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Evaluation of the antibacterial activity of *Rauwolfia vomitoria* leaf extracts against multidrug resistant *Escherichia coli* and *Klebsiella pneumoniae*

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Abstract
As resistance to conventional drugs continues to increase, the growing knowledge on the medicinal values of herbs and herbal products and their subsequent use have endeared researchers to intensified study of medicinal plants. The estimated composition of *Rauwolfia vomitoria* extracts and its traditional use in the treatment of many infections necessitates the evaluation of its actual composition and effectiveness towards the management of multidrug resistant infections. In this study, bacteria were isolated from urine samples collected from patients in University of Nigeria Medical Centre and Chinemerem Specialist Hospital in Nsukka metropolis. Methanol, chloroform and aqueous extractions of *Rauwolfia vomitoria* leaves were done. The methanol extract was further fractionated into n-hexane, ethyl acetate and aqueous partitions. Antibiotics sensitivity was done using agar disc diffusion method and Multiple Antibiotics Resistance (MAR) index calculated. Antimicrobial testing of the plant extracts was done using agar well diffusion method. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were also determined. The resistance induction potential of the plant extract was carried out. The MAR indices ranged from 0.25 to 0.5. Some of the resistant isolates were susceptible to the plant extracts though the MIC (25-50mg/ml) and MBC (200mg/ml) were at high concentrations. This study provides evidence that extracts of *R. vomitoria* have moderate antibacterial effect against MDR *E. coli* and *K. pneumoniae*.

Keywords: *rauwolfia vomitoria*, extracts, multidrug resistance, resistance induction

Introduction
Antibiotic resistance has risen from the stance of a simple medication mishap to an impending epidemiologic disaster. All over the world, reports of multi-drug resistance are becoming increasingly prevalent [1] and lactamase producing Enterobacteriaceae are almost untreatable with standard therapies. The commonly implicated microorganisms include *Escherichia coli* and *Klebsiella pneumoniae.* The patterns of resistance are magnified by transfer of genetic traits among species [2]. Studies suggest that resistant bacteria are persistent in nature due to the stability of resistant genes and transfer elements [3]. In addition, several researches have shown that over dependence on conventional drugs as the sole remedies for the management of infectious diseases have contributed to the malfunctioning of vital organs in the body [4-6]. As resistance to conventional drugs continues to increase with drug costs getting beyond the reach of most patients, in addition to side effects of drugs, there is urgent need for potent, reliable and cheap alternatives [7]. The growing knowledge on the medicinal values of herbs and herbal products and their subsequent use has encouraged researchers to study herbal pharmacology [5, 8, 9]. *Rauwolfia vomitoria* (family Apocynaceae) commonly known as serpent wood or swizzler stick has been used in traditional folk medicine to treat a variety of ailments including fever, general weakness, gastrointestinal diseases, liver diseases, psychosis, pain and cancers [10, 11, 4]. Some of the plant parts have been investigated and reported to contain alkaloids and saponin among others [12, 13]. The reported phytochemical composition of *Rauwolfia vomitoria* and its traditional use in the treatment of infections necessitate further evaluation of its effectiveness towards the management of multidrug-resistant infections and possible resistance induction potential. This study, therefore, evaluated the antimicrobial properties of *Rauwolfia vomitoria* against multidrug-resistant *Escherichia coli and Klebsiella pneumoniae* and the resistance induction potential of the plant extract.

Materials and Methods
Bacterial Isolation and Antibiotic Susceptibility Testing
The clinical isolates used were obtained from hospitalized patients at the University of Nigeria Medical Centre and Chinecherem Specialist Hospital, Nsukka, Nigeria. Fifty bacteria (*Escherichia coli* and *Klebsiella pneumoniae*) isolates were obtained from urine samples. The isolates were identified by colony morphology, growth characteristics on chromogenic agar and biochemical tests.
Susceptibility of the test organisms to conventional antibiotics was determined by the Kirby Bauer disk diffusion method [14] using 8 different antibiotics [Cefuroxime (30 µg), Ceftazidime (30 µg), Gentamicin (10 µg), Ciprofloxacin (5 µg), Ofloxacin (5 µg), Augmentin (30 µg), Nitrofurantoin (300 µg) and Ampicillin (10 µg)]. The zones of inhibition diameter in mm were measured and interpreted according to the NCCLS guidelines [14]. Isolates were considered multidrug resistant (MDR) when they showed resistance to ≥ 2 antibiotics [15]. For further study,

10 resistant *E. coli* and 13 resistant *K. pneumoniae* isolates were used.

Plant formulations preparation

The leaves of *Rauwolfia vomitoria* were collected from the botanical garden of the University of Nigeria, Nsukka and taxonomically identified at the plant herbarium (registration number = UNH 262b). Properly washed plant leaves were dried, ground to powder and subjected to Soxhlet extraction. Seventy grams each of ground plant material was used for methanol, chloroform and aqueous extractions. The resultant extracts were concentrated under vacuum with the aid of a rotary evaporator. Extracts were stored in air-tight containers and refrigerated at 4 ºC until needed for analysis [16]. Eight grams of methanolic extract was further partitioned into n-hexane, ethyl acetate, butanol and aqueous fractions with the aid of a separating funnel [17].

Percentage Yield of Extract

The method as modified by Oluwagbenga *et al.* [18], was used. After rinsing and drying in the oven, the McCartney bottles used were weighed. After evaporating the solvent, the extracts were put into the McCartney bottles and the weights of the bottles again taken. The percentage yields of extracts were calculated as follows

Percentage yield= $\frac{W_1 - W_2}{X_g} \times \frac{100}{1}$ Where X_g = initial weight of dried plant sample, W_1 = weight of McCartney bottles and pasty extracts, W_2 = weight of empty McCartney bottles

Determination of Total Phenolic Content

The total phenolic content in aqueous, chloroform and methanolic extracts was determined using Folin-Ciocaletu reagent colorimetric method [19]. A reaction mixture which contained 2.5 ml of 0.2 M freshly prepared FC reagent, 500 µl of 0.1% aqueous dilution of the extracts and 2 ml of sodium carbonate solution was kept for 30 min under ambient condition in the dark for the completion of reaction. At 760 nm in a UV-vis spectrophotometer, absorbance of the resulting solution was measured. Using a standard curve of gallic acid, the total phenolic content was expressed as mg of gallic acid equivalent per gram of extracts.

Total Flavonoid Content

Total flavonoids content from methanol, chloroform and aqueous extract was determined by aluminium chloride colorimetric assay method as described by Lodhi *et al* [20]. Aliquots of 3.4 ml of 30% methanol, 0.15 ml of 0.5 M $NaNO_2$ and 0.15 ml of 0.3 M $AlCl_3.6H_2O$ were added to a test tube containing 0.3 ml of plant extract. The resulting mixture was mixed completely by shaking. After 5 min, 1 ml of 1 M NaOH was added in the mixing well and the absorbance was measured at 510 nm. The total flavonoid content was expressed as mg of quercetin equivalents per 100 g of dried extract after the standard curve of quercetin was made.

Antimicrobial Activity Testing of the plant extracts

The antimicrobial activity of the plant extracts was assessed against test isolates using agar well diffusion method as described by Balouiri *et al* [21]. The Petri dish preparation was achieved by pouring 20 ml of pre-sterilized molten Mueller Hinton agar (MHA) seeded with 200µl of standardized test culture. Using a sterile cork borer, wells of 6 mm were bored aseptically at equidistant positions on the solidified agar. Forty microlitres each of the plant extracts at different concentrations was separately introduced into the wells except the well at the center (control). At 37 ºC, all the agar plates were incubated for 24 h. The plates were examined subsequently and results recorded by measuring the zones of inhibition around the well. The tests were carried out in triplicate and the calculated mean values recorded.

Minimum Inhibitory Concentration (MIC)

The MIC of the methanol, ethyl acetate, n-hexane and aqueous partitions of *R. vomitoria* was determined using the broth dilution method [22, 23]. In test tubes, different concentrations of extracts and standard drug were prepared by dilution. An aliquot of standardized bacteria inoculum from overnight culture of multidrug resistant isolates (*E. coli* and *K. pneumoniae*) was added to each test tube and control tube containing bacterial inoculum and nutrient broth was prepared for each bacterial species. The test tubes were incubated at 37 ºC for 24 h. Afterwards all the tubes were compared with the control to determine inhibition of bacteria growth. The MIC for each plant extract were determined and recorded. All tests were performed in triplicates and the mean MIC calculated.

Minimum Bactericidal Concentration (MBC)

Extract concentration that showed no visible bacterial growth was seeded into freshly prepared nutrient agar plates and incubated at 37 ºC for 24 h. The MBC was regarded as the lowest concentration that did not yield any bacterial growth [24].

Induction of Resistance

Sub-inhibitory concentration of each extract was introduced into broth cultures of drug sensitive bacteria isolates and incubated for 48 h with aliquots taken at intervals of 18, 24 and 48 h. Each aliquot was purified and subjected to susceptibility testing [25].

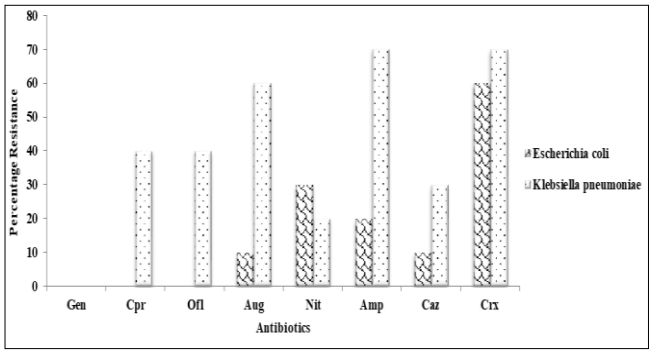
Results and Discussions

Antibiotic resistance pattern and Multiple Antibiotics Resistance (MAR) Index for the isolates

Varying degrees of resistance were observed with the various groups of antibiotics used as shown in Figure 1. Among the isolates, the highest resistance was observed against Cefuroxime: *E. coli* (60%) and *K. pneumoniae* (70%) followed by Ampicillin (30% for *E. coli* and 70% for *K. pneumoniae*) then Augmentin (20% for *E. coli* and 60% for *K. pneumoniae*). The MAR index (Table 1) of *Escherichia coli* ranged from 0.125 to 0.5 while that for *Klebsiella pneumoniae* ranged from 0.125 to 0.75. The MAR index value when above 0.2 is an indication that the organism is multi-drug resistant and most of the isolates have MAR index greater than 0.2 for both species.

From this study, the percentage resistance displayed by the organisms especially *K. pneumoniae* to the various antibiotics assayed is high. This is really worrisome. Some of the *K. pneumoniae* isolates for instance, were resistant to all the tested antibiotics except gentamicin. This is a cause for concern because most clinicians fall back on the quinolones for the treatment of multidrug resistant Gram-negative pathogens [26]. The use of fluoroquinolones has increased in many countries and emergence of resistance

among bacterial isolates to fluoroquinolones is alarming. Consistent step up of resistance among *E. coli* and *K. pneumoniae* strains against ciprofloxacin has been observed from 1995 (0.7%) to 2001 (2.5%) [27]. This increasing resistance to multiple antibiotics has been attributed tos inappropriate use (overuse, under use, misuse and non-compliance with the treatment duration) of antimicrobial drugs which leads to selective pressure. Lewis and Shan [28] reported that the abuse and misuse of antimicrobial agents for growth promotion and disease prevention has impressed a selective pressure that induces more resistance among bacteria.



Key: CRX=Cefuroxime, CAZ=Ceftazidime, GEN=Gentamicin, CPR=Ciprofloxacin, OFL=Ofloxacin, AUG=Augmentin, NIT=Nitrofurantoin and AMP=Ampicillin.

Fig 1: Percentage resistance of bacterial isolates against test antibiotics

Table 1: Multiple Antibiotics Resistance (MAR) Index for *Escherichia coli* and *Klebsiella pneumoniae*

Test organism	Number of antibiotics resisted	Number of resistant isolates	Total antibiotics used	MAR index
Escherichia coli	1	2	8	0.125
	2	9	8	0.25
	4	1	8	0.5
Klebsiella pneumoniae	1	4	8	0.125
	4	8	8	0.5
	5	4	8	0.625
	6	1	8	0.75

Percentage Yield of Extracts

The yield of extract obtained from 70g of the leaves of *R. vomitoria* was deduced as previously mentioned (section 2.3). The percentage yield of the methanol extract was the highest (table 2a) and this suggests that methanol as an organic solvent dissolves more organic compounds hence liberating the active compounds in the plant [29, 30]. A report by Francois *et al* [31]. Posited that the lower yield of aqueous extract suggests lower proportion of water-soluble plant components. After solvent partitioning of the methanol extract, the yield of the n-hexane and ethyl acetate fractions depicted that non-polar compounds where present in the plant which agrees with the study done by Ekwealor *et al.* [32] and Adeogun *et al* [33].

Table 2: Crude extract Yield

Solvent	Ground leaf used (g)	Yield of extract (g)	Yield of extract (%)
Methanol	70	18.50	26.43
Chloroform	70	5.73	8.19
Aqueous	70	3.56	5.09

Table 3: Fraction Yield of Solvent Partition

Solvent	Yield of fraction (g)	Yield of fraction (%)
n-Hexane	1.15	14.38
Ethyl acetate	1.73	21.63
Aqueous	2.56	32.00

Total phenolic and flavonoid content

Phytochemicals are compounds derived from plants. They are responsible for the medicinal benefits associated with the consumption of the plant-based diets and concoctions [34]. The quantitative phytochemical analysis of the test plant material showed high percentage composition of 9.20% for flavonoid and 10.88% for phenol. Phenolic compounds like flavonoid, tannin and phenol have been studied in a different clime and shown to have physiological functions like antimicrobial, anti-carcinogenic and anti-inflammatory activity [35, 36, 23]. The presence of these phytochemicals in the extracts of the plant is indicative that they will play significant role in the prevention of degenerative diseases and this may explain the wide-spread folklore use of the plant.

Antimicrobial activity of *Rauwolfia vomitoria* leaf extracts

Table 4 shows the antimicrobial activity of *Rauwolfia vomitoria*. Different concentrations of the leaf extracts showed significant differences ($p < 0.05$) in their efficacy against the tested isolates. All the leaf extract showed inhibitory effect at 25mg/ml concentration on the test microorganisms, except for the aqueous partition that did not show activity against all the test isolates. The result of the agar well diffusion revealed that the ethyl acetate partition showed the highest zone of inhibition at all levels of concentration. The mean zone of inhibition increased as concentration of the extracts increased and reduced with reduction in concentration. The activities of the aqueous extract against the bacteria were quite ineffective at all analysed concentrations. Again, this agrees with Alo *et al* [37]. and Adeogun *et al* [33]. (who reported that aqueous extract of *Ocimum gratissimum* and *Thaumatococcus daniellii* respectively did not inhibit the growth of test organisms) but differed from a recent study by Taiwo *et al* [38], who reported that crude aqueous and ethanolic extracts of *Mangifera indica* leaf extracts have good activity against Gram positive and negative bacteria. In contrast, Ojo *et al* [39], reported that both the aqueous, ethanol and methanol extracts of *R. vomitoria* show marked antimicrobial activities against *Klebsiella pneumoniae, Staphylococcus aureus, Enterobacter, Pseudomonas aeruginosa*, and *E. coli*. The absence of activity in the aqueous extract of *R. vomitoria* leaves may be due to reduced solubility of the active principles in water or the presence of active

Component in insufficient quantities in the extract to show activity with the dose level employed [31, 33].

Table 4: Inhibition Zone Diameter of *Rauwolfia vomitoria* leaf extracts on MDR test organisms

Concentration	Organism	Methanol (mm)	Ethyl acetate (mm)	n-Hexane (mm)	Aqueous (mm)
100mg/ml	*E. coli*	16.33 ± 1.5	20.17 ± 2.65	19.5 ± 2.7	00.0 ± 0
	K. pneumoniae	13.85 ± 1.98	16.38 ± 1.6	15.77 ± 1.38	00.0 ± 0
50mg/ml	*E. coli*	15.5 ± 1.99	16.83 ± 1.44	14.01 ± 1.89	00.0 ± 0
	K. pneumoniae	11.21 ± 0.42	12.11 ± 0.44	11.4 ± 0.17	00.0 ± 0
25mg/ml	*E. coli*	9.6 ± 0.89	11.6 ± 1.22	8.1 ± 0	00.0 ± 0
	K. pneumoniae	7 ± 0	9.5 ± 0.08	7.5 ± 0	00.0 ± 0
12.5mg/ml	*E. coli*	00.0 ± 0	00.0 ± 0	00.0 ± 0	00.0 ± 0
	K. pneumoniae	00.0 ± 0	00.0 ± 0	00.0 ± 0	00.0 ± 0
6.25mg/ml	*E. coli*	00.0 ± 0	00.0 ± 0	00.0 ± 0	00.0 ± 0
	K. pneumoniae	00.0 ± 0	00.0 ± 0	00.0 ± 0	00.0 ± 0

Data are presented as mean ±SD as measurement of inhibition zone (mm)

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extracts of *Rauwolfia vomitoria*

Table 5 shows the result of the Minimum Inhibitory Concentration (MIC) of the extracts against *Escherichia coli* and *Klebsiella pneumoniae*. At the concentration of 25mg/ml, the extracts (methanol, ethyl acetate and n-hexane) inhibited all the test organisms, with an MIC of 25mg/ml against the test organisms.

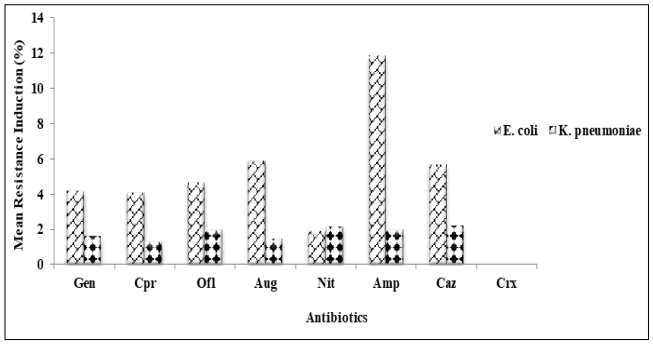
The Minimum Bactericidal Concentration (MBC) of the extracts against *Escherichia coli* and *Klebsiella pneumoniae* was at 200mg/ml concentration.

Table 5: The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Rauwolfia vomitoria* leaf Partitions

Organisms	Mean MIC and MBC values of different Partitions (mg/ml)							
	Methanol		Ethyl acetate		n-Hexane		Aqueous	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Escherichia coli	25.00	200.00	25.00	200.00	50.00	200.00	0.00	0.00
Klebsiella pneumoniae	50.00	200.00	50.00	200.00	50.00	200.00	0.00	0.00

Percentage Resistance Induction

Drug resistance induction assays revealed that three (35%) of the *E. coli* isolates used for this study developed resistance to ciprofloxacin, ofloxacin, Augmentin, ampicillin and ceftazidime after exposure to 12.5 mg/ml concentration of *R. vomitoria* for 48 h. Also 11% of the *E. coli* isolates developed resistance to nitrofurantoin and ampicillin, 8% developed resistance to gentamicin, while 52% remained susceptible after the exposure. When the methanolic extract of *Rauwolfia vomitoria* was applied on *K. pneumoniae*, 18% of the test isolates developed resistance to gentamicin, ciprofloxacin and ofloxacin. Nine percent developed resistance to nitrofurantoin, while the other bacterial clones recovered from the control nutrient broth cultures showed no variations from their initial antibiogram. The drug resistance induction occurred in varying degrees for the organisms against the different antibiotics assayed. *E. coli* demonstrated approximately 4% mean resistance induction to gentamicin and ciprofloxacin, 2% to nitrofurantoin, 5% to ofloxacin, 6% to Augmentin and ceftazidime, while the highest mean induction resistance of 11.84% was recorded for ampicillin. *K. pneumoniae* exhibited 1.61, 1.27, 1.95, 1.43, 2.10, 1.97 and 2.14% mean induced resistance to gentamicin, ciprofloxacin, ofloxacin, Augmentin, nitrofurantoin, ampicillin and ceftazidime respectively after exposure as shown in Figure 2.



Key: CRX=Cefuroxime, CAZ=Ceftazidime, GEN=Gentamicin, CPR=Ciprofloxacin, OFL=Ofloxacin, AUG=Augmentin, NIT=Nitrofurantoin and AMP=Ampicillin.

Fig 2: Percentage Resistance Induction

Conclusion

The need for novel and effective antimicrobials increases daily. However, it is imperative that the mechanism of action of discovered active components is fully studied and understood. This will help stakeholders to focus effort on medication of the principal active ingredient or on discovered better methods of delivery. The ethyl acetate partition of the methanolic extract of *Rauwolfia vomitoria* leaves showed the best antimicrobial activity promise. However, the antimicrobial moiety should be isolated and also the various components be investigated for their ability to induce antibiotics resistance in previously susceptible strains. Further research is therefore recommended to determine the active ingredient and the precise mechanism of action.

Conflict of interest

The author declares no competing interest in this work.

References

1. Gandhi NR, Nunn P, Dheda K, Schaaf HS, Zignol M, Van Soolingen D *et al*. Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis. The Lancet. 2010; 375(9728):1830-1843.
2. Pourmand A, Mazer-Amirshahi M, Jasani G, May L. Emerging trends in antibiotic resistance: Implications for emergency medicine. The American journal of emergency medicine. 2017; 35(8):1172-1176.

3. Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J *et al*. Call of the wild: antibiotic resistance genes in natural environments. Nature Reviews Microbiology. 2010; 8(4):251.
4. Ekong MB, Ekpene UU, Thompson FE, Peter AI, Udoh NB, Ekandem GJ et *al*. Effects of co-treatment of Rauwolfia vomitoria and Gongronema latifolium on neurobehaviour and the neurohistology of the cerebral cortex in mice. Internet Journal of Medical Update. 2015; 10(1):3-10.
5. Anyaegbunam ZKG, Amadi OC, Mba IE, Anyaegbunam TC, Onu W, Cosmas S *et al*. Allium cepa: an alternative medicine. International Journal of Botany Studies. 2020; 5(6):717-725.
6. Nillert N, Pannangrong W, Welbat JU, Chaijaroonkhanarak W, Sripanidkulchai K, Sripanidkulchai B *et al*. Neuroprotective effects of aged garlic extract on cognitive dysfunction and neuroinflammation induced by β-amyloid in rats. Nutrients. 2017; 9(24).
7. Osemene KP, Elujoba AA, Ilori MO. A Comparative Assessment of Herbal and Orthodox Medicines in Nigeria. Research Journal of Medical Science. 2011; 5(5):280-285.
8. Ip FC, Zhao YM, Chan KW, Cheng EY, Tong EP, Chandrashekar O *et al*. Neuroprotective effect of a novel Chinese herbal decoction on cultured neurons and cerebral ischemic rats. BMC Complement Alternative Medicine. 2016; 16(1):437.
9. Anyaegbunam KZ, Amaechi LO, Anyaegbunam Tito C, Wisdom OO, Henrietta CO, Cosmas S *et al*. Antibacterial Activity of Fresh Red and White Onion (Allium cepa) Extract against Some Drug Resistant Bacteria. Journal of Advances in Microbiology, 2019, 1-8.
10. Eluwa MA, Udofa MT, Vulley MBG, Ekanem TB, Akpantah AO, Asuquo OA *et al*. Comparative study of teratogenic potentials of crude ethanolic root bark and leaf extract of Rauwolfia vomitoria (Apocynaceae) on the fetal heart. North American Journal of Medical Sciences. 2010; 2(12):592-595.
11. Yu J, Ma Y, Drisko J, Chen Q. Antitumor activities of Rauwolfia vomitoria extract and potentiation of carboplatin effects against ovarian cancer. Current Therapeutic Research. 2013; 75:8-14.
12. Goel MK, Mehrotra S, Kukreja AK, Shanker K, Khanuja SP. *In vitro* propagation of Rauwolfia serpentina using liquid medium, assessment of genetic fidelity of micropropagated plants and simultaneous quantification of reserpine, ajmaline and ajmalacine. New York: Humana Press, 2009.
13. Mensah JK, Okoli RI, Turay AA, Ogie-Odia EA. Phytochemical analysis of medicinal plants used for the management of hypertension by Esan people of Edo State, Nigeria. Ethnobotanical Leaflets. 2009; 13:1273-1287.
14. Clinical and Laboratory Standard Institute (formerly NCCLS). Methods for dilution in antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A5 and informational supplement M100-S10. National Committee for Clinical Laboratory Standards, Wayne, Pa, 2000.
15. Gayathri G, Kathrireslian A, Kaliya P, Balagurunathan R. Prevalence of ESBLs in uropathogenic Escherichia coli in suburban tertiary care hospital and its antibiogram pattern. Research Journal of Microbiology. 2011; 6:796-804.
16. Alara OR, Abdurahman NH, Mudalip SA, Olalere OA. Effect of drying methods on the free radicals scavenging activity of Vernonia amygdalina growing in Malaysia. Journal of King Saud University-Science, 2017, 1-5.
17. Raynie DE. Modern extraction techniques. Analytical Chemistry. 2006; 78(12):3997-4004.
18. Oluwagbenga A, Adedotun A, Anofi A. Chemical composition, lethality and antifungal activities of the extracts of leaf of Thaumatococcus danielli against foodborne fungi. Beni-Seuf University Journal of Basic and Applied Sciences. 2016; 5:356-368.
19. Bozin B, Dukic N, Samojlik I, Igic R. Phenolics as antioxidants in garlic (Allium sativum L., Alliaceae). Food Chemistry. 2008; 4:1-5.
20. Lodhi S, Rai G, Shukla A, Mourya P. Hypoglycemic and hypolipidemic effect of ethanolic and aqueous extracts from Ziziphus oenoplia (L) Mill on alloxan-induced diabetic rats. Beni-Seuf University Journal of Basic and Applied Sciences. 2017; 6:1-9.
21. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. Journal of Pharmaceutical Analysis. 2016; 6:71-79.
22. Clinical and Laboratory Standard Institute (CLSI). Performance standards for antimicrobial disc susceptibility tests; approved standard- eleventh edition. CLSI document M02-A11, 2012.
23. Acheampong A, Okyem S, Akoto CO, Baah KA. Antioxidant, antimicrobial and FTIR analysis of methanol root extract of Cnestis ferruginea and ethanol root extract of Citrus limon. Journal of Pharmacognosy and Phytochemistry. 2018; 7(4):2938-2946.
24. Oro D, Heissler A, Rosci E, Scapin D, Malherios P, Boff E *et al*. Antifungal activity of natural compounds against Candida species isolated from HIV-positive patients. Asian Pacific Journal of Tropical Biomedicine. 2015; 5(9):781-784.
25. Eze EA, Ezeonu IM, Okafor JI. The flip side of Herbal Remedies. Journal of Rural and Tropical Public Health. 2010; 9:67-76.
26. Khaneja M, Naprawa J, Kumar A, Piecuch S. Successful treatment of late-onset infection due to resistant Klebsiella pneumoniae in an extremely low birth weight infant using ciprofloxacin. Journal of Perinatology. 1999; 19(4):311.
27. Christopher AF, Hora S, Ali Z. Investigation of plasmid profile, antibiotic susceptibility pattern multiple antibiotic resistance index calculation of Escherichia coli isolates obtained from different human clinical specimens at tertiary care hospital in Bareilly-India. Annals of Tropical medicine and public health. 2013; 6(3):285.
28. Lewis K, Shan Y. Why tolerance invites resistance. Science. 2017; 355(6327):796-796.
29. Anukworji CA, Putheti RR, Okigbo RN. Isolation of fungi causing rot of cocoyam (Colocasia esculenta (L.) Schott) and control with plant extracts: (Allium sativum, L., Garcinia kola, heckel., Azadirachta indica, L. and Carica papaya, L.). Global Advanced Research Journal of Agricultural Science. 2012; 1:33-47.

30. Onifade D. The experience of black/minority ethnic police officers, support staff, special constablesand resigners in Scotland. Scottish Executive: Central Research Unit, 2002.
31. Francois J, Manchin M, Norberg H, Pindyuk O, Tomberger P. Reducing transatlantic barriers to trade and investment: An economic assessment (No. 1503). Working Paper, Department of Economics, Johannes Kepler University of Linz, 2015.
32. Ekwealor CC, Oyeka CA, Okoli I. Antifungal activities of some Nigerian medicinal plants against non-dermatophyte molds isolated from cases of onychomycosis among rice farmers in Anambra State, Nigeria. British Microbiology Research Journal. 2012; 2(2):62.
33. Adeogun O, Adekunle A, Ashafa A. Chemical composition, lethality and antifungal activities of the extracts of leaf of Thaumatococcus daniellii against foodborne fungi. Beni-Suef University Journal of Basic and Applied Sciences. 2016; 5(4):356-368.
34. Lukmanul F, Girija A, Boopathy R. Characteristic of essential oil from Ocimum basilicum. Journal of Medicine and Plants Research. 2008; 2(9):250-257.
35. Ayoola GA, Coker HA, Adesegun SA, Adepoju-Bello AA, Obaweya K, Ezennia EC *et al.* Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in Southwestern Nigeria. Tropical Journal of Pharmaceutical Research. 2008; 7(3):1019-1024.
36. Adebiyi OE, Olayemi FO, Ning-Hua T, Guang-Zhi Z. In vitro antioxidant activity, total phenolic and flavonoid contents of ethanol extract of stem and leaf of Grewia carpinifolia. Beni-Suef University Journal of Basic and Applied Sciences. 2017; 6(1):10-14.
37. Alo MN, Anyim C, Igwe JC, Elom M, Uchenna DS. Antibacterial activity of water, ethanol and methanol extracts of Ocimum gratissimum, Vernonia amygdalina and Aframomum melegueta. Advances in Applied Science Research. 2012; 3(2):844-848.
38. Taiwo OS, Olasehinde GI, Sholotan KJ, Openibo JO, Bello OA, Ajayi JB *et al*. Phytochemical and Antimicrobial Properties of Mangifera indica Leaf Extracts. Covenant Journal of Physical and Life Sciences. 2018; 6(1):55-63.
39. Ojo OO, Ajayi SS, Owolabi LO. Phytochemical screening, anti-nutrient composition, proximate analyses and the antimicrobial activities of the aqueous and organic extracts of bark of Rauvolfia vomitoria and leaves of Peperomia pellucida. International Research Journal of Biochemistry and Bioinformatics. 2012; 2(6):127-134.