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Phytochemical Screening and Anti-Ovarian Cancer Properties of *Annona muricata* Linn. (Annonaceae) Seed Ethanol Extract

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Throughout medical history, plant products have been shown to be valuable sources of novel anti-cancer drugs. This present study was aimed determining the phyto-constituents and potency of ethanol extract of seed of Annona muricata Linn. on ovarian cancer tissues. Preliminary phyto-chemical screening of seeds revealed that it contains alkaloids, saponins, terpenes, flavonoids, anthraquinones, tannins, and cardiac glycosides. From the fractionation using column chromatographic technique, the fraction with alkaloids and flavonoids showed more anti -ovarian cancer activity when compared to other fractions. Mean survival time (MST) and percentage increase in life span were highest in group 4 with values 23 ± 0 .33 and 28 ± 0.30 respectively at 1000 mg / kg body weight (b.w) (P< 0.05, n = 8). Packed cell volume (PCV) showed progressive decrease as the dosage increased from 100 mg/kg to 1000 mg/kg in all the groups when compared with Ehrlich's Ascites Carcinoma cell (EAC) control groups with value 2.12 ± 0.10. Viable tumour cell counts (x 10^7 cells / ml) were 7.78 ± 0.18 (group 3 100 mg/kg), 5 .85 ± 0.23 (group 4 1000 mg/kg) and 4.90 ± 0.015 group 5 (vinblastin 0.8 mg / kg standard drug) and values are statistically different from the EAC control (group 2) with value 12.25 ± 0.01 (P< 0.05, ANOVA). Non-viable tumour cell counts (x 10^7 cells/ ml) were on the increase as the doses increased; 0.90 ± 0.24 (group 3 100 mg/kg), 1.47 ± 0.21 (group 4 1000 mg/kg) and 1.63 ± 0.81 (group 5 vinblastin 0.8 mg/kg standard drug) while the EAC control (group 2) was 0.8 ± 0. 02 and values were compared. All haematological parameters showed increase at the doses (intraperitoneal) investigated except total WBC white blood cells which slight decrease in values among the groups. Biochemical parameters in EAC- bearing Swiss female albino mice showed significance reduction in level of lipid perioxidation and increase in catalase and protein contents when compared to EAC control group (P< 0.05, ANOVA). The study therefore showed that seed ethanol extract of A. muricata Linn. had anti-ovarian cancer properties on the experimental animals and can therefore serve as a medication for ovarian cancer problems in females.

***Keywords**: Anti-ovarian cancer, Phytochemical, Tumour cells, Parameters, Annona muricata Linn.*

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INTRODUCTION

The use of plants in treating diseases dated back from the first century till date. Many orthodox medicines had been developed from plant and animal materials. These drugs include: chloroquine, artemisinins, nicotine, quinine, among others. Traditional medicinal products no doubts, present the cheapest and easily affordable therapy especially in Nigeria and other developing countries of the world. It is hope

that traditional medicine will in future provide the cure to many tropical diseases that have defied orthodox prescriptions.

A. muricata Linn. is a plant belonging to the family *Annonaceae*. It is commonly called ‘’Graviola or Soursop’’ in English and other various local names in Nigeria and other parts of the world where it is being used for one purpose or the other. The plant is a small tree (shrub) 5-6m high with glossy, dark green leaves. It produces a large, heart-shaped fruit that is 15-20cm in diameter which is yellowish when ripped but whitish inside. *A. muricata* L. is indigenous to most of the world’s tropical rainforest areas in South and North America as well as West Africa countries like Nigeria, Ghana, Ivory Coast, and the Gambia [1].

A. muricata L. had been used as phytotherapy for various ailments such as cancer (all types), as a broad spectrum internal and external antimicrobial to treat bacterial and fungal infections, for internal parasites and worms, for high blood pressure, for stress and nervous disorders [1, 2, 3, 4]. Other uses of the plant documented by traditional medicine practitioners are: antiviral, cardiotonic (tones, balances, strengthen the heart), decongestant, digestive stimulant, febrifuge (reduces fever), nervine (calm nerves), pediculicide (kill lice), vermifuge (expel worms) and uterine stimulant [5]. Chemically, the plant contains Acetogenins which is an anticancer, anti-tumour and antiviral agent [6, 7, 8].

The various histological subtypes of ovarian include serous, mucinous, clear cell, endometrioid, transitional cell/Brenner tumours and undifferentiated adeno carcinomas; these are different in their aetiology and genetic abnormalities [9]. Ovarian cancer constitute about 55-60 % gynaecological problems in females worldwide. It is clear that epithelial ovarian cancer have highly aneuploid cells with multiple marker chromosomes and high heterogeneity both within and between individual cases [10].

Novel therapeutic strategies have been investigated for ovarian cancer to reduce toxicity and to improve outcome for patients. Short intervening RNA (siRNA), which directs the sequence-specific degradation of target mRNA and provides specificity of gene knockdown, represents a unique class of potential therapeutics for ovarian cancer.

Nanocarriers can efficiently protect siRNA from *in vivo* degradation and are able to deliver these active macro-molecules to tumour cell even after intravenous administration. Nanocarriers for siRNA delivery for the treatment of cancer may be applied in two ways: active targeting and passive targeting [11]. We have to investigate first to know if the seed extract of *A. muricata* Linn. can reduce the growth of ovarian cancer in females.

Therefore, this research was conducted in order to determine the effects of the extract of *A.muricata* Linn. seeds on ovarian cancer tissues.

MATERIALS AND METHODS

Collection and Preparation of Plant Materials

The fruits of *A. muricata* Linn. were collected from tropical rainforest zone of South-East Nigeria, it was identified at the herbarium unit of the Department of Biological Sciences Ahmadu Bello University Zaria by Mr. U.S Galla where a voucher number was deposited for the plant. The seeds were removed from the fruits dried in free air for seven days and grind using electric grinder (Model ZWQ18764 made in China) and prepared following standard procedures and stored for onward use.

Extraction of Plant Materials

The powdered plant materials of weight 250 g was defatted using petroleum ether (60-70 ^{0}C) and then extracted in absolute ethanol (100 % v/v) containing 300 ml of distilled water following Soxhlet extraction processes for 96 hrs (4 days) to allow all the constituents dissolved in ethanol. The filtrate was then concentrated by boiling in a water bath 90 - 100 ^{o}C and then the residue was collected and weighed, stored in a bottle and kept in desiccators at room temperature for further analysis.

Preliminary Phytochemical Screening of Extract

Seed ethanol extract of A. muricata Linn. was screened for the following phyto constituents: alkaloids, saponins, terpenes, flavonoids, anthraquinones, tannins and cardiac glycosides. The methods described by Trease and Evans [12] and Sofowora [13] were used.

Anti-Ovarian Cancer Activity Evaluation of *A. muricata* Linn. Seeds

Anti-Ovarian cancer activity of the extract was evaluated following these procedures: toxicity study (LD_{50}), Isolation of ovarian cancer cell line, preparation of extract drug and administration, tumour transplantation, drug treatment schedule, tumour cell volume and packed cell volume determinations, viable and non-viable cell counts, mean survival time (MST) and percentage increase in life span of animals, evaluation of haematological parameters and biochemical assay of extract.

Toxicity Evaluation of Extract (LD_{50})

0.5 gram of the extract was weighed and prepared into appropriate millilitres in order to determined the lethal dose (LD_{50}) (acute toxicity) of the extract in Swiss white female albino mice numbering fifty four (54) and weighed between 18-25 g. The experiment was divided into two phases. In the phase one, the animals were divided into 9 groups including 4 for pet –ether extract and 4 for ethanol extract of the plant seed plus one control group each consisting of 6 mice . Doses 10 mg/kg, 100 mg/kg and 1000 mg/kg b.w ethanol extract administration i.p were administered to the 9 experimental groups while the control received vehicle. The animals were fed with ordinary water on the first day and afterwards with maize feeds, and then monitored for any deaths and changes in behaviour for one week during the working days.

In the second phase, the remaining animals were divided into 3 groups of one animal per group and administered the doses of 1600 mg/kg, 2900 mg/kg and 5000 mg/kg b.w ethanol extract (i.p) and observed for changes and death for 24 hrs. The LD_{50} (Acute toxicity) was calculated as the geometric mean of the dosage that resulted in 100 % mortality and that dosage which cause no mortality at all. Recovery and body weight gain after each investigation was taken as a sign of surviving the acute intoxication. The experiment was terminated after two weeks [14].

Ovarian Cancer Cell Line Isolation

Ehrlich's Ascites Carcinoma (EAC) cells were obtained from the Oncology Unit of the Ahmadu Bello University Teaching Hospital (ABUTH) Shika, Zaria. They were maintained by weekly intraperitoneal (i.p) inoculation of 10^6 cells/ mouse [15].

Preparation of Extract for Administration (i.p)

Ethanol extract of *A. muricata* (EEAM) in the dosage of 100 and 1000 mg/kg were prepared as suspension by dispersing the ethanol extract into mixture of ethylene

glycol and sterile physiological saline containing Twee 20 (1:3) to obtain the needed concentration [16].

Tumour Transplantation Procedure

EAC of the ovary was maintained by serial transplantation from tumour bearing Swiss albino female mice. Ascetic fluid was drawn out from the mice at log phase (day 80 of tumour bearing) of the tumour cells. Tumour cell number was adjusted to 2 x 10^6 tumour cell/ml. Samples showing more than 90 % viability were used for transplantation. Each mouse received 0.2 ml tumour suspension containing 2 x 10^6 cell /ml (i.p) [17].

Drug Treatment Protocols of Ethanol Extract of *A. muricata* Seed

Female Swiss albino mice were divided into 5 groups (n = 8) and all the groups except the normal group were administered with EAC cells (0.2 ml of 2 x 10^6 cell / mouse i.p). This was taken as day zero. From the first day, normal saline (0.9 % NaCl), 5 mg/kg b.w was administered to group 1 and ethylene glycol 5 mg/kg was also administered to group 2 (cancer control) for 2 weeks (i.p). Similarly, EEAM at various doses (100 and 1000 mg/kg/mouse/day) were administered to the animals of groups 3 and 4 respectively. Standard drug vinblastin 0.8 mg/kg/mouse/day were administered to group 5. After the administration of last dose followed by fasting, 4 mice from each group were sacrificed for studying anti-ovarian cancer activity, haematological and liver biochemical parameters. The remaining animals in each group were kept to check the mean survival time (MST) and percentage increase in life span of tumour bearing mice [18].

Various parameters like body weight of mice, cytological studies of cell lines, RBC, WBC and haemoglobin differential count were equally studied. Anti-ovarian cancer effect of EEAM was assayed by observation of changes with respect to body weight, ascetic tumour cell volume, PCV, viable and non-viable tumour cell counts, MST, and percentage increase in life span [19].

Tumour Cell Volume (TCV) and Packed Cell Volume (PCV)

The mice were dissected to collect ascetic fluid from peritoneal cavity, and centrifuged at 1000 rpm for 5 min to determine the PCV. The transplantable murrain tumour was carefully collected to measure the tumour volume [19].

Viable and Non-viable Cell Count

Viable and non-viable cell counting of ascetic cell was done by staining with tryphan blue (0.4 % in normal saline), dye exclusion test, and count was determined in a Neubauer counting chamber. The cells that did not take up the dye were considered viable and those that took up the stain were not viable [20].

Mean Survival Time (MST) and Percentage Increase in Life Span (% ILS)

The effect of EEAM on tumour growth was observed by MST and % ILS. MST of each group containing 4 mice was monitored by recording the daily mortality for 6 weeks while % ILS was calculated by using the equation below [20]:

MST = Day of first death + Day of last death / 2

$$\% \text{ ILS} = \frac{\text{MST of Treated Groups}}{\text{MST of Control Groups}} \times 100$$

Effect of Ethanol Extract of A. muricata Seeds on Haematological Parameters

Blood was collected from each mouse by intracardial puncture with blood anticoagulant (heparin), WBC, RBC, haemoglobin concentration and differential counts were determined in groups comprising tumour bearing mice (control), tumour bearing mice treated with ethanol extract of *A. muricata* (EEAM) seed (100 mg/kg/mouse/day),tumour bearing mice treated with EEAM (1000 mg/kg/mouse/day), and normal group.

Biochemical Assay of Animals

After the collection of blood samples, the mice were sacrificed, and their livers were excised. The isolated livers were rinsed in an ice-cold normal saline followed by cold phosphate buffer with pH 7.4, blotted dry and weighed. 10 % w/v homogenate of liver was prepared in an ice-cold phosphate buffer and a portion was utilised for the estimation of lipids, and the other portion was used after precipitation of proteins with trichloroacetic acid (TCA) for the estimation of glutathione. The remaining homogenate was centrifuged at 1800 rpm (4 ^{0}C) for 15 min. The supernatant obtained in this case was used for the estimation of superoxide –dismutase, catalase and protein contents [20].

Statistical Analysis

The experimental results were expressed as mean ± standard errors of means (SE). Data were assessed by analysis of variance (ANOVA) for significant difference. $P < 0.05$ was considered as statistically significant.

RESULTS

The Phytochemical screening showed that the seed of the plant contains mainly glycosides and alkaloids. These constituents invariably were responsible for the activities of plants either negatively or positively [**Table 1**].

TABLE 1 : Phytochemical Screening of *A. muricata* Seed Ethanol Extract

Phytoconstituent:	Alkaloids	Saponins	Terpenes	Flavonoids	Anthracene	Cgly
Ethanol Extract:	+	+	+	+	+	+

+ (present), - (absent), Cgly (cardiac glycosides).

Mean survival time and percentage increase in life span of the mice were on the increase as the doses were increase in the groups [**Table 2**]. At 1000 mg/ kg b.w (i.p), the MST and % ILS values were 23 ± 0.33 and 28.30 % respectively for group 4, and value was significantly different from the EEAM control ($P< 0.05$, ANOVA).

TABLE 2: Mean Survival Time (MST) and Percentage (%) Increased in Life Span (ILS) of Mice after Administration of Extract at Different Doses.

Experimental group (Grp)		**MST ± SE**	**% ILS**
Normal control	Grp 1	–	–
EEAM control	Grp 2	18 ± 0.14	–
100 mg/kg	Grp 3	20 ± 0.22	15.25
1000 mg/kg	Grp 4	23 ± 0.33	28.30
Vinblastin	Grp 5	25 ± 0.34	33.42

Values are means ± SE, experimental group was compared with EEAM control ($P< 0.05$; ANOVA), n = 8 in each group, - (not applicable).

Weights of mice tend to be stable at the doses administered whereas tumour cell volume, packed cell volume, viable tumour count values decreased as the dosage increased in the groups **[Table 3]**. However, nonviable tumour cell count was on the increase with increase dosage and significantly different from the EEAM control group (P< 0.05; ANOVA).

TABLE 3: Effects of ethanol extract of *A. muricata* on tumour cell volume (TCV**),** packed cell volume (PCV), viable and non-viable tumour cell count on EEAM bearing mice

Parameters	Normal Grp 1	EEAM control Grp 2	100 mg/kg Grp 3	1000 mg/kg Grp 4	Vinblastin Grp 5
Body weight	26.10±0.12	26.11±0.12	24.34±0.16	23.28±0.13	23.90±.02
TCV	0	6.82±0.04	5.22±0.05	4.42±0.08	3.42±0.13
PCV	0	2.12±0.10	1.75±0.04	0.05±0.09	0.15±0.03
VCC	0	12.25±0.09	7.78±0.18	5.85±0.23	4.90±0.15
NVC	0	0.8±0.12	0.90±0.24	1.47±0.21	1.63±0.81

Values are means ± SE, weight of normal mice = 20 ± 0.15, TCV (Tumour cell volume), PCV (Packed cell volume), VCC (Viable cell count), NVC (Non-viable cell count) x 10^7 cells/ml.

In **table 4**, the haematological parameters increase with increase dose of the the extract with more value increase in the number of lymphocytes in the experimental groups. The values were statistically significantly different from the control (EEAM) (P< 0.05; ANOVA). There was a reduction in the level of lipid peroxidation which showed also that the extract of *A. muricata* has anti-ovarian cancer properties [**Table 5**].

TABLE 4: Effect of Ethanol Extract of A. muricata (EEAM) Seed on Haematological Parameters of EEAM Treated Albino Mice

Parameter	**Normal Grp 1**	**EEAM Control Grp 2**	**100 mg/kg Grp 3**	**1000 mg/kg Grp 4**	**Vinblastin Grp 5**
Hb(g)	14.88±0.25	9.82±0.02	10.60±0.06	*11.45±0.18	*11.70±0.5
RBC	8.62±0.14	3.81±0.04	4.75±0.03	*5.42±0.22	5.81±0.05
WBC	7.82±0.05	*20.07±0.07	11.92±0.04	8.85±0.06	9.12±0.06
Lymphocyte	77.75±0.19	33.77±0.56	*53.71±0.50	*66.72±0.38	69.12±0.30
Monocyte	1.71±0.04	0.82±0.02	1.15±0.01	1.23±0.05	1.34±0.02
Granulocyte	33.88±0.37	52.61±0.37	40.87±0.22	33.70±0.63	30.63±0.30

Values are means ± SE, EEAM Control group compared to normal group, experimental group compared to EEAM Control,*Statistical significant difference (P< 0.05; ANOVA), RBC (red blood cell count), WBC (white blood cell count), n = 8.

TABLE 5: Effect of Extract Doses on Biochemical Parameters in EEAM Bearing Mice

Parameter	**Normal Grp 1**	**Extract Control Grp 2**	**100 mg/kg Grp 3**	**1000 mg/kg Grp 4**	**Vinblastin Grp 5**
LPO	0.92±0.02	*1.39±0.06	*1.27±0.04	1.11±0.02	2.45±0.25
CTS	2.51±0.72	*1.73±0.15	1.75±0.13	*2.34±0.23	3.56±0.63
PTN	12.66±69	17.30±0.81	18.50±0.51	18.60±0.51	20.24±0.47

Note: Values are means ± SE, LPO (Lipid perioxidation; MDA/g of tissue), CTS (Catalase; unit/mg tissue), PTN (Protein; content g/100 ml), Extract control group was compared to normal group; experimental group was compared to extract control group. *Significant difference ($P < 0.05$; ANOVA), n = 8.

DISCUSSION

Preliminary Phytochemical screening of extract showed that it contains alkaloids, saponins, terpenes, tannin, flavonoids, anthraquinones, and cardiac glycosides [**Table 1]**. In addition, steroids, mineral nutrients, proteins, oleic acid had been reported to be present in methanol extract of the seed [21]. These Phytoconstituent exhibit diverse pharmacological and biochemical actions when ingested by animals [22]. Therefore, the anti-ovarian cancer properties of ethanol extract of seed was as a result of these phytochemicals **[Table 1]**. Higher dose of the extract (1000 mg/kg) increases the mean survival time (MST) and percentage increase in life span (%ILS) of albino mice, and the results are significantly different from the control (group) ($P < 0.05$; ANOVA) **[Table 2]**. This implies that at higher dose a better result is achieved in the anti-ovarian cancer properties of the plant. The implication is that, as the dosage increases, body weight did not reduce significantly in all the groups, while tumour cell volume (TCV), packed cell volume (PCV) and viable tumour cell count (VCC) values decreased. Non-viable tumour cell count (NVC) values increases with increased dosage **[Table 3]**. Despite the slight reduction in total WBC and granulocyte in groups 3 and 4 , other parameters such as haemoglobin concentration, red blood cell (RBC), lymphocytes and monocyte had higher values which are significantly different from the extract control ($p < 0.05$; ANOVA) **[Table 3]**. The destruction of WBC and granulocytes by the extract as the dose increase was overcame by differential increase in lymphocyte and monocyte which are the major immune cells of WBC, and this contribute to the anti-ovarian cancer properties observed in the plant extract.

Biochemical assay indicated that the extract significantly reduced the elevated levels of lipid perioxidation making it a good anti-ovarian cancer agent. There were general increase in biochemical parameters of the mice at doses 100 and 1000 mg/kg b.w investigated **[Table 5]**, results which also make the plant ethanol extract of seed an alternative therapy for ovarian cancer patients in traditional medicine. Cancer cells had been reported to affect neighbouring healthy cells by destroying haematological and biochemical parameters of the host [24].

CONCLUSION

The study showed that ethanol extract of *A. muricata* Linn. seed possessed anti-ovarian cancer properties and can be used to treat ovarian cancer in traditional medicine. This plant part thus represents a source towards new drug discovering.
However, the fact that the ethanol extract of seed of the plant destroyed some of the parameters investigated in the mice, suggests that for effective treatment of the disease, there is the need to design and develop a drug delivery system for targeting genes (siRNA) to ovarian cancer tissues.

The use of nanocarriers especially, chitosan nanoparticles which is develop from *Annona muricata* plant parts, is suggested for further research, as these nanoparticles are capable of delivering drugs to ovarian cancer tissues without causing any damage to neighbouring cells or tissues.

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