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Biochemical Properties, *In-Vitro* Antimicrobial, and Free Radical Scavenging Activities of the Leaves of *Annona muricata*

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ABSTRACT: Fifteen (15) male Wistar rats were completely randomized into three (3) groups. Group A was normal control, Group B received 200 mg/kg weight of ethyl acetate extract of *Annona muricata* leaves (AMLE) and Group C received 100 mg/kg b.weight AMLE. Single daily dose of AMLE was administered orally for twenty one (21) days after which the animals were sacrificed. The blood samples were taken for haematological and biochemical analysis. Antibacterial and antifungal activities of n-hexane, ethyl acetate and methanol extracts of *Annona muricata* leaves were carried out. These extracts exhibited satisfactory inhibitory activities against bacterial and fungal strains, which include; *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis, Pseudomonas aeruginosa, Salmonella typhi, Klebsiellae pneumonae, Candida albicans, Aspergillus niger, penicillium notatum* and *Rhizopus stolonifer.* N-hexane extract of the plant exhibited antioxidant property by scavenging DPPH radicals with IC_{50} of 342.44 µg/mL. GC-MS analysis of n-hexane, ethyl acetate and methanol extracts of the plant principally revealed the presence of Urs-12-ene (23.15%), Squalene (48.80%) and Clionasterol (15.88%) respectively.

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***Keywords*:** biochemical properties, antimicrobial, antioxidant, urs-12-ene, squalene, clionasterol

Annona muricata, otherwise known as graviola, is an edible fruit which belongs to the family of Annonaceae known as custard apple tree. Graviola (*Annona muricata* L.) is a tropical fruit commonly grown in Asia, South America and many tropical islands (Lannuzel and Michel, 2009; Gajalakshmi *et al.,* 2012) *Annona muricata* is slightly acidic in taste when ripe and hence its name as soursop (Hutchinson *et al.,* 2011). A. muricata seeds may possess the characteristics to be employed as a feed supplement for cattle, sheeps, goats and horses, because of their protein, fiber, fat, carbohydrate and ash contents in the seed and seed coat. Moreover, the seeds showed a very low level of phytotoxins, which in turn can be very hazardous in animal feeds (Fasakin *et al.,* 2008).The leaves of *A. muricata* can be consumed as tea (Hansra *et al.,* 2014; Port's *et al.,* 2013) or as dietary supplement in capsules accompanied by side effects (Torres *et al.,* 2012). The leaves of Graviola (*Annona muricata*) are used by some people to treat or even cure cancer, however, reports of an atypical form of Parkinson's disease has resulted from the overconsumption of the fruit due to the presence of the neurotoxins, annonacin and squamocin (de Moraes *et al.,* 2016). The pharmacological and biochemical properties of the plant have not been fully reported. This paper is focused on the determination of the biochemical properties, antibacterial, antifungal and free radical scavenging activities of the leaves of *Annona muricata*.

MATERIALS AND METHODS

Plant sample preparation and extraction: *Annona muricata* plant sample was collected from Kwara state, Nigeria. The plant was identified and authenticated by a taxonomist, Mr. Bolu Ajayi of the Department of Plant Biology, University of Ilorin where voucher specimen (UILH/003/1106) was deposited in the herbarium. The leaves of *A. muricata* were air dried and crushed into smaller pieces using mortar and pestle. The plant sample was weighed and extracted with n-hexane, ethyl acetate and methanol by using serial exhaustive extraction method according to Das *et al.,* 2010.

Experimental Animals: Fifteen healthy male Wistar rats weighing 200 to 250 were used in this study. They were housed under standard laboratory conditions of light, temperature and relative humidity. The animals were given standard rat pellets and water *ad libitum.* The rats were randomly distributed into three experimental groups, each containing 5 rats:

Group A: (Normal control) Received vehicle (2mls/kg b.wt 5% DMSO in Normal Saline)
Group B: Received ethyl acetate leaf extract of *Annona muricata* (200mg/kg b.wts) in 5% DMSO in Normal Saline orally (2mls/kg b.wts)

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Group C: Received ethyl acetate leaf extract of *Annona muricata* (100mg/kg b.wts) in 5% DMSO in Normal Saline (2mls/kg).

Estimation of Biochemical parameters: At the end of the experiment, animals were sacrificed by cervical dislocation and blood samples collected through cardiac puncture were dispensed into EDTA bottles for immediate analysis of haematological parameters using packed cell volume (PCV), haemoglobin concentration (Hb), red blood cell (RBC) count, platelets (Plat) count, white blood cell (WBC) count and differentials, mean capsular volume(MCV), mean capsular haemoglobin (MCH), and mean capsular haemoglobin concentration (MCHC) were assayed for.

Plain bottles were filled with the portion of the blood and centrifuged at 4000rph for 10 minutes to separate plasma for biochemical estimations. Determinations included alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin concentrations, total cholesterol, high density lipoproteins (HDL), total triglycerides, glucose, creatinine and urea using standard kits. Low density lipoprotein (LDL) concentration was calculated using the Friedewald's equation (Crook, 2006).

Statistical Analysis: Data were expressed as the means + SEM five (5) animals. Statistical analysis was performed using One-way analysis of Variance (ANOVA) and Duncan Multiple Range Test (DMRT).The data was considered statistically significant at $p< 0.05$.

Phytochemical screening: Preliminary phytochemical screening of the crude extracts was carried out using the modified methods as described by Pranshant, *et al.,*2011.

Antimicrobial assay: Microorganisms**:** Cultures of six human pathogenic bacteria made up of four gram negatives and two gram positives were used for the antibacterial assays. These cultures include; *Salmonella typhii, Escherichia coli, Pseudomonas aeruginosa* and *Klebsiellae pneumonae* belong to the gram-negative, and *Bacillus subtilis* and *Staphylococcus aureus* which are grampositive bacteria. Four fungi were also utilized for the antifungal assays. These are; *Candida albicans, Aspergillus niger, Rhizopus stolon* and *Penicillum notatum.* All the microorganisms used were clinical strains from the Medical Microbiology (University College Hospital, Ibadan) and screened in the Laboratory of Pharmaceutical Microbiology Department, University of Ibadan, Ibadan, Nigeria.

*Media***:** Nutrient agar, Sabouraud dextrose agar, nutrient broth and tryptone soya agar were used in this study. Hexane, ethyl acetate and methanol were used in solubilizing the extracts and as negative controls in the assays.

***Antimicrobial agents used*:** Gentamycin (10 µg/mL) and Tioconazole (0.7 mg/mL) were employed as standard reference drugs in this study.

Determination of Antimicrobial activity: A*gar diffusion-Ditch method (for bacteria):* A 14hour culture of each organism was prepared by taking two wire-loop of the organism from the stock, each inoculated into 5ml of sterile nutrient broth and incubated for 24 hr at 37^{o}C. 0.1 mL of each organism was taken from the 14hour culture and put into the 9.9 mL of sterile distilled water to obtain 10^{-2} inoculum concentration of the test organism. 0.2 mL was taken from the diluted test organism (10^{-2}) into the prepared sterile nutrient agar cooled to about 45 ^{o}C and then poured into sterile petri dishes which were allowed to solidify for about 60 minutes. A sterile corkborer of 8mm diameter was used to make 8 wells on the media according to the number of the diluted extracts for the experiment. The graded concentrations (6.25 – 200 mg/mL) of the extracts were put into each well and separated from the controls. The studies were done in duplicates to ascertain the results obtained. The plates were left on the bench for about 2 hrs to allow the extract diffuse properly into the nutrient agar i.e. pre-diffusion. The plates were incubated for 24 hrs at 37^{o}C.

Agar diffusion-Surface method (fungi): A sterile sabouraud dextrose agar was prepared accordingly and aseptically poured into the sterile plates in triplicates and solidified. 0.2 mL of the 10-2 inoculum concentration of the test organism was spread on the surface of the agar using a sterile Petri dish to cover all the surface of the agar. Eight wells were bored by using a sterile cork-borer of 8mm diameter. The graded concentrations of the extracts were put into each well separately with the controls. All the plates were left on the bench for 2hr to allow the extract diffuse properly into the agar i.e. prediffusion. The plates were incubated at 25^{o}C for 72 hrs (Collins and lyne, 1970).

Antioxidant Activity: The free radical scavenging activity of the extracts was carried out using DPPH as the test radical, and was assessed by the standard method adopted with suitable modifications (Sies, 1997)**.** The stock solutions of extracts were prepared

in methanol to achieve the concentration of 1 mg/mL. Dilutions were made to obtain concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90 and 1.99 µg/mL. DPPH (2,2- diphenyl-1-hydrazine) is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity. The absorbance was measured in triplicate at varying concentrations and the mean absorbance was determined. Parallel to examination of the antioxidant activity of plant extracts, the value for the standard compound (Ascorbic acid) was obtained and compared to the values of the antioxidant activity, the percentage inhibitions of the serial concentrations of the methanol DPPH extracts and that of the standard which was determined at different concentrations using the expression as shown below.

$$\%inhibition = \left(\frac{A\,ofcontrol - Aofsample}{Aofcontrol}\right) \times 100$$

The IC_{50} values (Inhibition Concentration at 50%) were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm.

GC-MS analysis of the extracts: GC-MS was performed with Agilent 19091GC plus automatic sampler system coupled with a quadruple mass spectrometer 433HP-5MS. Compounds were separated in HP5MS column fused with phenyl methyl silox, (length; 30m x 250µm; film thickness 0.25µm). Samples were injected at a temperature of about 250 ^{0}C with a split ratio of 10:1 with a flow rate of helium 1mL/min. Extracts of *A. muricata* leaves were dissolved in the respective solvent (n-hexane, ethyl acetate and methanol) to form solution. After this, the extracts were inserted into GC-MS instruments for chromatographic separation of the respective constituents and mass spectra of these constituents were obtained.

RESULTS AND DISCUSSION

Oral administration of ethyl acetate extract of *Anonna muricata* leaves at the doses of 100 mg/kg body weight and 200 mg/kg body weight respectively, showed no significant differences *(p>0.05)* in all the hematological determinations when compared with the normal control. The general lack of significant changes in blood indices is an indication of safety of the extract.

The results of the effects of AMLE on Lipid profile are shown in Figure 2. There were significant *(p>0.05)* dose dependent decreases in Triglycerides (TG), Total Cholesterol, Low Density Lipoprotein (LDL) , Very Low Density Lipoprotein (VLDL) and increases in High Density Lipoprotein when compared with the normal control. The decrease in the plasma total cholesterol and triglycerides may be attributed to the presence of hypolipidaemic agents in the extract. However, increases in HDL levels and a reduction in LDL levels observed is an indication that the extract can reduce the cardiovascular risk factors.

Liver and Kidney Biochemical markers in the treated groups showed no significant differences *(p>0.05)* in all the determinations when compared with the normal control (Figure 3).

The liver releases alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). An elevation in plasma concentration is an indicator of liver damage. The non-significant changes observed in the treatment groups (Figure 3) indicate that the extract had no deleterious effect on liver function. Lack of significant changes in creatine and urea level shows that AMLE is safe and offers no deleterious effect on Kidney.

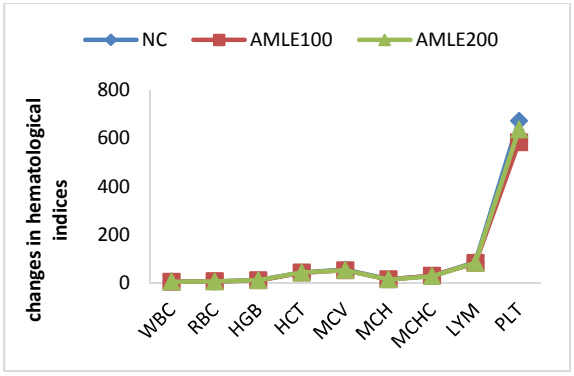

Fig 1: Effect of treatment with ethyl acetate leaf extract of *Anonna muricata* (AMLE) on hematological indices of Wistar rats

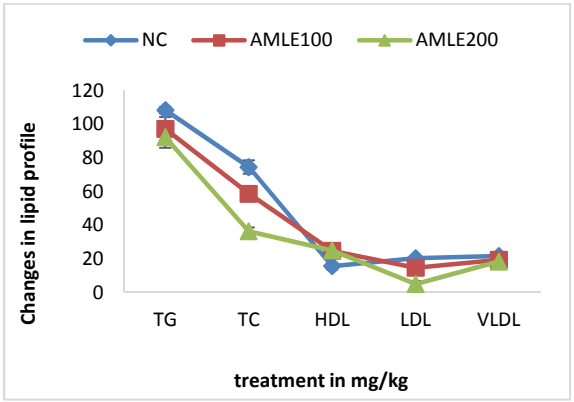

Fig 2: Effect of treatment with ethyl acetate leaf extract of *Anonna muricata* (AMLE) on lipid profile of Wistar rats

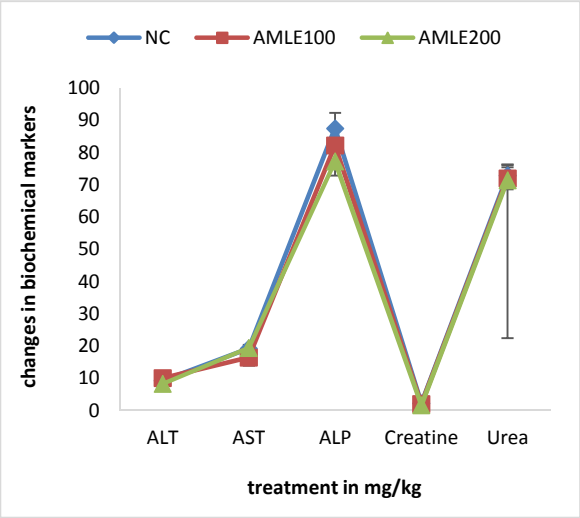

Fig 3: Effect of treatment with ethyl acetate leaf extract of *Anonna muricata* (AMLE) on Liver and Kidney Biochemical Markers of Wistar rats

Phytochemical screening: The preliminary phytochemical investigation of the crude extracts of n-hexane, ethyl acetate and methanol extracts of *A. muricata* leaves revealed the presence of saponins, flavonoids, terpenoids, steroids, tannin, glycosides and fats & oil as shown in table 1 below. The presence of these bioactive compounds especially, flavonoids, is an indication that these plants possess pharmacological activities, better still, anticancer activities.

Table 1: Results of phytochemical screening of the extracts of *A .muricata* leaves

chemical constituent fractions	AMLH	AMLE	AMLM
Alkaloids	-ve	-ve	-ve
Anthocyanins	-ve	-ve	-ve
Anthraquinones	-ve	-ve	-ve
Flavonoid	-ve	+ve	+ve
Glycosides	-ve	+ve	+ve
Saponins	-ve	-ve	+ve
Steroids	+ve	+ve	+ve
Terpenoid	+ve	+ve	+ve
Tanins	-ve	-ve	+ve
Fats n Oil	+ve	-ve	+ve
Phenols	-ve	-ve	-ve
Carbohydrate	-ve	-ve	-ve
Protein	-ve	-ve	-ve

KEYS: +ve – Present; -ve – Absent; AMLH: Hexane extract of *A. muricata* leaves; AMLE: Ethylacetate extract of *A. muricata* leaves; AMLM: Methanol extract of *A. muricata* leaves

Antimicrobial activity: The crude extracts of *A. muricata* leaves gave a clear zone of inhibition against the growth of the test bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Klebsiellae pneumonae*) at low concentrations for the hexane extract (6.25 mg/mL), modest concentrations for methanol extract (12.50 mg/mL) and high concentrations for ethyl acetate extract (25mg/ml) as well as test fungi (*Candida albicans*, *Aspergillus niger*, *Penicillium notatum* and *Rhizopus stolonifer*) at an analogous concentration. These inhibitions give credence to the fact that leaves of *Annona muricata* exhibit antibacterial and antifungal activities and hence can be used for the treatment of various illnesses caused by bacteria and fungi.

Antioxidant activity: N-hexane, ethyl acetate and methanol extracts of *A. muricata* leaves exhibited antioxidant activity by scavenging DPPH radicals with IC_{50} of 342.49, 729.67 and 433 µg/mL respectively (Figure 4). The results are shown in table 2 below.

Table 2: Absorbance and percentage inhibition of Ascorbic Acid Standard for DPPH Antioxidant activity of the extracts of *Annona muricata* leaves. Absorbance of control is 1.265

Conc. (µg/mL)	A1	A2	A3	MEAN±SD	%I A.A
1000	0.138	0.138	0.140	0.139±0.0012	89.02
500	0.150	0.150	0.150	0.152±0.0000	88.14
250	0.161	0.162	0.160	0.161±0.0010	87.26
125	0.180	0.180	0.180	0.180±0.0000	85.79
62.5	0.193	0.195	0.194	0.194±0.0010	84.26
31.25	0.245	0.245	0.245	0.245±0.0000	80.67
15.62	0.311	0.311	0.311	0.311±0.0000	75.44
7.81	0.453	0.452	0.454	0.453±0.0010	64.18
3.9	0.782	0.781	0.78	0.781±0.0010	38.26
1.95	0.991	0.991	0.991	0.991±0.0000	21.66

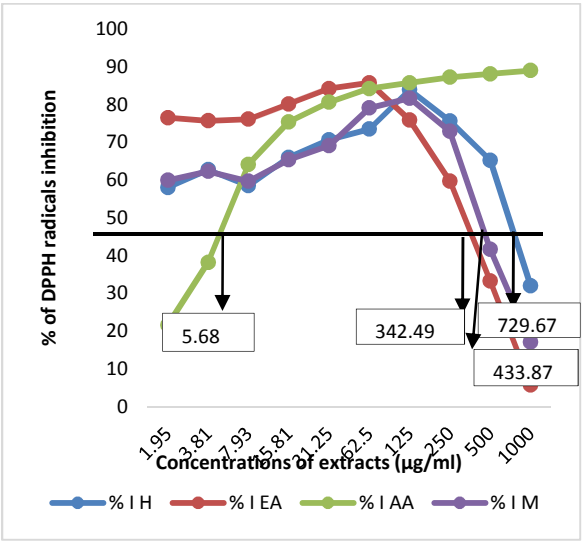

Fig 4: IC_{50} antioxidant activities of n-hexane, ethyl acetate and methanol extracts of *A. muricata* leaves.

Key: % I H = percentage inhibition by n-hexane extract; % IM = percentage inhibition by methanol extract; % I A A = percentage inhibition by Ascorbic Acid; % I EA = percentage inhibition by ethyl acetate extract; IC = Inhibition Concentration

GC-MS Analyses: GC-MS analysis of the *n*-hexane, ethyl acetate and methanol extracts of the leaves of *A. muricata* revealed twenty three (23), eleven (11) and seventeen (17) compounds respectively. The major compounds and their respective percentage abundance in these extracts are; urs-12-ene (23.15%), squalene (48.80%) and clionasterol (19.45%) for *n*-hexane, ethyl acetate and methanol extracts respectively.

Conclusion: The result of this study indicated that ethyl acetate extract of *Anonna muricata* leaves possessed hypolipidemic effect, reduces cardiovascular risk factors and safe. The presence of some bioactive metabolites in the plant extracts, which were analysed by using GCMS technique are responsible for antioxidant and antimicrobial properties of the plant. These properties also justified its use in the treatment of various bacterial infectious diseases like pneumonia, diarrhoea, urinary tract infections and even some skin infections.

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