Trop J Nat Prod Res, April 2025; 9(4): 1868 - 1877 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Antibacterial Potentiation Activities of *Newbouldia laevis* Against Some Members of ESKAPE Pathogens Isolated from Hospital Environments

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ARTICLE INFO

Article history:
Received 26 October 2024
Revised 03 March 2025
Accepted 12 April 2025
Published online 01 May 2025

ABSTRACT

The continued resistance of pathogens to a wide range of antibiotics has necessitated the search for herbal alternatives. This research investigates the antibacterial potential and antibiotic potentiation activity of *Newbouldia laevis* on some members of ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter* spp). Three stock 'ESKAPE' organisms isolated from hospital environment were tested for multidrug resistance using standard procedure. Leaves of *Newbouldia laevis* were extracted with methanol using standard method. Antibacterial activity of different concentrations of the methanol plant extracts was carried out using agar well diffusion method. Antibiotic potentiation activity of the extract was tested by comparing the Minimum Inhibitory Concentrations (MICs) of the antibiotics with the fractions of MICs of both antibiotics and extracts. Fractional inhibition concentration was measured as ratio of the two MICs while the strength of the synergism was measured using combination index (CI). Data were analyzed using one-way analysis of variance (ANOVA). There were reductions in the MICs of the antibiotics: ceftriaxone, erythromycin, ceftazidime, cefuroxime and ampicillin across all the isolates. The reductions in the MICs varied among the organisms and the antibiotics with ceftazidine showing lowest MIC to *P. aeruginosa.* However, significant ($P < 0.05$) reduction in the MICs was achieved only in ceftazidine and erythromycin at a combination ratio of 50:50. Combination index values were less than 0.1, signifying strong synergism between the extract and the antibiotics. *Newbouldia laevis* potentiates the activities of these antibiotics against the bacterial species. The potentiations were however MIC-dependent.

Key words: Newbouldia laevis, Antibiotic potentiation, Resistant pathogens, Multidrug resistance, Hospital environment

Introduction

A group of highly resistant pathogens with acronym 'ESKAPE' have been implicated in the surging hospital infections with the tendency to 'escape' the effect of commercial antimicrobial agents.[1, 2, 3] ESKAPE stands for *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter* spp. Their involvements in nosocomial infections present new paradigm of pathogenesis, transmission and resistance.[4, 2] The evolution of multidrug-resistance (MDR) among these isolates has remained a global threat to humanity with significantly high mortality and morbidity rates. [5, 6] Different classes of antibiotics, including cephalosporins, aminoglycosides, glycopeptides, and quinolones have been extensively used in treatment of different diseases.[7] Unfortunately, their overuse and misuse have birthed antibiotic resistant strains.[8]

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Citation: Ugwoke HU, Ezema JN, Adonu CC, Chinaka CB, Anyaegbunam ZG, Aduba CC, Eze EA. Antibacterial Potentiation Activities of *Newbouldia Laevis* Against Some Members of ESKAPE Pathogens Isolated From Hospital Environments. Trop J Nat Prod Res. 2025; 9(4): 1868 – 1877 https://doi.org/10.26538/tjnpr/v9i4.62

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

In a bid to overcome these challenges, the use of plant extracts and combination therapy have been adopted.[9, 10] Apart from their pharmacologically important phytochemical contents, each specie of plant has its own nutrient compositions which are essential for improved body physiological functions.[11]The use of natural medicines have remained a fundamental part of history and culture, particularly in Africa, and are gradually increasing in developed countries due to their varying degrees of antimicrobial activity and other beneficial effects.[12, 13, 14]Medicinal plants have been very useful in primary health care services globally.[15, 11] Thus, World Health Organization came up with the slogan, "save the plants which save life", to underscore the importance of medicinal plants.[16] Studies reveal that about 80% of the world population now depends on herbal medicine owing to their relative safety, affordability, availability and tolerance.[11, 16]*Newbouldi laevis*, atropical Africa plant has been reportedly used in the treatment such as malaria, sexual transmitted disease, worms, dental caries and also as a formula in dysentery and diarrhea, eye problems and wounds infection, sore feet, convulsion, skin ulcer, epilepsy, peptic ulcer, constipation, hemorrhoids, pelvic pain in women, snake bite and impotence and infertility.[17, 18, 19, 20, 21]

Combination ofantibiotics and plant bioactive extracts is a relatively new and novel concept in the treatment of antibiotic resistant pathogens.[9] The effects of combined therapy can however be complicated as different interactions can occur among the individual components. Potentiation is a process where two drugs are used in the same prescription. One of them serves as the principal drug while the other is seen as an adjunct or auxiliary drug. The latter helps to strengthen the effect of the former. Thus, developing synergistic antimicrobial activity could be an alternative to monotherapy for patients with severe infections.[22] Synergistic effect may be as a result cell wall inhibition by a formed complex or by cell lysis or death. Synergism can be contributed by sequential blockage of metabolic pathways, facilitation of the entry of drug into microorganism by the other drug or when one drug prevents the inactivation of the second drug by microbial enzymes.[22] Thus, this study is aimed at evaluating the antibacterial activities of *Newbouldia laevis* against some multidrug resistant ESKAPE pathogens isolated from hospital environments as well as its potentiation of antibiotics against the pathogens.

Materials and Methods

Stock organisms

Organisms used were *Staphylococcus aureus, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* previously isolated from hospital environment.

Antibiotic Sensitivity Testing

Mueller Hinton Agar (MHA) was prepared following manufacturer's instruction, autoclaved and dispensed into sterile Petri dishes, where they were allowed to gel. Standardized inoculums were spread evenly on the media surfaces, in duplicates using a sterile glass rod and allowed to stand for 15min with the lids in place. Gram positive and Gram negative antibiotic discs (Rapid Labs, Colchester, United Kingdom) were then placed and pressed down aseptically using forceps to make contacts with the surfaces of the inoculated plates. The plates were incubated at 37ºC for 24 h. The inhibition zone diameters which show the degree of susceptibility of the test organisms) were measured in milliliter and recorded. The isolates were classified into resistant, intermediate or susceptible according to Clinical Laboratory Standard Institute (CLSI) 2024 guideline.[23]

Plant Collection and Identification

Fresh leaves of *Newbouldia laevis* was collected in June 2020 in Umakashi in Nsukka, Enugu State, Nigeria (6.9 N, 7.4E). The plant was identified in the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka (UNN) and deposited at the University of Nigeria (UNN) Herbarium with the following collection details: *Newbouldia laevis* (P. Beau v.) Seem. Ex, Voucher No: UNN/11765.

Extraction of plant materials

The *Newbouldia laevis*leaves were washed and air dried at room temperature for 14days. The dried leaves were grinded to obtain fine particles and stored in a polythene bag until use. Extraction was carried out by soaking 500g of the plant powder in 1.5L of methanol and kept under room temperature for 72h with intermittent shaking to achieve proper extraction.[24] The extract was filtered using Whatman filter paper (No.1). The filtrates was allowed to cool and subsequently concentrated under vacuum with the aid of a rotary evaporator. The filtrate was weighed and stored in air-tight container and refrigerated at 4ºC until needed for analysis.

Plant Extracts Sensitivity Testing

Plant extract sensitivity test was carried out as described elsewhere.[14] Preparation was further diluted to yield 200mg/ml, 100mg/ml, 50mg/ml and 25mg/ml. Mueller Hinton Agar (MHA) was prepared according to the manufacturer's prescription (38g of MHA in 1000mLof distilled water). Spread plate method was used to inoculate, in triplicates, the standardized inoculums on Mueller Hinton agar. The plates were allowed to stand for 15min with the lids in place. Well-spaced holes were then made on the plates using sterile cork borer. A 0.1ml concentration of the plant extract was injected into the wells and allowed to stand for 45mins for proper diffusion before incubation for 24h at 37ºC. The plates were later examined for inhibition zones.

Antibiotic Potentiation Assay.

The synergistic effect of the combination of the herbal extract and the antibiotics was determined using Chekerboard method as described elsewhere. [25] *Newbouldia laevis* was used in association with the antibiotics. The different concentrations of the combination (antibiotics and plant extract) were prepared by using the plant extract at concentrations lower than the MIC (MIC/2 and MIC/4) with the different antibiotics in different ratios. Mueller Hinton Broth (MHB) were prepared according to manufacturers' standard (21g of Mueller Hinton Broth powder in 1000ml of distilled water), autoclaved at 121ºC for 15min at 15 lbs and used for the preparation of the different concentrations of the antibiotics. The plant extract at MIC/2 and MIC/4 in different ratios were added to the test tubes containing different concentrations of antibiotics. A 0.1ml of standardized inoculums were added to the test tubes and incubated for 24h at 37ºC. The test tubes were later examined for growth which is indicative of MIC of the combination. The MIC was recorded. Fractional Inhibition Concentration was calculated as the ratio of MIC $_{\text{antibiotics in combination}}$/ MIC $_{\text{antibiotics alone}}$ which is used to express the antibiotic potentiation effect of the plant extract and antibiotics. The results were described as synergy (≤ 0.5), indifferent (> 0.5 to 4), or antagonism (>4). All assays were performed in triplicates.

Statistical Analysis

The data obtained were analyzed with one-way-ANOVA using IBM SPSS version 21

Results and Discussion

Antibacterial effects of some selected antibiotics on the isolates

The inhibition zone diameters of five antibiotics selected for the purpose of this potentiation study were extracted and presented in Table 1. Different isolates of each of the organisms were inhibited at varying degrees with *S. aureus* showing the highest level of susceptibility to the three antibiotics applied. Ceftazidine exerted the lowest antibacterial effect of 2 on against *P. aeruginosa* isolates. Analysis of MICs of antibiotics used against the isolates (*S. aureus*, *P. aeruginosa* and *K. pneumonia*) shows that ceftriaxone, erythromycin and ceftazidime had an MIC range of 128 to 256µg/mg for *Staphylococcus aureus*; cefuroxime had an MIC range of 128 to 256µg/mg, ampicillin had 32 to 256µg/mg and ceftazidime had 2 to 64µg/mg for *P. aeruginosa* while cefuroxime and ampicillin showed an MIC range of 128 to 256µg/mg and ceftazidine had 64 to 256µg/mg for ceftazidime for *K. pneumoniae* (Table 1). The inhibitory effects of these antibiotics varied across the different isolates of the same specie. This may be due to extrinsic resistance acquired by these isolates in the course of various exposures to antiobitics.

Antibacterial effects of methanolic extract of Newbouldia laevis leaves on the isolates

Newbouldia laevis methanolic extract was found to inhibit the growths of *S. aureus, P. aeruginosa* and *K. pneumonia.* The minimum inhibitory concentration (MIC) varied among different isolates of the same species but no marked difference between different species. Generally concentration as low as 50mg/mL inhibited growth across the species (Table 2). *Newbouldia laevis* methanol extract inhibited *S. aureus, P. aeruginosa* and *K. pneumonia* at varying degrees with MICs of the extract varying across the isolates of the same specie*Newdouldia laevis* methanolic extract, on the other hand, showed reasonable activities against the three isolates. Its activities were however varied among the isolates of both same and different species. Recall that there were several isolates of each of the species which were all subjected to antibacterial testing of both the extract and the selected antibiotics. The varied susceptibilities suggest acquisitions

of independent resistance by the isolates which resulted in the response to different concentrations of the same extract. The reason for this is not clear but, may, again, be due to some extrinsic factors relating to absorption of the extract. Although some MICs were small enough to suggest great potential of the extract, the inhibition zone diameter of most of them were not enough to rely on, in achieving therapeutic results.

Table 1: Minimum Inhibitory Concentrations (MICs) (Mg/Ml) of Selected Antibiotics on the Isolates

Organisms	ABs	Is1	Is2	Is3	Is4	Is5	Is6	Is7	Is8
S. aureus	CTR	256	256	128	256	128	256	128	256
	ERY	256	128	256	128	256	256	128	128
	CAZ	256	128	256	128	256	256	128	128
P. aeruginosa	CAZ	2	32	16	64	8	32	16	-
	CRX	256	256	128	128	128	256	256	-
	AMP	64	32	64	128	64	256	128	-
K. pneumonia	CAZ	128	64	256	64	128	256	-	-
	CRX	256	128	256	256	128	256	-	-
	AMP	128	256	128	256	256	128	-	-

Key:Abs= Antibiotics; CTR= Ceftriaxone; ERY= Erythromycin; CAZ= Ceftazidime; CRX= Cefuroxime; AMP= Ampicillin; Is = Isolates

Table 2: MICs of Methanolic Extracts of *Newbouldia laevis* on the Susceptible Isolates

S. aureus	**Isolates**	**Is1**	**Is2**	**Is3**	**Is4**	**Is5**	**Is6**	**Is7**	**Is8**
	MIC of extract (mg/mL)	200	50	100	50	50	50	100	50
P. aeruginosa	Isolates	Is1	Is2	Is3	Is4	Is5	Is6	Is7	-
	MIC of extract (mg/mL)	50	50	100	50	50	100	200	-
K. pneumoniae	Isolates	Is1	Is2	Is3	Is4	Is5	Is6	-	-
	MIC of extract (mg/mL)	100	100	200	100	50	50	-	-

Key: Is = Isolate

Antibacteria Potentiation Activities of Newbouldia laevis

Three antibiotics were combined with *N. laevis* leaves extract on each of the bacterial strains. Potentiation effects were obtained with the tested extract and antibiotics on at least one of the selected bacterial strains at different ratios. The synergistic tests of the antibiotic-extract combination shows that the best percentage synergy (100%) is at MIC /2 with *N. laevis* leaf extract in combination with Erythromycin and Ceftazidime in the ratio 50:50 (Table 3) as well as *N. laevis* leaf extract in association with Ceftazidine in the ratio 70:30 (Table 4). The least effect of the combinations was observed in the ratios of 80:20. However, certain isolates responded well to ceftazidine-extract combination (Table5). The dose effect curve depicts the relationship between a given dose and the effect (inhibition) produced. While fa is fraction of cells killed, fu is fraction of cells unaffected. As shown in the dose-effect curve, 50, 100 and 300 mg/ml of ERYExt, NlExt and ERY respectively, inhibited 50% of the *S. aureus* cells; hence, ERYExt is more potent (Figure 1). Result in Figure 2 shows that *N. laevis* and ERY at ratio 50:50 are strongly synergistic with CI values; 0.406 to 0.218 for fa=0.22 to 0.88. The output also shows favourable dose reduction index (DRI>1). The isobologram indicate synergism at fa=0.5 to 0.9 (Figure 3). The result of CI and isobologram are experimental proof of synergism for ERY and *N. laevis* extract at fa=0.5 to 0.9. The best percentage synergistic effect was observed in *P. aeruginosa* at MIC/2 of *N. laevis* leaves extract in combination with cefdazidime (100%) in the ratio 50:50 (Table 6) and cefuroxime and *N. laevis* leaves extract (71.42%) in the ratio 70:30 (Table 7). For ceftazidme (CAZ) and *N. laevis* extract, Dm ranged from 3.21 to 447.19 mg/ml, with the combination, CAZNlExt having the lowest Dm value, hence, the highest potency (Figure 3). Combination index (CI) data in Figure 4 shows how much synergism, antagonism or additive effect results from the interaction. If CI=1 (additive), less than 1= synergism, greater than 1= antagonism. Combination index for all the actual data points (i.e. the experimental values) were synergistic. Isobologram (Figure 5) depicts very strong synergism between CAZ and NlEx (CI < 0.1). The best percentage synergistic effect on *K. pneumoniae* was at MIC/2 of *N. laevis* leaves extract in combination with ceftazidime and cefuroxime (100%) in the ratio 50:50 (Table 9) and cefuroxime and *N. laevis* leaves extract (83.33%) in the ratio 70:30 (Table 10). The dose effect values curves in Figure 7 showed that, CAZExt had the highest potency against *K. pneumonia* Combination index values obtained were <0.1, implying a very strong synergism between CAZ and *N. laevis* extract as shown in Figures 8 and 9.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

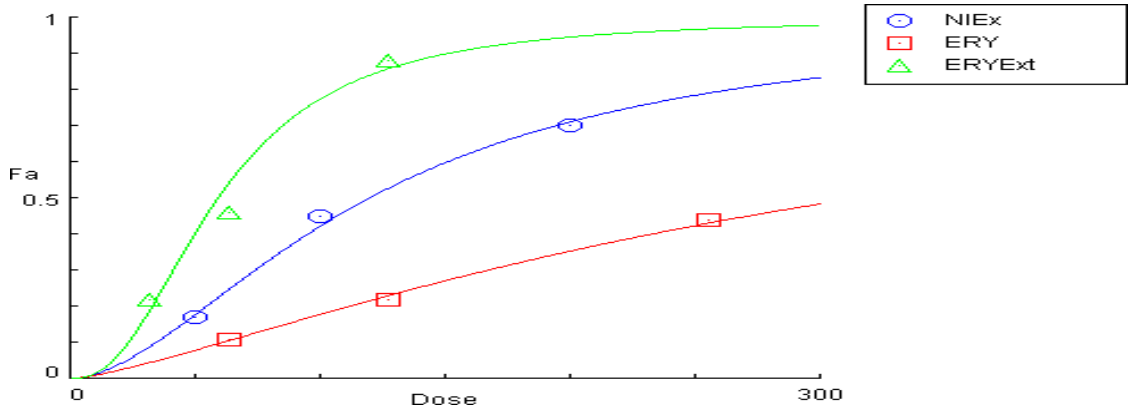

Figure 1: Dose-effect curve from compuSyn, for erythromycin and *N. laevis* combination against *S. aureus*

Table 3: MIC of Antibiotics-*Newbouldia laevis* Combination at MIC/2 and MIC/4 against Selected *S. aureus* at Ratio of 50:50 (v/v)

ABS	Extract Concentration	Is1	Is2	Is3	Is4	Is5	Is6	Is7	Is8	BSS (%)
CTR	0	256	256	128	256	128	256	128	256	
	MIC/2	64(0.25)	64(0.25)	32(0.25)	64(0.25)	64(0.5)	32(0.125)	128(1)	64(0.25)	7/8 (87.5)
	MIC/4	128(0.5)	128(0.5)	64(0.5)	64(0.25)	128(1)	64(0.25)	128(1)	128(0.5)	6/8 (75)
ERY	0	256	128	256	128	256	256	128	128	
	MIC/2	32(0.125)	64(0.5)	128(0.5)	64(0.5)	32(0.125)	128(0.5)	32(0.25)	64(0.5)	8/8 (100)
	MIC/4	128(0.5)	128(1)	128(0.5)	128(1)	128(0.5)	256(1)	64(0.5)	128(1)	4/8 (50)
CAZ	0	256	128	256	128	256	256	128	128	
	MIC/2	64(0.25)	64(0.5)	64(0.25)	64(0.5)	32(0.125)	128(0.5)	32(0.25)	64(0.5)	8/8 (100)
	MIC/4	128(0.5)	128(1)	128(0.5)	128(1)	256(1)	256(1)	64(0.5)	128(1)	3/8(37.5)

Key:Abs= Antibiotics; CTR= Ceftriaxone; ERY= Erythromycin; CAZ= Ceftazidime; 0= No extract (antibiotics alone), Is = Isolate, BSS = Bacterial strains on which synergy occurred.

Table 4: MIC of Antibiotics-*Newbouldia laevis* Combination at MIC/2 and MIC/4 against Selected *S. aureus* at Ratio of 70:30 (v/v)

ABS	Extract Concentration	Is1	Is2	Is3	Is4	Is5	Is6	Is7	Is8	BSS (%)
CTR	0	256	256	128	256	128	256	128	256	
	MIC/2	64(0.25)	128(0.5)^s	128(1)	128(0.5)^s	64(0.5)^s	128(0.5)^s	64(0.5)^s	128(0.5)^s	7/8(87.5)
	MIC/4	128(0.5)^s	128(0.5)^s	128(1)	128(0.5)^s	64(0.5)^s	128(0.5)^s	128(1)	256(1)	5/8(62.5)
ERY	0	256	128	256	128	256	256	128	128	
	MIC/2	64(0.25	64(0.25)	128(0.5)	64(0.25	128(0.5	128(0.5)	64(0.25	64(0.25	8/8(100)
	MIC/4	128(0.5)	128(1)	256(1)	128(1)	128(0.5)	256(1)	128(1)	128(1)	2/8(25)
CAZ	0	256	128	256	128	256	256	128	128	
	MIC/2	32(0.125)	128(1)	32(0.125)	64(0.5)	64(0.25)	128(0.5)^s	64(0.5)^s	64(0.5)^s	7/8(87.5)
	MIC/4	128(0.5)	256(2)	64(0.25)	128(1)	256(1)	256(1)	64(0.5)^s	256(2)	3/8(37.5)

Key:Abs= Antibiotics; CTR= Ceftriaxone; ERY= Erythromycin; CAZ= Ceftazidime; 0= No extract (antibiotics alone), Is = Isolate, BSS = Bacterial strains on which synergy occurred; S = Significant.

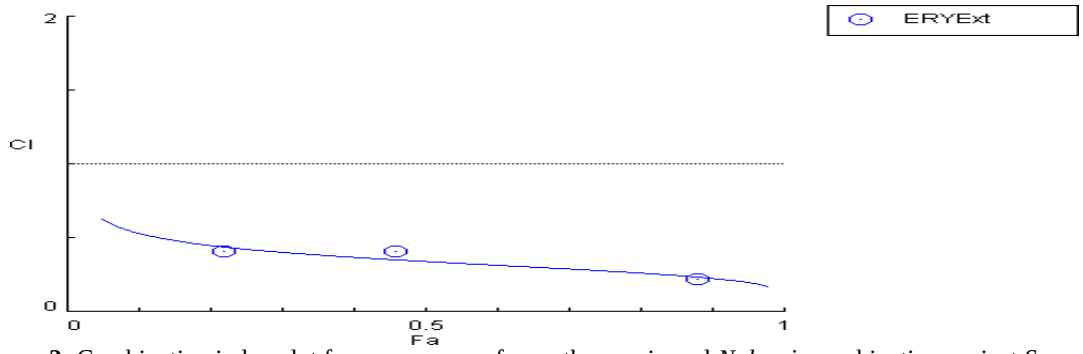

Figure 2: Combination index plot from compusyn, for erythromycin and *N. laevis* combination against *S. aureus*

Table 5: MIC of Antibiotics-*Newbouldia laevis* Combination at MIC/2 and MIC/4 against Selected *S. aureus* at Ratio 80:20

ABS	Extract Concentration	Is1	Is2	Is3	Is4	Is5	Is6	Is7	Is8	BSS (%)
CTR	0	256	256	128	256	128	256	128	256	
	MIC/2	128(0.5)^s	256(1)	128(1)	128(0.5)^s	64(0.5)^s	128(0.5)^s	128(1)	256(1)	4/8 (50
	MIC/4	256(1)	256(1)	128(1)	256(1)	128(1)	256(1)	128(1)	256(1)	5/8 (62.5)
ERY	0	256	128	256	128	256	256	128	128	
	MIC/2	128(0.5)^s	64(0.5)^s	128(0.5)^s	64(0.5)^s	64(0.25)^s	128(0.5)^s	64(0.5)^s	128(1)	7/8 (87.6)
	MIC/4	256(1)	128(1)	128(0.5)^s	256(2)	128(0.5)^s	256(1)	64(0.5)^s	128(1)	3/8 (37.5)
CAZ	0	256	128	256	128	256	256	128	128	
	MIC/2	3/8 (37.5)	128(1)	64(0.25)^s	64(0.5)^s	128(0.5)^s	128(0.5)^s	128(1)	64(0.5)^s	6/8 (75)
	MIC/4	128(0.5)^s	256(2)	128(0.5)^s	128(1)	256(1)	500(1.95)	128(1)	256(2)	2/8 (25)

Key:Abs= Antibiotics; CTR= Ceftriaxone; ERY= Erythromycin; CAZ= Ceftazidime; 0= No extract (antibiotics alone), Is = Isolate, BSS = Bacterial strains on which synergy occurred; S = Significant

Table 6: MIC of Antibiotics-*Newbouldia laevis* Combination at MIC/2 and MIC/4 against Selected *P. aeruginosa* at Ratio 50:50

ABS	Extract Concentration	Is1	Is2	Is3	Is4	Is5	Is6	Is7	BSS (%)
CAZ	0	2	32	16	64	8	32	16	
	MIC/2	0.5(0.25)^s	16(0.5)^s	8(0.5)^s	32(0.5)^s	4(0.5)^s	8(0.25)	8(0.5)	/7(100)
	MIC/4	1(0.5)^s	32(1)	8(0.5)^s	32(0.5)^s	8(1)	32(1)	16(1)	3/7(42.85
CRX	0	256	256	128	128	128	256	256	
	MIC/2	64(0.25)^s	128(0.5)^s	64(0.5)^s	128(1)	64(0.5)^s	128(0.5)^s	64(0.25)^s	6/7(85.71)
	MIC/4	128(0.5)^s	256(1)	128(1)	256(2)	64(0.5)^s	256(1)	128(0.5)^s	3/7(42.85)
AMP	0	64	32	64	128	64	256	128	
	MIC/2	64(1)	64(2)	32(0.5)^s	64(0.5)^s	32(0.5)^s	128(0.5)^s	128(1)	4/7(57.14)
	MIC/4	128(2)	64(2)	64(1)	256(2)	128(2)	500(1.95)	64(0.5)^s	1/7(14.28)

Key: ABS = Antibiotics; CAZ= Ceftazidime; CRX= Cefuroxime; AMP= Ampicillin; 0= No extract (antibiotics alone), Is = Isolate, BSS = Bacterial strains on which synergy occurred; S = Significant.

Table 7: MIC of Antibiotics-*Newbouldia laevis* Combination at MIC/2 and MIC/4 against Selected *P. aeruginosa* at Ratio 70:30

ABS	Extract Concentration	Is1	Is2	Is3	Is4	Is5	Is6	Is7	BSS (%)
CAZ	0	2	32	16	64	8	32	16	
	MIC/2	1(0.5)^s	16(0.5)^s	16(1)	32(0.5)^s	4(0.5)^s	32(1)	16(1)	4/7(57.14)
	MIC/4	2(1)	32(1)	32(2)	32(0.5)^s	8(1)	32(1)	16(1)	1/7(14.28)
CRX	0	256	256	128	128	128	256	256	
	MIC/2	128(0.5)^s	128(0.5)^s	128(1)	128(1)	64(0.5)^s	128(0.5)^s	128(0.5)^s	5/7(71.42)
	MIC/4	128(0.5)^s	256(1)	128(1)	256(2)	64(0.5)^s	256(1)	256(1)	2/7(28.57)

AMP	0	64	32	64	128	64	256	128	
	MIC/2	64(1)	64(2)	32(0.5)^s	64(0.5)^s	32(0.5)^s	128(0.5)^s	128(1)	4/7(57.14)
	MIC/4	128(2)	64(2)	64(1)	256(2)	128(2)	500(1.95)	64(0.5)^s	1/7(14.28)

Key: ABS = Antibiotics; CAZ= Ceftazidime; CRX= Cefuroxime; AMP= Ampicillin; 0= No extract (antibiotics alone), Is = Isolate, BSS = Bacterial strains on which synergy occurred; S = Significant.

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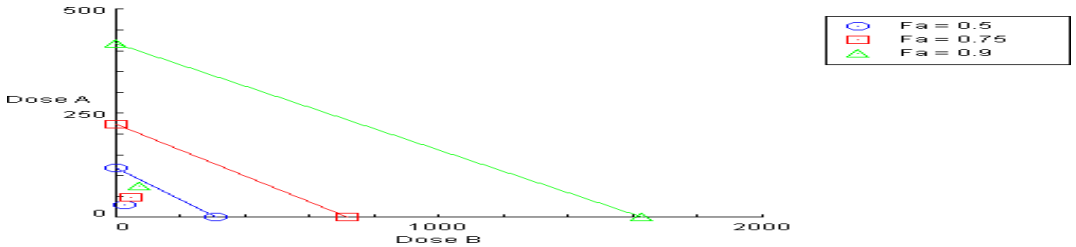

Figure 3: Isobologram obtained from compuSyn, for erythromycin and *N. laevis*combination against *S. aureus.*

In view of the individual limitations of both antibiotics and the extract, an attempt to establish synergy between them to achieve bacteriocidal effect became imperative.*Newbouldia laevis* methanolic crude extract potentiated the activity of Ceftriaxone, Erythromycin, Ceftazidime, Cefuroxime, Ampicillin at sub inhibitory concentration (MIC/2 and MIC/4) against the isolates, with the degree of potentiation increasing with increase in concentration of the extract (Tables 3-11). This finding is in line with the report of other authors on the potentiation activities of *Beilschmiedia acuta, Clausena anisata, Newbouldia laevis and Polyscias fulva* against resistant bacteria.[25] Combination of the individual inhibitory effects of both the extracts and the antibiotics results in microbial death. These synergistic effects however were dependent on the isolates, as different isolates were observed to respond in different in degrees. Erythromycin in combination with *Newbouldia laevis* showed strong synergism with combination index values of 0.406 to 0.218 (Figures 1 & 2) and isobologram values at 0.5 to 0.9 (Figure 3), for *S. aureus* at ratio 50:50. Ceftazidme-*N. laevis* extract also showed high synergism on *P. aeruginosa*, with combination index and isoblogram values at less than 0.1(Figures 4, 5 & 6), while ceftazidime-*N. laevis* extract on *K. pneumoniae* also showed synergism with combination index and isoblogram values at less than 0.1 (Figures 7, 8 & 9). The high synergistic effect obtained in ERY and CAZ suggests that the combination could improve activities resistant bacteria. The presence of some plant metabolites especially flavonoids could improve the activity of antibiotics against resistant bacterial strains.[26]

The mechanisms of these potentiation activities are thought to be as a result of synergistic effects of the individual antibacterial agents. Recall that medicinal plants contain several structurally diverse bioactive compounds which uses various mechanisms to exert bacteriostatic or bacteriocidal effects. Antibacterial activities of plant extracts may be due to complex formation, inhibition of cell wall synthesis, sequential blockage of metabolic pathways, facilitation of entry of drug into microorganism by the other drug and prevention of inactivation of the second drug by microbial enzymes.[22] In general, our findings on combination therapy shows that plant antimicrobial enhances bioactivity and suggest that the combination could improve fight against multidrug resistant bacterial infection.[27]

Table 8: MIC of antibiotics-*Newbouldia laevis* combination at MIC/2 and MIC/4 against selected *P. aeruginosa* at ratio 80:20

ABS	**Extract Concentration**	**Is1**	**Is2**	**Is3**	**Is4**	**Is5**	**Is6**	**Is7**	**BSS (%)**
CAZ	0	2	32	16	64	8	32	16	
	MIC/2	1(0.5)^s	16(0.5)^s	16(1)	32(0.5)^s	4(0.5)^s	32(1)	16(1)	4/7(57.14)
	MIC/4	2(1)	32(1)	32(2)	32(0.5)^s	8(1)	32(1)	16(1)	1/7(14.28)
CRX	0	256	256	128	128	128	256	256	
	MIC/2	128(0.5)^s	128(0.5)^s	128(1)	128(1)	64(0.5)^s	128(0.5)^s	128(0.5)^s	5/7(71.42)
	MIC/4	128(0.5)^s	256(1)	128(1)	256(2)	64(0.5)^s	256(1)	256(1)	2/7(28.57)
AMP	0	64	32	64	128	64	256	128	
	MIC/2	64(1)	64(2)	32(0.5)^s	64(0.5)^s	128(1)	128(0.5)^s	128(1)	3/7(42.85)
	MIC/4	128(2)	64(2)	64(1)	256(2)	128(1)	500(1.95)	128(1)	0/7(0.00)

Key: ABS = Antibiotics; CAZ= Ceftazidime; CRX= Cefuroxime; AMP= Ampicillin; 0= No extract (antibiotics alone), Is = Isolate, BSS = Bacterial strains on which synergy occurred; S = Significant.

Table 9: MIC of antibiotics-*Newbouldia laevis* combination at MIC/2 and MIC/4 against selected *K. pneumoniae* at ratio 50:50

ABS	**Extract Concentration**	**Is1**	**Is2**	**Is3**	**Is4**	**Is5**	**Is6**	**BSS (%)**
CAZ	0	128	64	256	64	128	256	
	MIC/2	64(0.5)^s	16(0.25)^s	64(0.25)	32(0.5)^s	64(0.5)^s	128(0.5)^s	6/6(100)
	MIC/4	64(0.5)^s	32(0.5)^s	128(0.5)^s	64(1)	128(1)	256(1)	3/6(50)
CRX	0	256	128	256	256	128	256	
	MIC/2	64(0.25)^s	32(0.25)^s	64(0.25)^s	128(0.5)^s	32(0.25)^s	64(0.25)^s	6/6(100)
	MIC/4	128(0.5)^s	128(1)	256(1)	128(0.5)^s	128(1)	128(0.5)^s	3/6(50)

AMP	0	128	256	128	256	256	128	
	MIC/2	128(1)	128(0.5)^s	32(0.25)^s	128(0.5)	256(1)	64(0.5)^s	4/6(66.66)
	MIC/4	128(1)	128(0.5)^s	64(0.5)^s	256(1)	256(1)	128(1)	2/6(33.33)

Key: Abs= Antibiotics; CAZ= Ceftazidime; CRX= Cefuroxime; AMP=Ampicillin; 0= No extract (antibiotics alone),), Is = Isolate, BSS = Bacterial strains on which synergy occurred; S = Significant

Table 10: MIC of antibiotics-*Newbouldia laevis*combination at MIC/2 and MIC/4 against selected *K. pneumoniae* at ratio 70:30

ABS	Extract Concentration	Is1	Is2	Is3	Is4	Is5	Is6	BSS (%)
CAZ	0	128	64	256	64	128	256	
	MIC/2	64(0.5)^s	32(0.5)^s	128(0.5)	64(1)	64(0.5)^s	256(1)	4/6(66.66)
	MIC/4	128(1)	32(0.5)^s	256(1)	64(1)	128(1)	256(1)	1/6(16.67)
CRX	0	256	128	256	256	128	256	
	MIC/2	128(0.5)^s	64(0.5)^s	128(0.5)^s	256(1)	64(0.5)^s	128(0.5)^s	5/6(83.33)
	MIC/4	256(1)	128(1)	128(0.5)^s	256(1)	64(0.5)^s	128(0.5)^s	3/6(50)
AMP	0	128	256	128	256	256	128	
	MIC/2	128(1)	128(0.5)^s	64(0.5)^s	256(1)	256(1)	64(0.5)^s	3/6(50)
	MIC/4	128(1)	256(1)	64(0.5)^s	256(1)	500(2)	128(1)	1/6(16.67)

Key: Abs= Antibiotics; CAZ= Ceftazidime; CRX= Cefuroxime; AMP=Ampicillin; 0= No extract (antibiotics alone),), Is = Isolate, BSS = Bacterial strains on which synergy occurred; S = Significant

Table 11: MIC of antibiotics-*Newbouldia laevis* combination at MIC/2 and MIC/4 against selected *K. pneumoniae* at ratio 80:20

ABS	Extract Concentration	Is1	Is2	Is3	Is4	Is5	Is6	BSS (%)
CAZ	0	128	64	256	64	128	256	
	MIC/2	64(0.5)^s	32(0.5)^s	128(0.5	64(1)	128(1)	256(1)	3/6(50)
	MIC/4	128(1)	32(0.5)^s	256(1)	64(1)	128(1)	256(1)	1/6(16.67)
CRX	0	256	128	256	256	128	256	
	MIC/2	128(0.5)^s	64(0.5)^s	128(0.5)	256(1)	128(1)	256(1)	3/6(50)
	MIC/4	256(1)	64(0.5)^s	256(1)	256(1)	128(1)	256(1)	1/6(16.67)
AMP	0	128	256	128	256	256	128	
	MIC/2	128(1)	128(0.5)^s	64(0.5)^s	256(1)	256(1)	128(1)	2/6(33.33)
	MIC/4	256(2)	256(1)	128(1)	256(1)	500(2)	128(1)	0/6(0)

Key: Abs= Antibiotics; CAZ= Ceftazidime; CRX= Cefuroxime; AMP=Ampicillin; 0= No extract (antibiotics alone),), Is = Isolate, BSS = Bacterial strains on which synergy occurred; S = Significant

Statistical analysis

The one-way analysis of variance showed the resulting MICs from association between the test antibiotics; CAZ, ERY, CRX, CTR and AMP, with *Newbouldia laevis* leaf extracts at ratios; 70:30, 50:50 and 80:20 (Table 12). All the test ratios exhibited statistically significant inhibition on the pathogens; *S. aureus*, *P. aeruginosa* and *K.pneumoniae*. However, result of Turkey test post-hoc analysis revealed that, ERY- *Newbouldia laevis* extract at ratio 50:50 was the most effective against *S. aureus*, hence its low MIC of 10 ± 4 mg/ml, *P. aeruginosa*, CAZ- *Newbouldia laevis* extract at ratio 50:50 was most effective (MIC = 10 ± 4 mg/ml) while for *K.pneumoniae*, at CAZ- *Newbouldia laevis* extract ratio of 50:50, the lowest MIC value of 89 ± 19 mg/ml was obtained, in contrast to associations with other test antibiotics at other ratios. Significant differences in the MICs ($p < 0.05$) were observed at 50:50 combinations of ceftazidine and erythromycin in all the isolates. There were, however no significant difference in the effects on MICs at 70:30 and 80:20 combinations in most cases.

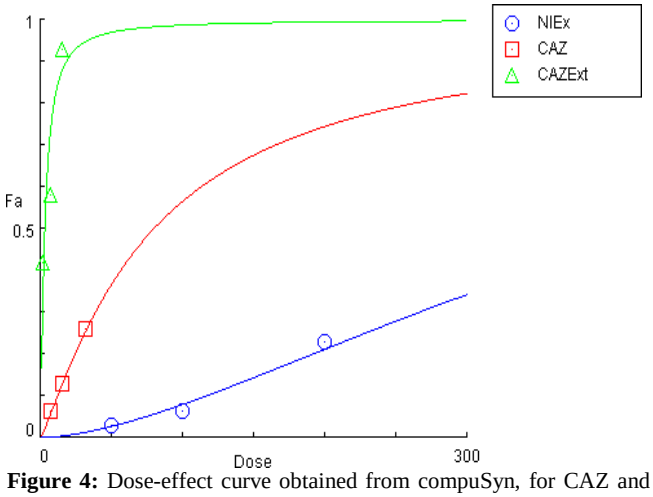

Figure 4: Dose-effect curve obtained from compuSyn, for CAZ and *N. laevis* combination against *P. aeruginosa*.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

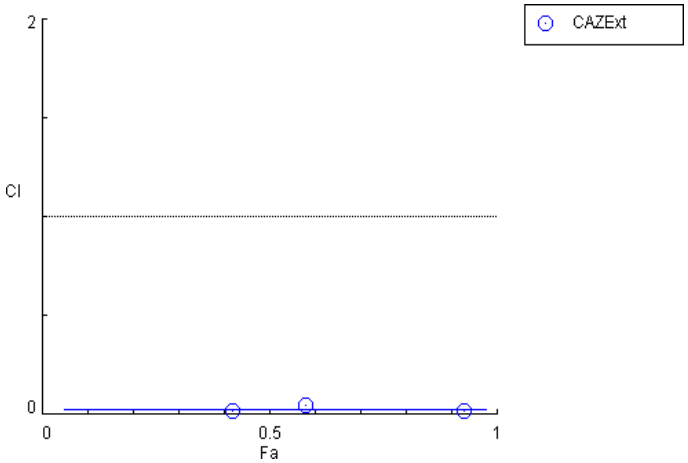

Figure 5: Combination index plot from compuSyn, for CAZ and *N. laevis* combination against *P. aeruginosa*.

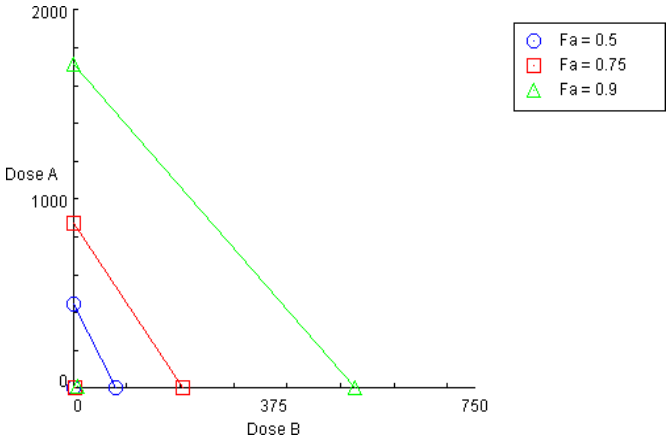

Figure 6: Isobologram obtained from compuSyn, for CAZ and *N. laevis* combination against *P. aeruginosa*.

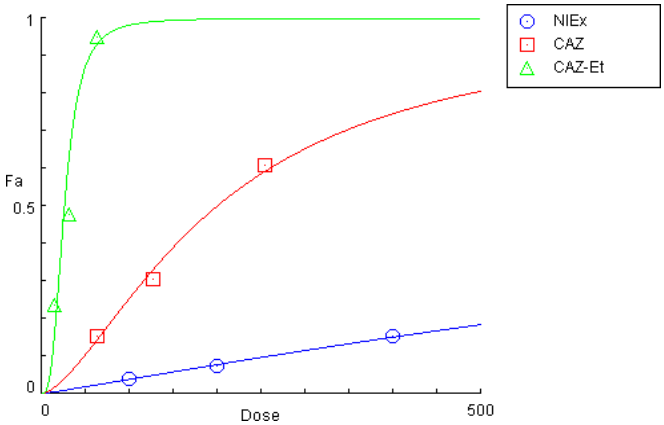

Figure 7: Dose-effect curve obtained from compuSyn, for CAZ and *N. laevis*combination against *K. pneumoniae*.

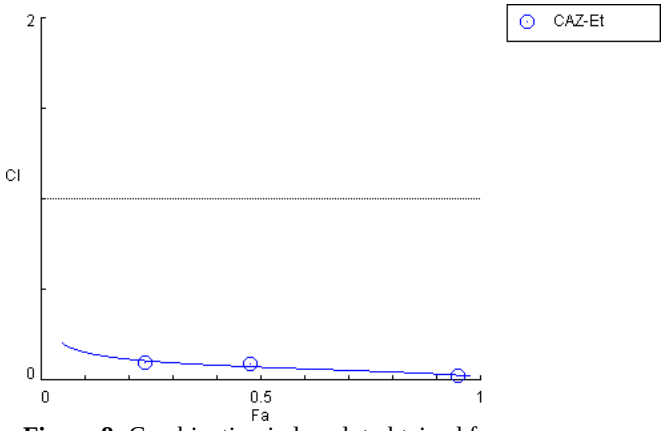

Figure 8: Combination index plot obtained from compusyn, for CAZ and *N. laevis* combination against *K. pneumoniae*

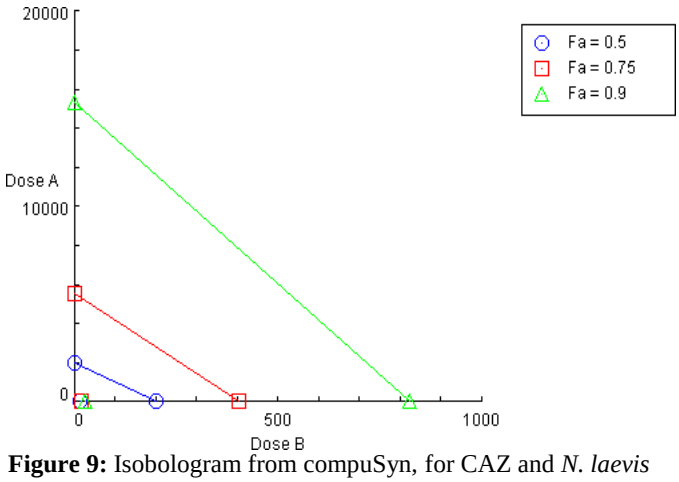

Figure 9: Isobologram from compuSyn, for CAZ and *N. laevis* combination against *K. pneumoniae*

Table 12: One-way ANOVA of the potentiation activities of plant extracton to the antibiotics

antibiotics	MIC (in mg/ml) of antibiotics-*Newbouldia laevis* extract ratio								
	S. aureus			*P. aeruginosa*			*K. pneumoniae*		
	70:30	50:50	80:20	70:30	50:50	80:20	70:30	50:50	80:20
CAZ	96 ± 18^{b}	85 ±11^{c}	117 ±15^{b}	19 ± 4^{d}	16 ± 4^{d}	19 ± 4^{d}	123 ± 25^{a}	89 ± 19^{c}	128 ±25^{a}
ERY	19 ± 3^{a}	10 ± 4^{b}	18 ± 3^{a}	NA	NA	NA	NA	NA	NA
CTR	180 ± 18^{a}	84 ± 9^{b}	180 ± 18^{a}	NA	NA	NA	NA	NA	NA

AMP	NA	NA	NA	144 ± 21^{c}	106 ± 18^{d}	181 ± 23^{b}	181 ± 34^{b}	141±22^{c}	207 ±34^{a}
CRX	NA	NA	NA	155 ± 19^{a}	132 ± 20^{b}	155 ± 19^{a}	122 ± 32^{a}	96 ± 20^{c}	134 ±23^{b}

Results are presented as mean ± SE. Values in bold represent most significant MICs. Means with the same letters are not significantly different from each other by Tukey test at $p < 0.05$.
CAZ: Ceftazidime, ERY: Erythromycin, CTR: Ceftriaxone, AMP: Ampicillin, CRX: Cefuroxime, NA: Not applicable

Conclusion

The results have shown that *Newbouldia laevis* leaf extracts can be used in the treatment of wound infections, urinary tract infections and other bacterial infections caused by ESKAPE pathogens (*P. aeruginosa, K. pneumonie*, and *S. aureus*). The ability of the extract to inhibit the growth of investigated pathogens further validates the folkloric uses of this plant against infectious diseases. *N. laevis*, therefore may be source of antimicrobial agent for the development of drugs against infections caused by *P. aeruginosa, K. pneumonie* and *S. aureus*. Its ability to lower the MICs of these antibiotics puts it forward as a good candidate for combination therapy in antibiotic treatment especially with those with failed efficacy. Being a natural product, this potentiation effect further raises hope of drastic reduction in side effects associated with high dose of antibiotics. More research into herbal medicine is therefore a veritable step in combating the persistent antibiotic resistance and its public health and economic implications.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that this work is original and that any liability for claims relating to the content of this article will be borne by them."

Acknowledgement

The authors acknowledge the Department of Microbiology, University of Nigeria, Nsukka, for provision of some materials needed for the study. We also acknowledge Dr Felix Nwafor of Department of Plant Science and Biotechnology, UNN for helping in the plant identification.

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