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Antimicrobial Susceptibility Patterns of Methanolic leaf extract of *Azadirachta Indica* and some selected antibiotics and Plasmid Profiles of *Escherichia Coli* Isolates Obtained from Different Human Clinical Specimens in Lagos- Nigeria

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ABSTRACT

Escherichia coli have been associated with severe and sometimes fatal infections like pyelonephritis, septicemia, endocarditis, meningitis, urinary tract infections (UTIs), epidemic diarrhea of adults and children. The situation is worsening due to increased antibiotic resistance plasmid genes of the bacteria. The study correlates plasmids with drug resistance of clinical isolates of *E.coli*. A total of 52 *E.coli* clinical isolates from different human clinical specimens (comprising of urine, blood, faeces, hand and wound swab) were obtained from patients in hospitals in Lagos, Nigeria and their antimicrobial susceptibility pattern and plasmid profiles were tested against methanolic leaf extract of *Azadirachta indica* and some selected antibiotics. Thirteen isolates of *E.coli* were selected and cultured in nutrient broth in the presence and absence of 1% SDS solution. Very high resistance levels (≥75) were detected against ciprofloxacin, amoxicillin. ampicillin, tetracycline, Co-Amoxiclavand imipenem,while nitrofurantoin (96.2%), gentamicin (88.5%), ofloxacin (78.9%), chloramphenicol (71.2%) and *A.indica* (65.4%) were highly sensitive to the *E.coli* strains. Some of the isolates possessed single sized plasmid while others had multiple plasmids with different sizes which ranged from 2.1 kb to 21.7 kb. High antibiotic resistances were detected from isolates with high molecular weight plasmids.

KEYWORDS: Antimicrobial susceptibility, *Azadirachta indica, Escherichia coli* and plasmid profiles.

INTRODUCTION

*Escherichia coli*is a bacterial organism that belongs to the family Enterobacteriaeceae. *E.coli* has been associated with severe and sometimes fatal infections like pyelonephritis, septicemia, endocarditis, meningitis, urinary tract infections (UTIs), and epidemic diarrhea of adults and children (Fine gold, 1982 and Daini, *et al* 2005). New strains of *E.coli* evolve through natural biological process of mutation. The organism possesses the ability to transfer DNA via bacterial conjugation, transduction or transformation, which allow genetic materials to spread horizontally through an existing population (Brussow et al., 2004). The organism is therefore of clinical importance and can be isolated from various human clinical specimens. It is one of the organisms most frequently isolated from the blood (Karlowskey*et al* 2004). *Azadirachta indica,* commonly known as Neem, is found in Nigeria and in most of the tropical and subtropical countries and is widely distributed in the world. The taxonomic classification of Neem is as follows:
Kingdom :*Plantae*, Order: *Rutales*, Suborder: *Rutinae*, Family: *Meliaceae*, Subfamily : *Melioideae*, Genus : *Azadirachta*, Species: *indica*(Girish*et al* 2008).All parts of the plant are useful and have been used in treatment of diseases ranging from teeth decay, swollen liver, ulcers, dysentery, diarrhea, malaria etc. (Allameh et al., 2002 and Mossini et al., 2004). The plant has great medicinal uses and has been used for the treatment of bacterial infections. Hassan (1995) observed that antibiotic susceptibility of bacterial isolates is not constant but dynamic and varies with time and environment.

Drug resistance is a severe problem worldwide and is spreading rapidly due to overuse, self-medication and non-therapeutic use of antimicrobials (Slama*et al*, 2005). Plasmid copies play an important role in imparting various characteristics to the pathogen such as resistance towards different antibiotics. The uses of antibiotics cause antibiotic resistant plasmids in Nigeria, since the intake of these antimicrobial agents are not restricted. Drug resistance property in bacteria is borne in R-plasmids, which can be disseminated to diverse population and regions causing worldwide problems. R-plasmid mediated antibiotic resistance which can spread in a population subjected to heavy antibiotic therapy. (Daini, *et al*.1995).

STUDY OBJECTIVES

To investigate the effect of Methanolic leaf extract of *Azadirachta indica* and some selected antibiotics on *Escherichia coli* Isolates before and after curing using 1% SDS solution.

MATERIALS AND METHODS

Collection and identification of plant material

The leaves of *Azadirachta indica* were gotten from Ikorodu in Lagos State, Nigeria and authenticated by Mrs Shokefun, a botanist from the Department of Science Laboratory Technology (Environmental Biology Unit), Lagos State Polytechnic inIkorodu-Lagos-,Nigeria.

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-Preparation of methanolic leaf extract of *Azadirachta indica*

The leaves were air dried under shade in the laboratory. The dried leaves were pounded to coarse powder in a mortar and then to fine powder with a blender. Extraction was carried out by dispersing 200g of the grounded plant material in 1L of 80% Methanol and shaking was done with GFL shaker for 72 hours. This was followed with vacuum filtration and evaporation at a temperature not exceeding 40°C. The concentrate was heated over a water bath to obtain a solvent-free extract, which was stored in a refrigerator at 4°C.

Phytochemical analysis of methanolic leaf extract of *Azadirachta indica*

Phytochemical tests for bioactive constituents were carried out on portions of the residual material using standard phytochemical procedures (Harborne (1993), Trease and Evans (1995) and Sofowora (1993).

Sample collection and identification

A total of 5 clinical specimens comprising urine, hand and wound swabs, feaces, and blood of patients attending Nigeria Institute of Medical research (NIMR), were screened for *E.coli.* Samples were screened using standard isolation and identification procedure for detection of *E.coli.* (Karlowskey*et al* 2004 and Cheesbrough, 2000).

The media used during the study were Eosin methylene blue agar, blood agar, MacConkey Agar, XLD and DCA agar, nutrient agar and nutrient broth. They were subjected to gram staining test; isolates were confirmed to be gram negative. Isolate were further subjected to biochemical tests. Isolates gave a positive test to indole, lysine decarboxylase, *Beta*-glucuronidase, urine nitrite test and gave a negative test to citrate and H_2S. Plasmid profile was carried out at the Nigeria Institute for Medical Research (NIMR) Yaba, Lagos-Nigeria.

Inoculum preparation

A loopful of isolated colonies was inoculated into 4 ml of peptone water, incubated at 37°C for 4 hours. This actively growing bacterial suspension was then adjusted with peptone water so as to obtain a turbidity visually comparable to that of 0.5 McFarland standard prepared by mixing 0.5 ml of 1.75% (w/v) barium chloride dihydrate ($BaCl_2$. $2H_2O$) with 99.5 ml of 1% (v/v) sulphuric acid (H_2SO_4). This turbidity is equivalent to approximately 1–2 × 10^8 colony forming units per ml (CFU/ml).

Antibiotic Susceptibility Testing

Susceptibility of isolates to *A.indica* and different antibiotics were tested using stokes disc diffusion method (Edwing, 1986 and Stokes FJ) on freshly prepared Mueller. Hinton agar and standardized by the method of National Committee for Clinical Laboratory Standard using *A.indica* and some selected antibiotics namely: Amoxicillin (30ug), Chloramphenicol (30ug), Ciprofloxacin (5ug), Ofloxacin (30ug), Gentamicin (10ug), Ampicillin (25ug), Tetracycline(30ug), Streptomycin (25ug), Nitrofurantoin (300ug), Co-Amoxiclav(30ug), Imipenem(10ug), and Cotrimoxazole(30ug). The sensitivity tests were standardized using *E.coli*NCTC no. 10418 as control. The Inhibition zones sizes were interpreted using Clinical Laboratory Standard Institute (CLSI).

Antibacterial susceptibility test of *E.coli* in the presence of 1% SDS solution.

13 isolates of the *E.coli* were selected and cultured in nutrient broth in the presence of 1% SDS solution and in the absence of SDS solution. SDS solution is used as the curing agent. Antibiotic susceptibility test for these 13 isolates were carried out in the presence and absence of 1% SDS by using stokes disc diffusion method (stokes, 1987). using the methanolic leaf extract of *A. indica* and 12 antibiotic strips. Plasmid profile analyses of the above samples were done using gel electrophoresis. The samples were processed using gel electrophoresis to identify the number of plasmid copies present in different isolates. The DNA was electrophoresed on 0.8% Agarose gel stained with ethidium bromide and visualized by UV trans illumination. Standard DNA molecular weight markers were used to estimate the plasmid size.

RESULTS AND DISCUSSION

Table 1. Below shows that the phytochemical constituent of *Azadirachta indica* contain secondary metabolites like alkaloids, steroids, saponin, flavonoids etc. These secondary metabolites are responsible for the antimicrobial activity of *A. Indica.* Out of the 124 clinical specimens analyzed, *E.coli* was present in 52 samples. Table 2. shows the distribution of *E.coli* from various clinical specimens.

The highest source of *E.coli* in this clinical specimens was faeces (36.50%) followed by hand (32.7%) and urine (23.1%). The resistance ability of these 52 *E.coli* isolates were tested against *A. indica* and some different antibiotics (Table 3).

Out of the *52 E.coli* isolates, 23(44.2%) were found to possess plasmids, which ranged in sizes from 2.1kb to 21.7kb. Plasmids were not detected in 29 (55.8%) of the resistant *E.coli* strains indicating that their resistances were probably chromosomal borne.

From plasmid analysis, bands for plasmid DNA were absent when multi drug resistant isolates of *E.coli* were cultured in nutrient broth in the presence of 1% SDS. The presence of 1% SDS in the agar caused all the plasmids to come out of the *E.coli* cells leading to the formation of small pores. No bands for plasmids DNA were obtained in the electrophoretic pattern (Fig 2). When the same isolates were cultured in nutrient agar in the absence of 1% SDS, the electrophoretic pattern showed the presence of plasmid DNA (Figure 1).

Medicinal plants constitute an effective source of both traditional and modern medicines, herbal medicine have been shown to have genuine utility and about 80% of rural population depends on it as primary health care.
The phytochemical screening of methanolic leaf extract of *Azadirachta indica* indicated the presence of tannins, saponins, alkaloids, flavonoids, etc (Table 1).

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In preliminary findings, Neem inhibited *Streptococcus mutans*(bacterium causing tooth decay) and reversed incipient carious lesions (that is, primary dental caries. Vanka,2001). In HIV/AIDS patients, a 12-week oral administration of acetone water Neem leaf extract (IRAB) had a significant influence *in vivo* on CD4 cells (which HIV reduces) without any adverse effects in the patients (Mbah, 2007).

A study on *Azadirachta indica* has revealed a chemo preventive capability by regressing the hepatocarcinogenesis induced by Diethyl Nitrosamine (DEN) / 2 Acetylaminofluorene (AAF) carcinogens on Spraque-Dawly rats (Manal, 2009). Resistance of bacteria isolates to antibiotics is onthe increase worldwide, particularly in developing countries.-*E.coli* isolates were collected from different pathological specimens like urine, blood,-faeces, hand and wound swab.(Table 2).

Methanolic leave extract of *A. indica* and twelve antimicrobial agents (antibiotics) were used to test the susceptibility. Pathogenic isolates of *E.coli* have relatively high potentials for developing resistance (Karlowsky*et al*.2002). 34(65.4%) of the isolates of*E.coli* were sensitive to methanolic leaf extract of *A. indica* while 18(34.6%) of the isolates were resistant to *A.indica* (Table 3).

Forty-six (78.8%) of the isolates were resistant to tetracycline. Forty-two (80.8%) of the isolates were resistant to both Co-Amoxiclav and imipenem. Forty-one (78.8%) were resistant to Ciprofloxacin, 40(76.9%) to Amoxicillin and 37(71.2%) to Cotrimoxazole. Resistance to Tetracycline and Cotrimoxazole observed in this study were similar to what was reported by Densenclos*et al*. 1988. They reported 53% of their *E.coli* isolates were resistant to Cotrimoxazole and 67% to Tetracycline. The high resistance of *E.coli* to some of these antibiotics may be due to indiscriminate abuse of the drugs. *E.coli* isolates were highly sensitive to Nitrofurantoin (96.2%). Extreme sensitivity of *E.coli* isolates to Nitrofurantoin has earlier been reported by Bonten*et al* 1990. The *E.coli* isolates showed high sensitivity to Gentamicin(88.5%), Ofloxacin(78.9%) and Chloramphenicol (71.2%) (Table 3).

Thirteen isolates out of the 23*E.coli* isolates with different sizes of plasmids were cured with 1% sodium dodecyl sulphate (SDS). After curing, these isolates lost their plasmid DNAs as shown in Figure 2. Antimicrobial susceptibility tests were carried out using Stokes disc diffusion method on freshly prepared nutrient agar containing 1% SDS solution against *A.indica* and some selected antibiotics. Isolates that were resistant to Amoxicillin, Imipenem, Gentamicin and Ciprofloxacin before curing became more sensitive when cultured in the presence of 1% SDS solution. This is an indication that the resistant genes may be present in the plasmid. Resistance to high level of antibiotics has been ascribed to be caused by the presence of plasmids (Daini, *et al*.1998).
Isolates that showed multiple drug resistance were found to harbor plasmids with sizes ranging from 2.1kb to 21,70kb. (Table 4).

This is similar to what was observed by Smith *et al*(2003) who reported that 47 of the *E.coli* isolated from animals in Lagos, Nigeria harbored detectable plasmids which ranged in sizes from 0.564kb to 23kb. This is an indication that animals could be a source of dissemination of this plasmid-resistant *E.coli* in the environment. It has been shown that emergence of R-plasmids particularly against drugs used for first line therapy could be due to lapses from institutional monitoring policies and antibiotic prescribing policy as many physicians in Nigeria usually prescribe without recourse to antibiotic sensitivity patterns(Montefiore *et al* 1989 and Ogunsola, *et al* 1997).

Table 4 also shows that the higher the molecular weight of the plasmids, the higher is the antibiotic resistance pattern of the *E.coli* isolates. This is also in agreement with the work done by Nasreen*et al* 2008. It was observed that some isolates possess single sized plasmid while others had multiple plasmids with different sizes ranging from 2.3kb to 26kb; very high antibiotic resistance was detected from isolates possessing high molecular weight plasmids (23kb).

CONCLUSION

The result of the present study showed that *E.coli* had multi-drug resistance to some antibiotics due to the presence of single and multiple sized plasmids with different sizes ranging from 2.1 kb to 21.7 kb.

RECOMMENDATIONS FOR FURTHER STUDIES

More research should be carried out to locate the gene responsible for multi-drug resistance in *E.coli.* Further research can be done to prevent *E.coli* from becoming resistance to antibiotics.

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TABLES AND FIGURES

Table 1. Phytochemical analysis of Methanolic leaf extract of *Azadirachtaindica*

Phytochemical constituents	Methanolic leaf extract of *Azadirachtaindica*
Alkaloid	.+
Steroids	.+
Saponin	.+
Tanin	.+
Flavonoids	.+
Terpenoids	.+
Glycoside	.+
Reducing sugar	.+

+ signifies the presence of a constituent

Table II. Distribution of *E coli* from different human clinical specimens.

S/N	Specimens	Number screened	No of positive samples	% of positive samples
1	Urine	31	12	23.1
2	Blood	15	3	5.8
3	Faeces	26	19	36.5
4	Hand	44	17	32.7
5	Wound Swab	8	1	1.9

Table III. The antibiotic/resistance of *E.coli* strains isolated from different human clinical specimens.

ANTIBIOTIC TEST	SENSITIVITY (%)	RESISTANT (%)
A.indica	34(65.4)	18(34.60)
Chloramphenicol	37(71.2)	15(28.8)
Ciprofloxacin	11(21.2)	41(78.8)
Ofloxacin	41(78.9)	11(21.2)
Gentamicin	46(88.5)	16(11.5)
Amoxicillin	12(23.1)	40(76.9)
Ampicillin	13(25)	39(75)
Tetracycline	6(11.5)	46(88.5)
Streptomycin	23(44.2)	29(55.8)
Nitrofurantoin	50(96.2)	2(3.8)
Augmentin	10(19.2)	42(80.8)
Imipenem	10(19.2)	42(80.8)
Cotrimoxazole	15(28.8)	37(71.2)

Table IV: Plasmid Profile of antibiotic resistance *E.coli* isolates from different human clinical specimens.

Level of resistance profile	Number of *E.coli* Strains	Plasmid sizes (kb)
Very low (1-2 antibiotics)	29	No plasmids
Low (2-3 antibiotics)	3	< 3.1
Medium (2-5 antibiotics)	8	≥3.1- 10.0
High (5-8 antibiotics)	12	>10 – 21.7

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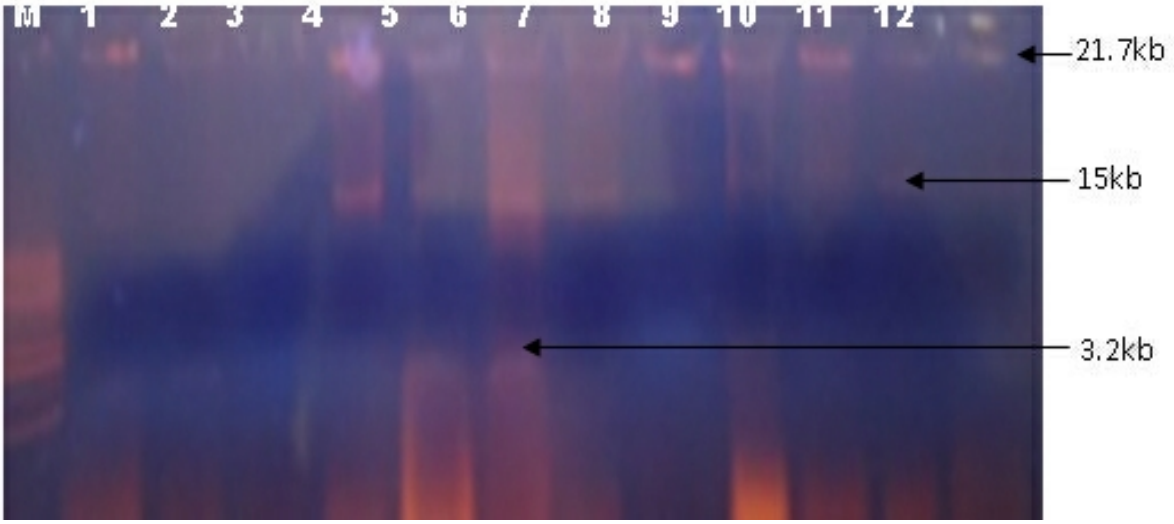

Figure I. Agarose gel electrophoresis of plasmids DNA from *E.coli*isolates grown in nutrient agar in the absence of 1 % SDS solution.

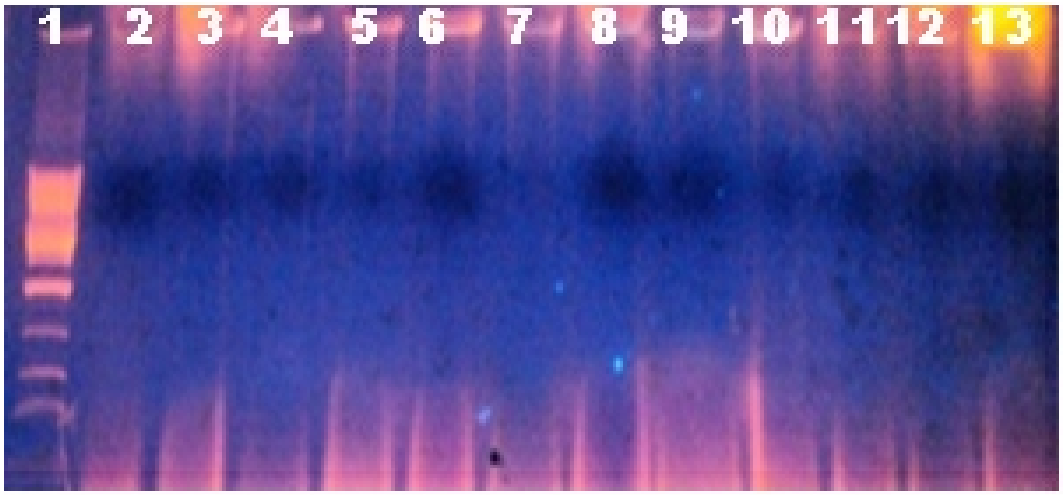

Figure II. Agarose gel electrophoresis of plasmids DNA from *E.coli*isolates grown in nutrient agar in the presence of 1 % SDS solution.

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