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Phytochemical screening & antibacterial activity of ethyl acetate & methanol extracts of *Annona muricata* aerial part

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Abstract
The medicinal plant *Annona muricata* is an evergreen plant that is mostly distributed in tropical and subtropical regions of the world and it has been deployed as an ethnomedicine against various illnesses. The ethyl acetate and methanol extracts of the plant were subjected to phytochemical screening and were also investigated for antibacterial activities against four gram positive which were *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Klebsiallae paeumonae* and two gram negative which were *Esherichia coli* and *Salmonellae typhii.* The ethyl acetate and methanol extracts were found to inhibit the growth of the test organisms, but the ethyl acetate extract proved to be a better antibacterial agent. The results showed that the ethyl acetate extract at 100.0mg/ml concentration exhibited the highest zone of inhibition of 25.0±1.00mm against four of the organisms. The phytochemical screening of the extracts both revealed the presence of tannins, resins, flavonoid and phenols. This study has shown that this plant contains certain phytochemical constituents of a great medicinal importance and that it has the potential for the treatment of various infections caused by bacteria.

Keywords: *Annona muricata*, bacteria, phytochemical screening, medicinal plant

Introduction
The world is facing the serious threat of antimicrobial resistance (AMR) today, as the microorganisms are developing AMR mechanisms at an alarming rate. This global threat calls for development of new, safe and effective antimicrobial drugs as to replace the existing ones in the market today. *Annona muricata* is a medicinal magic plant used since ancient times to cure variety of ailments and it is known to have a wide spectrum of biological activities. Treating of diseases through natural medicine is the most ancient treatment known to mankind [1, 2]. The medicinal property of the plants is due to the presence of various bioactive constituents like phenols, terpenoids and alkaloids. The diseases that have been managed traditionally using medicinal plant include epilepsy, malaria, convulsion, dysentery, fungal and bacteria infections [3]. *A. 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 [4]. It is known that the leaf extract of *Annnona muricata* is used in the treatment of various bacterial infectious diseases such as pneumonia, diarrhoea, urinary tract infection and even some skin diseases.
Annona muricata extract contains a wide spectrum of activity against a group of bacteria that are responsible for the most common bacterial diseases. The *Annona muricata* possesses an abundant of the antibacterial compounds [5]. It was reported that the presence of Annonaceous acetogenins, muricoreacin (1) and murihexocin C (2) (mono-tetrahydrofuran acetogenins) in the leaves of *A muricata* (Annonaceae) with significant cytotoxic activities targeting human prostate adenocarcinoma (PC-3) and pancreatic carcinoma (PACA-2) cell lines has been demonstrated [6]. Man has depended on plants and plants extracts as a source of medicine, food, shelter, clothing etcetera, since creation [7]. It has been revealed that the aqueous extracts of *A. muricata L* showed an antibacterial effect against *S. aureus* and *V. cholera* [8]. This research was carried out as to determine the phytochemical constituents and antimicrobial properties of *Annona muricata*. The results therefore have shown that *Annona muricata* possesses the healing potential over bacteria causing infections and it that will be a good alternative to the existing antibacterial drugs in the market today

Materials & Methods

Collection & authentication of plant material

The plant was obtained locally from a farmland in Lagos, Lagos State, Nigeria and the plant specimen was identified by a Taxonomist in the Department of Botany, University of Lagos, Lagos State, Herbarium. The voucher specimen was deposited at the Herbarium of the Department of Botany, University of Lagos, Lagos State, Nigeria.

The plant material was air dried under shade, grinded to coarse powder. The dried plant material was sequentially extracted using hexane, ethyl acetate and methanol respectively using the method of maceration at normal room temperature for three days according to Handa *et al*., 2008 [9]. The extract was filtered and then distilled off the extracting solvent by drying it on an evaporating dish under a mild temperature.

Phytochemical screening

The ethyl acetate and methanol extracts of *Annona muricata* aerial part phytochemical screening was carried out to determine the presence of the following compounds; flavonoids, tannins, saponins, glycosides, sterols, phenols, resins and alkaloids using standard procedures. The qualitative chemical tests of the phytochemicals in the extracts were done using the methods described by Abulude *et al.* 2007 [10] and Abulude 2010 [11].

Test for tannins

Two drops of 5% $FeCl_3$ was added to 1ml of the extract. A dirty green precipitate indicated positive test.

Test for glycosides

Ten (10) ml of 50% H_2SO_4 was added to 1ml of extract in a test tube, this mixture was heated in boiling water for 5 minutes. 10ml Fehling's solution A and B (5 ml each) were added and boiled. Brick red precipitate indicated positive test.

Test for resins

Two and a half (2.5) ml of Copper (II) Sulphate solution was added to 2.5 ml of the extract. The resulting solution was shaken vigorously and allowed to settle. A green colour indicated positive test

Test for saponins (Frothing test)

Two (2) ml of extract was vigorously shaken in test tube for two minutes. Frothing indicated positive test.

Test for phlobatannins

Five (5) ml of distilled water was added to 5 ml of extract solution and boiled with 1% HCl for two minutes. A deep green colour indicated positive test.

Test flavonoids

Alkaline Reagent Test: The extract was treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

Test for sterols (Salkowski test)

Two (2) ml of conc. H_2SO_4 was added 2 ml of extract solution. A red precipitate indicated steroidal ring.

Test for phenols.

Equal volumes of extract solution and $FeCl_3$ were mixed. A deep bluish green solution confirmed the presence of phenols.

Test for carbohydrate. (Fehling test)

Five (5) ml of the mixtures of equal volume Fehling solution A and B were added to 2 ml of the extract in a test tube. The resultant mixture was boiled for two minutes. A brick red precipitate of copper oxide indicated a positive test.

Test for alkaloids

One (1) ml of conc. H_2SO_4 was added to 3 ml of the extract, then treated with few drops of Wagner reagent. Reddish brown precipitate indicated positive test.

Test for terpenoid (Solkowski test)

0.2g of the extract sample was mixed with 2ml of chloroform ($CHCl_3$) and conc. H_2SO_4 (3ml) was carefully added to form a layer. A reddish brown colouration of the interface was formed to indicate positive result for the presence of terpenoids.

Microorganisms

Six strains of bacteria were used for this study, four were gram positive, which were *Bacillus subtilis*, *Pseudomonas aeruginosa* (ATCC 27583), *Staphylococcus aureus* (ATCC 29213) and *Klebsiollae pneumonae*. The two gram negative used were *Escherichia coli* (ATCC 35218) and *Salmonellae typhii.* Three of the bacteria used were clinical isolates obtained from Medical Microbiological Department of University College Hospital, University of Ibadan, Ibadan, Oyo State Nigeria. Single colony plates of nutrient agar medium of these organisms were maintained at 4˚C and sub-cultured on to nutrient broth for 24 hours prior to testing.

Antibacterial activity assay

Antibacterial activity of the *Annona muricata* extracts was determined by using pour plate method (agar diffusion) on sterile nutrient agar medium. Nutrient agar medium was poured into the sterile petri-plate and the medium was allowed to solidify for about 45 – 60 minutes. Gentamycin (10µg/ml) was used as positive control while the solvent of extraction was used as the negative control. Using a sterile cork borer of 6mm diameter, the wells were made according to the number of graded concentration of the sample. In each well, the different graded concentrations of the sample were prepared, this was done in duplicates. The plates were allowed to stay on the bench for 2hrs to allow pre-dilution. The plates were incubated uprightly at 37 ºC for 18-24 hrs. Then antibacterial activity was determined by measuring the diameter of zone of inhibition (ZI) in millimeter and activity index (AI) was also calculated as the division of zone of inhibition of the extract by that of the standard drug used.

Results & Discussion

Phytochemical screening

Table 1: Classes of Secondary Metabolites in *Annona muricata* Aerial Part

S/N	Constituent	Intensity	
		Amee	Amme
1	Tannins	+++	++
2	Glycosides	-	+++
3	Resins	+++	++
4	Saponins	-	-
5	Phlobatannins	+++	-
6	Flavonoids	+++	+++
7	Sterols	-	+
8	Phenols	+++	+
9	Carbohydrates	-	+++
10	Alkaloids	-	-
11	Terpenoids	-	-

Keywords: (+++) Intense, (++) Moderate, (+) Mild, (-) Absent, AMEE – *Annona muricata* ethyl acetate extract, AMME – *Annona muricata* methanol extract

Table 2: Antibacterial Properties of Ethyl Acetate Extract of *Annona muricata* at Different Concentrations Showing its Zone of Inhibition (ZI) and Activity Index (AI).

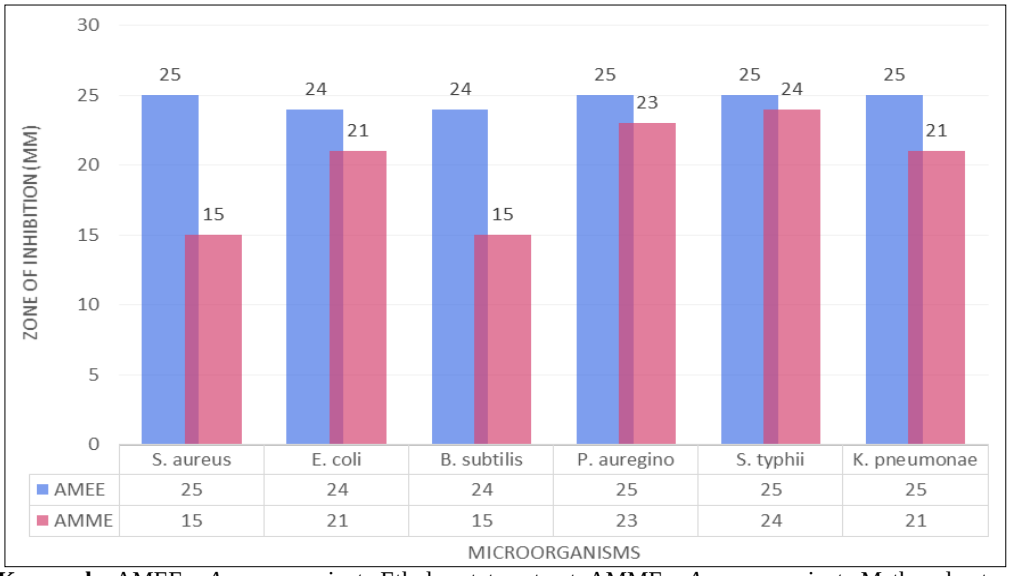
S/N	Organisms	Gentamycin	100.0mg/ml		50.0mg/ml		25.0mg/ml		12.5mg/ml	
			ZI	AI	ZI	AI	ZI	AI	ZI	AI
1	*Staphylococcus aureus*	36.0±0.00	25.0± 1.00	0.69	21.0± 1.00	0.58	18.0± 0.00	0.50	15.0± 1.00	0.42
2	*Escherichia coli*	36.0±0.00	24.0± 0.00	0.67	20.0± 0.00	0.56	18.0± 0.00	0.5	16.0± 0.00	0.44
3	*Bacillus subtilis*	37.0±1.00	24.0± 0.00	0.65	20.0± 0.00	0.54	18.0± 0.00	0.49	15.0± 1.00	0.41
4	*Pseudomonas aureginosa*	38.0±0.00	25.0± 1.00	0.66	22.0± 0.00	0.58	18.0± 0.00	0.47	16.0± 0.00	0.42
5	*Salmonellae typhii*	38.0±0.00	25.0± 1.00	0.66	21.0± 1.00	0.55	18.0± 0.00	0.47	16.0± 0.00	0.42
6	*Klebsiollae paeumonae*	38.0±0.00	25.0± 1.00	0.66	21.0± 1.00	0.55	17.0± 1.00	0.45	14.0± 0.00	0.37

Keywords: ZI – mean zone of inhibition in mm ± SEM, A.I – activity index with respect to Gentamycin

Table 3: Antibacterial Properties of Methanol Extract of *Annona muricata* at Different Concentrations Showing its Zone of Inhibition (ZI) and Activity Index (AI).

S/N	Organisms	Gentamicin	100.0mg/ml		50.0mg/ml		25.0mg/ml		12.5mg/ml	
			ZI	AI	ZI	AI	ZI	AI	ZI	AI
1	*Staphylococcus aureus*	39.0±1.00	15.0± 1.00	0.39	13.0± 1.00	0.33	10.0± 0.00	0.26	-	0.00
2	*Escherichia coli*	36.0±0.00	21.0± 1.00	0.58	18.0± 0.00	0.50	16.0± 0.00	0.28	14.0±0.00	0.39
3	*Bacillus subtilis*	38.0±2.00	15.0± 1.00	0.40	13.0± 1.00	0.34	10.0± 0.00	0.26	-	0.00
4	*Pseudomonas aureginosa*	38.0±0.00	23.0± 1.00	0.61	18.0± 0.00	0.47	16.0± 0.00	0.34	14.0± 0.00	0.37
5	*Salmonellae typhii*	38.0±0.00	24.0± 0.00	0.63	20.0± 0.00	0.53	18.0± 0.00	0.47	16.0± 0.00	0.42
6	*Klebsiillae pneumonae*	37.0±1.00	21.0± 1.00	0.57	18.0± 0.00	0.49	16.0± 0.00	0.43	14.0± 0.00	0.38

Keywords: Z.I – mean zone of inhibition in mm ± S.E.M, A.I – activity index with respect to Gentamycin



Keywords: AMEE – *Annona muricata* Ethyl acetate extract, AMME – *Annona muricata* Methanol extract

Fig 1: Histogram showing zone of inhibition exhibited by the organisms at 100mg/ml Concentration of *Annona muricata* extracts

Phytochemistry of the plant extracts

In Table 1 the results of the phytochemical screening of the ethyl acetate and methanol extracts of *Annona muricata* revealed the presence of bioactive agents in the extracts. There was presence of tannins, resins, flavonoids and phenols in both of them. In the two extracts (AMME & AMEE) the following saponins, alkaloids and terpenoids were found absent.

Antimicrobial Activity

The antibacterial activity of the ethyl acetate extract and methanol extract of *Annona muricata* aerial part were investigated against some organisms. The extracts were tested at different concentrations and were found to exhibit varying degrees of antibacterial properties. In comparism, at higher concentrations of 100.0mg/ml and 50.0mg/ml the ethyl acetate extract of *Annona muricata* showed higher antibacterial property than the methanolic extract of *Annona muricata* when tested against the six organisms. In Table 1, the standard drug used (gentamycin) highest zone of inhibition was 38.0mm against *Pseudomonas aureginosa*, *Salmonellae typhii* and *Klebsiollae paeumonae* while it lowest zone of inhibition was 36.0mm against *Staphylococcus aureus* and *Escherichia coli*. In Table 2, gentamycin exhibited the highest zone of inhibition of 39.0mm against *Staphyloccocus aureu*s and the lowest zone of inhibition was 36.0mm against *Escherichia coli*. The results in Table 1 & 2 showed that the zone of inhibition and activity index decreased as the concentration decreased.

The ethyl acetate extract of *Annona muricata* at 50.0mg/ml concentration exhibited 20.0mm zone of inhibition against *Bacillus subtilis* with the activity index of 0.54. The extract at 12.5mg/ml concentration exhibited 15.0mm zone of inhibition against the same organism with the activity index of 0.41. The methanol extract of *Annona muricata* at 12.5mg/ml concentration showed no inhibition against *Staphyloccocus aureus* and *Bacillus subtilis* while it exhibited zone of inhibition against the other tested organisms. Also at this concentration the two extracts exhibited the same zones of inhibition (16.0mm, 14.0mm) against *Salmonella typhii* and *Klebsiollae pneumonae* but with different activity index (AI) for *Klebsiollae pneumonae.*

Discussion

There are presence of bioactive compounds in the extracts as it is shown in Table 1, and they are responsible for its pharmacological activities, and especially its antibacterial properties. The phytochemical screening of the two extracts both revealed the presence of tannins, resins, flavonoids and phenols. Medicinal plants have been of age long remedies for human diseases because they contain component of therapeutic value [12]. Flavonoids was found present in the two extracts and flavonoids are known to have a wide range of biological activities such as the diuretic antibacterial [13, 14], antifungal, antiplasmodial and antimycobacterial activities [15, 16].

The aerial part of the plant was used for this experiment and the previous works done on the plant showed that the leaf, twigs, root, stem and fruit seed extracts of *A. muricata* have several biological activities such as antibacterial [8], antitumor [17] and anti-malarial [18]. It was reported that the leaves of *A. squamosal* L. contains a considerable quantity of phenolic compounds and they are responsible for their antioxidant and antibacterial activities [19]. The two extracts as shown in Table 1 and 2 showed significant antibacterial activity against *Pseudomonas aureginosa, Escherichia coli, Salmonellae typhii* and *Klebsiillae pneumonae* at 100.0mg/ml concentration. Also at 100mg/ml concentration, ethyl acetate extract was found to exhibit higher zone of inhibition, activity index more than the methanol extract and thereby proved to be a better antibacterial agent.

Conclusion

This study has revealed the presence of bioactive compounds like phenols, tannins and flavonoids in the two extracts and these are responsible for the broad antibacterial activities of *Annona muricata.* Therefore, with the antibacterial activities exhibited by the extracts against the tested organisms, this has shown that *Annona muricata* has the potential to treat various bacterial infections.

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