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Gas chromatography-mass spectrometry and acute toxicity studies of *Annona muricata* Ethanol stem bark extract

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Abstract

Plants with therapeutic potentials are considered to be the main source of phytoconstituents that can be used for the treatment of various ailments. This work focuses on determining the acute toxicity and the phytochemical constituents present in ethanol stem bark extracts of *Annona muricata* using Gas chromatography-mass spectroscopy. Lorke's toxicity testing method was used to determine the acute toxicity of the *A. muricata* stem bark extract. The result of the quantitative phytochemical GC-MS analysis of the stem bark showed that the ethanol stem bark extracts contained 13 compounds at different retention times. The dominant constituents present in the stem bark ethanol extracts of *A. muricata* were; Diisooctyl phthalate (25.81%), 1,2,3-Benzenetriol (14.41%),Phthalic acid (12.47%), Silane (7.60%) and 2-Heptanol (7.01) while the other constituents have less than 6% abundance respectively. Studies on acute toxicity revealed that the LD_{50} was > 5000 mg/kg of body weight.

Keywords: *Annona muricata* Stem Bark; GC-MS; Acute Toxicity; Phytochemicals; Phytomedicine

1. Introduction

Plant derived medicines popularly known as herbal drugs or phytiomedicine are recognized as the most common form of alternative medicine, hence almost 65% of the world's populations have incorporated traditional medicine (mainly herbs) into their primary modality of health care (Fabricant and Farnsworth, 2001). In recent years, there has been an increase in the study and use of medicinal plants most especially in developing countries where many people rely on traditional medicine for the treatment of different disease conditions and health challenges. This renewed interest by scientist especially biochemist and pharmacologists in the use of medicinal plants may be attributed to its availability, cheapness and accessibility by the local populace, high incidence of side effects of synthetic medicinesand most especially the environmental friendliness of natural plant extracts to the body (Gotep *et al.*, 2010). According to the World Health Organization (WHO, 2008) as reported by Opara *et al.,* (2021), 80% of people still rely on plant-based traditional medicines for primary health care and 80% of the plant derived drugs were related to their original ethnopharmacological purpose (Fabricant and Farnsworth, 2001). This is because medicinal plants are considered to be the main source of biologically active compounds that can be used for the treatment of various ailments including cancer, diabetes, hypertension, and ulcer. Out of the wide array of plant species on earth, only about 1-10% has been studied chemically and pharmacologically for their potential medicinal value especially for chemotherapeutic effect (WHO 2008) hence many natural products and synthetically modified natural product derivatives have been successfully developed for clinical use to treat human diseases in almost all therapeutic areas (Newman and Cragg, 2007). One of such plants with extensive traditional use is *Annona muricata* (sour sop).

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Annona muricata commonly known as sour sop (English), '*Ebo'* or '*Apekan* in Yoruba, '*Shawshopu'* or '*Sawansop'* in Igbo, 'Tuwon Biri' or '*Mama'* in Hausa, 'Graviola' (Portuguese) belong to the family *Annonaceae* comprising at approximately 130 genera and over 70 species (Moghadamtousi *et al.,* 2015; Makeri *et al*., 2015; Opara *et al.,* 2024) that has been widely studied in the last decade due to their therapeutic potentials.

Annona muricata is predominantly found in the Eastern part of Southern Nigeria and Africa in general. Although, research have shown that the plant is currently cultivated widely throughout the West Indies, and in some countries such as China, Australia, India, Southern and Northern America and it is becoming invasive in tropical climates throughout the world (Opara *et al.,* 2021).

Research on the various parts of *Annonamuricata* (leaf, stem bark, root and root bark, and fruit juice) has been documented in several journal publications worldwide which has significantly improved and elevated the primary health care system and interest in plant pharmacology, particularly because these plant extracts are thought to be safer than synthetic drugs. According to Okigbo and Mmeka (2006), the main shortcomings of traditional medicine are the imprecise diagnosis and lack of precision in dosing, which tend to be problematic for chronic and complex illnesses.

All parts of *Annona muricata* plant, similar to other *Annona* species including *A. squamosa* and *A. reticulate*, are extensively used as traditional medicine against an array of human ailments and diseases especially cancer and parasitic infections. The fruit is used as a natural medicine for arthritic pain, neuralgia, diarrhea, dysentery, fever, malaria, rheumatism, skin rashes, arthritis, worm and it is also eaten to elevate a mother's milk after childbirth (Moghadamtousi *et al*., 2015). . The leaves and the stem bark are used to make decoctions which are taken orally for intestinal malaise. A massage of the leaves is good for nervous shock, while a leaf or bark decoction is used for anxiety attacks. Flower bud tea mixed with honey are used for colds, chest pain and nerve disorders and the bark of the young fruit are used to treat diarrhea and dysentery, the green back is rubbed on wounds to stop bleeding (Orwa *et al.,* 2009). The anti-inflammatory, hypoglycemic, sedative, smooth muscle relaxant, hypotensive and anti-spasmodic effects of *A*. *muricata* are attributed to its leaves, barks and roots (Moghadamtousi *et al*., 2015), in addition to its ethnomedicinal uses, the fruits are widely employed for the preparation of beverages, candy, ice creams, shakes and syrups (Usunobun*et al.,* 2015).Several studies by different researchers have implicated the leaves as well as the bark as anti-hypertensive, vasodilator, anti-spasmodic (smooth muscle relaxant) and cardio depressant (slowing of heart rate) activities in animals (Coria-Tellez *et al*., 2016). According to (Usunobum and Okolie, 2015), the stem bark of *Annona muricata* are used in the treatment of diabetes and arthritis.

There have been several pharmacological attributes of *A. muricata* stem bark as used in ethnomedicines. Although most of the plant parts are used synergistically, hence studies by different researchers demonstrated that the leaf, stem bark, root and seed extracts of *A. muricata* are antibacterial invite against numerous pathogens (Adewole and Caton –Martins, 2006; Makeri *et al.,* 2015) and that the stem bark has antifungal properties, this is because of the presence of large number of biologically active compounds and chemicals generally referred to as annonaceous acetogenins (Makeri *et al.,* 2015)

Every part of *A. muricata* has rich deposit of plant chemicals which have healing effects. These phytochemicals include; alkaloids, tannin, megastigmanes, flavonol, triglycerides, flavonoids, alkaloids, steroids, triterpenoid (Hardoko *et al.,* 2015) and cyclopeptides (Moghadamtousi *et al.,* 2015). Besides the roles phytochemicals play in plants, they also play important roles in man and animals. Most phytochemicals possess antioxidant activities hence they help in cleaning up free radicals and also prevent diseases that manifest from reactive oxygen species (ROS) (Mith *et al.,* 2014; Oksana *et al.,* 2012). Phytochemicals prevent and ameliorate diseases such as diabetes, cancer, hyperlipidaemia, cardiovascular diseases, liver toxicity, Alzheimer, cataract, age related function decline, stroke and others (Onuah *et al.,* 2019).

Although scientists have identified thousand of different phytochemicals found in plants, the biological activities for most of these phytochemicals are yet unknown or are poorly understood in isolation or as part of foods, hence phytochemicals with established roles in the body are classified as essential nutrients (Molyneux *et al.,* 2007). Some are known as phyotoxins that are toxic to humans for example anstolochic acids which is thus carcinogenic at low doses, some other phytochemicals are anti-nutrients that interfere with the absorption of nutrients, while others such as some polyphenols and flavonoids may be pro-oxidants in large ingested amounts (Halliwell, 2007).

The stem bark of *A. muricata* are reportedly extensively employed for various ethnomedicinal purposes, with a particular emphasis on their notable antifungal, anti-inflammatory, hypoglycemic, sedative, smooth muscle relaxant, hypotensive and anti-spasmodic attributes (Moghadamtousi *et al.,* 2015; Makeri *et al.,* 2015) Thus, this study aims to identify using GC-MS, the industrial and pharmaceutical applications of the chemical components of ethanol stem bark extract of *A. muricata* and its acute toxicity on experimental rats.

2. Materials and methods

2.1. Sample Collection, Identification, and Preparation of *Annona muricata* stem bark

The stem bark of *A. muricata* were removed from the plant from a home garden in Umunnemochie Akabo in Ikeduru L.G.A of Imo State, Nigeria, and identified by a taxonomist Mr. Finan Iroka, of the Department of Botany, Nnamdi Azikiwe University, Awka, with the herbarium number NAUH-004B. The stem barks were also washed thoroughly in tap water and air dried at room temperature and constantly weighed using an electronic weighing balance until constant weights were observed, after which the dried stem bark were milled to fine powder using a mechanical grinder (Corona).

2.2. Procurement and Management of the Animals

A total of twelve (12) male Wistar rats weighing between70-100 g were obtained from the Faculty of Biological Sciences, University of Nigeria Nsukka, Enugu State, and housed in the animal house of Anatomy Department, Imo State University Owerri under standard conditions of light, temperature, and humidity of 12 hrs Light and 12 hrs Dark periods at room temperature. The animals were allowed free access to standard commercial rat pellets and drinking tap water *ad libitum* and were kept for 7 days to acclimatize

2.3. Ethical Clearance

Ethical clearance was obtained and granted by the Nnamdi Azikiwe University-Animal Research Ethics Committee and all animal studies were conducted in compliance with the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals (Pub. No. 85-23 Revised 1985) as approved by the Nnamdi Azikiwe University, Awka Ethical Committee for the use of Laboratory Animals.

2.4. Extraction from Plant Stem Bark

Ethanol extract from the plant stem bark was prepared by a modified method as described by Sanni *et al.,* (2014). One Hundred (100) grams of the dry-milled plant stem bark were soaked separately in 1000 ml of 70% ethanol solution at room temperature for 48 hrs. The extracts were cold macerated (filtered) using a muslin cloth, and then filtered through Whatman filter paper No.4 respectively. The extracts were thereafter concentrated by the aid of a water bath at 60 ^{o}C to one-tenth of its original volume. The crude extracts of the stem bark were then stored at 4 ^{o}C in the refrigerator and subsequently used for the studies. An aliquot portion of the crude plant extract residues was weighed and used for (GC-MS) phytochemical screening while the remaining extracts were reconstituted in distilled water for use in acute toxicity studies.

2.5. Quantitative Phytochemical Analysis

The phytochemical investigation of ethanol extracts of *A.muricata* stem bark was performed on GC-MS equipment, using the method as described by Kanthal *et al.* (2014). Exactly 2 ul of the sample extract was injected into the GC column for analysis. The GC (Thermo GC-TRACE ultra version: 5.0) and MS (Thermo MS DSQ II) is equipped with DB-5 ms capillary column (30 m×0.25 mm; film thickness 0.25 µm). The initial temperature was set at 40°C which increased to 150 °C at the rate of 10°C/min. The temperature subsequently increased to 230 °C at the rate of 5 °C/min. The process continued until the temperature reached 280 °C at the rate of 20 °C/min which was held for 8 minutes. The injector port temperature remained constant at 280 °C and detector temperature was 250°C then. Helium served as the carrier gas with a flow rate of 1 mL/min. Split ratio and ionization voltage were 110:1 and 70 eV respectively. To identify the unknown phytoconstituents present in the extract and their individual mass spectral peak value were compared with the database of National Institute of Science and Technology 2014 by using the Wiley Spectral library search programme.

2.6. Acute Toxicity Testing

The median lethal dose (LD_{50}) of *A. muricata* ethanol stem bark was determined in rats using a modified method of Lorke (1983), as reported by Aroma and Enegide (2014). Exactly 12 male Wistar rats were used for this study and the study was done in two phases. Nine (9) rats were used in the first phase and they were divided into three (3) groups of three (3) rats each and were administered 10, 100, and 1000 mg/kg body weight of the extract respectively, through oral administration. After administration of the extract, observations were made at regular intervals to check for the commencement of adverse effects, time of death, or time of recovery. This period lasted for 24 hrs. In the second phase, three (3) rats were randomly divided into three (3) groups, and the extract's doses were increased to 1600, 2900, and 5000 mg/kg of body weight. They were monitored for potential toxicity symptoms for a whole day as well as for potential delayed toxicity signs for a further fourteen days. Using the formula below, the lethal dose was determined.

$$LD_{50} = \sqrt{(D_o \times D_{100})}$$

Where
D_o = highest dose that gave no mortality
D_{100} = lowest dose that produced mortality

3. Results

3.1. GC-MS analysis results of ethanol stem bark extract of *A. muricata*

The GC-MS Chromatogram revealed a total of 50 peaks with different retention times. Though the peak number was 50, the identified compounds were 13 due to the reiteration of some compounds.

Table 1 Quantitative phytochemical constituents of ethanol stem bark extracts of *Annona muricata*

SN	RT (mins)	Phytochemicals	Abundance %	Molecular formular	Molecular weight (g/mol)
1	1.688	Cyclooctane	0.23	C_8H_{16}	112.21
2	1.798	2,4-Dinitro-1,3-dimethyl-benzene	0.16	$C_8H_8N_2O_4$	*196.16*
3	13.976	1,2,3-Benzenetriol	14.41	$C_6H_6O_3$	126.11
4	14.911	Acetophenone	0.50	C_8H_8O	120.40
5	15.815	Benzoic Acid	3.29	$C_7H_6O_2$	122.12
6	17.789	Triethyl Citrate	0.67	$C_{12}H_{20}O_7$	276.28
7	20.269	Phthalic acid	12.47	$C_8H_6O_4$	166.14
8	21.118	n-Hexadecanoic acid	1.25	$C_{16}H_{32}O_2$	256.43
9	21.275	Dibutyl Phthalate	1.54	$C_{16}H_{22}O_4$	278.34
10	21.468	1H-Imidazole-4,5-dicarboxylic acid	3.47	$C_5H_4N_2O_4$	*156.10*
11	22.506	Diisooctyl phthalate	25.81	$C_{24}H_{38}O_4$	390.56
12	24.318	2-Heptanol	7.01	$C_7H_{16}O$	116.20
13	24.882	Silane	7.60	SiH_4	32.12

3.2. Median lethal dose (LD_{50}) of the extract

Table 2 Median Lethal Dose (LD_{50}) of the Extracts

Group	Dose (mg/kg) Body weight	Quantity of Animals	Number of Death	% Mortality
A	10	3	0	0
B	100	3	0	0
C	1000	3	0	0
D	1600	1	0	0
E	2900	1	0	0
F	5000	1	0	0

When the extract was given orally to rats at doses of 10, 100, 1000, 1600, and 2900 mg/kg body weight, there was no mortality; however, at doses of 5000 mg/kg body weight, the rats experienced discomfort and became lethargic during a 72-hour observation period (Table 2). The calculated LD_{50} for ethanol stem bark extracts of *A. muricata* at the end of 72 hours of acute toxicity test was >5000 mg/kg body weight.

4. Discussion

The phytochemical screening carried out in this study revealed compounds with significant interest which have been reported to have both therapeutic and industrial uses.

The phytochemical result on the stem bark of *Annona muricata* in this study is in contrast with the study carried out by Olowofolahan *et al*., (2022) where the phytochemical result revealed that the stem bark of *Annona murcata* contains Cyclooctane, 2,4-Dinitro-1,3-dimethyl-benzene, 1,2,3-Benzenetriol, Acetophenone, Benzoic Acid, Triethyl Citrate, Phthalic acid, n-Hexadecanoic acid, Dibutyl Phthalate, 1H-Imidazole-4,5-dicarboxylic acid, 2-Heptanol, Silane and that these phytochemicals exhibit various pharmacological and biochemical actions when ingested by animals.

The stem bark extract has alcoholic and phenolic compounds. The phenolic compound, 1,2- Benzenedicarboxylic acid, also known as Phthalic acid, was found in the stem bark extract of *Annona muricata* (12.47%). Epidemiologic studies found that early phthalates exposure could induce significant neurodevelopmental damage (Miodovnik *et al.,* 2014). Some Phthalic acid esters have been proven to possess reproductive and developmental toxicities to animals and are suspected of causing endocrine-disrupting effects to humans (Huang *et al.,* 2021).

Di-n-butyl Phthalate present in the stem bark extract of *Annona muricata* has been reported to effectively activate zebrafish embryos' antioxidant system and lead to immunotoxicity and neurotoxicity as reported by Huang *et al.,* (2021). Zhao *et al*., (2014) reported that di-n-butyl phthalate causes a disruption of the antioxidant system of carps (Cyprinuscarpio).

A study carried out by Zhu *et al.,* (2014) on the allelopathic activities of a plant isolated two allelochemicals, di(2-ethylhexyl) phthalate and di-n-butyl phthalate, from the root exudates of the invasive plant, *Ageratina adenophora*. In a bioassay, di-n-butyl phthalate was found to possess a significant inhibitory effect on seed germination and seedling growth of *A. adenophora*. Meanwhile, these two compounds significantly increased the superoxide dismutase (SOD) activity of *A. adenophora*'s leaves and caused lipid peroxidation and cell membrane damage.

Physiological studies have indicated that phthalate can influence enzyme activity, which might be at least one of their phytotoxicity mechanisms. A study carried out by Dong *et al.,* (2016) and reported by Huang *et al.,* (2021) extracted diisobutyl phthalate and di-n-butyl phthalate from the ethyl acetate extract of *Cladophora fracta*, both of which show a strong inhibitory effect on the growth of *Heterosigma akashiwo* and *Gymnodinium breve*, which may be related to the production of reactive oxygen species (ROS) induced by diisobutyl phthalate and di-n-butyl phthalate in algal cells. Furthermore, Di-n-butyl phthalate has been reported to posses antibacterial properties. Di-n-butyl phthalate has been shown to inhibit the growth of gram-positive (*Bacillussubtilis* and *S. epidermidis*, MIC at 18.75 µg/mL for both) as well as gram-negative bacteria (*E. coli* and *P. aeroginosa*, MIC at 37.5 µg/mL for both) (Huang *et al.,* 2021).

One of the dominant phytoconstituents in the stem bark extract Diisooctyl phthalate is a phthalic acid diester which is primarily used as a plasticizer for synthetic rubber and vinyl, cellulosic and acrylate resins (Saillenfait *et al.,* 2013). Common reproductive and developmental effects have been associated with Diisooctyl phthalate as a result embryoletality and fetal malformations (e.g. skeletal, cardiovascular, nervous system) have been observed in rats after prenatal exposure to active Diisooctyl phthalate, at relatively high doses. Diisooctyl phthalate have also been documented to have heptatoxic effects and may cause central nervous system (CNS) depression after ingestion of large amount over a long period of time although no report has been documented when ingested for a short period of time (NICNAS, 2008).

Acetophenone present in the stem bark extract is used for fragrance in soaps and perfumes, as a flavoring agent in foods, and as a solvent for plastics and resins. Acute exposure of humans to acetophenone vapor may produce skin irritation and transient corneal injury. A study noted a decrease in light sensitivity in exposed humans. Acetophenone is hypnotic in high concentrations and may cause narcosis and central nervous system depression. Overexposure by inhalation is unlikely because of low volatility and odour-warning properties. Central nervous system (CNS) depression may include general discomfort, symptoms of giddiness, headache, dizziness, nausea, anaesthetic effects, slowed reaction time, slurred speech and may progress to unconsciousness. Serious poisonings may result in respiratory depression and may be fatal (Chemical Update Worksheet, 2016).

Acute toxicity study of the ethanol leaf and stem bark extracts of *A*. *muricata* on experimental rats, showed that the extract was not lethal to the animals even at a concentration of 5000 mg/kg, and this is in agreement with the study carried out by Olowofolahan *et al*., (2022); Agu *et al.*(2017), that no mortality was observed at all dose levels from the critical 24 hours post administration to the end of the seventh day and that the LD_{50} was estimated to be >5000 mg/kg.

However, this study is in contrast with the study carried out by Agu and Okolie(2017) that high dose of aqueous leaf extract of *A. muricata* were found to be toxic and or lethal to the animals.

Furthermore, the ethanolic leaf and stem bark extracts of *Annona muricata* are considered to have mild toxicity in rats by oral route according to the classification of Diezi, (1989) as reported by Alphonse *et al.,* (2018) thus this plant deserves to be used with caution in humans. This could however be as a result of the presence of oxalate, annonaceous acetogenins, acetophenone and octadecenoic acid methyl ester derivatives which studies have shown to possess deleterious consequences at high dose / concentration as reported by Opara *et al.,* (2021), Coria-Tellez *et al*., (2016) and Yu *et al.,* (2005).

5. Conclusion

The extract may be assumed to be non-toxic judging from the lack of serious alteration in the functional and behavioral observations, and lack of mortality following the administration of the stated doses in the acute toxicity studies, this may be as a result of the presence of chemical constituents in the plant extracts that may possess hepatoprotective, renal-protective and pancrea-protective effects.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

Ethical clearance was obtained and granted by the Nnamdi Azikiwe University-Animal Research Ethics Committee and all animal studies were conducted in compliance with the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals (Pub. No. 85-23 Revised 1985) as approved by the Nnamdi Azikiwe University, Awka Ethical Committee for the use of Laboratory Animals.

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