Synergistic Effect of some Selected Antibiotics with *Terminalia catappa* Leaves Extract against the Multi-Drug Resistant ETEC Isolated from Vegetables Sold within Kano metropolis, Kano State, Nigeria

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Abstract: This study was carried out in order to isolate and identify MDRs ETEC from vegetable samples, and test for the synergistic effect of umbrella leaves extract with some selected antibiotics on the resistant strains. Fifty (50) vegetables samples were collected which includes Cabbage, Lettuce, Spinach, Cucumber and Carrots, (10 samples each). Samples were chopped into smaller pieces using a sterile stainless steel knife prior to weighing and were then vigorously shaken in sterile peptone water in order to dislodged the bacteria cells from the vegetables samples; the suspension was spread-plated on both Eosin Methylene Blue (EMB) and MacConkey agars. The plates were incubated. Colonies exhibited metallic sheen on EMB agar and pink color on MacConkey agar were sub cultured to obtain pure culture, which were then subjected to Gram staining technique and biochemical tests prior to antibiotic sensitivity testing using the Kirby-Bauer disc diffusion technique. The isolates were then inoculated in skimmed milk media and was sent for Molecular detection of *ETEC* toxins. The results obtained showed that out of the 50 samples collected from different markets within Kano metropolis, *Escherichia coli* was isolated from 23 isolates representing 46% of the samples. Out of the 23 isolates of *E. coli*, (11) samples were found to be MDRs out of which seven (7) ETEC toxigenic genes were detected. With which two isolates responded to the synergistic activity of resisted antibiotics with the plant extract. The plant extract was screened for the presence of active secondary metabolites where: Alkaloid, Flavanoid, Steroid, Tannin were present and Saponins was absent.
Keywords: *Escherichia coli*, ETEC toxins, MDRs, EMB, MacConkey, Muller-Hinton.

INTRODUCTION

The persistent outbreaks of cholera-like diarrhoea and gastroenteritis in some northern states of Nigeria that annually claims several lives and hospitalizes many, coupled with the inadequate diagnosis of the actual causative agent is alarming (Shu'aibu *et al.*, 2016). Moreover, it has been widely asserted that vegetables consumed have been implicated and associated with the incidence of such diarrhoea without justification. This problem if left unchecked for a certain period of time could lead to loss of lives and economy of the nation (Steinsland *et al.*, 2012).

ETEC is known to have been responsible for the cholera-like diarrhoea. The rationale behind conducting this research lies on the fact to prove if such diarrhoea thought to have been contracted via consumption of vegetables is not cholera diarrhoea but the cholera-like diarrhoea caused by *ETEC*. This may be supported by a surveillance data supporting the consumption of vegetables as the source of the outbreaks (Steinsland *et al.*, 2012). And to determine the synergistic activity of some selected antibiotics with ethanolic leaves extract of umbrella (*Terminalia catappa)*.

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Emergence of resistance to multiple antimicrobial agents in pathogenic bacteria has become a significant public health threat as there are fewer, or even sometimes no, antimicrobial agents available for infections caused by these bacteria (Lazarus, 2009). The use of traditional medicine to cure infection has been practiced since the origin of mankind, and in the past it was the only method of available. Currently, due to inadequate health care system particularly in the rural areas, people prefer to visit traditional healers and herbal medicine (Sofowora, 2005). The development of drug resistance by microorganisms reduces the effectiveness of modern drugs. Thus, resistance to antibacterial agents poses threat in many areas of the world especially in the developing countries. The integration of traditional and modern medicine are gaining increase recognition globally (Adwan and Mhanna, 2008).

MATERIALS AND METHODS

Study area

Kano is located in North western Nigeria, with the coordinates $11^0$30'N $8^0$30'E, and a population of 9383682 (2006 census). Kano state has been a commercial and agricultural producing state, farming many varieties of vegetables. It has a total area of 18684km^2, and 44 local governments.

Sample collection

Lettuce, cabbage, cucumber and Carrots are among the major cultivated crops and most consumed vegetables in Kano. A total of fifty (50) samples (10) samples each were collected over a period of three months (January to March). The samples were collected randomly from Kano markets, the samples were collected into different sterile containers and transported to the laboratory within 2 hours and were analysed on the same day (Shu'aibu *et al.*, 2016).

Culture Media and Antibiotics

All the media used were prepared according to manufacturer's instruction, and the antibiotics used are CAZ = Ceflaxidine, AMC =Augumentine, CRO = Ceftriazone, TZP = Piperacillin Tazobactam, TE = Tetracycline, LEV = Levofloxicin, CN = Gentamycin and AMS = Amoxicillin.

Sample preparation

The vegetables samples were chopped into smaller pieces using a sterile stainless steel knife prior to weighing. The weighed vegetable samples were then vigorously shaken in sterile peptone water in order to dislodge the bacteria cells from the vegetables samples. And the suspension was spread-plated on a suitable media for incubation at 37ºC for 24 to 48 hours.

Isolation and Identification of *E. coli*

Each sample that was dislodged was then plated on both Eosin Methylene Blue (EMB) and Mac Conkey agars. The plates were incubated at 37ºC for 24 hours. Colonies which exhibited greenish metallic sheen on EMB agar and pink color on MacConkey agar were sub cultured to obtain pure culture. Pure cultures were then subjected to Gram staining technique and biochemical tests; Oxidation production, Indole test, Methyl Red, Citrate Utilization, Voges Proskauer, Triple Sugar Iron, Urease test, and Sugar fermentation. (Cheesebrough, 2006).

Innoculum Standardization /Sensitivity Testing

Few colonies of the bacterial isolates were emulsified in normal saline of about 2-3mls in test-tubes to match the 0.5 Mcfarland standard for sensitivity test as described by (Cheesebrough, 2006). The Kirby-Bauer disc diffusion technique was used (CLSI, 2009). Pure colonies of isolates were obtained from agar slants and were sub-cultured on already prepared culture plates. Isolates from each of the plates were subjected to 0.5 McFarland standards in 0.9% saline and was streaked uniformly on Muller-Hinton agar plates. Multi antibiotic sensitivity discs were placed on the surface media, using a pair of forceps. The plates were then inverted and incubated aerobically at 37^0C for 18-24 hours. Zone of inhibition was then measured (Dawoud *et al.,* 2013).

Detection of ETEC using Multiplex Polymerase Chain Reaction (mPCR)

Enterotoxins were detected from the isolates by PCR using the following procedures as follows;

DNA Extraction

The respective DNAs of the isolates were extracted using boiling method (Mohammed, 2012; Schimitt and Pawlita, 2009). There isolates were subcultured for on Luria Bertani (LB) broth and incubated overnight. One ml of the bacterial culture was subjected to centrifugation in an Effendorf centrifuge (Effendorf AG, 22331 Hamburg, Germany) at 13,400 x g for 1 minute. The supernatants were discarded and pellets re-suspended in 200 µl of Tris-EDTA (TE) buffer and the mixture was boiled at 100°C for 10 min. It was later cooled at -20°C for 10 min in a freezer. After cooling, it was centrifuged at 13,400 x g for 2 min. The supernatant containing the extracted template DNA was kept in a new sterile Effendorf tube at -20°C for further use.

Amplification of the Target LT and ST Genes and Visualization of the PCR products

The primers for the target genes of ETEC heat-labile and heat-stable enterotoxins were those previously published by (Gurama *et al* 2018). And same method was adopted.

Preparation of *T. catappa* leaves extract

The leaves of *T. catappa* were collected and washed thoroughly with tap-water and air-dried for one week under shade. The leaves were crushed to fine powder using clean laboratory motor and pestle and kept for further analysis. Extraction using Soxhlet Extractor, Disc preparation, phytochemicals screening, Inoculum Standardization, determination of minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) was done by method used by (Gurama *et al* 2018).

Determination of Synergism between the leaves Extract and the used Antibiotic

The combined activity of the plant extract and the used antibiotics was determined using well diffusion method described by Adwan *et al.* 2013).

The combined activity of the plant extract and used antibiotic was determined using well diffusion method where wells of 6mm in diameter were punched on the agar surface using Cork borer and filled with 30µl of plants extract and 30µl of antibiotics used then covered with remaining agar inverted and kept an incubated for 24hrs. The following morning combined activity zones of inhibition were measured using meter rule as described by NCCLS (2006).

RESULTS

The results of this work shows the physical properties of *T. catappa* leaves extract, the percent recovery of the extracts was 15.5%, the color and texture of the plant extract were dark greenish brown and soft. The secondary metabolites detected were Alkaloids, Flavanoids, Steroids, tannins while Saponins were absent. The classification of bacterial isolates based on gram staining protocol, 32 samples were gram negative while 18 samples were gram positive. The biochemical test results for *Escherichia coli.* Base on the Biochemical tests conducted which includes Indole, Mythyl Red, Voges-Proskauer, Citrate Utilization, Triple Sugar Iron, and Catalase. *Escherichia coli* was isolated in 23 samples representing 46% of the total samples collected, while others were 27 samples representing 54%.

Table 1: **%Frequency Occurrence of *E. coli* and *ETEC* genes from vegetable samples**

Samples Used	Samples Collected	%Occurrence of *E. coli*	Occurrence of *ETEC*
Cabbage	10	4 (40)	1 (LT)
Lettuce	10	7 (70)	2 (LT, LT)
Spinach	10	4 (40)	3 (LT, ST/LT, LT)
Cucumber	10	5 (50)	1 (LT)
Carrot	10	3 (30)	0
TOTAL	**50**	**23 (46)**	**7 (14)**

Key: LT = Heat labile and ST = Heat Stable

Table 1 shows the isolation of *E. coli* and *ETEC* toxigenic genes from vegetable samples sold within Kano metropolis, highest occurrence of *Escherichia coli* was observed in lettuce which is 70% and lowest occurrence of *Escherichia coli* was observed in carrot which is 30% of it. Out of the 50 samples of vegetables, *ETEC* toxigenic genes were detected in seven isolates with which six isolates possess LT toxigenic genes and 1 isolate possess both LT and ST amounting 14% of the total samples.

Table 2 and Table 3 shows Resistance pattern of the isolates against some selected antibiotics against the *Escherichia coli* isolates. 100% resistivity was observed in AMS, 47.8% in both TZP and TE, 4.3% in AMC, and zero or no resistivity at all against CAZ, LEV, CN, and CRO. Thus 11 samples were found to be multi-drug resistance organisms.

Table 2 Resistance pattern of the isolates against some selected antibiotics against the *Escherichia coli* isolates.

No.	CAZ (mm)	AMC (mm)	CRO (mm)	TZP (mm)	TE (mm)	LEV (mm)	CN (mm)	AMS (mm)
1	S (24)	S (13)	S (30)	R (0)	R (0)	S (12)	S (25)	R (0)
3	S (25)	S (15)	S (25)	R (0)	R (0)	S (15)	S (8)	R (0)
6	S (30)	S (10)	S (10)	R (0)	R (0)	S (14)	S (15)	R (0)
8	S (20)	S (12)	S (10)	S (20)	S (10)	S (10)	S (20)	R (0)
12	S (10)	S (12)	S (25)	R (0)	R (0)	S (10)	S (10)	R (0)
13	S (21)	S (12)	S (22)	R (0)	R (0)	S (20)	S (15)	R (0)
15	S (20)	S (11)	S (25)	S (20)	S (10)	S (10)	S (17)	R (0)
17	S (22)	S (12)	S (30)	S (24)	S (10)	S (10)	S (16)	R (0)
18	S (24)	S (14)	S (10)	S (16)	S (25)	S (25)	S (20)	R (0)
19	S (22)	S (14)	S (25)	S (20)	S (10)	S (12)	S (10)	R (0)
20	S (23)	S (12)	S (20)	S (20)	S (10)	S (20)	S (20)	R (0)
23	S (26)	S (12)	S (24)	R (0)	R (0)	S (20)	S (12)	R (0)
24	S (22)	R (0)	S (25)	R (0)	R (0)	S (10)	S (20)	R (0)
25	S (24)	S (12)	S (30)	S (18)	S (18)	S (11)	S (18)	R (0)
30	S (25)	S (12)	S (22)	R (0)	R (0)	S (16)	S (18)	R (0)
35	S (22)	S (12)	S (25)	R (0)	R (0)	S (20)	S (20)	R (0)
36	S (25)	S (11)	S (30)	S (18)	S (18)	S (20)	S (16)	R (0)
37	S (20)	S (14)	S (29)	S (20)	S (18)	S (24)	S (18)	R (0)
38	S (26)	S (14)	S (26)	S (18)	S (20)	S (16)	S (16)	R (0)
40	S (23)	S (12)	S (20)	S (19)	S (8)	S (14)	S (14)	R (0)
44	S (25)	S (11)	S (30)	S (18)	S (20)	S (12)	S (12)	R (0)
49	S (25)	S (11)	S (30)	R (0)	R (0)	S (16)	S 16)	R (0)
50	S (25)	S (12)	S (27)	R (0)	R (0)	S (19)	S (19)	R (0)
Total%	0	4.3	0	47.8	47.8	0	0	100

KEY: CAZ = Ceflaxidine, AMC =Augumentine, CRO = Ceftriazone, TZP = Piperacillin Tazobactam, TE = Tetracycline, LEV = Levofloxicin, CN = Gentamycin, AMS = Amoxicillin, R = Resistant and S = Sensitive.

Table 3 % Resistance of the used antibiotics

S/N	Antibiotic	% Resistance
1	CAZ	0
2	AMC	4.3
3	CRO	0
4	TZP	47.8
5	TE	47.8
6	LEV	0
7	CN	0
8	AMS	100

KEY: CAZ = Ceflaxidine, AMC =Augumentine, CRO = Ceftriazone, TZP = Piperacillin Tazobactam, TE = Tetracycline, LEV = Levofloxicin, CN = Gentamycin, AMS = Amoxicillin, R = Resistant and S = Sensitive.

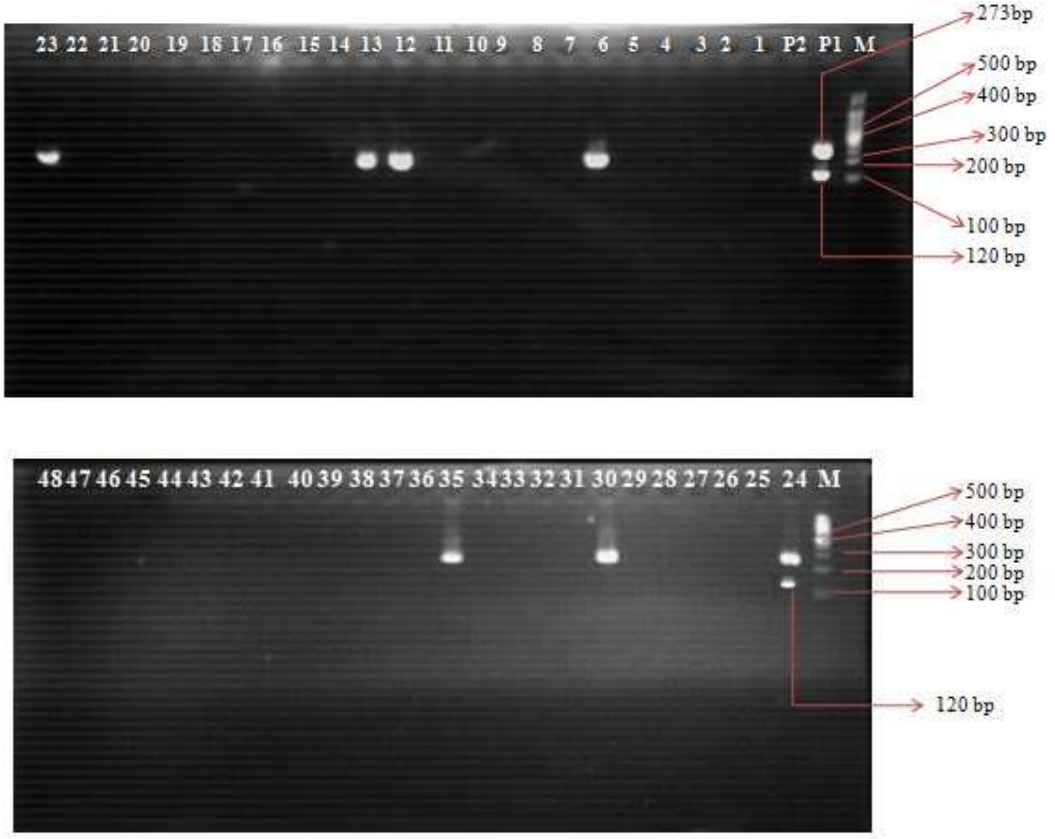

Plate 1: *ETEC* Toxigenic genes detected from the vegetables Isolates
Amplified mPCR products of the heat-labile (LT) and heat-stable (ST) genes of *Enterotoxigenic E. coli* Lane M; Molecular weight marker (Genedirex, Biohelix), P1; Positive control (*E. coli* ATCC 35401), P2; Negative Control (*E.* coli ATCC 25922). Isolates Nos. 06, 12, 13, 23, 30 and 35 were positive for only LT while only isolate 24 possessed both LT and ST.

Table 4 Antibacterial activity of the plant extract with varying concentrations against the MDRs isolates

N	25% Conc. V/v(mm)	50% Conc. V/v(mm)	75% Conc. V/v(mm)	100% Conc. V/v(mm)
1	R (0)	R (0)	R (0)	R (0)
3	R (0)	R (0)	R (0)	R (0)
6	R (0)	R (0)	R (0)	S (11)
12	R (0)	R (0)	R (0)	S (9)
13	R (0)	R (0)	R (0)	R (0)
23	R (0)	R (0)	R (0)	R (0)
24	R (0)	R (0)	R (0)	R (0)
30	R (0)	R (0)	R (0)	R (0)
35	R (0)	R (0)	R (0)	R (0)
49	R (0)	R (0)	R (0)	R (0)
50	R (0)	R (0)	R (0)	R (0)

Key: R = Resistant and S = Sensitive

Table 4 shows the antibacterial activity of *T. catappa* leaves extract against MDRs organisms. The isolates were almost all resistant to the extract, slightly activity was observed in two isolates which are (9 and 11 mm) zones of inhibition which was only observed in 100% concentration Vv.

Table 5: Synergistic activity of the plant extract and resisted antibiotics against MDRs isolates.

N	Activity (mm)
1	R (0)
3	R (0)
6	S (14)
12	S (11)
13	R (0)
23	R (0)
24	R (0)
30	R (0)
35	R (0)
49	R (0)
50	R (0)

Key: R = Resistant and S = Sensitive

Table 5 shows the synergistic activity of the plant extract and the resisted antibiotic against the MDRs isolates. The combined activity was only observed in two isolates which are (14 and 11 mm) zones of inhibition.

DISCUSSION

Enterotoxigenic Escherichia coli (ETEC) is the most common cause for travelers' disease and a major cause of diarrhoea for infants and new born animals in developing countries. It produces heat-labile and heat-stable enterotoxins and some other toxins that lead to diarrhoea. These enterotoxins are small peptides that are different in their structure and mechanism. Understanding the different structures and mechanisms underlying the process is important to know how *ETEC* interacts with the hosts and develops diarrhoea.

The phytochemicals result of this study is in accordance with the work done by (Mansur *et al*., 2015)., which screened ethanolic leaves extract of Umbrella for the presence of active secondary metabolites and confirmed that Alkaloid, Flavonoids, Steroid, Tannins were present and Saponins were absent, and the antimicrobial activity of the extract responded against *E. coli, and Salmonella*. Whereas is in contrast with the work done by Lawal *et al*. (2014), which detected saponins from the Umbrella leaves extract.

The investigation using mPCR to detect the presence of toxigenic genes in vegetables isolates demonstrated that *Escherichia coli* was isolated from 23 samples representing 46% of the samples, while the others 27 (54%) samples were negative to *Escherichia coli*. Out of the 23 isolates of *Escherichia coli*, 11 isolates were found to be MDRs out of which 7 *ETEC* toxigenic genes were detected (Isolates Nos. 06, 12, 13, 23, 30 and 35 were positive for only LT while only isolate 24 possessed both LT and ST). From the 50 vegetables samples *E. coli* isolates with which two isolates responded to the synergistic activity of resisted antibiotics with the plant extract.

The results of this study is in line with the work carried out in Malaysia by (Novak *et al*., 2015) on the Detection of major diarrheagenic bacterial pathogens of *E. coli* by multiplex PCR panels. Where their results of the percentage occurrence of the *ETEC* toxins isolated is slightly the same with the results of this study.

So also the results of this study is in accordance with the work done by (Savarino, 2015) who worked on the Comparative analyses of phenotypic and genotypic methods for detection of enterotoxigenic *Escherichia coli* toxins and colonization factors from Raw vegetables and fruits. Who was also able to detect heat-labile, heat-stable toxins and both from the isolates, and also the results of this study is in contrast with the work done by (Mohammed, 2012). Who worked on the Molecular characterization of diarrheagenic *Escherichia coli* isolated from meat products sold at Mansoura city, Egypt who were able to detect larger percentage of occurrence of *ETEC* toxigenic genes from the isolates (which could be as a result of the outbreak of the disease in Egypt then).

Enterotoxigenic Escherichia coli (ETEC) is the most common cause for travelers' disease and a major cause of diarrhoea for infants and new born animals in developing countries. *ETEC* is known to have been responsible for the cholera-like diarrhoea.

The rationale behind conducting this research lies on the fact to prove if such diarrhoea thought to have been contracted via consumption of vegetables is not cholera diarrhoea but the cholera-like diarrhoea caused by *ETEC*. Thus one can say that the persistent outbreaks of cholera-like diarrhoea and gastroenteritis in some northern states of Nigeria such as Katsina, Kano, Niger, Bauchi, Adamawa, and Gombe as reported by Premium Times 12 December 2013, WHO 2017, and Solayide, Abosede (Molecular Biology, & Biotechnology division, Nigerian Institute of Medical Research Yaba, Lagos). That annually claims several lives and hospitalizes many, coupled with the inadequate diagnosis of the actual causative agent is alarming. Many healthcare settings treat the problem by simple fluid and electrolyte replacement which in some instances yielded no result thereby leading to unnecessary loss of lives. Could be attributed to *ETEC* infection not really the suspected cholera.

CONCLUSION

Medicinal plant (*Terminalia catappa)* **c**ontains secondary active metabolites, which includes Alkaloid, Flavonoids, Steroids, Tannins, were present but Saponins were absent. Which has antimicrobial activity. Vegetables harbours microbes especially *Escherichia coli,* which was isolated from 23 samples representing 46% of the samples, while the others 27 (54%) samples were negative to *Escherichia coli*. Out of the 23 samples of *Escherichia coli*, 11 samples were found to be MDRs out of which 7 *ETEC* toxigenic genes were detected (Isolates Nos. 06, 12, 13, 23, 30 and 35 were positive for only LT while only isolate 24 possessed both LT and ST). With which two isolates responded to the synergistic effect or activity of the resisted antibiotics with the plant extract.

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