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Biocidal and Phytochemical Analysis of Leaf Extracts of Annona muricata (Linn.)

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Abstract

Annona muricata is used traditionally in the treatment of several human ailments. In the present study, the biocidal activities of A. muricata leaf extracts were evaluated against 10 clinical strains of bacteria and 6 fungal strains. The antimicrobial activity was determined by the disc diffusion method. The ethyl acetate fraction of the leaf extract produced the greatest antibacterial activity (mean zones of inhibition range 27.6 ± 1.2 to 31.3 ± 1.5 mm) followed by the n-butanol fraction (mean zones of inhibition range 21.0 ± 1.7 to 30.3 ± 1.5 mm). However, the n-butanol fraction exhibited the greatest antifungal effect.

Minimum inhibitory concentrations (MIC) of the two best fractions were determined by the broth microdilution method. The MIC values for the ethyl acetate and n-butanol fractions ranged from 0.0313 to 0.0125 µg/ml and 0.0625 to 0.125 µg/ml respectively.

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The ethyl acetate fraction also exhibited remarkable bactericidal activity against Enterococcus faecalis and Bacillus subtilis by achieving complete eradication (100% killing rate) of these organisms after 180 and 210 min, respectively. Phytochemical compounds detected in the plant extracts were alkaloids, anthraquinones, saponnins, flavonoids, tannins, reducing sugars, cardiac glycosides, carbohydrate (ketonic sugar) and phlobatannins. The results of this study indicate that A. muricata leaf extracts could be effectively used against diseases caused by selected human pathogens. It can therefore be further assessed for discovery of bioactive compounds with potential usefulness in antiinfective therapy.

Keywords: Annona muricata; Annonaceae; Biocidal activity; Ethnobotanical; Phytochemical analysis; Antibacterial; Antifungal.

1. Introduction

Ethnobotanical records show that plants have potentials as a natural source of medicinal compounds that may be employed in treating or controlling some infections globally [1,2]. Resistance developed by microorganisms against the available antibiotics and drugs has been on the increase leading to treatment failures [3,4]. There is a continuous search for new and effective antimicrobial agents, especially of natural origin, to combat this development. An appreciation of plants as natural products of importance in medicine necessitates a comprehensive investigation of their biological activities and key phytochemicals.

1.1. Annona muricata

Annona muricata (Linn) belongs to the family annonaceae comprising approximately 130 genera and 2,300 species [5]. A. muricata is variously known under the common names “graviola”, “soursop” and “guanabana” [6,7]. It is an upright, evergreen tree that grows between 5 to 7 m in height, with large, smooth dark green leaves. The plant is indigenous to the warmest tropical areas of South and North America but now has a wide distribution throughout the tropical and subtropical parts of the world including India, Malaysia and Nigeria [8,9]. A. muricata is a medicinal plant with several parts such as the leaves, bark, roots, fruits and seeds used as natural remedies for variety of illnesses. 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 effects [10,11,12,13]. A novel set of phytochemical compounds called acetogenins have been isolated from the leaf, stem, bark and fruits which have been demonstrated to have significant anticancerous properties, and selective toxicity against various types of cancer cells, without harming healthy cells [14,15]. In general, acetogenins in the plant family annonaceae have been documented with antidiabetic, antitumoral, antiparasitic, pesticidal, antiprotozoal, antihelminthic and antimicrobial activities [16,17].

1.2. Aim and Objectives of the Study

The study aimed at providing scientific basis for the traditional use of this plant in the treatment of several human ailments. The aim of the present study was to evaluate the *in vitro* antimicrobial activities of *A. muricata* leaf extracts against pathogenic microorganisms.

Figure 1: Annona muricata leaves on its tree

2. Materials and Methods

2.1. Plant Material

Annona muricata leaves were collected from locations in the Obafemi Awolowo University, Ile-Ife, Nigeria campus and authenticated taxonomically at the Ife herbarium in the University.

2.2. Extraction Preparation

The leaves were air dried for three weeks and then powdered. Extractions were performed by maceration by soaking the powdered A. muricata leaves (2000 g) in 50%v/v aqueous-methanol (5 L) at 25^{o}C for 72 h. The extracts were then filtered using Whatman No. 1 filter paper and concentrated in vacuo at 40^{o}C on a rotary evaporator (Heldolph, Germany) to about one-third of its original volume. Concentrated crude extract of the plant was in turn dissolved in distilled water and partitioned with n-hexane (3×1 L), dichloromethane (3×1 L), ethyl acetate (3×1 L) and n-butanol (3×0.7 L). The partitioned fractions obtained were concentrated to dryness on a rotary evaporator and then screened for their antimicrobial activities.

2.3. Test Microorganisms

Clinical isolates of Staphylococcus aureus, Enterococcus faecalis, Bacillus subtilis and Clostridium sporogenes (Gram-positive bacteria) and Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli and S. marcesens (Gram-negative bacteria) were collected from the Obafemi Awolowo University Teaching Hospitals, Ile-Ife, Nigeria. The fungal species, Aspergillus niger, A. flavus, Penicilium camemberti, Fusarium oxysporium and Candida albicans used were all soil environmental strains.

2.4. Antimicrobial Assays

2.4.1 Disc diffusion assay

Antimicrobial activities were determined by the disc diffusion method [18], with modifications. 200 µl of standardized microbial cell suspensions (10^6 CFU/ml) were inoculated into 20 ml of Mueller Hinton agar and Sabouraud dextrose agar, respectively, and poured into sterile Petri dishes. Dried solvent fractions of A. muricata leaf extract were reconstituted in 10% dimethylsulfoxide (DMSO) solution to give a final concentration of 1000 µg/ml. Sterile paper discs (6 mm) were impregnated with 10 µl of the sterile solutions of the solvent fractions and placed, aseptically, on the agar surface. Standard discs (6 mm) containing the broad spectrum antibiotic, streptomycin (10 µg/disc) (Oxoid, UK), and antifungal agent amphotericin B (10 µg/disc) (Abtek Biologicals Ltd, UK), were used as positive controls. The plates were incubated at 37ºC for 18-24 h for bacterial strains and 31ºC for 72 h for the fungal strains. The experiments were carried out in triplicate and zones of growth inhibition were recorded in millimetres. Statistical analysis were performed using SPSS software.

2.4.2 Determination of minimum inhibitory concentrations (MIC)

Broth microdilution method was used to determine the minimum inhibitory concentrations (MIC) of the two best solvent fractions [19]. The dried solvent fractions were dissolved in 10% DMSO solution to give a concentration of 400 µg/ml. Serial 2-fold dilutions were then made in concentrations ranging from 12.5 to 400.0 µg/ml. The 96-well microtiter plates were prepared by dispensing into each well 100 µl of Mueller Hinton broth. Inoculum (10 µl) of test bacteria suspensions and 50 µl (0.2 mg/ml) of p-iodonitrotetrazolium (INT) chloride were then added into each well. The plates were covered with parafilm, shaken to mix the contents and then incubated at 37^{o}C for 24 h. Each experiment was carried out in triplicates. The MIC was defined as the lowest concentration at which no visible growth was observed. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-colored formazan product by biologically active organisms [20]. Where bacterial growth was inhibited, the solution in the well remained clear after incubation with INT.

2.4.3 Determination of minimum bactericidal concentration (MBC)

Minimum bactericidal concentrations (MBC) of the two best solvent fractions were determined by removing 100 µl of the test bacteria suspension from cultures demonstrating no visible growth in the MIC assay and inoculating them on freshly prepared Mueller Hinton agar plates. Plates were incubated at 37^{o}C for 48 h with experiments carried out in triplicates. The MBC was taken as the concentration of the solvent fraction that did not show any growth on the new set of agar plates.

2.4.4 Determination of killing rate

Rate of killing studies on the most susceptible (Enterococcus faecalis) and least susceptible (Bacillus subtilis) bacterial isolates when exposed to the MBC of the ethyl acetate fraction were carried out using the method of [21]. Standardised inocula (10^6 CFU ml^{-1}) of test organisms (0.5ml) were mixed with 4.5ml of MBC of ethyl

acetate fraction. The preparations were allowed to stand at room temperature and the rate of killing was determined over 3 hours. At each 30 minute interval, 0.1ml of mixture was taken and transferred to 4.5 ml of brain heart infusion broth recovery medium containing 3% "Tween 80" to neutralize the effects of antimicrobial extract carry overs from the test organisms. The suspension was then serially diluted 10-fold with sterile normal saline and plated out on sterile Mueller Hinton agar in triplicates. The plates were incubated at 37^0C for 24 hours. Control plates containing organism suspension without extract were also set up. The number of surviving colonies were counted and recorded against time.

2.5. Phytochemical analysis

A small portion of the dried leaf extract was subjected to phytochemical analysis for the presence of alkaloids, tannins, flavonoids, steroids, saponins, reducing sugars and cardiac glycoside using standard methods described by [22,23].

3. Results

3.1 Antimicrobial activity

All the test bacterial isolates were susceptible to all the solvent fractions of A. muricata leaf extract. The ethyl acetate fraction exhibited the highest antibacterial activities with mean zones of inhibition range 27.6 ± 1.2 to 31.3 ± 1.5 mm (Table 1). This was followed by the n-butanol fraction with mean zones of inhibition range 21.0 ± 1.7 to 30.3 ± 1.5 mm and the dichloromethane solvent fraction with the mean zones of inhibition range 15.6 ± 1.0 to 24.6 ± 0.6 mm. The hexane fraction exhibited the least activity with mean zones of inhibition range 8.6 ± 1.2 to 23.0 ± 1.7 mm. The zones of inhibition range observed for the standard antibiotic streptomycin was 26.3 ± 1.2 to 28.6 ± 0.6 mm. The antibacterial activities of the ethyl acetate and n-butanol fractions compared favourably with that of streptomycin. The n-butanol solvent fraction exhibited the highest antifungal activity against all the test fungal isolates with mean zones of inhibition range 18.7 ± 1.2 to 22.0 ± 2.0 mm (Table 2). This compared well with the mean zones of inhibition produced by the standard antifungal agent amphotericin B. The ethyl acetate fraction had effect only on three of the isolates A. flavus, P. camemberti and T. mentagrophytes while the dichloromethane and hexane fractions had effect only on one of the fungal isolates (Table 2).

3.2 Minimum inhibitory concentration (MIC)

In view of the results of the antibacterial disc diffusion assay, the minimum inhibitory concentrations (MIC) of the active fractions ethyl acetate and n-butanol were determined (Table 3). The MIC values of ethyl acetate fraction ranged between 0.0313 µg/ml and 0.0625 µg/ml. It was most inhibitory against Salmonella typhi, S. aureus, E. faecalis, K. pneumonia and S. marcescens but least inhibitory against B. subtilis, E. coli, B. stearothermophillus, Cl. Sporogenes and P. aeruginosa. The n-butanol fraction exhibited MIC values with range 0.0625 to 0.125 µg/ml with S. aureus, E. faecalis, B. subtilis, S. marcescens, S. typhi and P. aeruginosa being the most inhibited isolates. The least inhibited isolates were K. pneumonia, E. coli, B. stearothermophillus and Cl sporogenes (Table 4).

Table 1: Antibacterial activity of crude extracts of the leaf of Annona muricata

Test bacteria	Fraction analyzed/ Mean zones of inhibition±SD (mm*)				
	HEX (1000 µg/ disc)	DCM (1000 µg/ disc)	ETAC (1000 µg/ disc)	BUT (1000 µg/ disc)	STR (10 µg/ disc)
S. aureaus	17.0±1.0	23.3±1.5	29.3±0.6	26.6±0.6	27.6±0.6
E. faecalis	17.0±1.5	19.7±1.5	29.3±0.6	26.3±1.2	28.6±0.6
B. subtilis	19.0±1.0	20.3±0.6	31.3±1.5	26.6±0.6	26.3±1.5
K. pneumonia	19.0±0.6	18.3±1.2	30.6±2.1	21.0±1.7	28.3±0.6
E. coli	17.3±0.6	23.3±2.1	30.3±0.6	22.3±2.1	27.6±1.5
B. stearothermophillus	19.7±0.6	24.6±0.6	30.6±0.6	24.6±0.6	27.0±2.0
Cl. Sporogenes	8.6±1.2	15.6±1.0	30.6±0.6	30.3±1.5	26.3±1.2
S. marcescens	20.3±1.2	19.7±1.2	30.6±1.5	28.0±0.6	28.3±1.2
S. typhi	23.0±1.7	20.3±0.6	30.3±1.5	28.3±1.2	27.3±1.5
P. aeruginosa	17.6±1.2	21.0±1.0	27.6±1.2	26.3±1.2	27.0±1.7

mm* - Mean of three replicates ± standard deviation; HEX – N-hexane, DCM – dichloromethane, ETAC – ethyl acetate, BUT – n- butanol, STR – streptomycin.

Table 2: Antifungal activity of crude extracts of the leaf of *Annona muricata*

Test fungi	Fraction analyzed/ Mean zones of inhibition±SD (mm*)				
	HEX (1000µg/disc)	DCM (1000µg/disc)	ETAC (1000µg/disc)	BUT (1000µg/disc)	AMPHOT (10µg/disc)
A. niger	0.0	0.0	0.0	22.0±2.0	24.7±1.2
A. flavus	0.0	17.3±2.3	18.3±2.1	21.3±1.2	20.7±1.2
P. camemberti	15.3±1.2	0.0	17.6±2.1	20.6±1.2	21.3±1.2
F. oxysporium	0.0	0.0	0.0	20.0±2.0	23.0±1.0
T.mentagrophytes	0.0	0.0	14.7±1.5	21.0±2.6	22.7±1.2
C. albicans	0.0	0.0	0.0	18.7±1.2	15.7±1.2

mm* - Mean of three replicates ± standard deviation; HEX – N-hexane, DCM – dichloromethane, ETAC – ethyl acetate, BUT – n-butanol, AMPHOT – amphotericin B.

3.3 Minimum bactericidal concentration (MBC)

The MBC values ranged between 0.0625 µg and 0.125 µg/ml for the ethyl acetate fraction and between 0.125 µg/ml and 0.250 µg/ml for the n-butanol fraction (Table 4).

Table 3: Minimum inhibitory concentrations of crude extracts from the leaf of Annona muricata. .

Test bacteria	Average MIC(µg/ml)/Fraction analyzed	
	Ethyl acetate	Butanol
S. aureaus	0.0313	0.0625
E. faecalis	0.0313	0.0625
B. subtilis	0.0625	0.0625
K. pneumonia	0.0313	0.125
E. coli	0.0625	0.125
B. stearothermophillus	0.0625	0.125
Cl. Sporogenes	0.0625	0.125
S. marcescens	0.0313	0.0625
S. typhi	0.0125	0.0625
P. aeruginosa	0.0625	0.0625

Table 4: Minimum bactericidal concentrations of crude extracts from the leaf of Annona muricata

Test bacteria	Average MBC (µg/ml)/Fraction analyzed	
	Ethyl acetate	Butanol
S. aureaus	0.125	0.125
E. faecalis	0.0625	0.250
B. subtilis	0.125	0.250
K. pneumonia	0.0625	0.250
E. coli	0.125	0.250
B. stearothermophillus	0.125	0.250
Cl. Sporogenes	0.125	0.250
S. marcescens	0.0625	0.125
S. typhi	0.125	0.250
P. aeruginosa	0.125	0.125

3.4 Killing rate

A linear relationship was observed between the percentage bacteria killed by the MBC of ethyl acetate fraction

of A. muricata leaf and exposure time (Figures 1 and 2). The killing rate of 25.0% of E. faecalis was observed after 30 min of exposure to MBC of the fraction for the organism and this increased until there was complete eradication after 150 min (Figure 1). A similar trend was observed when the killing rate of 20.0% of B. subtilis was recorded after 30 min exposure to MBC of the fraction for the same organism. The rate increased until there was complete eradication of the organism after 180 min (Figure 2).

3.5 Phytochemical analysis

Phytochemical analysis of leaf extract of A. muricata revealed the presence of all the phytochemical goups screened for such as alkaloids, anthraquinones, saponnins, flavonoids, tannins, reducing sugars, cardiac glycosides, carbohydrate (ketonic sugar) and phlobatannins (Table 5).

Table 5: Phytochemical compounds present in the leaf extract of Annona muricata

Phytochemical compound	Test	Observation	Indication
Tannins	Ferric Chloride	Blue-green colour	Positive
Anthraquinones	Borntrager	Bright pink colour	Positive
Saponins	Frothing test	Frothing formation	Positive
Flavonoids	HCl	Red or Orange colour	Positive
Alkaloids	Meyer	Absence of Turbidity	positive
Reducing sugar	Fehling test	Red precipitates	Positive
Cardiac glycoside	H_2SO_4	Pink colour	Positive
Carbohydrate (ketonic sugar)	HCl	Cherry red colour	Positive
Phlobatannis	HCl	Cloudy red colour	Positive

4. Discussion

The effectiveness of many of the currently used antimicrobial agents have been threatened by the development of multidrug-resistant pathogens [4,24]. This has necessitated interests in natural medicine as potential source of novel antimicrobial compounds. In this study, Annona muricata leaf extract was found to exhibit appreciable biocidal activities against all test bacterial and fungal isolates. The extract showed broad spectrum antimicrobial effects which compared favourably with those of reference drugs – streptomycin and amphotericin B. The ethyl acetate and n-butanol fractions of the leaf extract exhibited the highest antibacterial effects and this indicates that greater quantities of the inherent bioactive agents are extracted by these solvents. Moreover, the n-butanol could be a better solvent for the extraction of the antifungal compounds from A. muricata leaf.

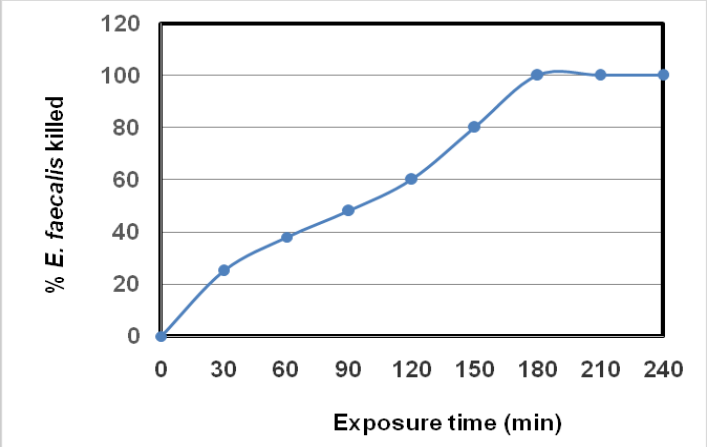

Figure 2: Rate of killing of E. faecalis by the minimum bactericidal concentration (MBC) of ethyl acetate fraction of A. muricata

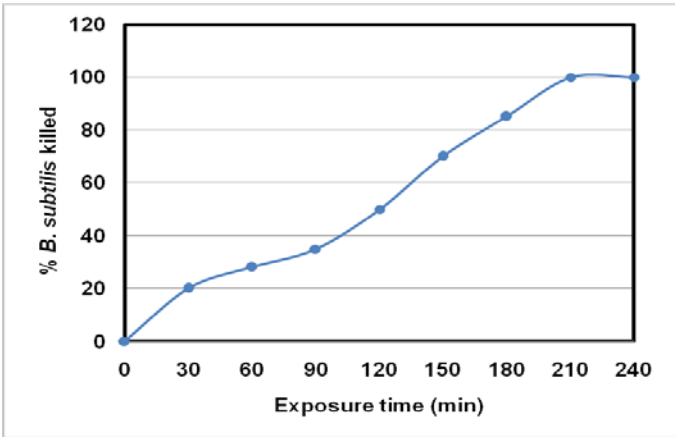

Figure 3: Rate of killing of *B. subtilis* by the minimum bactericidal concentration (MBC) of ethyl acetate fraction of *A. muricata*

These results are in accordance with similar findings obtained by previous workers. Fruit peel extract of A. muricata was found to inhibit S. aureus, Vibrio cholera and E. coli, which are bacterial species commonly associated with food intoxication [25]. Methanolic extracts of A. muricata leaf also produced inhibitory effects on S. aureus, B. subtilis, E. coli, K. pneumonia and Proteus vulgaris [26]. Seed extracts of A. muricata produced significant antifungal effects against Fusarium moniliforme, Alternaria solani and Helminthosporium sp. which were isolated from infected sorghum grains [27]. These findings corroborate the use of several parts of A. muricata in various human cultures for treatment of different bacterial and fungal infectious diseases such as pneumonia, diarrhoea, urinary tract infection and some skin diseases [11]. A direct proportion was observed between the killing rate of organism and exposure time to the MBC of solvent fraction for same organism. This finding corroborates the observations of [21] who reported the rate of killing of E. coli and B. subtilis by the aqueous fractions of Afzelia africana to be dependent on time and extract concentration.

Phytochemical compounds tannins, anthraquinones, saponnins, flavonoids, alkaloids, cardiac glycosides,

carbohydrates and phlobatannins were detected in A. muricata leaf extract. The significant antimicrobial properties of the leaf extract could be attributed to the presence of these bioactive compounds. Flavonoids are phenolic compounds known to form complexes with extracellular proteins and bacterial cell wall thus exhibiting antibacterial effects [28]. Flavonoids also exhibit antitrypanosomal, antileishmanial, anti-inflammatory, analgesic and antioxidant effects [28]. Tannins exert their antimicrobial effects through mechanisms such as membrane disruption, binding to proteins, enzyme inhibition, substrate deprivation and metal ion complexation [29]. Medicinal plants that have tannins as their main component are used in the treatment of intestinal disorders such as diarrhea and dysentery [30]. 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 [29]. Saponnins are classes of glycosides which demonstrate antifungal properties [30]. Synergistic interactions between some of these chemical groups may produce greater activity against pathogenic microorganisms.

This study has demonstrated appreciable biocidal activities of A. muricata leaf extracts which justify the ethnomedicinal use of the plant. The plant can be further assessed to discover active chemical components with potentials for use against infectious microorganisms.

5. Conclusion

Our results indicate that the ethyl acetate and n-butanol solvent fractions of A. muricata leaf extract presented in vitro broad spectrum biocidal activities against clinical strains of bacteria and fungal isolates. A. muricata may therefore be a potential source of new compounds useful in the control of microbial infections.

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