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SENSITIVITY OF EXTENDED-**SPECTRUM β**-LACTAMASES PRODUCING ENTEROBACTERIACEAE ISOLATES TO *ANNONA SQUAMOSA* EXTRACTS

***[1]Yusha'u, M., [1] Adakawa, M. I. and [2] Suleiman K.**
[1]Department of Biological Sciences, Bayero University, P. M. B. 3011, Kano, Nigeria
[2]Department of Biological Sciences, College of Arts, Science and Remedial Studies, Kano, Nigeria
*Correspondence author: *mryushau@gmail.com*

ABSTRACT

Powdered leaves of Annona squamosa (L.) were extracted with ethanol and methanol using percolation method. The extracts were tested for antimicrobial activity against clinical isolates of confirmed extended spectrum β-lactamase producing Escherichia coli, Klebsiella pneumoniae and Proteus specie using disc diffusion method. The extracts were further subjected to phytochemical screening for the presence of secondary metabolites. Sensitivity test results showed that methanol extract of the plant was only active on E. coli at 30 µg/disc concentration with 7mm zone of inhibition but inactive against P. specie even at 60 µg/disc concentration. Ethanol extract of the plant was active against all isolates only at 60 µg/disc concentration with P. specie forming slightly wider zone of inhibition (8mm) and the remaining isolates having 7mm zone diameter. The results of phytochemical screening indicated the presence of reducing sugars, saponins and steroids in either or both extracts.

Keywords: Sensitivity, ESBLs, Enterobacteriaceae, Annona squamosa, Extracts

INTRODUCTION

Extended spectrum β-lactamases (ESBLs) are enzymes that confer variable level of resistance to oxyiminocephalosporins such as cephotaxime, ceftazidime and monobactams. They occur predorminantly in the family enterobacteriaceae with *Klebsiella pneumoniae* been the most commonly reported worldwide and it is responsible for 5-20% of outbreaks of nosocomial infections in Hospital intensive care, burn, oncology and neonatal units (Kotra *et al.*, 2002).

At present there exist more than 200 different natural variants worldwide which constitute serious threat to current β-lactam therapies and represent major therapeutic challenges for clinicians (Lin *et al.*, 2005). Patients at risk of infection with ESBLs-producing organisms are seriously ill patients with prolonged hospital stays and those in whom invasive medical devices (such as catheters) are present for prolonged duration with the length of hospital stay prior to isolation of ESBL producer ranging from 11-67 days (Lautenbach *et al.*, 2001). Other risk factors for infection include presence of nasogastric tubes (Asensio *et al.*, 2000), recent surgery and poor nutritional status (Paterson and Bonomo, 2005), haemodialysis (D'Agata *et al*, 1998) as well as selective pressure on the use and overuse of antibiotics (Cosgrove *et al.*, 2002).

The use of herbal medicine is popular in several local communities in Nigeria as well as other developing countries. Prominent among the reasons is poverty among the populace as well as lack of basic primary health care system (Oke, 2000). Indeed generalized and highly exaggerated attributes have been associated with the potency of some of these plants against several ailments without any scientific and documented reports to backup some of the widely held claims. In any case plants provide about 80% of the remedies for several diseases and are cheap, readily available and safer alternative source of antibacterial agents (Sofowora, 1993).

Annona squamosa (sugar apple tree) ranges from 10 to 20 ft (3 – 6m) in height with open crown of irregular branches and zigzag twigs. Along the branch tips opposite the leaves, the fragrant flowers are borne single or in groups of 2 to 4. The fruit is nearly round, oval or conical, long its thick rind composed of knobby segments, separating when the fruit is ripe and revealing a conically segmented, creamy – white delightfully fragrant juice sweet delicious flesh (Morton, 1987).

Crushed leaves of *Annona squamosa* are used in India to overcome hysteria and faint spell while leaf decoction is used in the case of dysentery. Throughout tropical America, a decoction of leaves is imbibed as tonic cold remedy, digestive or to clarify urine whereas the crushed ripe fruit mixed with salt is applied on tumors while the bark and root are both highly astringent (Morton, 1987).

chloroform and distilled water. The extract and fractions were tested for antibacterial activity against clinical isolates of *Klebsiella pneumoniae*, *Proteus* species*, Pseudomonas* species, *Staphylococcus aureus, Streptococcus pneumoniae and α-haemolytic Streptococci* using disc diffusion method and micro-broth dilution technique. Sensitivity test results showed that water fraction of the plant was active on *Staphylococcus aureus and Streptococcus pneumoniae* at 500µg/disc concentration while ethanolic extract of the plant was active against *Klebsiella pneumoniae, Streptococcus pneumoniae* and *Proteus species* at 2000µg/disc concentration with zone diameter formed by *Klebsiella pneumoniae* being wider than that formed in response to standard Augmentin disc (Yusha'u, 2010).

This research was aimed at finding alternative treatment(s) to infections caused by ESBLs producers by determining antimicrobial activity of *Annona squamosa* extracts on clinical isolates of ESBLs producing enterobacteriaceae isolates.

EXPERIMENTAL

Collection of plant materials

The leaves of *Annona squamosa* were collected from the Department of Biological sciences, Bayero University, Kano, identified using the botanic guide, washed and air died at room temperature. Dried leaves were ground into fine powder using mortar and pestle in the laboratory as described by Mukhtar and Tukur (1999).

Extraction

Fifty grams each of powdered plant material was soaked in 500mls of ethanol and methanol in separate conical flasks and kept for two weeks in a shaker. The mixture was filtered and the filtrate evaporated at room temperature (Fatope *et al.*, 1993).

Phytochemical screening

The extracts were dissolved in their respective solvent for extraction in preparation for phytochemical studies.

Test for alkaloids

To 0.1ml each of the extract was measured in two separate test tubes, 2–3 drops of Dragendoff's reagent were added. An orange red precipitate with turbidity denoted the presence of alkaloids (Ciulci 1994).

Test for flavonoids

To 4mg/ml of each of the fractions measured in separate test tube, a piece of magnesium ribbon was added this was followed by concentrated HCl drop wise. A colour change ranging from orange to red indicates flavones; red to crimson indicates flavonoids (Sofowora, 1993)

Test for reducing sugars

One ml of each fraction measured in separate test tube was diluted with 2.0ml of distilled water. This was followed by addition of Fehling's solution (A + B) and the mixtures were warmed. Appearance of brick red precipitate at the bottom of the test tube indicates presence of reducing sugar (Brain and Turner 1975).

Test for saponins

Half gram of each of the extract was dispensed in a test tube each. 5.0ml of distilled water was added and shaken vigorously. A persistent froth that lasts for about 15 minutes would indicate the presence of saponins (Brain and Turner 1975)

Test for steroids

To 2mls of the extracts were taken into separate test tubes. The residues were dissolved in acetic anhydride and chloroform was then added. This was followed by the addition of concentrated sulphuric acid by the side of the test tubes using a pipette. A brown ring at the interface of the two liquids and a violet colour in the supernatant layer denoted the presence of steroids (Ciulci, 1994).

Test for tannins

Two mls of each of the extract in their respective solvent was diluted with distilled water in separate test tube and 2 – 3 drops of 5% ferric chloride ($FeCl_3$) solution was added. A green – black or blue colouration would indicate tannin (Ciulci, 1994).

Disc preparation

Improvised discs were punched from Whatman No. 1 filter paper, sterilized in bijou bottles by autoclaving at 121^0C for 15mins. Sensitivity disc were prepared by serial doubling dilution of the extract. In Dimethyl sulfoxide (DMSO) the paper disc were placed in the solution such that each disc took up 0.01m to make the disc potencies of 30, 60, 120, 240 and 480µg.

Test isolates

The test isolates were confirmed enterobacteriaceae isolates obtained from prevalence study at a Private Diagnostic Laboratory at Kano in 2009. The identity was confirmed by biochemical tests (Cheesbrough, 2000).

Inoculum Standardization

Few colonies of confirmed extended spectrum β-lactamase producers were dispensed in sterile normal saline to match the 0.5 McFarland standard for sensitivity tests as described by NCCLS (1999).

Bioassay

This was achieved by disc diffusion method (NCCLS, 1999). Standardized inocula of the confirmed ESBL producing isolates were swabbed onto the surface of prepared and solidified Mueller Hinton Agar in separate Petri-dishes. This was followed by placing the prepared discs of the extracts and ceftriaxone discs onto the surface of inoculated media at intervals. The plates were incubated at 37^oC for 24 hours before observation and measurement of zones of inhibition formed.

RESULTS AND DISCUSSION

The plant used yielded extracts amounting to 13.64% and 3.27% from ethanol and methanol respectively with the extracts having gummy texture and dark brown appearance as shown in Table 1.

Table 1: Physical properties of *Annona squamosa* extracts

Physical parameters	Ethanol	Methanol
Weight extracted (g)	55g	55g
Weight of extract (g)	7.5	1.8
Percentage yield (%)	13.64	3.27
Colour	Dark Brown	Dark Brown
Texture	Gummy	Gummy

The results of phytochemical screening of the two extracts of revealed the presence of reducing sugars, saponins and steroids. The plant secondary metabolites particularly flavonoids, steroids and tannins have been reported to posseess antimicrobial activity (Cowan, 1999).

Table 2: Phytochemical properties of *Annona squamosa* extracts

Extracts	Phytochemical Tests					
	Alkaloids	**Flavonoids**	**Reducing sugars**	**Saponins**	**Steroids**	**Tannins**
Ethanol	-	-	+	+	+	-
Methanol	-	-	+	+	+	-

Key: + - Present, **- -** Absent

Sensitivity test results showed that methanol extract (8mm) was more active than ethanol extract (7mm) most especially against *E. coli* with 7mm zone of inhibition when compared with standard ceftriaxone disc but inactive against the remaining isolates while ethanol extract inactive active against all isolates at 30 µg/disc concentration. Ethanol extract of the plant was active against all isolates with 7mm zone diameter except *Proteus specie* with slightly wider zone of inhibition (8mm) while methanol extract was only active against *E. coli* and *K. pneumoniae* but inactive against *Proteus specie* even at 60 µg/disc concentrations. The variation in the sensitivity of different species tested to both extracts may be as a result of the differences in the type of ESBLs harboured by these organisms since there were more than 200 different phenotypes identified worldwide (Jacoby and Muno-Price, 2005) and different ESBLs vary in their resistance to different antibiotic substances (Paterson and Bonomo, 2005). In general, the results of sensitivity tests indicated that both ethanol and methanol extracts of the plant were active on the isolates tested using disc diffusion method (Table 3).

Table 3: Sensitivity of ESBLs Producers to *Annona squamosa* extracts

Isolates	**EE**					**ME**					**CTX**
	30	**60**	**120**	**240**	**480**	**30**	**60**	**120**	**240**	**480**	**30**
	Inhibition Zones (*mm*)										
Escherichia coli	NZI	7	7	8	9	7	8	8	8	9	NZI
Klebsiella pneumoniae	NZI	7	7	8	9	NZI	7	8	8	9	NZI
Proteus specie	NZI	8	8	9	10	NZI	NZI	8	8	9	NZI

Key: EE – Ethanol extract, **ME –** Methanol extract, **CTX –** Ceftriaxone, **NZI –** No zone of inhibition

CONCLUSION

From the results of this work, it can be concluded that *Annona squamosa* used has the potential for the production of drug against extended spectrum β-lactamases (ESBLs) producing organisms. Further studies need to be carried out to establish the safety of the plant as well as to isolate and characterize the active compounds present in the extract.

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