O. gratissimum* and 14.50 ± 0.71 and 10.00 ± 0.00 respectively for *O. canum* (Table 1).

X. aethiopica was found to inhibit the growth of *E. coli and K. pneumonia* across all concentrations, at MIC of 85.51 mg/ml and 54.17 mg/ml respectively. The IZD ranged from 16 mm to 10 mm against *E. coli and* 15 mm to 10.5 mm against *K. pneumonia* (Table 1).

P. guineense showed no activity against any of the resistant microorganisms, evident in the absence of any zone of inhibition (Table 1).

Methanol and chloroform extracts of *N. latifolia* exhibited antimicrobial activity against *E. coli* at concentrations of 100 mg/ml and 50 mg/ml, with IZD of 22 mm and 15 mm respectively for ME against *E. coli* and 20 mm and 16 mm respectively for MCE against *E. coli* (Table 1).

Klebsiella pneumonia on the other hand had no reaction to both the Methanol and Methyl chloroform extracts of *N. latifolia* as observed in table 1.

		Mean IZD (mm)	
Conc. (mg/ml)	**Plant extract**	**E. coli (42)**	**K. pneumonia**
6.25	*N. latifolia* ME	0.00 ± 0.00a	0.00 ± 0.00a
	N. latifolia MCE	0.00 ± 0.00a	0.00 ± 0.00a
12.5	*N. latifolia* ME	0.00 ± 0.00a	0.00 ± 0.00a
	N. latifolia MCE	0.00 ± 0.00a	0.00 ± 0.00a
25	*N. latifolia* ME	0.00 ± 0.00a	0.00 ± 0.00a
	N. latifolia MCE	0.00 ± 0.00a	0.00 ± 0.00a
31.25	*O. gratissimum*	0.00 ± 0.00a	0.00 ± 0.00a
	O. canum	0.00 ± 0.00a	0.00 ± 0.00a
	X. aethiopica	10.00 ± 0.00b	10.50 ± 0.00b
	P. guineenses	0.00 ± 0.00a	0.00 ± 0.00a
50	*N. latifolia* ME	15.00 ± 0.00b	0.00 ± 0.00a
	N. latifolia MCE	16.00 ± 0.00b	0.00 ± 0.00a
62.5	*O. gratissimum*	0.00 ± 0.00a	0.00 ± 0.00a
	O. canum	0.00 ± 0.00a	0.00 ± 0.00a
	X. aethiopica	11.00 ± 0.00b	11.00 ± 0.00b
	P. guineenses	0.00 ± 0.00a	0.00 ± 0.00a
100	*N. latifolia* ME	22.00 ± 0.00b	0.00 ± 0.00a
	N. latifolia MCE	20.00 ± 0.00b	0.00 ± 0.00a
125	*O. gratissimum*	0.00 ± 0.00a	0.00 ± 0.00a
	O. canum	0.00 ± 0.00a	0.00 ± 0.00a
	X. aethiopica	12.00 ± 0.00b	12.00 ± 0.00b
	P. guineenses	0.00 ± 0.00a	0.00 ± 0.00a
250	*O. gratissimum*	0.00 ± 0.00a	10.50 ± 0.71b
	O. canum	0.00 ± 0.00a	10.00 ± 0.00b
	X. aethiopica	15.00 ± 0.00b	13.50 ± 0.00b
	P. guineenses	0.00 ± 0.00a	0.00 ± 0.00a
500	*O. gratissimum*	0.00 ± 0.00a	14.00 ± 1.41b
	O. canum	0.00 ± 0.00a	14.50 ± 0.71b
	X. aethiopica	16.00 ± 0.00b	15.00 ± 0.00b
	P. guineenses	0.00 ± 0.00a	0.00 ± 0.00a

Table 1: *Inhibition zone diameter (IZD in mm) of the sample plant extracts against E. coli and K. pneumonia.*

Results are in Mean ± Standard Deviation; Means with the same letter in a column are not significantly different.

Combined activity

Results of the combination assay revealed that *O. gratissimum* and *O. canum* extracts, when combined, had synergistic effect in the inhibition of *K. pneumonia* especially at the combination ratio of 7:3 with FIC index ranging from 0.1 to 0.19 (Table 2). In the combination of *Xylopia aethiopica and Piper guineense* against *E. coli*, antagonism was observed at combination (*Xylopia: Piper*) ratios of 9:1 with FIC index of 1.53 while the other combinations showed no activity (FIC index = 0). However, in the combination against *K. pneumonia*, combination ratios of 9:1 and 8:2, presented antagonistic activity with FIC index of 1.29 and 1.37 respectively, while combinations of 7:3, 6:4 and 5:5 exhibited synergistic activities with FIC index of 0.90, 0.79 and 0.84 respectively. The rest of the combinations had no activity (Table 3 and 4). In the combination of different ratios of the extracts (Methanol: Methyl Chloroform) of stem bark of *N. latifolia* against *E. coli,* combination ratio of 8:2 showed synergistic activity against ESBL producing *E. coli* with FIC index of 0.7, while ratios 9:1, 7:3, 5:5, 2:8 and 1:9 with FIC index of 1.5, 1.28, 1.29, 1.26 and 1.34 respectively, were antagonistic in activity. Combination ratios of 6:4, 4:6 and 3:7 showed no activity whatsoever (Table 5). In the combination against ESBL producing *K. pneumonia,* all the combinations showed synergistic activity against with FIC Index of ≤ 0.5 ≤ 1. However, the combination ratio of 8:2 presented with the highest FIC index of 0.57 as seen on table 6.

Combination ratio A:B	Sum (FIC index)	Inference
10:0	-	
9:1	0.1	Synergism
8:2	0.09	Synergism
7:3	0.19	Synergism
6:4	0.12	Synergism
5:5	0.16	Synergism
4:6	0.15	Synergism
3:7	0.15	Synergism
2:8	0.11	Synergism
1:9	0.17	Synergism
0:10	-	

Table 2: *Combined Activity of O. gratissimum (Scent leaf) and O. canum (Curry leaf) against ESBL Producing Klebsiella pneumonia.*

Combination Ratio (A:B)	MIC of A (mg/ml)	MIC of B (mg/ml)	FIC A	FIC B	FIC index	Inference
10:0	54.17					
9:1	68.35	8.6	1.26	0.03	1.29	Indifference
8:2	70.8	17.8	1.31	0.06	1.37	Indifference
7:3	45.83	16.9	0.84	0.06	0.90	Synergism
6:4	37.32	29.64	0.69	0.10	0.79	Synergism
5:5	38.67	38.67	0.71	0.12	0.84	Synergism
4:6	0	0	0		0	No activity
3:7	0	0	0		0	No activity
2:8	0	0	0		0	No activity
1:9	0	0	0		0	No activity
0:10	0	300	0		0	No activity

Table 3: *Combined Activity of Xylopia aethiopica and Piper guineense against representative ESBL Producing Klebsiella pneumonia. A: Xylopia aethiopica; B: Piper guineense*

Combination Ratio (A:B)	MIC of A (mg/ml)	MIC of B (mg/ml)	FIC A	FIC B	FIC index	Inference
10:0	85.51					
9:1	125.9	15.85	1.47	0.05	1.53	Antagonism
8:2	0	0	0	0	0	No activity
7:3	0	0	0	0	0	No activity
6:4	0	0	0	0	0	No activity
5:5	0	0	0	0	0	No activity
4:6	0	0	0	0	0	No activity
3:7	0	0	0	0	0	No activity
2:8	0	0	0	0	0	No activity
1:9	0	0	0	0	0	No activity
0:10	0	300	0	0	0	No activity

Table 4: *Combined Activity of Xylopia aethiopica and Piper guineense against representative ESBL Producing Escherichia coli. A: Xylopia aethiopica; B: Piper guineense*

Combination ratio A:B	Combination ratio	MIC of A (mg/ml)	MIC of B (mg/ml)	FIC A	FIC B	FIC Index	Inference
10:0	10:00	28.67	-	-	-	-	-
9:1	9	39.81	3.16	1.388559	0.120427	1.508986	Antagonism
8:2	8	15.4	6.31	0.537147	0.240473	0.777619	Synergism
7:3	7	31.62	4.87	1.102895	0.185595	1.28849	Antagonism
6:4	6	0	0	0	0	0	No activity
5:5	5	19.95	15.85	0.695849	0.60404	1.299889	Antagonism
4:6	4	0	0	0	0	0	No activity
3:7	3	0	0	0	0	0	No activity
2:8	2	7.94	25.9	0.276945	0.987043	1.263987	Antagonism
1:9	1	3.98	31.63	0.138821	1.205412	1.344233	Antagonism
0:10	0		26.24	-	-	-	-

Table 5: *Combined Activity of Methanol and Methyl Chloroform Extract of N. latifolia against ESBL Producing Escherichia coli. A: Methanol Extract of N. latifolia; B: Methyl Chloroform of N. latifolia*

Combination Ratio A:B	MIC of A (mg/ml)	MIC of B (mg/ml)	FIC A	FIC B	FIC Index	Inference
10:0	83.16	-	-	-	-	-
9:1	20.47	1.82	0.246152	0.072251	0.318403	Synergism
8:2	31.63	6.31	0.380351	0.199557	0.579908	Synergism
7:3	31.62	10	0.380231	0.159642	0.539873	Synergism
6:4	25.12	12.59	0.302068	0.20099	0.503058	Synergism
5:5	19.95	15.85	0.239899	0.253033	0.492932	Synergism
4:6	15.85	19.95	0.190596	0.318487	0.509083	Synergism
3:7	12.59	19.95	0.151395	0.318487	0.469881	Synergism
2:8	7.94	25.19	0.095479	0.402139	0.497618	Synergism
1:9	3.98	31.62	0.04786	0.504789	0.552649	Synergism
0:10		62.64	-	-	-	-

Table 6: *Combined Activity of Methanol and Methyl Chloroform Extract of N. latifolia against ESBL Producing Klebsiella pneumonia.*
A: Methanol Extract of N. latifolia; B: Methyl Chloroform Extract of N. latifolia
FIC: Fractional Inhibitory Concentration; MIC: Minimum Inhibitory Concentration

Discussion

Infectious diseases have been known to be one of the serious causes of death in Africa. With antibacterial resistance on the rise, there is need for an aggressive approach using all means possible. The use of herbal products, phytonutrients and supplements has increased greatly over the past decades with many people now resorting to these products for the treatment of various diseases [26], infectious diseases inclusive.

In this study, extracts from some medicinal African plants were evaluated for their antimicrobial activity, singly and combined. *O. canum* and *O. gratissimum* showed no antibiotic activity against resistant strains of *E. coli* at different concentrations. This suggests that the strain of *E. coli* has higher resistance than *K. pneumonia* to the plant extracts. The higher ability of *E. coli* to develop resistant to various antibiotics and plant extracts has already been recorded by previous studies [27].

X. aethiopica was found to inhibit the growth of *E. coli* and *K. pneumonia* at all concentrations. However, in a work done on the antimicrobial activity of ethanol fruit extract of *X. aethiopica* showed no activity against *E. coli* and *K. pneumonia* [18]. This disparity could be attributed to the presence of alkaloids in the leaf extract which was absent in the fruit extract. Alkaloids are a type of plant derived organic compound, they are commonly found to have antimicrobial properties.

P. guineense showed no activity against any of the resistant microorganisms, contrary to studies carried out on the antimicrobial activity of aqueous and ethanolic extracts of *P. guineense* leaves on some bacteria including *E. coli* [15]. This lack of activity may be linked to the differences in the solvents used as well as the strain of microorganisms.

Methanol and chloroform extracts of *N. latifolia* exhibited antimicrobial activity against *E. coli* at concentrations of 100 mg/ml and 50 mg/ml. This agrees with findings made by Falodun., *et al*. (2007) when they stated that Chloroform and methanol extracts of the stem bark revealed significant antibacterial activity against clinical isolates of *Escherichia coli* [28]. Methanol extract however showed higher antimicrobial activity at 100 mg/ml (with IZD of 22 mm) than Methyl Chloroform extract (IZD; 20 mm), contrary to results by Falodun., *et al.* (2007) [28], in which the chloroform extract showed higher antimicrobial activity than methanol extract. Other studies [29] on the other hand, support the fact that the chloroform extract of the stem bark of the plant inhibit the growth of Gram-negative bacteria such as *E. coli.* Interestingly, both plant extracts had no effect on *K. pneumonia,* manifested by lack of observable zones of inhibition. This is contrary to the observations made by Falodun., *et al.* (2007) [28] in which Chloroform and methanol extracts of the stem bark revealed significant antibacterial effects against clinical isolates of *Klebsiella pneumoniae*. This resistance could be attributed to the organism being a resistant strain, i.e. it has the ability to produce extended-spectrum ß- lactamases (ESBLs), an enzyme which is a major source of antimicrobial resistance in gram-negative pathogens [4].

The ability of the extracts of *O. canum, O. gratissimum* and *X. aethiopica* to inhibit the growth of *K. pneumonia* means that these plants can serve as a useful product in the pharmaceutical industries for the control of this organism when used at appropriate concentrations.

The antimicrobial activities of these plant extracts in this study were found to be dependent on the concentration. Wider zones of inhibition were produced at high concentrations and reduced at lower concentrations. This in line with observations made by Okigbo and Igwe (2007) in the Antimicrobial Effects of *Piper guineense* 'Uziza' and *Phyllantus amarus* 'Ebe-Benzo' on *Candida albicans* and *Streptococcus faecalis* [30] and Okigbo and Omodamiro (2006) in the effects of aqueous, petroleum-ether, ethanol and chloroform/methanol extracts on the diameter zones of inhibition (mm) at varying concentrations (mg/ml) of both dry and fresh leaf extracts of Pigeon pea (*Cajanus cajan* (L) Mill sp) on some human pathogens [31]. It could therefore be inferred that higher concentrations have the tendency of breaking down resistance of the microorganisms.

Combined Activity

Combination of plant extracts to treat infectious diseases is an alternative approach [32] which promises more effective treatment options for the control of infections caused by multi-resistant pathogens [7]. With the increase in antimicrobial resistance in disease pathogens, combination therapy seems to have a promising potential as synergistic interactions could increase efficacy, reduce toxicity, cure faster, prevent the emergence of resistance, and provide broader-spectrum of activity than monotherapy regimens [21]. Results of the combination assay revealed that *O. gratissimum* and *O. canum* extracts, when combined, had synergistic effect in the inhibition of *K. pneumonia* especially at the combination ratio of 7:3 with FIC index ranging from 0.1 to 0.19. *K. pneumonia* is a leading cause of hospital-acquired (HA) infections and neonatal sepsis globally [33,34] and has been known to develop resistance to third-generation cephalosporins and fluoroquinolones [35].

In the combination of *Xylopia aethiopica and Piper guineense* against *E. coli*, all but one combination showed no activity. However, in the combination against *K. pneumonia*, two combinations presented antagonistic activity with FIC index > 1, while three combinations exhibited synergistic activities with FIC index > 0 < 0.5 < 1. The rest of the combinations had no activity. In the combination of different ratios of the extracts (Methanol: Methyl Chloroform) of stem bark of *N. latifolia,* only one combination ratio had a synergistic effect on *E. coli.* It could be inferred that this combination gave just the right concentration of phytoconstituents that are responsible for inhibiting *E. coli.* Three combinations had no activity whatsoever on the bacteria while the others were antagonistic. Antagonism is an interaction between two or more individual compounds that produces an injurious effect that is less than either of the substances alone would have produced. When two drugs are antagonistic, their individual effect is better than their combined effect. All combinations showed synergistic activity against *Klebsiella pneumonia.* It is safe to surmise that these combinations that yielded synergistic activity could possess β lactamase inhibiting properties. This is a welcome solution to the urgent threat that resistant *K. pneumonia* poses to human health.

Conclusions

The findings of this study showed antimicrobial activity of combinations of *O. canum* and *O. gratissimum*, *X. aethiopica* and *P. guineense* and Methanol and Methyl Chloroform extracts of stem bark of *N. latifolia* against resistant strains of *E. coli* and *K. pneumonia.* The synergistic activity obtained from the combination of '*O. canum* and *O. gratissimum*' and also 'Methanol and Methyl Chloroform extracts of *N. latifolia* is a breakthrough in the ongoing research to bring solution to the urgent threat that *K. pneumonia* poses to human health. However, further clinical application of combinations among plant extracts and toxicology assays is required for treatment of infectious diseases in human.

Bibliography

1. Rawat M and Parma N. "Medicinal plants used as antimicrobial agents - a review". *International Journal of Pharmacy* 3.1 (2012): 31-40.

2. Keeney KM., *et al*. "Effects of Antibiotics on the Microbiota". *Annual Review of Microbiology* 68 (2014): 217-235.

3. Thenmozhi S., *et al*. "Antibiotic Resistance Mechanism of ESBL Producing Enterobacteriaceae in Clinical Field: A Review". *International Journal of Pure and Applied Biosciences* 2.3 (2014): 207-226.

4. Shaikh S., *et al*. "Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment". *Saudi Journal of Biological Sciences* 22.1 (2015): 90-101.

5. Balogun ME., *et al*. "A. Nauclea latifolia: A Medicinal, Economic and Pharmacological Review". *International Journal of Plant Research* 6.2 (2016): 34-52.

6. Pandey AK and Chowdhry PK. "Propagation techniques and harvesting time on productivity and root quality of Withaniasomnifera". *Journal of Tropical Medicinal Plants* 7 (2006): 79-81.

7. Van Vuuren S and Viljoen A. "Plant-based antimicrobial studies -Methods and approaches to study the interaction between natural products". *Planta Medica* 77 (2011): 1168-1182.

8. Dash DK., *et al*. "Antidiabetic activity and modulation of antioxidant status by Ocimum canum in streptozotocin-induced diabetic rats". *European Scientific Journal* 10.6 (2014): 168-177.

9. Effraim KD., *et al*. "Histopathological studies on the toxicity of Ocimum gratissimum leaf extract on some organs of rabbit". *African Journal of Biomedical Research* 6.1 (2003): 21-25.

10. Mbata TI and Saikia A. "Antibacterial Activity of Essential oil from Ocimum gratissimum on Listeria monocytogenes". *International Journal of Food Safety* 7 (2007): 15-19.

11. Nwinyi SA., *et al*. "Antimicrobial activities of leaf extracts of Ocimum gratissimum on selected diarrhea causing bacteria in Southwestern Nigeria". *African Journal of Biotechnology* 4.7 (2009): 682-684.

12. Bunrathep S., *et al*. "Chemical composition and antioxidative activity of essential oils from four Ocimum species endemic to Thailand". *Journal of Health Research* 21 (2011): 201-206.

13. Tania U., *et al*. "Anti-leishmanial activity of Eugenol-rich essential oil from Ocimum gratissimum". *Parasitology International* 55.2 (2006): 99-105.

14. Kiin-Kabari DB., *et al*. "Effects of extracts from three indigenous spices on the chemical stability of smoke-dried catfish (Clariaslezera) during storage". *African Journal of Food, Agriculture, Nutrition and Development* 11.6 (2011).

15. Anyanwu CU and Nwosu GC. "Assessment of antimicrobial activity of aqueous and ethanolic extracts of Piper guineense leaves". *Journal of Medicinal Plant Research* 8.10 (2014): 337-439.

16. Fleischer TC., *et al*. "Antimicrobial activity of essential oils of Xylopia aethiopica". *African Journal of Traditional, Complementary and Alternative Medicines* 5.4 (2008): 391-393.

17. Lawal IO., *et al*. "Ethno medicinal information on collation and identification of some medicinal plants in Research Institutes of Southwest Nigeria". *African Journal of Pharmacy and Pharmacology* 4.1 (2010): 1-7.

18. Ilusanya OAF., *et al*. "Antimicrobial Activity of Fruit Extracts of Xylopia aethiopica and its Combination with Antibiotics against Clinical Bacterial Pathogens". *Journal of Biology, Agriculture and Healthcare* 2.9 (2012): 2224-2233.

19. Gidado A., *et al*. "Effect of Nauclea latifolia leaves aqueous extracts on blood glucose levels of normal and alloxan-induced diabetic rats". *African Journal of Biotechnology* 4.1 (2005): 91-93.

20. Muanya C. "Herbal cures for malaria show promise in treating resistant strains". Nigeria: The Guardian Newspapers (2009).

21. Marr KA., *et al*. "Combination antifungal therapy for invasive aspergillosis". *Clinical Infectious Diseases* 39.6 (2004): 797-802.

22. Clinical Laboratory Standard Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing Fifteenth Informational Supplement. CLSI document M100-S15. CLSI, Wayne, PA (2005).

23. Ejikeugwu C., *et al*. "Susceptibility and Detection of Extended Spectrum β-Lactamase Enzymes from Otitis Media Pathogens". *American Journal of Infectious Diseases* 9.1 (2013): 24-29.

24. Aibinu I., *et al*. "Occurrence of ESBL and MBL in clinical isolates of Pseudomonas aeruginosa from Lagos, Nigeria". *Journal of American Sciences* 3.4 (2007): 81-85.

25. Afunwa RA., *et al*. "Antimicrobial resistance status and prevalence rates of extended spectrum beta-lactamase producers isolated from a mixed human population". *Bosnian Journal of Basic Medical Sciences* 11.2 (2011): 91-96.

26. World Health Organization. "WHO guidelines on safety monitoring of herbal medicines in pharmacovigilance systems". Geneva, Switzerland (2004).

27. Zander DS and Farver CF. "Pulmonary Pathology: A Volume in Foundations in Diagnostic Pathology Series". Elsevier Health Sciences, Elsevier (2016).

28. Falodun A., *et al*. "Anti-microbial evaluation of an herbal dental remedy stem bark of Nauclea latifolia-Family Rubiaceae". *Journal of Applied Sciences* 7.18 (2007): 2696-2700.

29. Anowi CF., *et al*. "Antimicrobial properties of the chloroform extract of the stem bark of nauclea latifolia". *International Journal of Pharmacy and Pharmaceutical Sciences* 4.2 (2012): 744-750.

30. Okigbo RN and Igwe DI. "The Antimicrobial Effects of Piper guineense 'Uziza' and Phyllantusamarus 'Ebe-Benzo' on Candida albicans and Streptococcus faecalis". *Acta Microbiologica et Immunologica Hungarica* 54.4 (2007): 353-366.

31. Okigbo RN and Omodamiro OD. "Antimicrobial effects of aqueous, petroleum-ether, ethanol and chloroform/methanol extracts on the diameter zones of inhibition(mm) at varying concentrations (mg/ml) of both dry and fresh leaf extracts of Pigeon pea (Cajanus cajan(L) Mill sp) on some human pathogens". *Journal of Herbs, Spices and Medicinal Plants* 12.1-2 (2006): 117-127.

32. Olayinka AA., *et al*. "Synergistic interaction of Helichrysum pedunculatum leaf extracts with antibiotics against wound infection associated bacteria". *Biological Research* 42.3 (2009): 327-338.

33. Jones RN. "Microbial etiologies of hospital-acquired bacterial pneumonia and ventilator-associated bacterial pneumonia". *Clinical Infectious Diseases* 51.1 (2010): S81-S87.

34. Falade AG and Ayede AI. "Epidemiology, aetiology and management of childhood acute community-acquired pneumonia in developing countries-a review". *African Journal of Medical Sciences* 40.4 (2011): 293-308.

35. World Health Organization. "Antimicrobial Resistance Global Report on Surveillance 2014". WHO Library Cataloguing-in-Publication Data (2014).

Volume 14 Issue 7 July 2018