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Cytotoxicity, Total Phenolic Contents and Antioxidant Activity of the Leaves Extract of *Annona Muricata*

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ABSTRACT

The leaves of *Annona muricata* were extracted using ethanol and the extracts were evaluated for cytotoxicity using Brine Shrimp Lethality Assay, total phenolic content (TPC) and antioxidant activity using DPPH radical scavenging assay. The crude extract showed 73.33 % mortality at 1000 µg/mL concentration and its LC_{50}-value was 1.59 µg/mL against brine shrimp larvae. The crude extract exhibited a significant toxicity in comparison with the positive control, the potassium dichromate ($K_2Cr_2O_7$) with LC_{50}-value 0.57 µg/mL. The TPC assay of the extracts was expressed as mg of tannic acid equivalent per gram of sample and mg of gallic acid equivalent per gram of sample, the result showed the content of phenolic compounds as 266.86 ± 2.21 mg·TAE/g and 278.60 ± 4.21 mg·GAE/g respectively. The DPPH radical scavenging activity of the ethanol extracts displayed a signigicant free radical scavenging capacity with the IC_{50} value of 2.58 µg/ml compared with the standard drugs, the ascorbic acid (IC_{50} 0.563 µg/mL) and the butylated hydroxytoluene (IC_{50} 0.570 µg/mL). The result suggested that the antioxidant property exhibited by the ethanol extract could be associated with its high total phenolic contents.

Keywords: *Annona muricata*, Antioxidants, Cytotoxicity, Total Phenolic Contents

INTRODUCTION

Traditional people have relied on medicinal plants to combat various `ailments caused by microorganisms such as bacteria, fungi and viruses (Ram *et al.*, 2004; Gurib-Fakim, 2006). According to the world health organization (WHO), more than 80% of the world's population relies on traditional medicines for their primary health care needs. Higher plants as sources of therapeutic agents continue to play a vital role in maintenance of human health since antiquities. Over 50% of all modern clinical drugs are of natural products origin clearly indicating its important role in drug development programs of the pharmaceutical industry (Sofowora, 1993 and Aliyu, 2004). Despite the availability of different approaches for the discovery of therapeutically active compounds, natural products still remain as one of the best reservoirs of new structural types. The extracts from plants provide unlimited opportunities for new drugs discoveries due to the unmatched availability of chemical diversity (Cos *et al.*, 2006; Tripathi *et al.*, 2012).

Annona muricata, commonly known as graviola or soursop, is a small tree of the Annonaceae family that could reach up to 7 m in height. The leaves are oblong in shape with ovoid scattered spine-like shaped fruits, while the flowers are large and solitary, yellowish or greenish-yellow in color. The pulp is soft, white in color and rather fibrous with an agreeable sour flavor (Rajeswari *et al.*, 2012). The leaves are traditionally used in the treatment of viral infection, headaches, influenza, hypertension, cough, asthma and sedative as well as nervine for heart condition **(**Padma *et al.*, (1998); Tripathi *et al.*, (2012))**.** Moreover, the juice of the fruit of *A. muricata* is a remedy for jaundice, diuretic and a remedy for hematuria and urethritis **(**Lans, 2006**).** The leaves of *Annona muricata* have been reported to possess powerful anticancer properties on pancreatic cancer, having the ability to stop the cancer pancreatic tumor cells from replicating (Oberlies *et al.*, 1997). Recently, the leaf, bark, root, stem, and fruit seed extracts of *Annona muricata* have been reported to possess various pharmacological activities some of which include; antimicrobial, antitumor, cytotoxicity, antiparasitic, anti-malarial and antiviral properties among others (Luna *et al.*, (2006); Rajeswari *et al.*, (2007); Isela *et al.*, (2008); Pathak *et al.*, (2010); Rajeswari *et al.*, (2012))**.** Moreover, other biological activities reported include anti-inflammatory, analgesic effects and antileishmanial activity **(**Oberlies *et al.*, (1997); Padma *et al.*, (1998); De Sousa *et al.*, (2010); Roslida *et al.*, (2010)). In addition, the roots of these species are used in traditional medicine due to their antiparasitical and pesticidal properties (Gleye *et al.*, 1997).

Since traditional medicine involves the use of plant extracts which contains an extensive diversity of compounds, often with indefinite biological effects there is need to determine the bioactivity of medicinal plants. The aim of this study is to evaluate the cytotoxicity using Brine-Shrimp lethality assay (BSLA) and antioxidant activity, using the total phenolic content and DPPH radical scavenging activity of the ethanol extracts of *A. muricata*.

MATERIALS AND METHODS

Collection and Identification of Plant Materials

Fresh leaves of *Annona muricata* were collected from Naibawa, Kumbotso local government area of Kano state, Nigeria and were identified at the Department of Plant Science, Bayero University Kano, Nigeria (BUKHAN0341).

Extraction of the Plant

The plant materials were air dried in the laboratory at room temperature (26°C) for 2 weeks, after which it was then pulverized into uniform fine powder. The powdered plant material was weighed using an electric weighing machine and recorded before extraction.

The powdered plant material was extracted using soxhlet method of extraction using ethanol as the extracting solvent. 60 g of the powdered leaves of the plant sample was packed in a muslin cloth (thimble) and inserted into the soxhlet extractor. 200 ml methanol was added and heated for 4 hrs at relatively lower boiling point below the boiling point of the solvent to avoid denaturing. At the end of the extraction period, the solvent was allowed to evaporate to obtain the crude extracts. The method was repeated in order to obtain enough extracts for analysis.

CYTOTOXICITY ASSAY AGAINST BRINE SHRIMP

Hatching Shrimp

Brine shrimp eggs, *Artemia salina* were hatched in seawater. After 48 h incubation at room temperature, the larvae was attracted to one side of the vessel with a light source and collected by pipette. Larvae were separated from eggs by aliquoting them three times in small beakers containing seawater (Lilybeth and Olga, 2013).

Brine Shrimp Assay

Toxicity of the extract was monitored by the brine shrimp lethality test according to the method described by Lilybeth and his co-workers, with slight modification (Lilybeth and Olga, 2013). Each of the extract (1 mg/mL) was dissolved in methanol, from which 5 000, 500 and 50 µL of each solution was transferred into vials corresponding to 1000, 100 and 10 µg/mL respectively. This was allowed to evaporate to dryness in about 24 h at room temperature. Each dosage was tested in triplicate (9 per test sample). Sea water (4 mL) and 10 larvae were introduced into each vial. The final volume of solution in each vial was adjusted to 5 mL with sea water immediately after adding the shrimps. A negative control was prepared as a drug-free and potassium dichromate was used as positive control. Survivors were counted after 24 h, and LC_{50} was determined by probit analysis using SPSS version 16 to establish the therapeutic index.

TPC Assay

The total phenolic contents of the plant extracts were determined by employing the methods reported in the literature (De Mello, *et al.*, 2013) involving Folin-Ciocalteu's reagent, Gallic acid and Tannic acid were used as standard. A solution of the sample (5 µL) containing 1 mg of extract was pipetted into a 96 well plate containing methanol (44 µL), then Folin-Ciocalteu's reagent (2.5 µL) was added, and the flask was thoroughly shaken. After 3 min, 5% Na_2CO_3 solution (25 µL) was added and the mixture was allowed to stand for 2 h with intermittent shaking. Then, methanol (123 µL) was added and left to stand in the dark for 1 h. The absorbance measurements were recorded at 715 nm.

The total phenolic contents in the extracts were expressed as mg of gallic acid equivalent per gram of sample and mg of tannic acid equivalent per gram of sample. Test was carried out in duplicate.

DPPH Assay

The free radical scavenging activity of the plant extracts against 2,2-diphe-nyl-1-picrylhydrazyl (DPPH) radical was determined according to the method described by de Oliveira and his co-workers, with slight modification (de Oliveira, *et al., 2012*). Each sample of stock solution (1.0 mg/L) was diluted to final concentration of 1000, 500, 250, 125, 31.3, 15.63 and 7.82 µg/mL. Then, a total of 100 µM DPPH methanolic solution (160 µL) was added to sample solution (40 µL) and allowed to react at room temperature for 30 min in dark. The absorbance of the mixtures was measured at 517 nm. Ascorbic acid (AA) and butylated hydroxytoluene (BHT) were used as positive controls. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity, and vice versa. Inhibitions of DPPH radical in percent (I%) were calculated using the formula:

$$I\% = \frac{A_{blank} - A_{sample}}{A_{blank}} \, x \, 100$$

Where; A_{blank} is the absorbance value of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance values of the test compounds. The sample concentration that

provides 50% inhibition (IC_{50}) was determined by probit analysis using SPSS 16.

RESULTS AND DISCUSSIONS

Brine Shrimp Lethality Assay

The extent of lethality was observed to be directly proportional to the concentration of the extracts. The highest observed mortality is 100 % at 1000 µg/mL in the standard. The Brine shrimp lethality activity of the crude extract is shown in Table 1. The crude extract showed 73.33 % mortality at 1000 µg/mL concentration with LC_{50}-value of 1.59µg/mL. The crude extract was considered significantly toxic in comparison with the standard drug potassium dichromate ($K_2Cr_2O_7$) which showed LC_{50}-value 0.57 µg/mL. No mortality was observed in the negative control (DMSO) group.

The Brine Shrimp Lethality Assay (BSLA) is inadequate to determine the mechanism of action of various bioactive compounds present in the crude extract of *A. muricata*. However its preliminary usefulness cannot be neglected as it supports the bioassays of different plant extracts.

Table 1: Result of the Brine Shrimp Lethality Assay of the leaves extracts of *A. muricata*

Sample	Concentration (µg/mL)	Number of Surviving Nuplii After 24 h			Total Number of Survivors	% Mortality	LC_{50} Value (SPSS)
		T1	T2	T3			
EtOH extracts of *A. muricata*	10	6	7	5	12	40	1.59
	100	5	4	4	17	56.67	
	1000	3	2	3	22	73.33	
$K_2Cr_2O_7$	10	4	4	3	19	63.33	0.57
	100	2	1	1	26	86.67	
	1000	0	0	0	30	100	

TPC Assay

Folin-Ciocalteu reagent comprises of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids which upon oxidation of phenolics were reduced to blue oxides of tungstene (W_8O_{23}) and molybdene (Mo_8O_{23}) oxides. This reaction occurs under alkaline medium of sodium carbonate. The intensity of blue colour reflects the quantity of phenolic compounds, which can be measured using a spectrophotometer (Kamarul, *et al*., 2010).

The total phenolic content (TPC) of the plant extract was determined using spectrophotometric method using tannic acid and gallic acid as the standards. The results were expressed as mg of tannic acid equivalent per gram of sample and mg of gallic acid equivalent per gram of sample. A linear calibration curve of Tannic acid and Gallic acid with regression coefficient value (R^2) of 0.9872 and 0.9898 respectively (Fig. 1). The mean TPC of the plants' leave extracts was calculated using the TAE equation of $y = 0.0007x + 0.3835$ ($R^2 = 0.9872$) and GAE equation of $y = 0.001x + 0.28$ ($R^2 = 0.9898$), whereby y = absorbance at 715 nm and x = concentration of total phenolic compounds in mg per mL of the extract.

In this study, the ethanol extracts of *A. muricata* displayed high TPC as observed in the ANOVA analysis, a significant difference ($p < 0.05$) between TPCs of the sample. The content of phenolic compounds were 266.86 ± 2.21 mg·TAE/g and 278.60 ± 4.21 mg·GAE/g for the ethanol extracts of *A. muricata*.

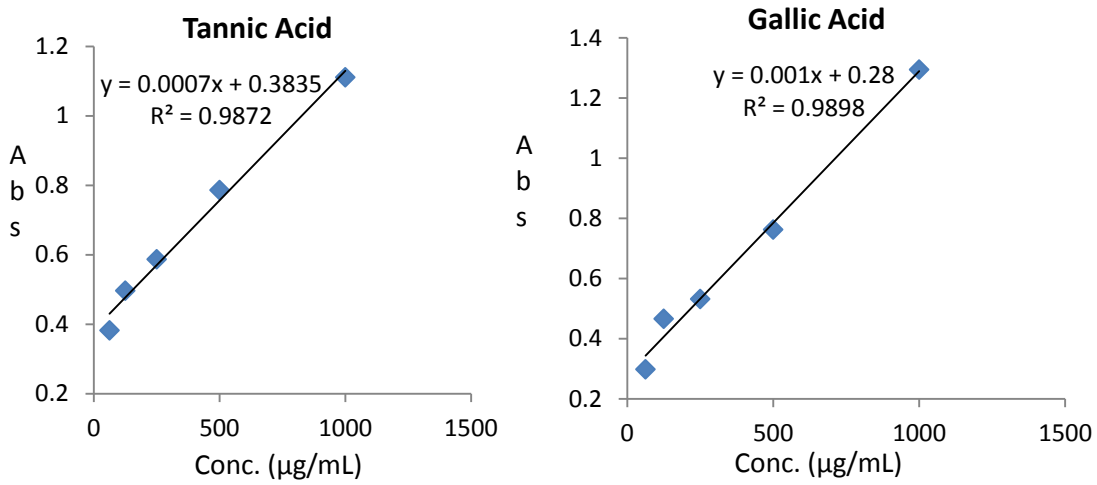

Fig. 1: A Linear Calibration Curve of Tannic Acid and Gallic Acid

DPPH Assay

DPPH radical scavenging activity assay assess the ability of the extract to donate hydrogen or to scavenge free radicals. DPPH radical is a stable free radical and when it reacts with an antioxidant compound which can donate hydrogen, it is reduced to diphenylpicrylhydrazine. The changes in colour (i.e. from deep-violet to light-yellow) can be measured spectrophotometrically. The DPPH radical scavenging activity was evaluated for the EtOH extracts of *Annona muricata*, Ascorbic acid (AA) and butylated hydroxytoluene (BHT) and their scavenging activities increased with increasing concentration (≥ 85 %, Fig. 2)**.** However, the ethanol extract of *A. muricata* showed no activity at the concentrations 31.3, 15.63 and 7.82 µg/mL.

DPPH radicals, is a parameter used to measure antioxidant activity of a drug; smaller IC_{50} value implies strong antioxidant activity of the plant extract. In this present study, the ethanol extracts of *A. muricata* displayed a significant free radical scavenging ability with IC_{50} value of 2.58 µg/mL compared with the standard drugs, ascorbic acid (IC_{50} 0.563 µg/mL) and butylated hydroxytoluene (IC_{50} 0.570 µg/mL).

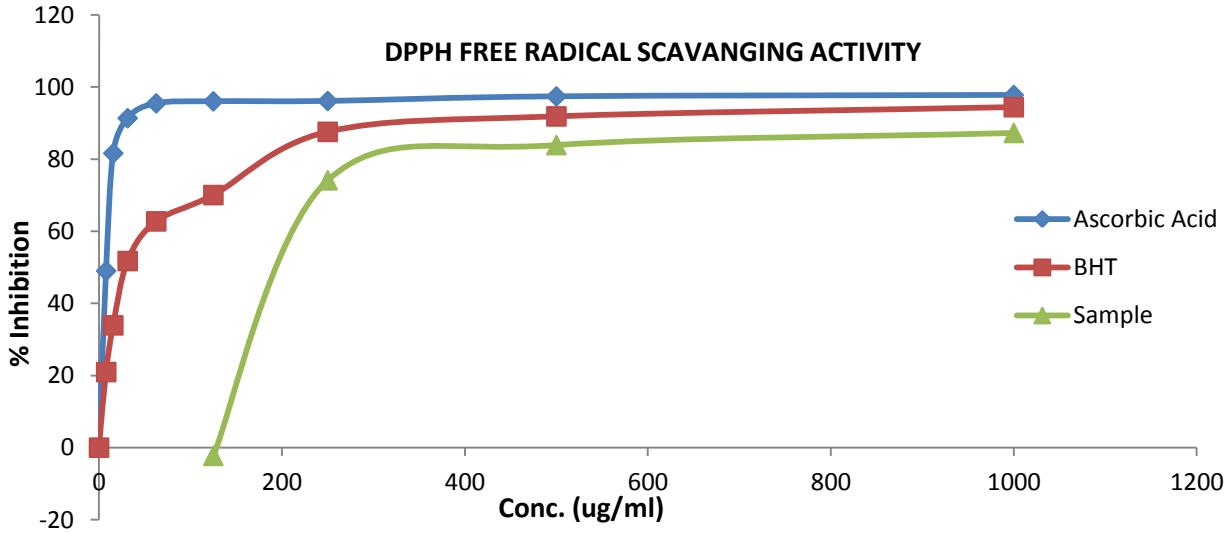

Fig. 2: DPPH radical scavenging activity of ethanol extract of the leaves of *A. muricata*

The Folin-Ciocalteu assay gives a crude estimate of the TPC present in an extract, whereas the free radical scavenging assay is not only specific to polyphenols. However, various phenolic compounds respond differently in DPPH assay, depending on the number of phenolic groups present (Prior *et al*., 2005). In this study, it was observed that, the higher total phenolic content of plants' extracts resulted in higher antioxidant activity as similarly reported by Cai *et al*., (2004) and Amina *et al*., (2006). In general, there was a good positive correlation between the TPC and antioxidant activity as assessed by DPPH-scavenging assays of the ethanol extracts of *A. muricata*. The positive correlation between TPC and antioxidant activity may probably be due to the ability of the phenolics to incorporate all the antioxidants that may be present in an extract. These findings suggest that polyphenols are

important contributors to the antioxidant and free-radical scavenging activities of the ethanol extracts.

CONCLUSION

The results of the brine shrimp lethality assay of the ethanol extracts of *A. muricata* shows LC_{50} value of 1.59 µg/mL indicates its potentiality of the plant to cure several human pathogens and suggest its importance in traditional medicine of tropical region of Africa. The antioxidant capacity of the leaves extracts of *Annona muricata* could be related to its phenolic constituents. The results showed that the potent antioxidant activity is justified by the high concentration of phenolic constituents present in the extract. For further studies, effort should be devoted to the isolation and characterization of the bioactive components through activity-guided assays.

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