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Full Length Research Paper

Antibacterial activity of ethanolic extracts *of Phyllanthus amarus* against extended spectrum β-lactamase producing *Escherichia coli* isolated from stool samples of HIV sero-positive patients with or without diarrhoea

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The antibacterial activity of extracts of the root and leaf of *Phyllanthus amarus* was assessed against extend spectrum β-lactamase (ESBL) producing *Escherichia coli* isolated from the stool samples of HIV sero- positive patients with or without diarrhoea between January, 2009 and April, 2009 using Bauer disc diffusion method. The phenotypic confirmation of ESBL-*E. coli* were done by *Double Disc Synergistic Methods (DDST).* The phytochemical analysis of both root and leaf revealed the presence of alkaloids, flavonoids, saponins, tannins, cardiac glycoside, terpenes and anthraquinones. The strains isolated from both HIV sero- positive patients were susceptible to various concentrations of the extracts (5, 10, 20, 40 and 80 mg ml^{-1}). In view of the efficacy of these extracts in inhibiting the growth of extend spectrum β-lactamase producing *E. coli* in HIV sero-positive patients, the utilization of the extracts in the formulation of new antibacterial drugs for the treatment of gastroenteritis in HIV positive patients caused by this organism is strongly recommended especially when the availability and low cost of these medicinal plants are put into strong consideration.

Key words: *Escherichia coli*, susceptibility, *Phyllanthus amarus,* HIV, cephalosporin, beta-lactamase.

INTRODUCTION

In recent years, drug resistance to human pathogenic bacteria has been reported from all over the world (Piddock and Wise, 1989; Akinjogunla et al., 2009). Among the wide array of antibiotics, β-lactams are the most varied and widely used agents with over 50% of all systemic antibiotics in use (Bronson et al., 2001). The most common cause of bacterial resistance to β-lactam antibiotics is the production of β-lactamases, especially extended spectrum betalactamase (ESBL) which mediates the resistance to extended spectrum of third and fourth-generation cephalosporins such as cephalothin cefotaxime, ceftazidime, cefepime etc. (Livermore, 1995). *Escherichia coli* are prominent members of family Enterobacteriaceae, widely distributed in nature and occurring in the intestinal tract of man and animals (Nataro et al., 1987; Smith et al., 2003; Akinjogunla et al., 2009b). Strains of *E. coli* that acquire invasion factors become virulent and consequently increase their ability to adapt to new niches and allow them to cause either a plain, watery diarrhoea or inflammatory dysentery (Yah et al., 2006; Akinjogunla et al., 2009b). Human immunodeficiency virus (HIV) is the aetiological agent of the Acquired Immunodeficiency Syndromes (Georges and

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Georges-Courboj, 1990; Talaro and Talaro, 1996; Prescott et al., 2008). The HIV epidemic has continued to be a burden globally and presents serious public health problems in developing countries like Nigeria. The AIDS pandemic claimed an estimated 2.1 million (range 1.9 - 2.4 million) lives in 2007 of which an estimated 330,000 were children under 15 years Globally, an estimated 33.2 million people lived with HIV in 2007, including 2.5 million children and an estimated 2.5 million (range 1.8 - 4.1 million) people were newly infected in 2007, including 420,000 children (Pennap et al., 2006; Akinjogunla and Adegoke, 2009). Sub-Saharan Africa remains by far the worst affected region. The number of HIV-positive is increasing and infections of gastro-intestinal tract are common in patients with Acquired Immunodeficiency Syndromes and it has been estimated that about 90% of patients with AIDS in Africa suffer from diarrhoea caused by enteric pathogenic bacteria which are resistant to antibiotics especially broad spectrum and some extended spectrum betalactamase drugs (Sanders et al., 1987). The use of medicinal plants by man for the treatment of diseases has been in practice for a very long time. Screening of compounds obtained from plants for their pharmacological activity has resulted in the isolation of innumerable therapeutic agents representing molecular diversity engineered by nature. One of the ways to prevent antibiotic resistance of pathogenic species is to use new compounds that are not based on existing antimicrobial agents (Shah, 2005; Adebayo and Adegoke, 2008; Akinjogunla et al., 2009a). The *Phyllanthus* genus of the family Euphorbiaceae was first identified in central and southern India in 18th century but is now found in many countries including Philippines, China, Cuba and Nigeria among others (Bharatiya, 1992). It is commonly called 'carry me seed', 'stone-breaker', 'windbreaker', 'gulf leaf flower' or 'gala of wind' (Bharatiya, 1992). *P. amarus* is an erect annual herb of not more than one and half feet tall and has small leaves and yellow flowers. In folk medicine *P. amarus* has reportedly been used to treat jaundice, diabetes, otitis, diarrhoea, swelling, skin ulcer, gastrointestinal disturbances, and weakness of male organ and blocks DNA polymerase in the case of hepatitis B virus during reproduction (Oluwafemi and Debiri, 2008). There are no reports on the antimicrobial potential of both the leaf and root extracts of *P. amarus* on isolates from stool samples especially *E. coli* from HIV positive patients. The authors investigated the in vitro antibacterial activity of root and leaf extracts of *P. amarus* against ESBL producing *E. coli* obtained from HIV Sero-positive patients.

MATERIALS AND METHODS

Bacterial cultures

E. coli were isolated from stool samples of confirmed HIV sero-positive patients attending University of Uyo Teaching Hospital and University of Uyo Health Centre between January and April, 2009. Eosin Methylene Blue (EMB) agar was used for the isolation of the organism and the culture was incubated at 37°C for 24 h. Green colonies with metallic metallic sheen, positive for *E. coli,* were further sub-cultured onto nutrient agar and incubated for 24 h. Stock cultures were maintained on nutrient agar slants at 4°C. The cultures on nutrient agar plates were subjected to tests such as Gram staining, motility, urease production, indole production, glucose, sucrose, mannitol, lactose, citrate utilization, oxidase and Voges-Proskauer tests. All Gram-negative, rod-shaped, motile, indole negative, urease-negative isolates that produced acid on Triple Sugar Iron agar slants were identified as species of the genus *E. coli* (Cowan and Steel, 1985; Fawole and Oso, 1988; Cheesbrough, 2004).

Antibiotic sensitivity testing

In vitro susceptibility of the *E. coli* to different antibiotics was determined using Bauer disk-diffusion technique (Bauer et al., 1996). Sterile Petri dishes of Mueller Hinton agar were prepared. 0.1 ml of *E. coli* was seeded into Mueller-Hinton agar plates and allowed to stand for 45 min.

The commercial discs containing gentamycin (Gen,10 µg), ofloxacin (Ofl, 30 µg), ampicillin (Amp, 10 g), tetracycline (Tet, 30 µg), cephalothin (Cep, 30 µg), cefotaxime (Cef, 30 µg), ceftazidime (Cft, 30 µg), cefepime (Cfp, 30 µg), ciprofloxacin (Cip, 5 µg), chloramphenicol (Crp, 30 µg) (Oxoid, UK) were aseptically placed on the surface of the sensitivity agar plates and these were incubated for 18 - 24 hrs at 37°C. Zones of inhibition after incubation were measured in millimeters. The interpretation of the measurement as sensitive, intermediate and resistant was made according to the manufacturer's standard zone size interpretive manual. The intermediate readings were considered as sensitive for the assessment of the data.

Confirmatory test for ESBL-producing *Escherichia coli*

All the *E. coli* that are resistant to cephalothin, cefotaxime, ceftazidime, cefepime (≤15) were screened using double-disc synergy tests (DDST) to detect ESBL-producing isolates, cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg) discs were placed The tests were performed in triplicate.

Plant material

The leaves and roots of *Phyllanthus amarus* were collected from Ikot Ekpene (about 20 km from Uyo) in Akwa Ibom State. The leaves and roots were repeatedly rinsed thoroughly under running distilled water for further analysis.

Preparation of plant extracts

A sample (50 g) of the powder of the shade-dried leaves and roots of *P. amarus* was macerated in 95% ethanol (200 ml) for 72 h and filtered. The filtrate was concentrated under vacuum at 30°C. The dry extract was weighed and preserved at 5°C. The graded concentrations (5, 10, 20, 40 and 80 mg/ml) of the extracts were prepared and tested for antibacterial activity against extended spectrum beta-lactamase *E. coli.*

Bioassay

The ethanolic extracts were tested against extended spectrum beta-lactamase *Escherichia coli* by the disc diffusion method using

Oxoid-Mueller Hinton agar (Difco Laboratories, Detroit, Mich) supplemented with 2% NaCl. Sterile filter paper discs of 6 mm diameter were separately impregnated with root and leaf extracts of graded concentrations (5, 10, 20, 40 and 80 mg mL^{-1}) and then applied on to the agar plates. Control experiments comprising ciprofloxacin and ofloxacin were set up. The plates were incubated at 37°C for 24 h.

The diameters of the inhibitory zones were measured in millimeters. Assays were performed in triplicate and the data are shown as the mean ± standard deviation (SD).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The Minimum Inhibitory Concentration (MIC) of the extracts was determined for each of the test strains in test tubes. 0.5 ml each of the test isolate was added to the following varying concentrations of the extracts 5, 10, 20, 40 and 80 mg mL^{-1} containing 2 ml of nutrient broth. Similar tubes were set containing streptomycin used as the control group.

The cultures were then incubated at 37°C for 24 h. After incubation the tubes were examined for microbial growth by observing for turbidity. The tube containing the least concentration of extract showing no visible sign of growth was considered as the minimum inhibitory concentration.

To determine the MBC, for each of the test isolate 1ml of the broth was collected from the tubes that showed no growth and inoculated onto sterile nutrient agar. The plates were then incubated at 37°C for 24 h. After incubation the concentration that showed no visible growth was considered as the Minimum Bactericidal Concentration (MBC). Both MIC and MBC for the test bacteria were determined in triplicate assays and the data were shown as the mean ± SD.

Phytochemical screening

The preliminary qualitative phytochemical analysis of the plant extracts of *Phyllanthus amarus* was performed to screen for the presence of bio-active components in the leaves and roots (Evans, 1989; Sofowora, 1993).

Test for tannins

i.) 1 cm^3 of freshly prepared 10% KOH was added to 1 cm^3 of the extract. A dirty white precipitate indicated the presence of tannins.
ii.) Powdered plant parts (leaf and root) of the test plant (1.0 g) were weighed into a beaker and 10 ml of distilled water added. The mixture was boiled for five minutes. Two drops of 5% $FeCl_3$ were then added. Production of a greenish precipitate was an indication of the presence of tannins.

Test for alkaloids

i) 0.5 g of the extract of the plant (leaf and root) was stirred with 5 ml of 1% HCl on a steam bath. The solution was filtered and 1 ml of the filtrate was treated with two drops of Mayer's reagent. Development of turbidity on addition of Mayer's reagent was regarded as evidence for the presence of alkaloids in the extract
ii) A few drops of the freshly prepared Drangendorff's reagent were added to 0.5 g of the plant extract in the test tube and a brown colour was observed.
iii) A few drops of freshly prepared Picric reagent were added to 0.5 g of the plant extract and a brown coloured solution was observed, showing the presence of alkaloids.

Test for saponins

0.5 g of plant extract was introduced into a tube containing 5.0 ml of distilled water, the mixture was vigorously shaken for 2 min, and formation of froth indicated the presence of saponins.

Test for flavonoids

A small piece of magnesium ribbon was added to ethanolic extracts of the plant material followed by drop wise addition of concentrated hydrochloric acid. Colours varying from orange to red indicated flavones, red to crimson indicated flavonoids, crimson to magenta indicated flavonones.

Test for terpenes

To 0.5 g of the extract, 3.0 ml chloroform were added and filtered, 10 drops of acetic anhydride and 2 drops of H_2SO_4 were added to the filtrate and the colour change from blue to green was observed.

Cardiac glycosides

0.5 g of the plant extract was dissolved in 2 ml of acetic anhydride and cooled in ice bath. Concentrated H_2SO_4 was carefully added drop by drop. A colour change from violet to blue to green indicates the presence cardiac glycoside. Also 0.5 g of the plant extract was dissolved in 2 ml of chloroform, Concentrated H_2SO_4 was carefully added drop by drop to form a lower layer. A reddish- brown colour at the interface indicated the presence of cardiac glycoside

Anthraquinones

0.5 g of plant extract was shaken with 10ml of benzene and filtered and 5 ml of 10% ammonia was added to the filtrate. The mixture was shaken and the presence of pink, red or violet colour indicates the presence of anthraquinones.

RESULTS

Phenotypic confirmation of ESBL- *E. coli* was carried out by the double-disc synergy tests. A total of eight strains of *E. coli* were confirmed to be ESBL producers. Out of them five were isolated from HIV patients with diarrhoea while three were isolated from HIV patients without diarrhoea. The results of the *in vitro* antimicrobial activity of the crude ethanolic extracts of root and leaf of *P. amarus* using Bauer agar diffusion method on the isolates varied as shown in Table 1. The mean zones of inhibition of the root extracts ranged from 8.0 ± 0.33 mm to 25.0 ± 1.50 mm against ESBL-*E. coli* (Table1) while the mean zones of inhibition the of the leaf extracts ranged from 8.0 ± 0.50mm to 26.0 ± 1.00 mm against ESBL-*E. coli* (Table 1). The root extracts showed the highest zone of inhibition (25 ± 1.50 mm) against ESBL-*E. coli* at 80 mg/ml while the leaf extracts showed the highest zone of inhibition highest (26 ± 1.00 mm) against ESBL-*E. coli* at 80 mg/ml (Table 1).The results indicate that the roots and leaf extracts of *P. amarus* are both bacteriostatic and bactericidal with the minimum inhibitory

Table 1. Antibacterial activity of crude extract of *Phyllanthus amarus* against extended spectrum beta-lactamase producing *E. coli*

E. coli	Mean zones of Inhibition (MM) ±SD (Leaf extract)					Mean zones of Inhibition (MM) ±SD (Root extract)					Antibiotics	
(strain)	a	b	c	d	e	a	b	c	d	e	Cip (30 ug)	Ofl (30 ug)
EEC 1	9.0±0.41	9.0 ± 0.50	12.0 ± 0.26	21.0 ± 0.25	26.0 ± 1.00	8.0±0.33	8.0 ± 0.50	10.0 ± 0.67	20.0 ± 1.50	25.0 ± 1.50	22.0 ± 1.00	22.0 ± 1.00
EEC 2	12.0±0.33	12.0 ± 0.65	17.0 ± 0.50	21.0 ± 0.33	21.0 ± 0.50	9.0±0.50	12.0 ± 0.67	14.0 ± 0.50	18.0 ± 0.60	21.0 ± 0.50	20.0 ± 0.67	18.0 ± 1.00
EEC 3	10.0±0.50	10.0 ± 0.50	10.0 ± 1.50	17.0 ± 0.33	19.0 ± 0.82	8.0±0.50	8.0 ± 0.50	10.0 ± 1.0	15.0 ± 0.30	20.0 ± 0.67	22.0 ± 1.50	21.0 ± 0.75
EEC 4	8.0± 0.50	14.0 ± 0.33	18.0 ± 1.00	24.0 ± 1.00	24.0 ± 1.50	10.0± 0.50	10.0 ± 0.65	12.0 ± 0.71	15.0 ± 0.50	15.0 ± 1.50	24.0 ± 2.00	25.0 ± 1.00
EEC 5	8.0±0.50	10.0 ± 0.33	12.0 ± 0.42	15.0 ± 0.67	15.0 ± 1.00	10.0±0.50	15.0 ± 0.42	15.0 ± 0.50	15.0 ± 0.71	22.0 ± 0.50	20.0 ± 0.73	18.0 ± 1.50
EEC 6	NZ	NZ	8.0 ± 1.00	8.0 ± 1.50	10.0 ± 0.72	8.0 ± 0.50	12.0 ± 1.50	15.0 ± 1.00	21.0 ± 1.50	24.0 ± 1.50	16.0 ± 0.33	18.0 ± 1.00
EEC 7	8.0±0.50	8.0 ± 1.00	12.0 ± 0.42	12.0 ± 1.0	21.0 ± 1.0	8.0±0.50	8.0 ± 1.00	12.0 ± 1.50	12.0 ± 1.50	21.0 ± 1.00	25.0 ± 1.00	21.0 ± 1.50
EEC 8	NZ	10.0 ± 0.5	10.0 ± 0.67	14.0 ± 0.50	18.0 ± 1.0	8.0±0.50	10.0 ± 0.50	10.0 ± 1.00	14.0 ± 0.67	18.0 ± 1.50	21.0 ± 1.50	20.0 ± 0.50

EEC1-3: Extended Spectrum Betalactamase *E .coli* isolated from HIV patients without Diarrhea
EEC 5-8: Extended Spectrum Betalactamase *E .coli* isolated from HIV patients with Diarrhea
NZ –Antibacterial activity was not detected; a: 5 mg $mL^{-1;}$ b: 10 mg mL^{-1}; c: 20 mg mL^{-1}; d: 40 mg mL^{-1}; e: 80 mg mL^{-1}.

concentration (MIC) and minimum bacterial concentration (MBC) of the plant extracts ranging from 5 - 20 $mgml^{-1}$ to 5 - 30 $mgml^{-1}$ respectively (Table 2).The ratio of the minimum bactericidal concentration (MBC) and minimum inhibitory concentration was found to be between 1 - 3.

The results of the preliminary phytochemical analysis of the extracts showed the presence of Anthraquinones, terpenes, and cardiac glycosides in the ethanolic crude extracts of leaves and roots (Table 3).

DISCUSSION

While the battle between man and microbes continues, starting with the defeat suffered by penicillin, methicillin, vancomycin and other antibiotics especially ESBL-antibiotics. It is important and valuable to find compounds that potentiate antimicrobial activity against extended spectrum betalactamase organisms such as *E. coli.* Preliminary phytochemical tests of the roots and leaf extracts of *P. amarus* revealed the presence of secondary metabolites such as anthraquinones, cardiac glycosides, saponins, tannins, alkaloids, flavonoids , which is in conformity with the earlier reports on *P. amarus* (Adebayo-Tayo and Adegoke, 2008; Olufemi and Debiri, 2008; Akinjogunla et al., 2009a).The antimicrobial effect of plant extracts could be due to the presence of some of these phyto-constituents (Sofowora, 1986; Ebana et al., 2005; Cushnie and Lamb, 2005). According to Ebana et al. (1991) and Cushnie and Lamb (2005), both alkaloids and flavonoids have antimicrobial activities. Phyto-constituents such as saponins and phenolics compounds have also been reported to inhibit bacterial growth.

These secondary metabolites exert antimicrobial activity through different mechanisms. Tannins form irreversible complexes with proline rich protein, resulting in the inhibition of cell protein synthesis and the flavonoids complex with extracellular- soluble proteins and with bacterial cell wall proteins while the lipophilic flavonoids exert antimicrobial activity by disrupting microbial cells membranes (Tsuchiya et al., 1996; Olowusulu and Ibrahim, 2006). The results obtained showed that ethanolic extracts of *P amarus* exhibited inhibitory activities against ESBL- *E. coli* at varying degrees of concentration as demonstrated by the diameters of the zones of inhibitions. These results were in conformity with

Table 2. Minimum Inhibitory Concentration (MIC) and minimum bactericidal concentration (MBC) of crude extract of leaf and root of *Phyllanthus amarus* against extended spectrum beta-lactamase producing *E. coli*.

E. coli (strain)	Leaves (mg mL^{-1})			Root (mg mL^{-1})		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
EEC 1	5.0 ± 0.33	10.0 ± 0.50	10/5	5.0 ± 0.67	5.0 ± 1.00	5/5
EEC 2	10.0 ± 0.67	20.0 ± 1.00	20/10	5.0 ± 1.00	20.0 ± 0.94	20/5
EEC 3	10.0 ± 0.50	10.0 ± 1.50	10/10	10.0 ± 1.00	20.0 ± 1.00	20/10
EEC 4	5.0 ± 0.67	10.0 ± 1.00	10/5	5.0 ± 1.50	10.0 ± 1.00	10/5
EEC 5	10.0 ± 0.50	20.0 ± 1.00	20/10	20.0 ± 1.50	30.0 ± 1.00	30/20
EEC 6	10.0 ± 0.33	30.0 ± 1.00	30/10	10.0 ± 1.50	20.0 ± 1.50	20/10
EEC *7*	10.0 ± 0.5	20.0 ± 0.94	20/10	5.0 ± 0.67	20.0 ± 1.00	20/5
EEC 8	5.0 ± 1.00	20.0 ± 0.50	20/5	10.0 ± 1.00	30.0 ± 1.50	30/5

EEC 1-3: Extended spectrum betalactamase *E. coli* isolated from HIV patients without diarrhea.
EEC 5-8: Extended spectrum betalactamase *E. coli* isolated from HIV patients with diarrhea.

Table 3. Phytochemical tests on the crude ethanolic extracts of the leaf and root of *Phyllanthus amarus.*

Plant Constituents	Test	Occurrence	
		Root extract	Leaf extract
Alkaloids	Drangendorff's test	++	+
	Mayer's reagent	+	+
	Picric acid test	-	±
Flavonoids	General test	+	+
Terpenes	General test	±	±
Saponins	General test	+	++
Tannins	General test	++	++
Anthraquinones	General test	++	++
Cardiac glycoside	General test	+	+

those earlier reported by Akinjobi et al. (2006); Abu-Zaida et al. (2008) and Olufemi and Debiri (2008). Ethanolic extracts of roots and leaves can be used as efficacious antibacterial agents against ESBL- *E. coli* that causes diarrhoea in Human immunodeficiency Virus (HIV) infected patients. In conclusion, toxicological studies, and purification should be embarked upon in addition to investigating extracts' activity on a wider range of bacteria. There is a need to consider the use of these potent root and leaf extracts that have shown some measures of antimicrobial potency, judging by the antimicrobial activity, activity index (A.I), low Minimum Inhibitory Concentration (MIC) and low Minimum Bactericidal Concentration (MBC) on ESBL-*E. coli.*

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