Original Research Article

Antibacterial Potential of Annona muricata Leaf Ethyl Acetate Extract: A Promising Natural Source for Novel Antimicrobial Therapies

ABSTRACT

Aims: Bacterial infections pose a significant public health challenge, necessitating the search for new antimicrobial agents. This study investigated the antibacterial potential of *Annona muricata* (Linn.) leaf ethyl acetate extract against various bacteria strains.
Study design: An experimental design was adopted for this study.
Place and Duration of Study: Fresh leaves of *Annona muricata* (Linn.) were collected from Modakeke, Osun State, South-West, Nigeria. Proper identification and authentication of the leaves were performed at the herbarium unit of the Department of Botany, Obafemi Awolowo University (OAU), Ile-Ife, Osun State, Nigeria.
Methodology: The plant material was extracted using ethyl acetate, and concentration-dependent assays determined antibacterial activity, including minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Phytochemical analysis identified bioactive compounds.
Results: Results showed significant antibacterial activity of the extract against nine out of fifteen tested organisms, including *Staphylococcus aureus, Aerococcus suis, Limonella grimontii, Limorella richardii, Globicatella suifidifaciens, Salinicoccus roseus, Staphylococcus muscae, Proteus hauseri,* and *Staphylococcus membrensis*. Susceptibility to the extract increased with higher concentrations. Comparative analysis with ampicillin indicated superior inhibitory properties. Phytochemical analysis revealed terpenoids, flavonoids, alkaloids, and tannins, known for their antimicrobial properties. These bioactive constituents likely contribute to the observed antibacterial effects.
Conclusion: The ethyl acetate extract from *A. muricata* leaf holds promise as a natural and effective alternative for developing antimicrobial therapies. Future research should focus on isolating and characterizing the active compounds and investigating their mechanisms of action. This study supports the traditional use of *A. muricata* as a medicinal plant and highlights its potential as a source for novel antibacterial agents. Further exploration in this area could contribute to alternative strategies against bacterial infections, especially in light of antibiotic resistance concerns.

Keywords: Annona muricata; antibacterial; bacterial; extract

1. INTRODUCTION

Throughout history, disease has posed a significant challenge to human health. Medicinal plants have long been recognized for their therapeutic properties and have been used for centuries to combat the effects of disease. The utilization of plant-based remedies in medical practice has evolved from traditional approaches to modern medicine, aiming to enhance the quality of life for patients worldwide. Nature-derived medicines derived from plants are often considered safer alternatives with reduced or no side effects [1]. Plants have played a crucial role in human existence since the dawn of life, and various plant species and parts have been employed to treat a wide range of diseases [2-4].

Numerous studies conducted worldwide have investigated the antibacterial properties of plants, leading to the identification of potential therapeutic alternatives [5-8]. These properties are attributed to various compounds found in plants, including quinine, tannins, alkaloids (such as anonaine and anoniine), acetogenins, flavonoids, and others. The exploration of these plant-derived products, either as pure compounds or standardized extracts, offers a vast array of opportunities for the development of new drug leads due to the immense chemical diversity available in plants. Consequently, many medicinal plants used in traditional medicine have been scientifically evaluated and shown to possess bactericidal properties.

Annona muricata (Linn)., belonging to the family *Annonaceae* and the genus *Annona*, is a well-known species primarily recognized for its edible fruits commonly referred to as "sour sop." The name "sour sop" is derived from its slightly acidic flavor when fully ripe. In English, it is commonly known as 'Graviola' or 'sour sop', and in some regions like India and Australia, it is also referred to as custard apple (although this term also encompasses Annona reticulata, a closely related species). Spanish-speaking countries refer to it as guanabana or guanaba, while other regions use names such as sorsaka, zuurzak, corossol, and graviola.

Annona muricata L., has a significant history of traditional use in herbal medicine. Various parts of the plant, including the bark, leaves, roots, fruit, and seeds, are utilized in natural medicine practices in tropical regions [9,10]. These different plant parts possess distinct medicinal properties and are attributed to various therapeutic uses. Annona muricata exhibits a wide range of pharmacological activities, such as antifungal, antitumor, analgesic, hypotensive, anti-inflammatory, and immune-enhancing effects [6,9,11]. Additionally, it has been recognized for its sedative, ulcer treatment, hypotensive, nervine, and oxytocic properties [10,12]. Phytochemical analysis of Annona muricata has identified alkaloids, flavonoids, carbohydrates, cardiac glycosides, saponins, tannins, phytosterols, terpenoids, and proteins [13]. These observations underscore the potential therapeutic value of Annona muricata in traditional medicine.

Therefore, the aim of this study was to investigate the antibacterial activities of the ethyl acetate extract of *Annona muricata*, determine the minimum inhibitory concentration and minimum bactericidal concentration of the leaf extract, and analyze the phytochemical constituents of *Annona muricata*. By exploring these aspects, the study aims to contribute to our understanding of the medicinal properties and potential applications of *Annona muricata*, thereby providing valuable insights for further research and development in the field of natural medicine.

2. METHODOLOGY

2.1 Collection and Authentication of Plant Materials

Fresh leaves of *Annona muricata* (Linn.) were obtained from Modakeke, Osun State, South-West, Nigeria. The leaves were carefully identified and authenticated at the herbarium unit of the Department of Botany, Obafemi Awolowo University (OAU), Ile-Ife, Osun State, Nigeria. Subsequently, the leaves were air dried at room temperature, ground to a fine powder [14] in the drug research and production unit at the Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, and stored properly until further use.

2.2 Source of Test Organisms

The bacterial strains used in this study were sourced from the Department of Microbiology's research laboratory, OAU, Ile-Ife, Nigeria. The test organisms were sub-cultured on nutrient agar and incubated at 37ºC for 24 hours. Colonies from the cultures were aseptically transferred to 5ml of nutrient broth in test tubes and incubated at 37ºC for 18 hours.

2.3 Serial Dilution and Colony Counting

Serial dilutions were performed using 1 ml of the test organism. Subsequently, 0.4 ml of the 10µl serial dilution was poured into Petri dishes and incubated for 18 hours. The resulting colonies were then counted using an electronic colony counter.

2.4 Preparation of Plant Powder

The leaves of *Annona muricata* were dried under shade for 2-3 weeks and subsequently ground into a powdered form.

2.5 Preparation of Plant Extracts

To prepare the plant extracts, 2.5 L of ethyl acetate was used to soak 1 kg of the powdered leaves in a glass jar for 5 days. The suspension was thoroughly agitated every 12 hours. On the fifth day, the suspension was filtered, and the solvent was evaporated to dryness at 40ºC using a rotary evaporator [15] at the Central Science Laboratory, OAU, Ile-Ife, Osun State, Nigeria. The resulting crude extract was stored in glass Petri dishes.

2.6 Reconstitution of the Plant Extract

The crude extract was reconstituted to a concentration of 35 mg/ml. Serial dilutions were then performed to obtain subsequent concentrations of 17.50 mg/ml, 8.75 mg/ml, 4.38 mg/ml, and 2.19 mg/ml, which were utilized for determining the minimum inhibitory concentration (MIC).

A total of 1.4 g of the crude extract was dissolved in a mixture of water and dimethyl sulfoxide (DMSO) in a ratio of 6:4. Two-fold serial dilutions were carried out using 5 ml of the diluted extract (stock) and 5 ml of water to obtain five dilutions. Each dilution was added to McCartney bottles containing 15 ml of sterilized nutrient agar, thoroughly mixed, and then poured into labeled Petri dishes. The Petri dishes were divided based on the number of test organisms used, and each dilution was appropriately labeled on the dishes.

The reconstituted concentrations were calculated as follows:

Concentration = mass/volume
Concentration of the crude extract = Unknown
Concentration of the crude extract = 1400 mg/10 ml = 140 mg/ml
Mass of the crude extract = 1400 mg
Volume of solvent (water and DMSO in a ratio of 6:4) used for dissolving = 10 ml
Using the dilution formula:
$C_1V_1 = C_2V_2$
C_1 = Concentration of the crude extract = 140 mg/ml
C_2 = Reconstituted concentration = Unknown
V_1 = Volume of the crude extract = 5 ml
V_2 = Volume of nutrient agar + dissolved extract = (15 ml + 5 ml) = 20 ml
140 mg/ml × 5 ml = 20 ml × C
C_2 = 140 mg/ml × 5 ml / 20 ml = 35 mg/ml

2.7 Culture and Maintenance of Microorganisms

Pure cultures of the experimental bacteria were maintained on nutrient agar medium. Each bacterial strain was regularly sub-cultured on the same medium and stored at 4ºC before use in the experiments.

2.8 Antibacterial Susceptibility Test

2.8.1 Agar Well Diffusion Method

A 24-hour culture of the test organisms was inoculated into nutrient broth and incubated for 4-18 hours. The cultures were then spread on Mueller Hilton agar plates. Wells were made on the plates, and varying concentrations of the leaf extract (35 mg/ml, 17.5 mg/ml, 8.75 mg/ml) were introduced separately into three wells. Ampicillin and dimethyl sulfoxide (DMSO) were introduced into wells 4 and 5, respectively, serving as positive and negative controls. The plates were incubated at 37ºC for 24 hours, and the diameters of the zones of inhibition were measured to the nearest millimeter using a transparent ruler.

2.8.2 Measurement of Antibacterial Activity of Annona muricata Leaf Extract Using Agar Well Diffusion

The antibacterial activity of the *Annona muricata* leaf extract was assessed by measuring the zones of inhibition against the tested organisms. The results were compared to the activity of the standard antibiotic, ampicillin.

2.8.3 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration was determined using a serial dilution method to obtain various concentrations of the leaf extract. Nutrient agar plates containing different concentrations of the leaf extract were inoculated with the test organisms. The plates were then incubated at 28ºC for 72 hours. The lowest concentration of the extract that showed no visible growth of the organisms was defined as the MIC.

2.8.4 Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration was determined using a similar serial dilution technique to obtain different concentrations of the *Annona muricata* leaf extract. Nutrient agar plates containing varying concentrations of the leaf extract were inoculated with the test organisms. After incubation at 28ºC for 72 hours, the MBC was determined as the lowest concentration of the extract at which no growth of the organisms was observed.

2.9 Phytochemical Analysis

Phytochemical analysis was conducted to determine the presence or absence of secondary metabolites, including phenols, saponins, flavonoids, alkaloids, tannins, etc., in the Annona muricata leaf extract. These compounds contribute to the antibacterial properties exhibited by the leaf.

2.9.1 Test for Alkaloids (Dragendorff's Test)
A solution of 0.5 g of the plant extract in 5 ml of 1% hydrochloric acid was prepared and subjected to Dragendorff's reagent. The appearance of turbidity or precipitation indicates the presence of alkaloids [16].

2.9.2 Test for Terpenoids (Salkowski Test)
A mixture of 0.2 g of the extract, 2 ml of chloroform, and 3 ml of concentrated sulfuric acid was prepared. The formation of a reddish-brown interface indicates the presence of terpenoids [16].

2.9.3 Test for Sterols
A solution of 0.5 g of the extract in 3 ml of chloroform was prepared and filtered. To the filtrate, 2 ml of concentrated sulfuric acid was added to form a lower layer. The chloroform layer appears red, while the acid layer exhibits a greenish-yellow fluorescence, confirming the presence of sterols [17].

2.9.4 Test for Flavonoids (Shinoda Test)
A solution of 0.2g of the extract in 2 ml of methanol was prepared and heated. Magnesium metal chips were added to the mixture, followed by a few drops of concentrated hydrochloric acid. The appearance of a red or orange coloration indicates the presence of flavonoids [16].

2.9.5 Test for Saponins (Hemolysis Test)
Freshly prepared 7% blood agar medium was used, and wells were created in it. Methanol extracts were applied, with distilled water and methanol serving as negative controls, while a commercial saponins solution was used as a positive control. The plates were incubated at 35ºC for 6 hours, and complete hemolysis of the blood around the extract indicates the presence of saponins [16].

2.9.6 Test for Phenols (Ferric Chloride Test)
Exactly 2 mg of the extract was dissolved in 4 ml of distilled water. A few drops of 10% ferric chloride solution were added to the solution. The observation of a blue or green coloration indicates the presence of phenols [16,17].

2.9.7 Test for Phlobatannins (Hydrochloric Acid Test)
Approximately 2 mg of the extract was dissolved in 4 ml of distilled water and subjected to boiling with 1% aqueous hydrochloric acid. The formation of a red precipitate serves as evidence for the presence of phlobatannins [16,17].

3. RESULTS

3.1 Antibacterial Activity of Ethyl Acetate Extract from *Annona muricata*

Table 1 illustrates the antibacterial efficacy of ethyl acetate extract derived from *Annona muricata* against a panel of test bacteria. The extract concentrations of 35.00 mg/ml, 17.50 mg/ml, 8.75 mg/ml, and 4.38 mg/ml exhibited significant antibacterial activity against ten out of twenty-one tested bacterial strains. Notably, these strains encompass *Klebsiella pneumoniae, Proteus vulgaris, Staphylococcus mariliensis, Salinicoccus roseus, Enterobacter asburiae, Streptococcus canis, Streptococcus pyogenes, Streptococcus equi, Limonorella grimontii, Proteus hauseri, Citrobacter werkmanii, Staphylococcus rosterii, Versinia enteroculitica, Macrococcus bruensis, Precoccus suis, Limorella richardii, Staphylococcus aureus, Macroccocus lavnae, Salmonella enterica, Streptococcus salviohxodontae*, and *Klebsiella ozanae.*

3.2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Annona muricata* Leaf Extract

Tables 2 and 3 present the MIC and MBC values of the Annona muricata leaf extract against susceptible test organisms. In certain cases, the MIC and MBC values could not be determined due to growth inhibition observed at all tested concentrations, including the highest concentration. The MIC values ranged from 4.48 mg/ml to 35.00 mg/ml, while the MBC values ranged from 2.19 mg/ml to 17.5 mg/ml.

3.3 Phytochemical Analysis of *Annona muricata* Leaf Extract

Table 4 elucidates the findings of phytochemical analysis performed on the leaf extract of *Annona muricata*. The analysis revealed the presence of diverse phytochemical constituents, notably alkaloids, sterols, and saponins, in the leaf extract.

Table 1: Diameter of Zone of inhibition of *Anonna muricata* extract on test bacteria

Tests Organisms	DMSO (mg/ml)	70 mg/ml	35 mg/ml	17.5 mg/ml	8.75 mg/ml	4.38 mg/ml	Ampicillin (mg/ml)
Staphylococcus aureus	0.00	22.00	13.00	11.00	19.00	0.00	0.00
Aerococcus suis	0.00	7.00	0.00	0.00	0.00	0.00	0.00
Limonellagrimontii	0.00	12.00	0.00	0.00	0.00	0.00	0.00
Limorellarichardii	0.00	11.00	0.00	0.00	0.00	0.00	0.00
Globicatellasuifidifaciens	0.00	15.00	0.00	0.00	0.00	0.00	0.00
Salinincoccusroseus	0.00	13.00	0.00	0.00	0.00	0.00	10.00
Staphylococcus muscae	0.00	6.00	0.00	0.00	0.00	0.00	0.00
Proteus hauseri	0.00	12.00	6.00	0.00	0.00	0.00	0.00

Key:

- Ampicillin = positive control
- DMSO = negative control

Table 2: Minimum Inhibitory Concentration (MIC) of *Annona muricata* extract

Test Organisms	35 mg/ml	17.5 mg/ml	8.75 mg/ml	4.38 mg/ml	2.19 mg/ml
Klebsiella pneumonia	-	-	+	+	+
Proteus vulgaris	-	-	+	+	+
Staphlococcus massiiliensis	-	-	+	-	+
Streptococcus canis	-	-	+	+	+
Streptococcus equi	-	-	+	+	+
Proteus hauseri	-	-	-	+	+
Proteus vulgaris	-	-	+	+	+
Staphlococcus muscae	-	-	+	+	+
Salinicoccus roseus	-	-	+	+	+
Citrobacter werkmanii	-	-	+	-	-
Globicatella sulfifaciens	-	-	+	+	+
Yersinia enterocolitica	-	-	+	+	+
Aerococcus suis	-	-	+	+	+
Liminorella richardia	-	-	-	+	+

Key:
+ represents growth at that concentration
- represents no growth at that concentration

Table 3: Minimum Bactericidal Concentrations of extract on test organisms

Test Organisms	35 mg/ml	17.5 mg/ml	8.75 mg/ml	4.38 mg/ml	2.19 mg/ml
Klebsiella pneumonia	-	+	+	+	+
Proteus vulgaris	-	+	+	+	+
Staphlococcus mariliensis	-	+	+	+	+
Streptococcus canis	-	-	+	+	+
Streptococcus equi	-	-	+	+	+
Proteus hauserii	-	-	+	+	+
Klebsiella ozanae	-	+	+	+	+
Staphlococcus muscae	-	+	+	+	+
Salinicoccus roseus	-	+	+	+	+
Citrobacter werkmanii	-	-	+	-	+
Glabicatella sulfifaciens	-	+	+	+	+
Yersinia enterocolitica	-	-	+	+	+
Aerococcus suis	-	-	+	+	+
Limonella richardii	-	-	+	-	+
Staphylococcus muscae	-	-	+	+	+

Key

\+ represents Growth

\- represents Inhibition (no growth)

Table 4: Phytochemical constituents of *Annona muricata* leaf extract

Phytochemical constituents	Observation
Alkaloids	-
Sterols	-
Terpenoids	+
Tannins	-
Flavonoids	-
Saponins	-
Phlobatannins	+
Phenols	-

Key:

+ The constituent is present

- The constituent is absent

4. DISCUSSION

The concept of the "magic bullet," introduced by Paul Ehrlich, marked the beginning of the antimicrobial era. However, over time, the irrational use of antimicrobial therapies has led to the development of resistance in microorganisms [18]. While antibiotics aim to eliminate or inhibit the growth of pathogenic microorganisms, they can also have detrimental effects on the host. Generalized adverse events are commonly associated with antibiotic use, necessitating the exploration of alternative treatment options and therapies.

Plants have been utilized as medicinal agents since ancient times. The use of plants as alternative medicine offers several advantages, including their diverse and flexible applications, regional availability, and affordability. One key advantage is the potential to reduce adverse reactions. In low- and middle-income countries, where plants enjoy widespread acceptance, their cost-effectiveness and relatively low technological requirements make them an ideal alternative to expensive therapies. Therefore, plant extracts have the potential to serve as superior and safer alternatives, provided they are supported by scientific evidence [19, 20].

The present study investigated the antibacterial effects of the ethyl acetate extract derived from *Annona muricata* (Linn.) leaf. The results demonstrate that the extract exhibited significant antibacterial activity against a subset of the tested bacteria. Out of the fifteen organisms included in the research, nine displayed susceptibilities to the extract, namely *Staphylococcus aureus, Aerococcus suis, Limonella grimontii, Limorella richardii, Globicatella suifidifaciens, Salinincoccus roseus, Staphylococcus muscae, Proteus hauseri*, and *Staphylococcus membrensis*. These findings are consistent with previous studies on *Annona muricata* [21, 22].

The susceptibility test performed using a concentration of 35 mg/ml revealed that Proteus vulgaris was the least susceptible organism, while *Staphylococcus aureus* exhibited the highest susceptibility to the plant extract. Notably, *Staphylococcus aureus* displayed a zone of inhibition measuring 22 mm.

Analysis of the susceptibility test results revealed a correlation between lower extract concentrations and reduced susceptibility of the organisms. Furthermore, the *Annona muricata* leaf extract demonstrated stronger inhibitory properties against the test isolates compared to the reference standard antibiotic (ampicillin). This difference may be attributed to the lower concentration of the antibiotic relative to the extract concentrations used.

Evaluation of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the leaf extract revealed a concentration-dependent effect on the test organisms, suggesting bactericidal activity. The presence of secondary metabolites such as terpenoids, phlobatannins, flavonoids, and alkaloids in the Annona muricata leaf contributes to its antibacterial characteristics.

These findings highlight the potential of *Annona muricata* as a source of antibacterial agents. Further studies should explore the specific bioactive compounds responsible for the observed antibacterial activity and their mechanisms of action.

5. CONCLUSION

The findings of this study demonstrate the significant antibacterial activity of the ethyl acetate extract obtained from *Annona muricata* (Linn.) leaf. The extract exhibited promising inhibitory effects against a panel of tested bacterial isolates, highlighting its potential as an alternative therapeutic agent for combating bacterial infections.

The results revealed that the susceptibility of the bacterial isolates to the Annona muricata leaf extract was concentration-dependent, with higher concentrations showing increased inhibitory effects. This suggests that the extract contains bioactive compounds that possess antibacterial properties.

Furthermore, the phytochemical analysis of the extract revealed the presence of secondary metabolites such as terpenoids, flavonoids, alkaloids, and tannins, which are known for their antimicrobial properties. These bioactive constituents likely contribute to the observed antibacterial activity of the extract.

The findings of this study support the traditional use of *Annona muricata* as a therapeutic plant and provide scientific evidence for its potential as a source of novel antibacterial agents. Further studies are warranted to isolate and characterize the active compounds responsible for the observed antibacterial activity and to evaluate their mechanisms of action.

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