

Screening of *Annona squamosa* extracts for antibacterial activity against some respiratory tract isolates

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Annona squamosa (L.) leaves were extracted with ethanol using percolation method and the extract obtained was partitioned in chloroform and distilled water at a ratio of 1:1 using 30ml volume. The extract and fractions were tested for antibacterial activity against clinical respiratory tract isolates of *Klebsiella pneumoniae*, *Proteus* species, *Pseudomonas* species, *Staphylococcus aureus*, *Streptococcus pneumoniae* and α -haemolytic *Streptococci* using disc diffusion and micro-broth dilution techniques. The extract and fractions were also screened for the presence of plant secondary metabolites using standard procedures. Sensitivity test results showed that water fraction of the plant was active on *Staphylococcus aureus* and *Streptococcus pneumoniae* (10 mm) at 50µg/disc concentration while ethanolic extract of the plant was active against *Klebsiella pneumoniae*, *Streptococcus pneumoniae* and *Proteus* species at 200µg/disc concentration with zone diameter formed by *Klebsiella pneumoniae* (11 mm) being wider than that formed in response to standard Augmentin disc (06 mm). The results of phytochemical screening indicated the presence of alkaloids, flavonoids, glycosides, reducing sugars, steroids and tannins in either the ethanolic extract, fraction(s) or both.

Keywords: Screening, *Annona squamosa*, Extracts, Biological activity, Disc diffusion, Broth dilution, Bacteria, Clinical isolates, Secondary metabolites.

INTRODUCTION

Herbal medicines can treat the diseases where chemicals and other drugs have failed. These include common illnesses, which lead to drowsiness and other side effects when treated through the regular medicines. Herbal remedies have the capacity to bring a certain amount of effect in the body and prove to be effective in treating health problem (Janny, 2009). 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 population as well as lack of basic primary health care system (Oke, 2000).

Annona squamosa (sugar apple) is commonly cultivated in tropical South America but not often in Central America, very frequently in southern Mexico, West Indies, Bahamas, Bermuda and occasionally southern Florida as well as in Jamaica, Puerto Rico,

Barbados and in dry regions of Australia. It was growing in Indonesia early in the 17th century and has been widely adopted in southern china, tropical Africa, Egypt and lowlands of Palestine. Cultivation is most extensive in India where the tree is exceedingly popular. The sugar apple is one of the most important fruits in the interior of Brazil (Morton, 1987).

Sugar apple tree ranges from 10 to 20 ft (3 – 6m) in height with open crown of irregular branches and zigzag twigs. A 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 the plant 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

Table 1: Phytochemical constituents of *A. squamosa* extract and fractions

Phytochemical			
Test(s)	EE	CF	WF
Alkaloids	-	+	-
Flavonoids	-	+	-
Glycosides	+	-	-
Reducing sugars	+	+	-
Saponins	+	-	+
Steroids	+	+	-
Tannins	+	-	-

Key: EE = Ethanol Extract, CF = Chloroform Fraction, WF = Water Fraction, + = Present, - = Absent

crushed ripe fruit mixed with salt is applied on tumors while the bark and root are both highly astringent (Morton, 1987).

The traditional claim that concoctions of *A. squamosa* can be used in the treatment of bacterial diseases need to be substantiated with scientific facts that could either support or negate this claim which necessitates the need for this study.

This study was aimed at extracting the plant material and evaluating the presence of secondary metabolites as well as the potency of the extracts on some clinical bacterial isolates.

MATERIALS AND METHODS

Collection of plant materials

A. squamosa leaves were collected at Hausawa quarters in Tarauni Local Government Area of Kano state. The plant leaves were washed, air-dried and ground into fine powder using mortar and pestle in the laboratory as described by Mukhtar and Tukur (1999).

Extraction

Eighty grams of the powdered plant material was soaked in 800 ml of ethanol, kept for two weeks in a shaker after which the mixture was filtered. The filtrate was evaporated at room temperature and a portion of the crude extract was mixed with 30ml of chloroform and 30 ml of water (1:1). The mixture was shaken properly, placed in a separating funnel and allowed to separate before collection in separate beakers. Both water and chloroform extracts were allowed to evaporate at room temperature (Fatope et al., 1993).

Phytochemical screening

Test for alkaloids

To 0.1ml of each extract in the test tube, 2 – 3 drops of Dragendoff's reagent was added. An orange red precipitate with turbidity denoted the presence of alkaloids (Ciulci, 1994). Table 1

Test for flavonoids

To 4mg/ml of each fractions, a piece of magnesium ribbon was added 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 glycosides

Ten milliliters of 50% H₂SO₄ was added to 1ml of the filtrates in separate test tubes and the mixtures heated for 15mins followed by addition of 10 ml of Fehling's solution and boiled. A brick red precipitate indicated presence of glycosides (Sofowora, 1993).

Test for reducing sugars

To 1ml of each fraction in separate test tubes, 2.0 mls of distilled water were added 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 the presence of reducing sugar in accordance with Brain and Turner (1976).

Test for saponins

Half gram of the powdered plant material was dispensed in a test-tube and 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

Two milliliters of the extracts were evaporated to dryness in separate test tubes and the residues dissolved in acetic anhydride followed by addition of chloroform. Concentrated sulphuric acid was added by means of a pipette via the side of the test tubes. Formation of brown ring at the interface of the two

Table 2: Sensitivity (mm) of clinical isolates to *A. squamosa* extract and fractions using disc diffusion method

Isolates	EE					CF					WF				
	(µg/ disc)					(µg/ disc)					(µg/ disc)				
	50	100	200	400	Aug 30	50	100	200	400	Aug 30	50	100	200	400	Aug 30
<i>S. aureus</i>	06	06	06	06	06	09	08	06	06	20	06	06	06	06	06
<i>S. pneumoniae</i>	06	06	09	06	12	07	08	09	09	18	10	09	06	06	15
<i>α-hemolytic strep.</i>	06	06	06	09	11	06	06	06	06	06	10	06	11	06	12
<i>K. pneumoniae</i>	06	06	11	06	06	06	06	06	06	06	06	06	06	06	06
<i>P. aeruginosa</i>	06	06	06	06	06	06	06	06	10	09	06	06	06	06	15
<i>Proteus spp.</i>	06	06	09	06	14	06	06	06	06	20	06	06	06	06	20

Key: EE = Ethanol Extract, CF = Chloroform Fraction, WF = Water Fraction

liquids and violet colour in the supernatant layer denotes the presence of steroids (Ciulci, 1994). Table 2

Test for tannins

Two ml of each extract was diluted with distilled water in separate test tubes, 2 – 3 drop of 5% ferric chloride (FeCl₃) solution was added. A green – black or blue colouration would indicate tannin (Ciulci, 1994).

Disc preparation

Sensitivity discs were punched from Whatman No. 1 filter paper, sterilized in bijou bottles by autoclaving at 121°C for 15mins. Sensitivity discs were prepared by weighing 40 milligrams of the extract or fraction and dissolving in 1 ml of Dimethyl-sulfoxide (DMSO) followed serial doubling dilution and placing 50 improvised paper discs in 0.5ml of each concentration such that each disc took up 0.01 ml to make the disc potency of 50, 100, 200 and 400 µg, respectively.

Test isolates

Respiratory tract isolates were collected from the microbiology laboratory of Aminu Kano Teaching Hospital (AKTH) and were re-identified using the following biochemical tests; catalase, oxidase, indole, motility, citrate utilization, urease production, hydrogen sulfide production, as well as acid and gas production according to standard procedures (Cheesbrough, 2005). They were maintained on nutrient agar slants in the refrigerator prior to use.

Inoculum standardization

A loopful of the test isolate was picked using a sterile wire loop and emulsified in 3 – 4 mls of sterile physiological saline followed by proper shaking. The turbidity of the suspension was matched with that of 0.5 McFarland Standard (Cheesebrough, 2000).

Bioassay

Agar Disc Diffusion Method

Using sterile swab stick, standardized inoculate of each isolate was swabbed onto the surface of Mueller Hinton Agar in separate Petri dishes. Discs of the extracts and standard antibiotic (Augmentin 30µg) were placed to the surface of the inoculated media. The plates were inverted and allowed to stand for 30 mins for the extract to diffuse into the agar after which the plates were incubated aerobically at 35°C for 18 hrs. This was followed by measurement of zone of inhibition formed by the test organisms around the each extract and standard antibiotic discs (NCCLS, 2008).

Micro-broth dilution technique

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations of the extract was prepared by serial doubling dilution using distilled water to obtain concentrations of 4000, 2000 and 1000 µg/ml. Equal volume of the extract and Mueller – Hinton broth i.e. 2ml each were dispensed into sterilized test tubes. Specifically 0.1 ml of standardized inocula (3.3×10^6

Table 3: Sensitivity of Clinical Isolates to *A. squamosa* Extracts using Microbroth dilution technique

Test isolates	MIC ($\mu\text{g/ml}$)			MBC ($\mu\text{g/ml}$)		
	EE	CF	WF	EE	CF	WF
<i>S. aureus</i>	4000	1000	**	**	4000	**
<i>S. pneumoniae</i>	4000	2000	1000	**	**	4000
α -hemolytic streptococci	4000	**	2000	**	**	**
<i>K. pneumoniae</i>	**	**	**	**	**	**
<i>P. aeruginosa</i>	4000	**	**	**	**	**
<i>Proteus spp.</i>	**	**	**	**	**	**

Key: EE = Ethanol Extract, CF = Chloroform Fraction, WF = Water Fraction, MIC = Minimum Inhibitory Concentration, MBC = Minimum Bactericidal Concentration, ** = MIC or MBC value greater than 8000 $\mu\text{g/ml}$

CFU/ml) was added to each of the test tubes above. The tubes were incubated aerobically at 35°C for 24 hrs. Tubes containing broth and plant extracts without inoculate served as positive control while tubes containing broth and inoculate served as negative control. The tubes were observed after 24 hrs of incubation to determine minimum inhibitory concentration. The lowest concentration that show no evidence of growth (NCCLS, 2008).

Minimum Bactericidal Concentration (MBC)

Sterile Mueller-Hinton agar plates were separately inoculated with sample from each of the test tubes that showed no evidence of growth. The plates were further incubated at 35°C for 24 hrs and observed. The highest dilution that yielded no bacterial colony was taken as MBC (NCCLS, 2008).

RESULTS

Sensitivity of the test isolates to *A. squamosa* extract and fractions were indicated by observation and measurement of inhibition zones formed around discs prepared from various concentrations of the extracts. Absence of turbidity in tube cultures indicates the activity of the extract or fraction using micro-broth dilution technique, the least concentration amongst the tubes without evidence of turbidity was considered the minimum inhibitory concentration (MIC).

DISCUSSION

The results of phytochemical screening of ethanolic extract, chloroform and water fractions of the plant revealed the presence of alkaloids, flavonoids, reducing sugars, saponins, steroids, tannins and glycosides. These metabolites have been reported to possess antimicrobial activity (Cowan, 1999). In particular the flavonoids were reported to be responsible for antimicrobial activity associated with some ethnomedicinal plants (Singh and Bhat, 2003).

It was observed that water fraction were active against *S. pneumoniae* and α -haemolytic streptococci with inhibition zone diameters of 10mm but inactive against the other test isolates while chloroform fraction was active against *S. aureus* and *S. pneumoniae* with inhibition zone diameters of 9 mm and 7 mm respectively but inactive against all other test isolates. In contrast, ethanolic extract was inactive against the other test isolates at the same disc concentration of 50 μg . The activity may be related to the polarity of the active compound(s) which make them to be soluble in the most highly polar (water) and the least polar (chloroform) solvents used in the study. The result is different from that exhibited by *Chrozophora senegalensis* extracts using acetone and hexane as extraction solvents and against the same isolates as reported by Yusha'u, 2011. It also varied with that obtained by Yusha'u (2010) when ethanol and methanol extracts of *Psidium guajava* were tested against extended spectrum betalactamase producing isolates of *E. coli*, *Klebsiella pneumoniae* and *Proteus vulgaris*.

Ethanol extract *A. squamosa* was only active against *S. pneumoniae*, *K. pneumoniae* and *Proteus* spp. at concentrations of 200 µg/disc and active on α -hemolytic *Streptococci* at 400 µg/disc but inactive against the remaining isolates. Chloroform fraction of the plant extract was active against *S. aureus* and *S. pneumoniae* at 50 µg/disc concentrations while inactive against the remaining isolates at all concentrations tested. Water fraction of the plant showed activity against *S. pneumoniae*, and α -hemolytic *Streptococci* at 50 µg/disc concentrations but inactive on the remaining test isolates at all concentrations.

The plant extract and fractions were found to have MIC values ranging from 1000 – 4000 µg/ml with *K. pneumoniae* and *Proteus* spp. being the least sensitive having both MIC and MBC values greater than 4000 µg/ml (Table 3) which may be due to the presence of capsules that give additional protection to the cells of *K. pneumoniae*.

Chloroform and water fractions exhibited MIC values of 1000 µg/ml against *S. aureus* and *S. pneumoniae* as well as against 2000 µg/ml against *S. pneumoniae* and α -haemolytic streptococci respectively whereas ethanol extract had MIC value of 4000 µg/ml against *S. aureus*, *S. pneumoniae* and α -haemolytic streptococci. With the exception of *S. aureus* and *S. pneumoniae* both of which showed MBC values of 4000 µg/ml in response to chloroform and water fractions respectively, all other isolates had MIC and MBC values greater than the highest (4000 µg/ml) concentration used in his study.

The results of sensitivity tests using both procedures indicated that ethanolic extract of *A. squamosa* was least active than the fractions on isolates tested. The activity exhibited by the fractions which may be related to the presence of alkaloids and saponins that are well documented for antimicrobial activity (Oyeleke and Manga, 2008) in addition to flavonoids which was reported to be responsible for antimicrobial properties of some ethno medicinal plants (Singh and Bhat, 2003).

CONCLUSION

The demonstration of activity against the different isolates by the leaf extract of *A. squamosa* is a justification for the basis of its use in traditional medicine and antimicrobial activities of the ethanolic extract of the plant suggest the presence of lead

compound(s) for the synthesis of an effective antibacterial agent.

RECOMMENDATIONS

In-vivo study on the plant extracts and fractions should be carried out to determine the presence or otherwise anti-nutrient and toxicological properties.

Further research should be carried out on this plant to determine the active components of the plant extract.

Further research needs to be carried out to determine the antimicrobial activity of the plant against a wider group of pathogens including fungi and parasites (protozoa and helminthes).

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