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Proximate Analysis and the Assessment of the Level of Toxicity of Hexane, Ethyl Acetate and Methanol Extracts of *Annona muricata* Aerial Part Using Brine Shrimp Lethality Test

Tojola O.B.
Ph.D. Student, Department of Chemistry, Federal University of Technology, Nigeria
Lajide L.
Professor, Department of Chemistry, University of Technology, Nigeria
Dr. Owolabi B.J
Lecturer, Department of Chemistry Federal University of Technology, Nigeria
Olaleye M.T
Lecturer, Department of Biochemistry, Federal, University of Technology, Akure, Nigeria
Okoh S.O
Chief Research Officer, Department of CFET, Federal Institute of Industrial Research,

Abstract

In this research work the medicinal plant Annona muricata was extracted with three different solvents and the extracts were subjected to toxicity test using Brine Shrimp Lethality Test at different concentrations. at 100 µg/ml concentration the hexane extract gave 80.0% mortality rate, ethyl acetate extract gave 75.0% mortality rate and methanol extract equally gave 75.0% mortality rate. the extracts showed different percentage mortality rates at different concentrations and the methanol extract was found to exhibit the most significant cytotoxicity activity against brine shrimp (Artemia salina) with the lethal concentration value (LC_{50}) of 32.515µg/ml. The dried plant material was characterized for proximate analysis as to determine its nutritional composition and it exhibited 2.0% moisture content, 7.40% ash content and 15.89% crude fibre content. This research results have shown that the plant extracts were toxic to brine shrimp due to the presence of bioactive compounds of various pharmacological activities. this is an indication that the plant extracts can exhibit good anticancer property when tested against some cancer cell lines.

Keywords: *Annona muricata, brine shrimp lethality test, mortality rate, proximate analysis*

1. Introduction

Medicinal plants are known to contain different bioactive components with enormous therapeutic potential to cure many diseases in human beings**.** A medicinal plant is a plant that is used with the intention of maintaining health, to be administered for a specific condition, or both, whether in modern medicine or in traditional medicine[1][2]. Medicinal plants have been of age long remedies for human diseases because they contain components of therapeutic value[3]. *Annona 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 was reported that the seeds and leaves of the plant possess enzymatic antioxidants, including catalase and superoxide dismutase, and non-enzymatic antioxidants, including vitamin C and E [5]. In the traditional pharmacopeia of South America and Africa, the *Annona muricata* fruit is used as a natural remedy for a variety of ailments such as malaria, fever, diabetes, insomnia, rheumatism, hypertension, arthritis among others [6]**.** *Annona muricata* extracts from leaf, pericarp, seed, and stem have each shown cytotoxicity towards hematological malignant cells such as the leukemia U-937 cell line [7, 8].

Proximate analysis is a type of scientific inquiry done to determine the approximate amounts of substances within a material. This information can be used to create quality controls for various materials, ensure that they do not contain hazardous chemicals, and determine whether they are healthy enough to be consumed by humans or animals [9]. Brine shrimp lethality test (BSLT) is based on the killing ability of test compounds on a simple zoological organism-brine shrimp *(Artemia salina)* [10], BSLT is a very useful method for the assessment of the toxic potential of various plant extracts [11,12].

The main objective of this research work was to investigate the toxicity potential of this medicinal plant as claimed by the traditional people that it has the ability to cure cancer related diseases in human.

2. Materials and Methods

2.1. Collection and 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 Herbarium. The voucher specimen was deposited at the Herbarium of Department of Botany, University of Lagos, Lagos State

2.2. Preparation of Extracts

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[13]. The extract was filtered and then distilled off the extracting solvent by dryingit on an evaporating dish under a mild temperature.

2.3. Proximate Analysis

2.3.1. *Moisture*

A crucible was dried in an oven to remove any moisture present and cooled in a desiccator. the weight of the crucible was recorded. About 1.0g of the sample was weighed into the crucible and placed in an oven at 105^{0}C for about 2-3hrs until almost constant weight of the dried sample and crucible was observed. The weight of the dried crucible and the sample was noted. The weight of empty crucible is represented as W1, the weight of the sample and the crucible before drying is W2 and the weight of crucible and the sample after dried is W3.The % moisture content was calculated from the differences in the weight of the dried crucible that contain sample and the crucible that contain sample before drying in relation to the initial weight of the sample.

2.3.2. *Ashing*

A crucible was dried in oven to remove any moisture present and cooled in a desiccator. The weight of the crucible was recorded. About 2.0g of the sample was weighed into the crucible and placed in a muffle furnace at about 500-600^{0}C until the sample turned slightly whitish which can take about 4-5hrs.The weight of empty crucible is represented as W1. The weight of the sample and the crucible before ashing is W2 and the weight of crucible and the sample after ashing is W3. The % ashing was calculated from the differences in the weight of the crucible that contain ashed sample and the crucible that contain sample before ashing in relation to the initial weight of the sample.

2.3.3 Crude Protein Determination

The micro Kjeldahl method described by A.O.A.C (1990)[14] was used. Two grams of each of the samples was mixed with 10ml of concentrated H_2SO_4 in a heating tube. One tablet of selenium catalyst was added to the tube and mixture heated inside a fume cupboard. The digest was transferred into distilled water. Ten-millimeter portion of the digest mixed with equal volume of 45% NaOH solution and poured into a kjeldahl distillation apparatus. The mixture was distilled and the distillate collected into 4% boric acid solution containing 3 drops of methyl red indicator. A total of 50ml distillate was collected and titrated as well. The sample was duplicated and the average value taken. The Nitrogen content was calculated and multiplied with 6.25 to obtain the crude protein content. This is given as percentage Nitrogen

= (100 x N x 14 x VF) T **/** 100 x Va

Where

N= Normality of the titrate (0.1N)

VF= Total volume of the digest = 100ml

T= Titre Value

Va= Aliquot Volume distilled

2.3.4. *Lipid (Fat)*

To about 0.5g of the samples were weighed into Whatman filter papers. The sample were held tightly inside soxhlet extractor. 250ml of round bottom flasks were weighed (W1) and petroleum ether (40-60^{0}C b. p) was added to about two-third (W2) and the set up was allowed to boil on heating mantle for about 4-6hrs.The petroleum ether siphoned over the barrel and the condenser was detached. The weight of the flask after extraction was taken (W3).

$$\% \text{ Fat} = \frac{W2-W3}{W2-W1} \times 100$$

2.3.5. *Crude Fibre*

The defatted samples (about 0.4g) were weighed into pre weighed conical flask. 25ml of dilute sulphuric was added and allowed to boil for 30mins. It was filtered through the filter paper and the residue was collected into conical flask and

about 100ml of dilute sodium hydroxide was added and allowed to boil for another 30mins, filtered through a filter paper and washed with hot distilled water, rinsed four times with distilled water and once with 10% HCl, rinsed again with hot distilled water and twice with ethanol, also three times with petroleum ether.
When the water had drained off, the residue was placed inside a pre- weighed crucible. The sample was placed inside an oven at about 105^{0}C to dry until a constant weight was achieved. The sample was placed in desiccators, and the weight was taken as W2. The sample was later placed inside a muffle furnace at about 300-400^{0}C for 1hr. The crucible containing the residue was allowed to cool and the weight was taken as W3.

$$\% \text{ Crude Fibre} = \frac{W2-W3}{W1} \times 100$$

2.4. Brine Shrimp Lethality Test

The lethal concentration (LC_{50}) was determined using Brine Shrimp Lethality Test (BSLT) as to determine the level of bio-activity of the extracts at different concentration of application. Brine Shrimp *(Artemia salina)* were hatched using brine Shrimp eggs in a vessel filled with sterile artificial seawater under constant aeration for 48 hrs. After hatching active nauplii free from egg shell were collected from brighter portion of the hatching chamber and used for the assay. Ten nauplii were drawn through a glass capillary and placed in each vial containing 4.5ml of brine solution to give different concentration (20,40,60,80 and 100 *µg/ml*) and maintained at room temperature for 24hrs under the light and surviving ;larvae were counted.
Experiments were conducted along with control and test substances in a set of three tubes per dose.

3. Results and Discussion

3.1. Proximate Analysis

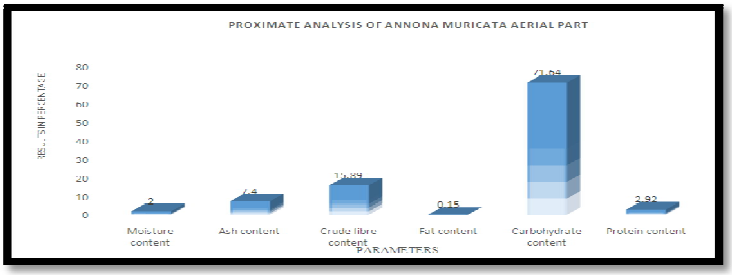

Figure 1: The Proximate Analysis of Annona muricata Aerial Part

3.2. Brine Shrimp Lethality Test

Sample	Concentration(µg/ml)	Number Exposed	Number Responded	% Mortality Rate	LC_{50} (µg/ml)
Hexane Extract	20.0	20.0	8.0	40.0%	38.577
	40.0	20.0	10.0	50.0%	
	60.0	20.0	14.0	70.0%	
	80.0	20.0	15.0	75.0%	
	100.0	20.0	16.0	80.0%	
Ethyl Acetate Extract	20.0	20.0	7.0	35.0%	52.875
	40.0	20.0	9.0	45.0%	
	60.0	20.0	11.0	55.0%	
	80.0	20.0	13.0	65.0%	
	100.0	20.0	15.0	75.0%	
Methanol Extract	20.0	20.0	10.0	50.0%	32.515
	40.0	20.0	11.0	55.0%	
	60.0	20.0	12.0	60.0%	
	80.0	20.0	15.0	75.0%	
	100.0	20.0	15.0	75.0%	
Standard ($K_2Cr_2O_7$)	25.0	20.0	17.0	85.0%	5.653
	100.0	20.0	20.0	100%	
Standard (Thymol)	12.5	20.0	20.0	100%	0.531
	25.0	20.0	20.0	100.0%	
	100.0	20.0	20.0	100.0%	

Table 1: Percentage Mortality Rate of Brine Shrimps at Different Sample Concentrations

4. Results

4.1. Proximate Analysis

The proximate analysis of the plant aerial part revealed its carbohydrate content to be above 70.0% of the entire plant material. This was followed by the crude fibre having the percentage content of 15.89% of the tested plant material while the fat content had the lowest percentage of the material. The results of the proximate analysis showed that the parameters were in this decreasing order, (Carbohydrate >Crude fibre>Ash content>Protein content>Moisture content>Fat content) with fat content having the least value.

4.2. Brine Shrimp Lethality Test

The cytotoxicity properties of the extracts were determined by subjecting them to brine shrimp lethality test at different extract concentrations. The cytotoxicity properties of the three were compared to the known standard drugs (Thymol and Potassium dichromate) at different concentrations and their respective LC_{50} were determined**.** At 60.0µg/ml concentration, hexane extract led to the death of 14 brine shrimps (70.0% mortality rate) out of 20 shrimps experimented, the ethyl acetate extract led to 11 deaths (55.0% mortality rate) out of the 20 brine shrimps experimented and the methanol extract led to 12 deaths only (60.0% mortality rate). At 100.0µg/ml concentration hexane extract gave 80.0% mortality rate, ethyl acetate extract gave 75.0% mortality rate, methanol extract gave 75.0% mortality rate while the two standards gave 100.0% mortality rate each. The LC_{50} of the extracts were higher than the standards while ethyl acetate had the highest LC_{50} value of 52.875µg/ml.

5. Discussion

Medicinal plants are known to be source of bioactive compounds that have therapeutic values, the World Health Organization (WHO) encourages the inclusion of herbal medicine in health care because of the great potential they possess [15]. One of the aims of this study was to investigate the proximate analysis of the plant, as to known the percentage composition of the components present. The ash content of the aerial part of *A. muricata* was 7.40% and this revealed the amount of minerals present in the plant aerial part. The microbiological stability of any food is known to be depended on the level of its mineral contents. The crude fibre was found to be 15.89%, fibre when consumed adds bulk to the diet and therefore prevents the intake of excess starchy food [16] and it also helps in the management of diabetes mellitus. The moisture content of the *A .muricata* aerial part was 2.0%, this will enhance the shelf life of the plant by hindering the growth of spoilage microorganisms on it after harvesting [17]. It was reported that the *A. squamosa* peel contained 3.96% moisture content by loss on drying method [18].

In Table 1, it was shown that at 60.0µg/ml concentration, the hexane extract gave 70.0% mortality rate, ethyl acetate gave 55.0% mortality rate while methanol extract gave 60.0% mortality rate. At 100.0µg/ml concentration, hexane extract gave the highest percentage mortality rate of 80.0%, while the other ones had 75.0% mortality rate each. The methanol extract had the lowest LC_{50} value of 32.515µg/ml and it was followed by hexane extract having LC_{50} value of 38.577µg/ml, the LC_{50} value of all extracts were found to be higher than that of the standard drugs used. It was reported that the leaves of *A. squamosa* hexane extract exhibited LC_{50} of 42.65µg/ml while its methanol extract exhibited LC_{50} of 14.12µg/ml[19].The results in Table 1 showed that the extracts showed different mortality rate at different concentrations and that the extracts were toxic to brine shrimps as they exhibited low LC_{50} value [20]. This showed the presence of bioactive compounds of cytotoxicity property in the plant extracts.

6. Conclusion

This study has revealed the nutritional composition of *A. muricata* aerial part, the high value of carbohydrate in it, shows that the plant is a good source of energy to animals and man. The three extracts were active against the brine shrimp, having good LC_{50} values, due to the presence of the cytotoxicity compounds in them. Therefore, it an indication that those bioactive compounds present in the plant extracts may exhibit anticancer property when tested against some cancer lines. Therefore, further research is essential as to determine the anticancer property of the bioactive compounds present in the extracts using different cancer cell lines.

7. References

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