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Antimicrobial activity of some medicinal plants against Avian Pathogenic *Escherichia coli (E.coli*).

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ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) is a pathosubgroup of ExPEC that afflicts birds. The APEC isolates have been found to be highly resistant to routine antibiotics, a situation which has been linked to the indiscriminate use of antibiotics on the farms as growth promoter. Thus, has been speculatively implicated in the emergence of antibiotic resistance strains in humans. This has necessitated a need to find an alternative which will produce desired results in farm with minimal or no side effects to humans and plant antimicrobial is a promising prospect. This study is thus, aimed at determining the susceptibility pattern of clinical isolates of Avian pathogenic *Escherichia coli* (APEC) to methanolic extracts of some medicinal plants. Twenty (20) isolates were subjected to antimicrobial susceptibility testing using well agar diffusion method with methanolic extracts of five (5) medicinal plants *(Thevetia nerifolia, Zingiber officinale, Alchornea cordifolia, Asystecia giganticum, Dialium guineense)* and Gentamicin as control. *Thevetia nerifolia* and *Zingiber officinale* extracts showed no antibacterial activity while *Asystecia giganticum, Alchornea cordifolia, Dialium guineense* possessed antibacterial activity ranked in the order *Asystecia giganticum > Alchornea cordifolia > Dialium guineense*. The highest activity was observed in the 500mg/ml dilution. The study reveals that *Alchornea cordifolia, Asystecia giganticum* and *Dialium guineense* possess antibacterial activity against APEC that can be harvested for use on poultry farms.
Keywords: Antimicrobial, activity, medicinal, plants, Avian, pathogenic, *Eschericha coli.*

INTRODUCTION

The effectiveness of currently available antibiotics is decreasing due to the increasing number of resistant strains causing infections [1]. Avian pathogenic *E. coli* (APEC), a patho-sub group of ExPEC that afflicts birds have been reported to possess very high resistance to multiple antibiotics classes, a situation which has been linked to the indiscriminate use of antibiotics on poultry farms both for clinical purposes and as well as growth promoters [2]; [3]. Excessive exposure of commensals like *E. coli* to antibiotics increases the breed of resistant bacteria and, if the resistance is plasmid-mediated as often is the case, resistance might be transferred to a more virulent acquired bacteria, then treatment for infection becomes increasingly complicated by the emergence of these resistant bacteria especially to most first-line antimicrobial agents [4] [5]. The implication of such is a high rise in the cost of treatment of what should have been minor infections and

the cumulative effect of a rise in mortalities. This has occasioned a search for antibiotics alternatives for use on poultry farms. This alternative will serve as a means for combating antibiotics resistance both on the farms and in humans by implication.

The use of medicinal plants to treat ailments has been in practice for as long as man and employed all over the world [6]. Plants are known to contain phytochemical such as tannins, terpenoids, alkaloids, flavonoids etc which are responsible for their therapeutic activities [7]. These phytochemicals are often constitutive in nature or are as a response to stimuli in the environment and often act in synergy to give the plant its therapeutic benefits.

The advent of drug resistance by bacteria has revived interest in research in medicinal plants as possible Antimicrobial agents and studies have demonstrated some of these plants as possessing antibacterial activities. Medicinal plants such as *Thevetia nerifolia (*exile tree), *Alchornea nerifolia (Christmas bush), Dialium guineense (*velvet/black tamarind), *Asystecia giganticum (*creeping foxglove) and *Zingiber officinale* (ginger) have been reported to have antibacterial activity especially against *E. coli* isolates [8]; [9]; [10]; [11]; [12]; [13]. This gives a basis to determine if the highly antibiotics resistant APEC would be susceptible to the methanolic extracts of these medicinal plants.

MATERIALS AND METHODS

Collection and Preparation of Leaf Samples

Leaves of *Thevetia nerifolia, Asystecia giganticum, Dialium guineense* and *Alchornea cordifolia* were collected from the campus of Michael Okpara University of Agriculture, Umudike (MOUAU) and its environ while the Rhizome of *Zingiber officinale* (Ginger) was purchased from Ahiaeke Market Umudike, Umuahia Abia State. The samples were taken to the Department of Forestry in MOUAU for identification. The collected leaves were cleaned while the rhizome of *Zingiber officinale* was cleaned, diced, placed in a flat basket and shade dried for three weeks. They were milled and passed through a 1 mm sieve to obtain a fine powder which was stored in a clean dry airtight glass bottle at ambient temperature until analysed.

Extraction and Phytochemical Analysis of the Leaves

The dried powder (200 g) of each milled sample was extracted with 400 ml of methanol in a stoppered container and allowed to stand at room temperature for a period of 72 h with frequent agitation. The resultant mixture was decanted and filtered through a muslin cloth and the filtrates were evaporated to dryness in a hot air oven (Lamington Scientific and Engg Products) at 40 °C. They were then stored at 4 °C in a refrigerator until ready for use.

Preliminary phytochemical analysis of the methanol extract of the leaves was carried out according to the methods outlined by [14] [15].

Determination of Susceptibility Pattern of Isolates to Routine Antibiotics

Antimicrobial susceptibility pattern of isolates to routine antibiotics were done on Mueller-Hinton agar (Oxoid, Hampshire, England) using Kirby Bauer disk diffusion method (Bauer and Kirby, 1996) The antimicrobial agents tested were: tarivid (10 mcg), reflacine (10 mcg), Augmentine (30 mcg), streptomycin (30 mcg), gentamicin (10 mcg), ciprofloxacin (10 mcg), ceporex (10 mcg), nalidixic acid (30mcg), septrin (30 mcg), and ampicilline (30 mcg) (Oxoid, England). Resistance data were interpreted according to Clinical laboratory Standards (CLS, 2018). Reference strains of *E. coli* ATCC 11175 was used for quality control for antimicrobial susceptibility tests.

Reconstitution of extracts

The extracts were reconstituted by redissolving in 10% DMSO at 5 g to 10 ml to get a concentration of 500 mg/ml.

Which were further diluted by carrying out a 2 fold dilution using 10% DMSO to obtain 250, 125, 62.5, 31.25 mg/ml respectively.

Determination of *In-vitro* Antimicrobial Activity

The antibacterial activity was conducted using the Agar diffusion method as described by [16]. From the extract stock, two fold dilutions were made from 500 to 32.25 using sterile distilled water. Sterilized Muller Hinton agar was poured into sterile Petri dishes and allowed to solidify. A sterile swab stick was dipped into the standardized innocula and spread on the solidified Muller Hinton agar aseptically and labelled. The inoculated plate was allowed to stay for 30 min to enable the organisms stick properly to the surface of the agar. Seven wells were bored aseptically with the use of a sterile cork borer of 6 mm diameter. The wells were then filled with 0.1 ml of the serially diluted solution of each plant extract. Gentamicin (10 mcg/ml) was placed inside the sixth hole and used as a positive control while the seventh hole contained sterile water and was used as negative control. The plates were in duplicates and incubated at 37 °C for 24 h. After which zones of inhibition observed, was measured and recorded in millimeter using a transparent meter rule.

Determination of Minimum Inhibitory Concentration (MIC) of the Extracts

This was done by broth dilution assay as reported by [17]. 1 ml of the reconstituted extract solution at a concentration of 500 mg/ml was added to another test tube containing 1 ml of sterile broth so as to obtain a concentration of 250 mg/ml. 1 ml of this dilution was transferred to another test tube till the 7th test tube is reached. The 8th test tube did not contain any extract, but a solution of pure methanol which served as negative control. Then 1 ml of an overnight culture of the bacterial suspension (ATCC 11175) earlier adjusted at 10^8 cfu/1ml was put into each tube and thoroughly votexed. The test tubes were incubated at 37°C for 24 h and observed for turbidity. The lowest concentration at which no detectable bacterial growth is observed was recorded as the MIC.

Statistical Analysis

The data obtained were analyzed using SPSS version 16.0, and group means was the unit for statistical analysis. Data was expressed as mean ± standard deviation. Comparison between the zones of inhibition diameters was done by analysis of variance (ANOVA) and differences were considered significant at 95% confident interval ($p<0.05$).

RESULTS AND DISCUSSION

Phytochemical Screening.

The phytochemical analysis of the various plants yielded many of plants secondary metabolites such as alkaloids, flavonoids, terpene, saponins, tannins etc. with *T. nerifolia* having the least of these metabolites when compared with the other plants. But has a greater amount of glycoside than the other plants in the study, just as reported by [18], who reported the presence of glucoside and hypothsied it as being responsible for the plant being known more for its toxic .This high glycoside and less of other Phytochemicals may be responsible for its undetectable antibacterial activity against the isolates or perhaps the isolates possess resistant genes to it. Tannins precipitate bacteria proteins, destroying their configuration while terpenoids display their action through membrane disruption mechanisms [19]. Phenols and phenolic compounds have been extensively used in disinfections and remain the standard with which other bactericides are compared [20]. Phenolic compounds act as electron donors and are readily oxidized to phenolate ion or quinine, an electron acceptor [21].. The presence of these secondary metabolites are believed to be responsible for the

antibacterial activities of the plants and studies have reported that antimicrobial properties of plants depended on the amount and type of secondary metabolites present in the extracts [22]. Often these metabolites work in synergy to bring about a more pronounced antibacterial activity [23]. This suggests that the huge array of Phytochemicals in *A. giganticum* may be responsible for its highantibacterialactivity.

Percentage susceptibility of isolates to individual antibiotics

Very high multiple resistances to routine antibiotics was observed in the isolates, most being susceptible to only one or two of the antibiotics, majority of the isolates showed no zone of inhibition around it or insignificant inhibition amounting to resistance as interpreted using CLS guidelines. On percentage level, ofloxacin had the highest percentage susceptibility with 65% of the isolate being totally susceptible and 35% only intermediately susceptible and only 5% of the isolates not susceptible. While none of the isolates showed any susceptibility to ampicillin (i.e. 100% non-susceptibility). Cephalexinhad the least of susceptibility of only 5%. Resistance to the individual antibiotics were as follows: Nalidixic acid (80%), Pefloxacin (85%), Gentamicin (65%), Augmentin (80%), Ciprofloxacin (65%), Streptomycin (80%), Septrin (70%), Ampicillin (100%), Cephalexin (95%) and Ofloxacin (35%). This is in accordance with the works of [23]; [24]; [25]; [26] [27] who also recorded resistance to the cephalosporins, the aminoglycosides and the fluoroquinolones, especially the first generation antibiotics. But the pattern and grade of such resistance appears to be related to the particular antibiotics and frequency with which the antibiotics are being employed on the farms thus were the fluoroquinolones are frequently used, the isolates develop resistance to that group progressively starting with the drug in that group used. This is what is observed in streptomycin (80%) and gentamicin (65%). The result clearly shows Ofloxacin as the drug with lowest resistance and better for use on these isolates but resistance to it is also gradually building up. The APEC isolates when subjected to antimicrobial susceptibility test to different dilutions of the methanolic leaves extract of the selected plants, inhibition were only observed around *A. cordifolia, A. giganticum,* and *Dialium guineense* while *T. nerifolia and Zingiber officinale* did not inhibit any of the isolates. The inhibition was observed around the first two dilutions (500, and 250 mg/ml) while a few of the isolates were also inhibited by the third dilution (125 mg/ml) but none of the isolates were inhibited by the fourth and fifth dilutions (62.5 and 31.25 mg/ml). At 500 mg/ml, *A. cordifolia* gave inhibition zones range of (8 – 21 mm), *A. giganticum*, (10 – 28 mm), *D. guineense* (7 – 16 mm), at 250 mg/ml, *A. cordifolia* (7 – 15 mm), *A. giganticum* (7 – 18 mm), *D. guineense* (7 – 12 mm) while at 125 mg/ml, *A. cordifolia had* (7 – 9 mm), *A. giganticum*, (7 – 12 mm) and *D. guineense* (7 -8 mm).

Comparing the plants activities, at 500 mg/ml, *A. giganticum* showed a significantly higher activity greater than that of *A. cordifolia* and *D. guineense* , while *A. cordifolia* also had a significant higher activity than *D. guineense.* At 250mg/ml, *A. giganticum* also had a significantly higher activity than the others while at that dilution, there was no significant difference in the activities of *A. cordifolia* and *D. guineense.* At 125 mg/ml, the activities recorded of the three plants were negligible and showed no significant difference amongst the three. Thus, in the order of activity *A. giganticum* ranked first followed by *A. cordifolia* , then *D. guineense.* In all, *A. giganticum* proved to be the plant with the highest antibacterial activity against the APEC isolates probably because of the interplay of abundance of its store of Phytochemicals.

Determination of the minimum inhibitory concentration (MIC) of the different selected plants to standard APEC strain

Interestingly the minimum inhibitory concentration (MIC) determination of the three plants using standard APEC strain (ATCC 11175) yielded the same value 250mg/ml which is high in comparison to those reported of non APEC strains. For *D. guineense,* [28] reported the MIC of 2.5mg/ml ethanolic root extract against non APEC, [29] recorded 200mg/ml for ethanolic/aqueous extract of *the* fruit pulp, [30] obtained a value of 0.63 mg/ml ethanolic extract of stem bark against *E. coli* (ATCC 25922), [31] reported MIC values for the crude leaf and stem back aqueous and ethanol extract to *S. aureus* and *K. pneumoniae* also at high concentration of 200mg/ml. For *A. cordifolia*, [3] reported MIC value of 50mg/ml aqueous and ethylacetate leaf extracts against *E.coli,* [9] obtained an MIC value of 3.125mg/ml of ethanolic stem *extract*. This variation could have arisen from the variety of strains of microbial isolates used, extraction methods as well as varying phytochemical components of plant parts.

CONCLUSION

The study has shown a high resistant of the clinical APEC isolates to routine antibiotics which is believed to be a function of the use of antibiotics often at sub – therapeutical doses as growth promoters. This has made our commercial farms big reservoirs of antibiotics resistance and places us in great danger because asides the consumption of the poultry products, the faecal droppings are often employed as cheap manure for plants by the locals and some of farm operators for agricultural purposes. Also, other animals raised on these farms are sometimes fed with the carcasses of dead birds from the farms placing us at a more dangerous position of possible antibiotics resistance transfer from these animals. Thus, there is need for caution in the use of antibiotics in our commercial poultry farms.

This work also revealed that the methanolic leaves extracts of *Asystecia giganticum, Alchornea cordifolia* and *Dialium guineense* contain potential antibacterial agents that can be explored as remedy for APEC infections. The antibacterial activity of the extracts could be ranked as *Asystecia giganticum > Alchornea cordifolia > Dialium guineense*. Based on results from this work, it appears that the methanolic extracts of *Zingiber officinale* and *Thevetia nerifolia* possess no antibacterial activity against APEC and may not be employed in the control of it.

The methanolic extracts contained phytochemicals such as alkaloids, flavonoids, terpene, saponins, tannins, phenol, glycoside and steroid.

The study provides valuable information for further detailed studies of the potency

of *Asystecia giganticum, Alchornea cordifolia* and *Dialium guineense* as viable replacements for antibiotics our farms.

RECOMMENDATIONS

Based on results from this work it is recommended that an information system be set up to educate the operators of farms and populace on eminent danger of indiscriminate use of antibiotics on farms. The Government should set up rules and regulations on the use of antibiotics on commercial farms; and adherence should be enforced through monitoring team. Further studies should also be conducted on the serotypes and resistance genes associated with antibiotics resistance on poultry farms to get a clearer picture. It is also necessary to investigate the toxicities of these plant extracts and *in vivo* antibacterial kinetics with a view to establishing their safety.

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Table 1: Classes of compounds found in the methanolic extracts of selected plants

PLANT SPECIE	ALK	FLA	TER	TAN	STE	PHE	GLY	SAP
T. nerifolia	-	+	-	-	+	-	+	+
A. cordifolia	++	+	++	+	-	-	-	++
D. guineense	+++	+	-	++	+	+	-	++
A. gigantic	+	-	++	-	+	+	++	+++
Z. officinale	++	+	+	++	++	-	-	+

+ - trace; ++ - intermediate; +++ - abundant; - - absent; Alk – alkaloids; Fla – flavonoids; Ter – terpene; Sap – saponins; Tan – tannins; Phe – phenol; Gly - glycoside; Ste - steroid.

Table 2: Percentage susceptibility of APEC isolates to individual antibodies

Antibiotics	*NCLSI resistance cut off range (mm)*	*Sensitive (S) %*	*Intermediate (I)%*	*Resistant (R)%*
Nalidixic acid	≤ 13	20	0	80
Pefloxacin	≤ 23	15	0	85
Gentamycin	≤ 12	25	10	65
Augmentin	≤13	10	10	80
Ciprofloxacin	≤ 15	10	25	65
Septromycin	≤11	15	5	80
Septrin	≤10	10	20	70
Ampicillin	≤ 13	0	0	100
Cephalexin	≤ 19	5	0	95
Ofloxacin	≤12	60	5	35

Table 3: Antimicrobial susceptibility of isolates to the different dilutions of the plants

Plants	**Zone of inhibition at different dilutions (mm)**			**Control Gentamicin (0.001mg/ml)**
	500mg/ml	**250mg/ml**	**125mg/ml**	
A. cordifolia	14.67±3.69	9.19 ±3.37	3.10 ± 4.10	7.52 ± 9.35
A. giganticum	17.76 ± 4.08	11.43 ± 4.09	3.86 ± 5.22	7.52 ±9.35
D. guineense	11.43 ± 2.25	8.24 ±2.36	2.76 ± 3.62	7.52 ±9.35
Z. officinale	00.00	00.00	00.00	7.52 ±9.35
T. nerifolia	00.00	00.00	00.00	7.52 ±9.35

Table 4: Comparison of antimicrobial activities between plants

Plant	Zone of inhibition at different dilutions (mm)			*P - value*
	500mg/ml	250mg/ml	125mg/ml	
A. cordifolia	14.67±3.69[b]	9.19 ±3.37[b]	3.10 ± 4.10[a]	0.05
A. giganticum	17.76 ± 4.08[a]	11.43 ± 4.09[a]	3.86 ± 5.22[a]	0.05
D. guineense	11.43 ± 2.25[c]	8.24 ± 2.36[b]	2.76 ± 3.62[a]	0.05
Z. officinale	00.00	00.00	00.00	00.00
T. nerifolia	00.00	00.00	00.00	00.00

Groups mean ± standard deviation, Mean values in a column with different superscripts are significantly different ($p<0.05$), n = 21

Table 5: Minimum inhibitory concentration of the selected plants to standard APEC strain (ATCC 11175)

Plant	MIC (mg/ml)
Alchornea cordifolia	250
Asystecia giganticum	250
Dialium guineense	250
Z. officinale	00
T. nerifolia	00