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Research Article

Antimicrobial Activities and Proportional indices of *Annona muricata* leaves extract on Nosocomial *Staphylococcus aureus* isolated from NAUTH, NNEWI

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Abstract

The study examined the antibacterial effect and proportional indices of methanolic and aqueous leaf extract of *Annona muricata* (Soursop) on *Staphylococcus aureus*. Fresh tender leaves of this plant were collected, air-dried, powdered, and percolated in methanol and aqueous solvents. Qualitative phytochemical analysis was done following an established protocol. The antimicrobial activities of the extract against test organism were done using agar well diffusion assay and MIC and MBC values were determined. *Staphylococcus aureus* was isolated from Nnamdi Azikiwe University Teaching Hospital, Nnewi. The activity index of the extracts compared to ciprofloxacin was calculated. Qualitative phytochemical analysis revealed the presence of tannins, saponins, cardiac glycoside, terpenoids, steroids, phenols, flavonoids, and alkaloids. The antimicrobial screening test revealed that aqueous and methanol extracts of *A. muricata* had antibacterial activity with minimum inhibitory concentration of 25 mg/ml and 12.5 mg/ml respectively, however, only the methanolic extract showed a minimum bactericidal concentration of 6.25 mg/ml. The maximum value of activity index was recorded in 400 mg/ml of methanolic extract while the least value of activity index was noted in 50 mg/ml of aqueous extract. The antibacterial effect of this plant extract could be due to the presence of these bioactive compounds that can be used in drug formulation.

Keywords: Antimicrobial; *annona muricata*; ciprofloxacin; proportional indices; *staphylococcus aureus*

Introduction

In recent years, there has been an increasing awareness about the importance of medicinal plants. Drugs from these plants are easily available, inexpensive, safe, efficient, and rarely accompanied by side effects. Plants which have been selected for medical use over thousands of years constitute the most obvious starting point for new therapeutically effective drugs such as anticancer drugs and antimicrobial drugs. Recently, medicinal plants usage has increased in spite of the advances made in the field of chemotherapy [1]. Due to the increasing use of herbal medicines around the world, their safety has often become a medical issue [2]. Despite this, the role of plants as producers of natural antimicrobial agents is often understated or even ignored [3]. During the last decade, the use of traditional medicine has expanded globally, and it is gaining more popularity. Plants are used medicinally in different countries and considered as a source of many potent and powerful drugs.

According to the world health organization (WHO), greater than 80% of the total world's population depends on traditional medicines in order to satisfy their primary health care needs. It also

suggested improving the technologies for cultivation of medicinal plants. *Staphylococcus aureus* represents one of the most serious gram-positive bacterial infections in nosocomial and community settings. Hospitalized patients show a high frequency of *S. aureus* infections due to their weak immune system and frequent injections and catheterizations [4]. The organism is well known for its ability to acquire resistance to various antibiotic classes. *Annona muricata*, also known as soursop or graviola or guanabana, is an evergreen plant that is mostly distributed in tropical and subtropical regions of the world.

muricata is native to the warmest tropical areas in South and North America and is now widely distributed throughout tropical and subtropical parts of the world, including India, Malaysia and Nigeria, Australia, Africa [5]. *Annona muricata* belongs to the family of Annonaceae, has a widespread pan tropical distribution and has been pridely known as corossol [6]. The fruits and seeds are used for the treatment of worms and parasitic infestations and for their analgesic and antidiarrhoeal effects. The bark, roots and leaves are used for their anti-inflammatory, antispasmodic, anticonvulsant, sedative, and antimalarial effect [7]. The aim of the study was to determine the antimicrobial activity of extracts from Soursop leaves on *Staphylococcus aureus* acquired from the hospital and its Minimum inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

Materials and Methods

Plant Sample Collection and Identification

Fresh leaves of *Annona muricata* was obtained from different compounds in Seaview Estate 3-3 Onitsha, Anambra state, Nigeria. All the leaves collected were identified and authenticated at the Department of Botany, Faculty of Natural sciences, Nnamdi Azikiwe University, Awka where the sample was deposited. The leaf spread out and dried on a clean surface under a shade at room temperature to exclude direct Sunlight in order to prevent the active constituents of the leaf from being degraded due to photochemical reactions. It was air dried for about eight days after which, it was observed to be dried. The dried leaves were gathered and crushed with a grinder. The powder was weighed using an electric weighing balance by Kern ALS 220 – 4. The powder was then stored in an airtight bag at room temperature and used for further extraction.

Extraction of Crude Extracts

Active components in the Soursop Leaves were extracted using aqueous (water) and methanol solvents.

Aqueous and Methanol Extract preparation

Twenty grams of grounded leaves were soaked in 200 ml sterile distilled water and methanol for 72hrs with interval shaking. Then the mixtures were centrifuged for 5 min at 5000 rpm. The brown colored aqueous and dark green colored methanol extracts were filtered using British standard mesh filter and concentrated by air drying under constant air current and water bath at 50°C. 1.2g of the extracted powder of the plant leaves under investigation were then dissolved in 3ml of distilled water and 10% dimethyl sulfoxide (DMSO) to a final concentration equal to 400 mg/ml [8].

Phytochemical Analysis

Qualitative Determination of Phytochemical Constituents were done using Standard methods.

Bacterial Sample Isolation and Identification

Samples were collected aseptically from the Laboratory Culture benches and fomites of Nnamdi Azikiwe University Teaching Hospital, (NAUTH) Nnewi using sterile disposable swab sticks. The swab sticks were used to swab surfaces of culture benches in NAUTH and were transferred immediately to the Microbiology laboratory for analysis. Swabs collected from the hospital fomites and environmental surfaces were inoculated into Mannitol Salt Agar (MSA) plates and incubated at 37°C for 48 hours. Golden yellow color colonies showed the fermentation of mannitol which is a presumptive test for *Staphylococcus aureus* [9].

Preparation of McFarland Standard and Standardized Bacterial Inoculum

.5 McFarland standard was prepared by mixing 0.5ml of barium chloride hydrate ($BaCl2.2H2O$) with 99.5 ml of 1% sulfuric acid ($H2SO4$) with constant stirring. This is expected to give an approximate bacterial cell density of 1.0 x 108 cfu/ml and an absorbance of 0.132 at 600 nm wavelength. The standard was distributed into screw cap tubes which was tightly sealed to prevent loss by evaporation and stored protected from light at room temperature. Before use, the standard was vigorously shaken. The direct colony suspension method was used in making a standardized suspension of the cultured organism. A colony from overnight growth (24 hours) on the Mannitol salt agar was suspended in 5 ml of sterile Nutrient broth using a sterile wire loop and incubated at 37°C for 4 hours. The turbidity standard was shaken vigorously before use and used to make a visual comparison with the density of the bacterial suspension against a white background with black lines. The density of the suspension was adjusted to 0.5 McFarland by adding more broth.

Antimicrobial Susceptibility Testing

The antibacterial activity of the investigated extracts was determined by agar well diffusion method according to CLSI 2009 guideline. 0.1ml of the standardized broth was added to a sterile petri dish and 20 ml of molten Mueller Hinton agar was poured to the plate with gentle mixing. It was allowed to gel. After 10 minutes, six 7mm wells were bored in the agar using a sterile corn borer. The plant extract under study was checked for antibacterial activity by introducing different concentrations into each well. The plates were allowed to stand at room temperature for 30 minutes for extract to diffuse into the agar and then the plates were incubated at 37°C for 24 hours. The plates were examined for bacterial growth inhibition by measuring the inhibition zone diameter (IZD) to the

nearest mm with a transparent ruler. The test was performed in duplicates and the average of the two calculated. The Antibiotic Ciprofloxacin tablet diluted to give 200 mg/ml was used as a positive control while distilled water and 10 % DMSO was used as a negative control for aqueous and methanol extract respectively (Lubna and Shurooq, 2017).

Determination of Minimum Inhibitory Concentration (MIC) and MBC

Minimum Inhibitory concentration (MIC) is the least or minimum dilution of the extract which inhibits the visible growth of organism.

Procedure

Two colonies of the test isolate were inoculated into 5ml of sterile nutrient broth and incubated for 30 minutes. The stock solution (400mg/ml) was serially diluted to various concentrations of 200, 100, 50, 25, 12.5, 6.25 and 3.125 mg/ml with diluents water and DMSO for aqueous and methanol extract respectively. 0.5 ml of the test organism was added to each dilution of the extract. The suspension was incubated at 37°C for 24 hours. After incubation, the tubes were observed for turbidity. From the tubes showing no visible sign of growth/turbidity in MIC, 0.1ml of the sample was inoculated onto sterile Mueller Hinton agar using streak plate method. The plates were incubated at 37ºC for 48 hours. The MBC is the lowest concentration that results in killing 99.9% bacteria.

Activity Index

The activity index was calculated to show the relationship between the zone of inhibition of the leaf extracts to that of reference drug. The activity index of the extract was calculated against that of a standard antibiotic, Ciprofloxacin.

$$Activity\ Index = \frac{Diameter\ Zone\ of\ Inhibition\ of\ the\ test\ extract}{Diameter\ Zone\ of\ Inhibition\ of\ the\ standard\ antibiotic}$$

Statistical analysis

SPSS software version 21 was used for the analysis. The data collected was analyzed and presented as mean and standard deviation.

Results

a) Table 1 shows the phytochemical analysis of *Annona muricata* leaf extract studied. The result shows the presence of various bioactive compounds such as alkaloids, saponins, tannins, flavonoids, cardiac glycoside, reducing sugar, paleobotanic and steroids.

b) Table 2 shows the result of antimicrobial activities of aqueous and methanol extracts of *Annona muricata* against *Staphylococcus aureus* using agar well diffusion method by measuring the diameter of growth inhibition zones in duplicates. From the result aqueous extract of *Annona muricata* had little inhibitory effect with mean values of 18 mm, 16.5 mm, 14 mm and 10 mm in 400 mg/ml, 200 mg/ml, 100 mg/ml, 50 mg/ml respectively. Then the methanol extract had more inhibitory effect with mean values of 25.5 mm, 23.5 mm, 20 mm, 16 mm, 14.5 mm and 11 mm in 400 mg/ml, 200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, and 12.5 mg/ml respectively. Also, Ciprofloxacin as positive control drug had inhibitory effect of 25 mm and DMSO and water as negative control, for methanolic extract had no inhibitory effect.

c) Table 3 showed the Minimum inhibitory concentration (MIC) and MBC of leaf extracts of *Annona muricata* against *Staphylococcus aureus*. The result revealed that methanol extract of *Annona muricata* had MIC of 12.5 mg/ml and aqueous extract had MIC of 25 mg/ml. The methanolic extract of the plant had an MBC of 6.25 mg/ml while the MBC of aqueous leaf extract was uncertain.

d) Table 4 shows the result of activity index of the extract against the isolated organism. The activity index was calculated to show the relationship between the zone of inhibition of the leaf extracts to that of reference drug (Ciprofloxacin). The result revealed that the maximum value of activity index was recorded in 400 mg/ml for methanol extract while the least value of activity index was noted in 50 mg/ml for aqueous extract.

Table 1: Phytochemical Compounds Present in the Leaf Extract of Annona Muricata.

Phytochemical compound	Test	Observation	Indication	Intensity
Tannins	Ferric Chloride	Blue green colour	Positive	+++
Phlobatannins	HCl	Cloudy red colour	Positive	+
Saponins	Frothing test	Frothing formation	Positive	+
Flavonoids	HCl	Red or Orange colour	Positive	++
Cardiac glycoside	Keller-Kilani	Brown ring	Positive	++
Reducing sugar	Fehling test	Red precipitates	Positive	+
Alkaloids	Meyer	Turbidity	Positive	+
Anthraquinone	Ammonia test	No colour change	Negative	-

Table 2: Average Diameter of Zones of Inhibition of Aqueous and Methanolic Leaf Extract of Annona Muricata on Isolated S Aureus.

Extract	Concentration (mg/ml)								Positive control (Ciprofloxacin)	Negative control (DMSO/ Water)
	Diameter of inhibition (mm)									
	400	200	100	50	25	12.5	6.25	3.125		
Aqueous	18.0	16.5	14.0	10.0	-	-	-	-	25.0	0.0
Methanol	25.5	23.5	20.0	16.0	14.5	11.0	-	-	25.0	0.0

Table 3: The MIC and MBC of Leaf Extracts against isolated *S aureus*.

Extract	MIC (mg/ml)	MBC (mg/ml)
Aqueous leaf extract	25	-
Methanol leaf extract	12.5	6.25

Table 4: Activity index of extracts on *S aureus*.

Concentration (mg/ml)	Aqueous extract	Methanol extract
400	0.72	1.02
200	0.66	0.94
100	0.56	0.80
50	0.40	0.64
25	-	0.58
12.5	-	0.44

Discussion

The increased rate of antibiotic resistance found in *S. aureus* has rendered some of the previously discovered synthetic antimicrobial agents ineffective in the treatment of infections caused by this microorganism. These infections range from postsurgical wound infection, osteomyelitis, folliculitis, cellulitis, boils, endocarditis, staphylococcal pneumonia, conjunctivitis, metastatic abscesses, and staphylococcal food poisoning. In order to remedy this situation, scientists have sought for another alternative which is herbal medicine (the use of plant materials to treat infectious diseases) to kill this ubiquitous microorganism and at the same time reduce the high rate of infectivity and morbidity caused by them especially in hospital settings. This is based on the knowledge that plants are important sources of potentially useful structures for the development of new chemotherapeutic agents and the first step towards this goal is in vitro antibacterial activity assay.

Furthermore, there has been a gradual revival of interest in the use of medicinal plants in developing countries because herbal medicines have been reported safe and without any adverse side effect especially when compared with synthetic drugs. In Table 1, the qualitative phytochemical analysis of the present study revealed the presence of tannins, alkaloids, steroids, paleobotanies, cardiac glycosides, saponins, terpenoids and flavonoids which have been found in vitro to have antimicrobial properties. Findings from this research have shown that methanol yielded a higher-end concentration compared to aqueous extraction solvent used. Methanol has the ability to extract bioactive compounds like Tannins, saponins, flavonoid, alkaloid, phenol, terpenoid and steroid which have higher concentrations in leaves of the plant used in this study as shown in Table 1. In Table 2, the mean diameter of zones of inhibition of aqueous and methanolic extracts showed significant antibacterial activity against the test organism, *S. aureus*. The comparative antimicrobial activity between methanolic and aqueous extracts of *Annona muricata* and the standard antibiotic Ciprofloxacin revealed that the methanolic extract showed significant antimicrobial efficacy.

In Table 3, both methanolic and aqueous extract exhibited a minimum inhibitory concentration of 12.5 mg/ml and 25 mg/ml respectively. At this concentration, there was no turbidity which indicates bacterial growth in the tubes with these dilutions. This is in accordance with the work done by Inyanda-Joel et al., (2014) and [10]. The Minimum bactericidal concentration of methanolic extract was 6.25 mg/ml; at this concentration, there was no growth on the inoculated agar. In Table 4, maximum value of activity index was recorded in 400 mg/ml of methanolic extract while the least value of activity index was noted in 50 mg/ml of aqueous extract. The activity index values are helpful in estimating the potential of antimicrobial activity quantitatively compared to the respective standards. The methanolic extract has shown higher activity index values against the test *S. aureus* which means that the methanolic leaf extract of Annona muricata has good activity against the Gram-positive *Staphylococcus aureus*.

Conclusion

The present finding showed that *Annona muricata* leaves extract possess interesting inhibitory properties against *S. aureus* species. Methanolic extract exhibited appreciable antibacterial activity. This data is promising and could encourage further research on toxicological and pharmacological aspects of these extract-products in order to support their possible rational use in antimicrobial therapy.

Ethical Approval

The Ethical Approval was gotten from Nnamdi Azikiwe University Teaching Hospital Health Research Ethics Committee.

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