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Antibacterial Activity of Annona Muricata Leaf Extract Against Clinical Isolates of Staphylococcus Aureus and Escherichia Coli

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Abstract

The increasing resistance of pathogenic bacteria to conventional antibiotics has necessitated the search for alternative antimicrobial agents, particularly from medicinal plants. This study investigates the antibacterial activity of Annona muricata (soursop) leaf extract against clinical isolates of Staphylococcus aureus and Escherichia coli. Phytochemical analysis revealed the presence of bioactive compounds including tannins, alkaloids, flavonoids, steroids and saponins (absent in aqueous extract). Using agar well diffusion methods, ethanolic and aqueous extracts demonstrated dose-dependent antibacterial activity, with ethanolic extract showing greater efficacy. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for the ethanolic extract were 25 mg/ml and 50 mg/ml respectively while the acqueous extract recorded higher values at 50 mg/ml and 100 mg/ml. the findings suggest that Annona muricata leaves possess potent antibacterial properties and may serve as a natural source for the development of new antimicrobial agents.

Keywords: antibacterial activity, antibiotic resistance, Annona muricata (soursop), S. aureus, E. coli, MIC and MBC.

Introduction

The use of plants as medicine is a worldwide phenomenon, plants not only provide safe and cost-effective remedies, they are also available and accessible at affordable prices. The use of natural ingredients as treatments for various diseases is increasing. Plants are a source of natural ingredients that are widely used as medicines (Amarasinghe *et al.,* 2020). The compounds present in plants are responsible for their activities against various diseases, and studies can be performed to identify the active compounds in plants and determine their pharmacological activities against diseases (Moghadamtousi *et al.,* 2015). The alarming increase in bacterial resistance to existing antibiotics as well as public interest in herbal medicine demands that a renewed effort be made to seek antibacterial agents effective against pathogenic bacteria (Sultana *et al*., 2015). It is common knowledge that novel pathogens resistance mechanisms against antimicrobials are emerging and spreading worldwide increasing bacterial resistance due to the dissemination of antibiotic resistance genes (ARGs) via plasmids and transposable element between microbial communities, hindering the effectiveness of the treatment of common infectious diseases and causing prolonged illness, disability and death (Le *et al.,*

2018; WHO, 2019). Over usage of antimicrobial agents in hospitals and by the community is a strong impetus for antimicrobial-resistant pathogens (Nagel *et al*., 2016).

Further, the use of antimicrobial agents in stockbreeding and agriculture contributes towards the selection of potentially resistant bacteria transferred to humans, directly or indirectly, through the food chain, representing a public health hazard (Lhermie *et al*., 2019). Indeed, sub-therapeutic antimicrobial concentrations may promote the development of acquired bacterial resistance by non-specific mutagenesis (Le *et al*., 2018; Kohanski *et al*., 2010). Natural products, especially those derived from plants have been used to help mankind sustain its health since the dawn of medicine. The emergence and spread of antibiotic resistance and the evolution of new strains of pathogenic agents are a great concern to community health worldwide wide and entail the development of new antimicrobials or potential sources of novel drugs (Manandhar *et al*., 2019). Over the past century, the phytochemicals in plants have been a pivotal pipeline for pharmaceutical discovery. The importance of the active ingredients of plants in agriculture and medicine has stimulated significant scientific interest in the biological activities of these substances (Moghadamtousi *et al*., 2015).

Plants are not merely chemically complex compounds, but their components may act synergistically on multiple targets. They may not only increase the efficacy but also minimize the possibility of resistance-developing pathogens (Wagner and Ulrich-Merzenich, 2009). Over the last decades, various plant-derived compounds and their active principles have been analyzed for phytochemicals with anti-bacterial activity (Chowdaiah *et al*., 2019). In a pharmaceutical landscape, plants with a long history of use in ethno-medicine are a rich source of active phytoconstituents that provide medicinal or health benefits against various ailments and diseases. One such plants with extensive traditional use is *Annona muricata* (Moghadamtousi *et al*., 2015).

The genus *Annona* comprises over 70 species among which *Annona muricata* is the most widely grown. *Annona muricata* has been empirically employed in tropical regions to prevent and alleviate diverse ailments such as fever, pain, respiratory and skin diseases, parasites, bacterial infections, hypertension, inflammation, diabetes and cancer (Coria-Tellez *et al*., 2018). The use of *Annona muricata* in medicine has been reportedly by researchers to have an antimicrobial effect against common pathogen and providing the solutions related to human" diseases (Agunloye *et al*., 2020). *Annona muricata* can be consumed raw or used as teas, smoothies, desserts and juices. The fruit provides a variety of heath supportive nutrients such as fiber and vitamin C, which may protect against several common health conditions. The benefit of *Annona muricata* and its leaves are mainly derived from its high fiber content, as it can reduce how quickly sugar is absorbed as well as help with constipation (Rady *et al*., 2018). *Annona muricata* (*Annonaceae*) has been utilized as a medicinal remedy for many years, attracting many scientists to investigate this plant such as leaves and bark, have been used for medicinal purposes (Agunlooye *et al.,* 2020). Over 200 chemical compounds have been discovered and extracted, including phenolics, steroids, and alkaloids (Zubaidi *et al.,* 2023). It is also cheap, can be easily accessed, and has environmental friendliness compared to current commercialized medications, which is a good package to be considered for new potential medications (Zubaidi *et al*., 2023).

The aim of this study was to determine the antibacterial activity of the leaves of Annona muricata against clinical isolates of *Staphylococcus aureus* and *Escherichia coli*.

Materials and Methods

Sample Collection

Fresh leaves of *Annona muricata* were collected from the garden at Kaduna Polytechnic housing quarters, Kaduna State, Nigeria and transported to the laboratory in a clean polythene bag. The leaf was taken for identification and authentication at the herbarium of the Department of Biological Science, Kaduna State University, Kaduna State, Nigeria.

Collection of Bacterial Isolates

The clinical test isolates used in this study are *Staphylococcus aureus* and *Escherichia coli*. Pure culture of the bacterial isolates was sourced from the Microbiology Laboratory, Shehu

Muhammad Kangiwa Medical Center, Kaduna Polytechnic, Kaduna State, Nigeria.

Preparation of the Plant Material

Using the method adopted in similar surveys by Adeleye *et al*. (2016), the leave samples were thoroughly washed with clean water. The washed leaves were then air dried at room temperature for one week and grinded to powder using a clean mortar and pestle. The powdered leaves was stored in a clean air tight container.

Extraction Method

The maceration method of extraction was used. The maceration process was done by soaking 300g of the dried powdered sample in a clean container containing 600ml of the solvent (ethanol and distilled water) respectively for 3 days. The extract were filtered using Whatman's filter paper and the extract were concentrated by drying in a water bath.

Preparation of the Extract Concentration

Stock solutions of the ethanol and aqueous extracts was prepared by dissolving two gram (2g) of the extract in 9ml of distilled water in a test tube respectively to obtained 200mg/ml. Using serial dilution method, 1ml was taken using a sterile syringe from the 200mg/ml and transferred into a test tube containing 9ml of sterile distilled water to obtain 100mg/ml. One milliliter (1ml) was taken from 100mg/mL concentration and transferred into a test tube containing 9ml of sterile distilled water to obtain 50mg/mL concentration. Another one milliliter (1 ml) was taken from 50mg/mL concentration and transferred into a test tube containing 9ml of sterile distilled water to obtain 25mg/mL concentration (Olorunnisola *et al*., 2018).

Preparation of McFarland Standard

Ten percent (10%) of Barium chloride (BaCl) solution was prepared by mixing one gram (1g) of anhydrous Barium chloride (BaCl) in 100ml of distilled water. One percent (1%) Sulfuric acid (H_2SO_4) solution was prepared by mixing 1ml of concentrated Sulfuric acid in 99ml of distilled water. The 1% BaCI and 1% H_2SO_4 were mixed in an appropriate proportion as per the concentration required. The resulting mixture was placed in a foil-covered screw cap and was stored at room temperature (25°C).

Standardization of Inoculum

Normal saline solution was prepared (0.9% Sodium chloride, NaCl) by dissolving 9g of NaCl in 100ml of distilled water in a clean beaker, and autoclave at 121°C for 15 minutes. About 10, ml of each overnight broth culture of *Staphylococcus aureus* and *Escherichia coli* were dispensed into separate test tubes containing the sterile normal saline. The density of the suspension of the bacterial cell was compared to the 0.5 McFarland turbidity Standards in front of a light against a white background with contrasting black lines. The turbidity of the bacterial cell suspension was adjusted to match 0.5 McFarland standards. The standardized inocula was used for the antibacterial assay (Aliyu *et al*., 2018).

Phytochemical Analysis of the leave Extract

The quantitative chemical analysis of the leave extract was carried out for the presence of tannins, alkaloids, flavonoids and saponins (Ajetunmobi and Towolawi, 2014). A small amount of the extract was mixed with distilled water and heated on a water bath, the mixture was filtered and ferric chloride was added to the filtrate, a blue solution indicated the absence of Tannins and dark green colour indicating the presence of Tannins. The aqueous extract (3ml) was stirred with 3ml of 1% Hydrochloric acid (HCI) on a steam bath. Mayer's reagent was then added to the mixture, turbidity of the resulting

precipitate was taken as positive evidence of alkaloids on the extract. About 0.2g of the extract was dissolve in dilute Sodium hydroxide solution and 2ml of Hydrochloric acid was added, a yellow solution that turned colorless indicated the presence of Flavonoids in the extract. About 0.2g of plant extract was mixed with distilled water and heated to boil, frothing (appearance of creamy mix of small bubbles) showed the presence of Saponins. Acetic anhydride (2ml) was added to 0.5g of the extract in a test tube. It was then followed by the addition of 2ml of Sulfuric acid, a color change from violet to blue or green indicated the presence of steroids on the extract.

Determination of Antibacterial Activity

The agar-well diffusion assays described by Aliyu *et al*. (2018) was used to test the antibacterial activity of the leave extracts. Nutrient agar was prepared and 20ml of the Nutrient agar was poured into a sterile petri dish and was allowed to solidify. The standardized inoculum was pipetted (0.1ml) and spread on the surface of the solidified Muller Hinton agar. Five (5) equidistant wells of 6mm in diameter and 4mm in depth were punched on the solidified agar plates using a sterile cork-borer and each hole was labeled with the number of concentration of the leave extract (200mg/ml, 100, 50 and 25mg/ml). Varied concentrations of the leave extracts-were dispensed into the labelled hole accordingly with the aid of a sterile micropipette. One more hole was made for the standard antibiotic (ciprofloxacin) in which the zone of inhibition was measured so as to compare with the zone of inhibition of the test extract. The plates were allowed to stand for about 30 minutes at room temperature to pre-diffuse into the agar. The plates were then incubated at 37°C for 24 hours. This was done in duplicates. Susceptibility of the bacteria to the extracts were recorded by measuring the zones diameter of inhibition in millimeter (mm) as described by Aliyu *et al*. (2018).

Determination of Minimum Inhibitory Concentration (MIC)

The MIC for the ethanolic and aqueous leave extracts of *Annona muricata* were determined by the broth dilution methods. Each concentration of the extract was dispensed into sterile nutrient broth in separate test tube. Each test tube was inoculated with 1.0ml of the test organisms. This was done in duplicate and incubated at 37°C for 24 hours. The tubes were examined for bacterial growth by observing turbidity. The lowest concentration where there was no turbidity was observed and recorded as the MIC for the particular extract (Aliyu *et al*., 2018).

Determination of Minimum Bactericidal Concentration (MBC)

For each set of test tubes in MIC determined, a loopful of the broth was collected from those tubes that showed no turbidity and was inoculated in duplicate on sterile Nutrient agar plates by streaking and was incubated at 37°C for 24 hours. The concentration at which no visible growth was observed and was recorded as MBC (Aliyu *et al*., 2018).

Results

The ethanol extract of *Annona muricata* leaf was green in colour, sticky and the percentage yield of the extract was 3.2% while the aqueous extract of *Annona muricata* was dark brown in color, watery and the percentage yield of the extract was 4.23% as shown below in table 1.

Table 1: Physical Characteristics and Percentage Yield of Ethanolic and Aqueous Extract of *Annona muricata* leaf

Characteristics	Ethanolic Extract	Aqueous Extract
Color	Greenish	Dark brown
Texture	Sticky	Watery
Initial Weight of Sample	300 grams	300 grams
Final Weight of Sample	9.6 grams	12.69 grams
Percentage Yield	3.2%	4.23%

Table 2, shows the phytochemical constituents detected in the aqueous extract of *Annona muricata* leaf were tannins, alkaloids, flavonoids and steroids in both the extract but saponins was not detected in the aqueous leaf extract.

Table 2: Phytochemical Constituents of Ethanolic and Aqueous Extract of *Annona muricata* leaf

Phytochemical constituents	Ethanolic extract	Aqueous extract
Alkaloids	+	+
Flavonoids	+	+
Saponins	+	-
Tannins	+	+
Steroids	+	+

Keys: + detected
- not detected

Table 3, presented the antibacterial activity of the extract on the test isolates. All the concentrations of the extracts showed activity on *Escherichia coli* and *Staphylococcus aureus*. The organisms were susceptible at different concentrations of the extract and the activities were dose dependent.

Table 3: antibacterial Activities of Ethanolic and Aqueous Extracts of *Annona muricata* leaf against the test Isolates

Test Organisms	Mean zone of Inhibition (mm) Concentrations (mg/ml)				
		Ethanolic Extract			Control
	200	100	50	25	Cipro25mg
E. coli	24.5 ± 0.4	13.4 ± 1.5	9.0 ± 3.0	6.0 ± 2.2	29.0 ± 0.0
S. aureus	28.0 ± 1.0	17.4 ± 2.3	13.0 ± 3.6	10.0 ± 3.2	29.0 ± 2.0
P-value	1.00	0.812	0.064	0.089	0.028
		Aqueous Extract			
E. coli	16.0 ± 4.0	10.0 ± 0.0	9.0 ± 3.0	6.0 ± 2.5	22.5 ± 4.7
S. aureus	20.0 ±0.0	15.0 ± 4.7	9.0 ± 3.0	6.0 ± 2.2	30.0 ± 0.5
P-value	0.041	0.098	0.036	0.976	0.048

Key: ± = Standard Deviation; mg/ml = milligram per mill; mm = milimetre; *S. aureus* = *Staphylococcus aureus*; *E. coli* = *Escherichia coli.*

Table 4, The Minimum Inhibitory Concentration (MIC) for the ethanol extract of *Annona muricata* leaf was 25mg/ml and the Minimum Bactericidal Concentration (MBC) was 50mg/ml on both the test Isolates. The Minimum Inhibitory Concentration (MIC) for the aqueous extract of *Annona muricata* leaf was 50mg/ml and the Minimum Bactericidal Concentration (MBC) was 100mg/ml on both the test Isolates.

Table 4: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Ethanolic and Aqueous Extract of Annona muricata leaf Against the Test Organisms

Test Organisms	Ethanolic Extract (mg/ml) MIC (MBC)	Aqueous Extract (mg/ml) MIC (MBC)
E. coli	25 (50)	50 (100)
S. aureus	25 (50)	50 (100)

Discussion

The Phytochemical compounds Tannins, Alkaloids, Flavonoids, Saponins and Steroids were detected in Ethanolic and Aqueous Extract of *Annona muricata* leaf with the exception of saponins which was

not detected in the aqueous extract of *Annona muricata* leaf. These results concur with the findings of Alatas *et al*. (2020) and Adeleye *et al*. (2016) were Alkaloids, Flavonoids, Saponins and Tannins were detected in the Ethanolic and Aqueous extract of *Annona muricata* leaf with the exception of saponins which was not detected in the aqueous extract of *Annona muricata* leaf. The significant Antimicrobial properties of the leaf extract could be attributed to the presence of these bioactive compounds. Tannins exert their antimicrobial effects through mechanisms such as membrane disruption, binding to proteins, enzyme inhibition, substrate deprivation and metal ion complexation (Mutakin *et al*., 2022).

Medicinal Plants that have tannins as their main component are used in the treatment of intestinal disorder such as diarrhoea and dysentery (Mutakin *et al*., 2022). Alkaloids produce antimicrobial effects by interfering with processes such as deoxyribonucleic acid (DNA) replication and ribonucleic acid (RNA) transcription which are vital to microbial functioning.

Saponins are classes of glycosides which demonstrate antifungal properties. Saponins inhibit the growth or kill microbes by interacting with membrane sterols (Rady *et al*., 2018). Synergistic interactions between some of these chemical groups may produce greater activity against pathogenic microorganisms (Mutakin *et al*., 2022). The mechanism of action of steroids as antibacterial actually is by destroying bacterial cell membranes as observed by Bun *et al*. (2020). Carmona *et al*. (2020) in their work proved that the antibacterial action of saponins causes increase in the permeability of cell membranes and thus inhibit bacterial growth, increase the efficiency of protein synthesis and renders cells unstable which leads to cell lysis. Flavonoid compounds contained in the extract of the leaves of *Annona muricata* potential as an antibacterial for being able to inhibit the growth of bacteria by destroying the wall permeability, microscopes, lysosomes and bacterial cells as a result of interaction between flavonoids with DNA (Carmona *et al*., 2020).

In this study, the leaf extracts of *Annona muricata* inhibited the growth of the bacteria tested *Escherichia coli* and *Staphylococcus aureus*, this is in agreement with the report of Rady *et al*. (2018), who reported the effectiveness of *Annona muricata* leaf extract against *S. aureus* and *E. coli* with values of 20mm-25mm for *S. aureus* and 17mm-21mm for *E. coli*. This shows that *Annona muricata* leaf could be a source of new drug effective against the infectious bacteria tested. The results of the antibacterial screening have shown that at varying concentrations, all the extracts have antibacterial activity on the test organisms; *Escherichia coli* and *Staphylococcus aureus*. The extract are more active at high concentration and less active at low concentration. This result agrees with the results obtained from the research carried out by Evangelista *et al.* (2017). The MIC of the ethanolic extract of *Annona muricata* leaf was 25mg/ml and the MBC was 50mg/ml for both *Escherichia coli* and *Staphylococcus aureus*. The MIC of aqueous extract of *Annona muricata* leaf was 50mg/ml and the MBC was 100mg/ml for both *Escherichia coli* and *Staphylococcus aureus*. The MBC results for the leaf extract of the Plant was found to be higher than the MIC value. That is, the extracts are bacteriostatic at lower concentrations and bactericidal at higher concentrations (Sun *et al*., 2021).

Conclusion

The study demonstrated that the ethanolic and aqueous extract of *Annona muricata* leaves are rich in potent bioactive constituents including tannins, alkaloids, flavonoids, saponins and steroids with saponins absent in the aqueous extract. All the bacterial isolates tested responded to both types of extracts, and their antibacterial effects were found to increase with concentration, as reflected in the MIC and MBC results. This shows that *Annona muricata* leaf could be potential sources of new drug effective against bacteria in fighting infectious disease and overcoming the menace of antibiotic resistance.

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