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Susceptibility of Multidrug Resistant Enterotoxigenic *Escherichia coli* to Saponin Extract from *Phyllanthus niruri*

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Abstract. *Escherichia coli* were isolated from 140 samples of blood, urine, stool and water made up of 15.7%, 42.9%, 30.0% and 25.7%, respectively. From the samples, 71.9% enterotoxigenic *E. coli* (ETEC), 14.3% enteropathogenic *E. coli* (EPEC), 7.1% enterohemorrhagic *E. coli* (EHEC) and 7.1% enteroinvasive *E. coli* (EIEC) occurred as diarrheagenic *E. coli*. Of the ETEC (240) isolates tested for susceptibility to eight conventional antibiotics, 110 (46.0%) showed resistance to all the tested antimicrobial agents. However, of the resistant strains; 24 (22.0%) were multidrug resistant. These were tested against 3.0 mg/mL of saponin extract from *Phyllanthus niruri* and 13 (55.0%) of these were susceptible to the saponin. The antimicrobial activities of saponin from *P. niruri* are of interest since the crude extract was effective at concentration of 3.0 mg/mL to multiple resistant isolates of ETEC.

Keywords: diarrheogenic *E. coli*, multidrug resistant, *E. coli* pathotypes, saponin, *Phyllanthus niruri*

Introduction

Different strains of *Escherichia coli* that cause diarrhoea are classified into pathogenic groups (pathotype) according to their virulence determinants (Wang *et al*., 2007; Robins-Browne *et al*., 2004). The specific nature of these virulence determinants imbues each pathotype with the capacity to cause clinical syndromes with distinctive epidemiologic and pathologic characteristics (Robins-Browne *et al*., 2004). For example, Enterotoxigenic *E. coli* (ETEC) causes watery diarrhoea in children in developing countries, whereas Enterohemorrhagic *E. coli* (EHEC) may cause haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) owing to the production of shiga toxins (Smet *et al*., 2011). Enteropathogenic *E. coli* (EPEC) shares several key virulence determinants with the most common varieties of EHEC but neither produces shiga toxins nor causes HC or HUS. Instead, it causes non-specific gastroenteritis, particularly in children in developing countries (Ramachandani *et al*., 2005; Robins-Browne *et al*., 2004). Pathogenic isolates of *E. coli* have a relatively large potential for developing resistance to antimicrobials (Dubos *et al*., 2010; Kumarasamy *et al*., 2010; Robins-Browne *et al*., 2004) and report of multiple antibiotic resistance is not infrequent (Kumarasamy *et al*., 2010). Because of the increasing resistance of most pathogenic bacteria to conventional antibiotics, it becomes imperative to search nature for alternatives. During the past decade, drug resistance in Enterobacteriaceae has increased dramatically worldwide. This increase has been caused mainly by an increased prevalence of extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae (Smet *et al*., 2011) and has increased the use of last-resort antimicrobial drugs.

Phyllanthus niruri L. (Syn. *P. fraternus* Webster) is a plant that has been used in folk medicine to treat a variety of illnesses particularly viral hepatitis (Qudhia and Tripathi, 2002). The plant has been widely recognized as a diuretic used in the treatment of urinary tract infections (Ajibade and Falegan, 2007) as well as fever (Barros *et al*., 2003). It is also believed to be helpful in treating oedema, anorexia, jaundice and diabetese (George and Roger, 2002), while its methanol and aqueous extracts have shown high efficacy on clinical isolates of *E. coli* (Ajibade and Egbebi, 2006).

Saponins are naturally occurring surface active glycosides, produced by plants, but also by lower marine animals and some bacteria (George and Roger, 2002; Haralampidis, 2002). They derive their names from their ability to develop stable, soap-like forms in aqueous solutions. Their logical role in plants is not yet fully

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understood, but they have been found to show multiple effects in animal cell and on fungi and bacteria (Haralampidis, 2002). Many saponins are known to be antimicrobial and inhibit moulds (George and Roger, 2002; Haralampidis, 2002). This study therefore, investigated the frequency of occurrence of ETEC in Ado-Ekiti and determined the effect of crude saponin extract from *P. niruri* on the multiple antibiotic resistant strains.

Materials and Methods

Collection, identification of plants and extract of saponin. *Phyllanthus niruri* was collected from farmlands at Ado-Ekiti, Nigeria between the months of March and June, 2009 and identified in the Herbarium, Plant Science Department, Ekiti State University, Ado-Ekiti, Nigeria. Voucher specimen No. UNAD/PLT 2009/00231 was deposited with Herbarium. Transplantation was carried out in the green house of the Department of Science Technology, Federal Polytechnic, Ado-Ekiti, Nigeria.

The methods of Marston *et al.* (2002) were employed for the extraction of saponin. Briefly, the plant material was air-dried at room temperature 31 °C ± 1 °C for 2 weeks. Milling to powder was done with a miller (Model-Restsch GM bit Zml). A 170 g portion of the milled plant material sample was defatted using 700 mL of petroleum ether for 72 h using Soxhlet apparatus. The defatted sample was air-dried for 24 h, and extraction carried out in 600 mL methanol for 48 h and the solvent was evaporated using a rotatory evaporator. The yield was dissolved in 600 mL *n*-butanol-distilled water (1:1v/v) and subsequently separated in a separating funnel. The upper layer of the separator mixture was collected and saponin was precipitated with diethyl ether. The precipitate was evaporated in a water bath and stored at about 4 °C until required.

Isolation of *Escherichia coli* and identification of ETEC. The method described by Wang *et al.* (2007) was employed to isolate *E. coli* strains from 140 samples each of water, stool, urine and blood by direct plating on MacConkey agar (Oxoid Ltd, Bashingstoke, UK). After overnight incubation at 37 °C, a sterile cotton swab was used to transfer the entire growth from each plate into Luria broth containing 30% (v/v) glycerol, which was then frozen at -70 °C until required. *Escherichia coli* pathotype were identified by polymerase chain reaction (PCR) and Southern hybridization (Robins-Browne *et al*., 2004) using primers FFLICI (5,-ATGGCACAAGTCATTAATACCCAAC-3) and R-FLIC2 (5,-CTAACCCTGCAGCAGAGACA-3). PCR positive bacteria were assigned to a pathotype. Ambiguous assays were repeated and if still unclear, were excluded from further analysis. ETEC strains were isolated in pure culture from the original sample and then serotyped by using hyperimmunized rabbit antisera to O - antigens (Robins-Browne *et al*., 2004).

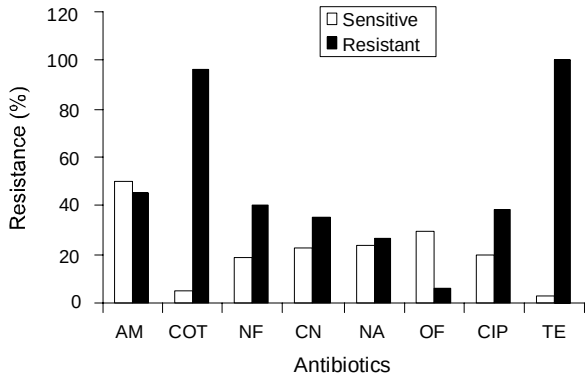
Antibacterial susceptibility. Antimicrobial susceptibility profiles of the test organisms were determined by disk diffusion method as specified by the Clinical and Laboratory Standards Institute (CLSI, 2008). Disk containing ampicilin (10 μg), trimethoprim (5 μg), gentamycin (5 μg), ciprofloxacin (5 μg), and nalidixic acid (30 μg), ofloxacin (5 μg), tetracycline (30 μg), nitrofurantoin (10 μg) and cotrimoxazole (10 μg) were used for testing on Mueller-Hinton agar (Oxoid). Antibacterial activity was demonstrated using a modification of the method originally described by Clinical and Laboratory Stan-dards Institute (CLSI, 2008). A bacterial colony was picked with a loop from a stock culture suspended in 0.1 mL of saline and standardized with 5% barium sulphate. All the tests were performed by placing the disc impregnated with crude extract (3.0 mg/mL) of saponin and multidisk (Roche Diagnostic Ltd) of various antibiotics on the Sensitivity Test Agar surface previously inoculated with bacterial suspension of ETEC isolates. Plates were incubated at 37 °C for 24 h to observe formation of inhibition zones around the disk. Zones were measured in millimeters and represent the dia-meter of the margins of inhibition. Results of antibiotic assay scored as susceptible (≥ 5.0 mm), intermediate (5.0 mm) and resistant (≤ 4.0 mm) according to criteria established by the Clinical and Laboratory Standards Institute (CLSI, 2008).

Results and Discussion

From four sources of 140 samples each, 42% of *E. coli* was isolated from urine, 15% from blood, 25% from water and 30% from stool specimens (Table 1). This showed that urine was the most frequent source of *E. coli* while blood sample showed the lowest frequency of occurrence in this study. The incidence of diarrheogenic *E. coli* indicated that from 42 (30.0%) isolates from stool, ETEC was highest with 30 (71.4%) (Table 2). However, no STEC strain was isolated while the incidence of EHEC, EIEC and EPEC was 3 (7.1%),

3 (7.1%) and 6 (14.3%), respectively. Diarrheogenic *E. coli* prototypes cause high rates of persistent diarrhoea (Cohen *et al*., 2005) which has been associated with malnutrition, growth impairment and death, in developing countries. The prevalence of ETEC in this area and the associated diseases is an indication of its cause of diarrhoea in the study area and agrees with the findings of Hedican *et al*. (2009); Ajibade and Falegan (2006) and Cohen *et al*. (2005) and continues to be the most prevalent cause of enteric infections and a major public health problem in developing countries.

The antimicrobial susceptibility testing of the ETEC showed that resistance to tetracycline, cotrimoxazole, ampicillin, ciprofloxacin and gentamicin was at the rate of 97%, 93%, 33% and 30%, respectively (Fig. 1).



AM = ampicillin; COT = cotrimoxazole; NF = nitrofurantoin; CN = gentamycin; NA = nalidixic acid; OF = ofloxacin; CIP = ciprofloxacin; TE = tetracycline

Fig. 1. Susceptibility profile of ETEC strains (*n* = 30).

Of the 240 isolates tested for susceptibility to 8 different antibiotics, 110 (45.8%) were resistant. The highest resistance was comparable to those of Kumarasamy *et al*. (2010) and Putnam *et al*. (2004). Tetracycline was the only agent among those studied that demonstrated a consistent stepwise increase in resistance. Thus, of the 110 resistant strains, 24 (21.8%) were multiple antibiotic resistant (Table 3) while 9 (8.1%) was resistant to ampicillin, cotrimoxazole, nitrofurantoin, 5 (4.5%) were resistant to nitrofurantoin, gentamycin, nalidixic acid and 10 (9.0%) were resistant to tetracycline, ampicillin, cotrimoxazole and nitrofurantoin. Only 1 (0.9%) was however resistant to ofloxacin. This resistance to tetracycline may be due to rising, community use and easy accessibility to the antibiotic in the study area.

The results show the need for new, affordable, and safe oral antimicrobial drugs to treat enterobacterial infections.

The antimicrobial susceptibility of multidrug-resistant strains to saponin (3.0 mg/mL) extracted from *P. niruri* (Table 4) revealed that of the 24 multidrug resistant strains, 11 (45%) were resistant to the extract preparation. It is envisaged that infection control measures and exploitation of plant extract for new drug development will avert the upsurge of emerging infectious diseases and their multidrug resistant agents. The discovery of

Table 1. Number of *Escherichia coli* isolated from different sources (n = 560)

Sources (*n*=140)	No (%)
Urine	60 (42.9)
Blood	22 (15.7)
Water	36 (25.7)
Stool	42 (30.0)

Table 2. Incidence of diarrheogenic *E. coli* pathotype from stool sample (*n* = 42)

Pathotype	No (%)
ETEC	30 (71.9)
EHEC	3 (7.1)
EIEC	3 (7.1)
EPEC	6 (14.3)

ETEC = enterotoxigenic *E. coli*; EHEC = enterohemorrhagic *E. coli*; EIEC = enteroinvasive *E. coli*; EPEC = enteropathogenic *E. coli*

Table 3. Multiple antibiotic resistance pattern of ETEC

No of antibiotics	*n* (%)	Drug-types
1	–	OF
2	3 (10)*	TE, COT
3	3 (10)*	TE, COT, AM
4	19 (63)*	TE, COT, AM, NF
5	3 (10)*	TE, COT, AM, NF, CIP
6	2 (7)*	TE, COT, AM, NF, CIP, NA

*Multidrug resistant (resistance to two or more antibiotics); TE = tetracycline; COT = cotrimoxazole; AM = ampicillin; NF = nitrofurantoin; CIP = ciprofloxacin; OF = ofloxacin; NA = nalidixic acid

higher resistance of isolates of ETEC strains is not unexpected. Given the ambiguity of *E. coli* as a commensal pathogen in the human gut and animal population, resistance among ETEC strains may be a sensitive indication of distinct therapeutic and non-therapeutic, appropriate and inappropriate use of antimicrobial drugs (Harbarth and Samone, 2005).

Table 4. Susceptibility of multiple-antibiotic resistant strain of ETEC to saponin extract (3.0 mg/mL)

Diameter of zone on inhibition (mm)	No (%)
≥ 9.0	2 (8)
8.0	1 (4)
7.0	1 (4)
6.0	6 (25)
5.0	3 (15)
≤ 4.0	11 (45)*

* Resistant

Prevalence of resistance is usually positively correlated with prescribed outpatient drug use, consumption of drugs obtained without prescription and use without medical guidance. This is inappropriate because using insufficient dosages or incorrect or unnecessary drugs increases bacterial resistance (Hedican *et al*., 2009; Bettelhein, 2007) and the spread of antimicrobial drug resistance (Harbarth and Samone, 2005). Since some DE infections appear indistinguishable from viral gastroenteritis, isolation and identification of DE strains could allow caretakers to provide appropriate treatment for pathogen-specific illness. Oral rehydration therapy (ORT) in children with dehydrating forms of diarrhoea has reduced death rates worldwide. ORT however, does not shorten duration of illness and shedding, whereas herbal-therapy may be of value for some forms of DE diarrhoea. The antimicrobial activities of saponin from *P. nururi* are of interest since it was effective at concentration of 3.0 mg/mL to multiple resistant isolates of ETEC. The fact that it was active against 55% of these isolates is worthy of note. Further studies on this extract are on-going in the laboratory.

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