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ISOLATION, ANTI-TUMOR AND ANTI-OVARIAN CANCER ANALYSIS OF PURE COMPOUND FROM THE LEAF OF *ANNONA MURICATA* LINN. (ANNONACEAE)

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ABSTRACT

Ovarian cancer remains the leading cause of death from gynaecological malignancy. More than 60% of the patients are presenting the disease in stage III or IV. In spite of combination of chemotherapy and surgery, the prognosis stays poor for therapy regimen. The leaves of Annona muricata were extracted and then fractionated by column chromatography. In vitro cytotoxicity tests were performed with fractions and with an isolated compound on ovarian cancer cell line, at concentrations of 6.25-200 µg/mL for crude extract and pure compound. Cytotoxicity was

Introduction:

Cancer is known medically as a malignant neoplasm, is a broad group of diseases involving unregulated cell growth. In cancer, cells divide and grow uncontrollably, forming malignant tumors, and invading nearby parts of the body. Breast and Ovarian cancers are the commonest of all cancer types; with the ovarian cancer (OVC) a major gynecological problem in

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measured after 24, 48 and 72 hours using MTT assay. In vivo cytotoxicity was tested on ascites, developed in the abdomen of albino mice after inoculation with human epithelia OV7-CDNA-20µL cells intraperitoneally. Cytotoxicity of the crude extract and pure compound for the cell line at all time points IC_{50} was greater than 10 µg/mL, at 48 hours (95% confidence interval ; t-test). Both the crude plant extract as well as the pure compound of A. muricata leaf killed the cancer cell at a final concentration of 200 µg/mL and 100µg/ mL respectively at all concentrations. Mean survival time (MST) and percentage increase in life span were highest in group IV with values 3.0 ± 0 .03 and 5±0.05 respectively at 1000 mg / kg body weight (b.w).Packed cell volume (PCV) and Non-viable tumors cell counts (NVC) showed progressive increase as the dosage increased from 10 -1000 mg/kg in all the groups while viable tumor cell counts decreased in all the groups as compared to doxorubicin. The study showed that A. muricata leaf has potent anti-ovarian cancer effects against ovarian cancer cell line, and represents a source for ovarian cancer therapy.

Keywords: *Ovarian cancer, Annona muricata, MTT assay, Cytotoxicity, tumor cell*

developing countries[1]. There are over 100 different types of cancer, and each is classified by the type of cell that is initially affected [2].

Mutation in FOXL2 is the major cause of ovarian cancer. FOXL2 is Forkhead transcription factor which is responsible for normal development of eyelids in newborns, it is also needed to form a full complement of eggs in ovaries before birth is the primary cancer of ovarian cancer. Mutation of FOXL2 can be caused by radiation, chemotherapy and autoimmune disorder . FOXL2 is a transcription factor; meaning it stimulates other genes to turn on or off in the

eyelids and the ovaries[3]. FOXL2 gene is located from base pair **138,944,223** to base pair **138,947,139** on chromosome **3**. Blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) is a genetic disorder characterized by eyelid malformation and ovarian dysfunction[4].

Annona muricata is a small, upright, evergreen tree that can grow to about 4 m (13 ft) tall. It is called soursop (Graviola) in English due to the sweet and sour flavor of the large fruit, 'Gwanda Masar' in Hausa , and a lowland tropical fruit-bearing tree in the Annonaceae family mainly found in South-East Nigerian States of Anambra, Imo, Abia and Enugu. The plant has many uses in traditional medicine. It protects the immune system and avoid deadly infections. Effectively target and kill malignant cells in 12 types of cancer, including colon, breast, prostate, lung and pancreatic cancer. The tree compounds (annonacin, annonins, bullatacin, etc) proved to be up to 10,000 times stronger in slowing the growth of cancer cells than Adriamycin (Doxorubicin), a commonly used drug [5].

Figure 1 : Pictorial view of *Annona muricata* in its natural habitat. Source: Takum Forest Taraba State, Nigeria (June ,2014).

This study was carried out in order to determine the anti-ovarian cancer activities of *Annona*

muricata isolated compound and to study the cytotoxicity of crude extract of leaf and pure compound on ovarian cancer cell line by *in vitro* and *in vivo* methods.

Materials and Methods

Collection and identification of plant

Leaves of *Annona muricata* were collected from forests in Takum and Bali L.G.A Taraba State and identified at the herbarium unit of Biological Science Department, Ahmadu Bello University, Zaria, Nigeria where voucher number was deposited for the plant.

Extraction and Isolation of *Annona muricata* leaf Powder

Dried ground leaves of *Annona muricata* weighing 5000g (5kg) were macerated with methanol at room temperature. After 24 hours a crude extract of the leaves was removed and evaporated using rotary evaporator under reduced pressure to yield a dark green extract (500 g, 10 % yield). 3 g portion of the extracts were fractionated using column chromatography (CC) with 4 mm thickness silica gel(60-120 Merck, Germany) on a round glass plate eluted with increasing polarity. Elution was carried out with a stepwise gradient consisting of hexane:dioxane, 98:2 (v/v 400 mL); hexane:chloroform:dioxane, 88:10:2 (v/v 600 mL); hexane:chloroform:dioxane: ethyl- acetate:2-propanol, 80:10:2:6:1, (v/v 600 mL) and hexane: chloroform: acetone: methanol, 56:20:16:8, (v/v 400 mL). A total of 125 fractions (10 mL each) were collected .The eluents that showed the same profile on thin layer chromatography (TLC) chromatogram were combined to give four fractions (I-IV). The isolated compound's purity was demonstrated through the observation of only one spot in TLC plate as well as a single peak on HPLC apparatus [5].

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HPLC Analysis

HPLC analysis of methanol extract was carried out with chromatographic system (YL 9100,Korea) consist of autosampler (YL 9150) with 100 µL fixed loop and an YL9120 UV-Visible detector. The separation was performed on a SGE Protocol PC18GP120 (250mm×4.6 mm, 5µm) column at ambient temperature. The mobile phase consists of methanol to ethylacetate (70:30 v/v) and the separations were performed by using isocratic mode, elution performed at a flow rate of 1 mL/min. The samples were run for 15min. and detection was done at 254 nm by UV detector. All chromatographic data were recorded and processed using autochro-3000 software[5-8].

Anti-tumor study of leaf methanol extract

Solution of formaldehyde(10%) was added to 50 mL distilled water in drinking bottles. The animals were allowed freely to drink the solution *ad libitum* for one month (i.e. 4 weeks) . Prior to tumor induction, the initial weights of the mice were recorded and animals were divided into five groups of six animals each, and were housed separately in cages. Each day the residual drinking solution was measured so that the consumption of each test solution could be calculated; solution pH was 6.8 to 7.0 [9]. After tumor development, the animals were administered methanol leaf extracts of the crude and pure compounds intraperitoneally. Group I received 5mL/kg normal saline, group II, III and IV received 10mg/kg, 100mg/kg and 1000mg/kg of crude and pure compound, while group V received Doxorubicin (Adriamycin® USP) 10mg/kg i.p [10]. The animals were observed for two weeks. Tumor parameters were recorded . Haematological parameters such as PCV, Hb, WBC, MCV, MCHC, and RBC were also determined. The investigations here were based on pure compound isolated from the leaves of *Annona muricata*

methanol extract. Mean survival time(MST) and increase in life span (%ILS) were calculated using the formulae below respectively:

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

%ILS = MST of treated group/MST of control group x 100

In-vitro Cytotoxicity Assay

Cytotoxicity assay was carried out in accordance with previously published protocol by [11]. Ehrlich ascites carcinoma cells (EAC) drawn from the animals ($5x10^3$ cells/well) were cultured on a flat-bottomed 96-well plate. After 48 h incubation, 20µl of MTT solution (5mg/mL) was added to each well of the assay plate, which was then incubated for 4 h at 37ºC. After incubation, the formazan crystals formed by the reduction of tetrazolium salt by the mitochondria of living cells, were dissolved in DMSO. The plates were read in ELISA plate reader at wavelength of 540 nm.

Cell lines culture

Cell line used in the assays was epithelial ovarian cancer OV7-96020764-CDNA-(20uL)
obtained on demand from Zayo-Sigma, Jos Nigeria.

In vitro cytotoxicity tests with different fractions of *Annona muricata*

In vitro cytotoxicity tests were performed using MTT assay as described by Xu-jie and Chu [11]. Briefly, ovarian cell line (5×10^4) was seeded in 24-wells plates (Costar, USA) and grown in RPMI-1640, supplemented with 6 mM L-glutamine, 10% foetal bovine serum (FBS) (Gibco, Invitrogen, UK) and penicillin (100 units/mL) and streptomycin (100 µg/mL), while normal fibroblasts were grown in Dulbecco's modified Eagle medium (DMEM), also supplemented with L-glutamine and FBS. Cultures were maintained in a humidified

atmosphere of 5% CO_2 at 37 °C. Cell cultures, in triplicates, in exponential growth were treated with the different dried fractions of the plant extract, re-dissolved in di-methyl sulfoxide (DMSO) and added at final concentrations of 1, 10 and 100 µg/mL. The control cultures had 0.02% (1 µg/mL) 0.2% (10 µg/ mL) and 2% (100 µg/mL) DMSO added to the medium. In 2 mL medium/well 10% MTT was added and 100 µL of the supernatants of the 24-well plates after 24, 48 and 72 h incubations were pipetted into 96-well plates. Cell viability was measured with a 96-well plate reader. In a later stage, after identifying fractions with high cytotoxic effects, the final concentrations of extracts tested ranged from 6.25-200 µg/mL, with final concentrations of 0.02 up to 0.2% DMSO.

In vivo pilot experiment

An *in vivo* pilot experiment was performed with thirty Swiss albino mice consisting of all females (average weights 18-25g) . In order to mimic advanced ovarian cancer, the mice were injected intraperitoneally (i.p.) with 1 x 10^4 OV7-96020764-CDNA-(20uL) cell line into the abdominal cavity to form ascites. Five groups of mice were examined: four control mice (no treatment), twelve mice treated with crude and pure compound from *A. muricata* leaf and four mice treated with doxorubicin (10mg) after ascites had formed. Cells of ascites of two mice were frozen and stored for future experiments. To study the reduction of swollen abdomen, 5 mg/kg doxorubicin (Pharma Chemie, The Netherlands) and the isolated compound at a final concentration of 20 mg/kg were administered i.p.

RESULTS

Table1: Effect of *A. muricata* crude/pure compound on body weight after tumor induction/extract treatment

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Groups	weight before Induction (g)	weight after induction of Tumor / extract treatment (g)		
Group1	29.5	12.0	**15.3**	19.2*
Group2	19.6	17.5	**23.5**	23.8*
Group3	20.4	14.7	**18.2**	19.4*
Group4	30.2	19.4	**20.6**	22.5*
Group5(Control)	17.8	16.3	**22.0**	22.2*

$^{\delta}$Results are means of group, figures in bold represent treatment values for crude extracts,

* Values for treatment from pure compounds.

Table2: Survival and tumor incidence in mice given FDH in drinking water

Animals groups	№ of survival at following wk after initiation of treatment/wk	№ of mice with tumors NC	LU	LT	ST	MK
Group1	6/1	4	2	2	4	0
Group2	6/1	4	3	4	3	2
Group3	4/2	4	2	3	4	0
Group4	4/3	4	4	2	5	0
Group5(Control)	4/4	3	0	0	1	0

$^{\delta}$NC(nasal cavity), LU(lung), LT(larynx and trachea), ST(stomach), MK(metastasis to kidney)

Table 3: Mean survival time and percentage increase in life span after tumor treatment with *A. muricata* leaf methanol extract

Experimental group	MST	% ILS
Normal control group1	-	-
10 mg/kg group 2	2.2	35.10
100mg/kg group3	2.5	37.50
1000mg/kg group4	3.0	50.50
Grp5doxo.10mg/kg	3.2	53.62

*Mean survival time (MST), Increase in life span(%ILS), doxo (doxorubicin control drug).

Table4: Effects of methanol extract of *Annona muricata* leaf extracts on tumor cell volume (TCV), packed cell volume(PCV), viable and non viable cell count on MEAM pure extract tumor- bearing mice

		Tumor Parameters(%)			
Group	Body wt (g)	TCV	PCV	VCC	NVCC
Group1(normal)	29.50	0	0	0	0
Group2(10mg/kg)	19.60	36.50	32.00	28.20	5.00
Group3(100mg/kg)	20.40	35.40	33.10	22.20	5.10
Group4(1000mg/kg)	30.20	33.50	34.50	20.10	6.50
Group5(10mg DRN)	17.80	32.13	31.00	6.47	7.24

Weight of mice tend not to be stable at the doses administered whereas tumor cell volume (TCV) packed cell volume, viable tumor count values increased as the dosage increased in the groups . However, non viable tumor cell count was on the decrease and significantly different from the normal control group at $p \leq 0.05$(one-way ANOVA).

Table5: Effect pure methanol extract of *Annona muricata* (MEAM) leaves on haematological parameters in albino mice

Animal group	Haematological parameters		
	Hb(g)	RBC(%)	WBC(%)
Group1(normal)	14.88	8.62	7.82
Group2(10mg/kg)	9.82	3.81	20.07
Group3(100mg/kg)	10.60	4.75	32.92
Group4(1000mg/kg)	11.45	5.42	49.85
Group5(10mgDRN)	11.70	5.81	16.12

* Control (doxorubicin USP).

Table 6: Growth inhibition assay(using human epithelial ovarian cancer cell line OV7-96020764-CDNA-(20uL) from crude plant extracts

Extract	Concentration (μg/mL)						
	6.25	12.5	25	50	100	200	IC50(μg/mL
AML	38.49	58.33	56.72	89.88	92.75	91.60	10.60
AMSt	12.93	26.46	34.07	35.93	47.14	59.63	108.35
Control	19.41	52.32	77.17	78.55	68.10	66.20	120.15

ᐞAML= *A. muricata* leaf extract, AMSt= *A. muricata* stembark, IC_{50} = concentration of tested sample to inhibit 50% growth of cell line.

Table 7: Growth inhibition assay(using human epithelial ovarian cancer cell line OV7-96020764-CDNA-(20uL) from pure compound

Extract	Concentration (μg/mL)						
	6.25	12.5	25	50	100	200	IC50(μg/mL)
AML	21.88	60.52	93.85	93.34	92.75	90.37	11.29
AMSt	0.43	1.73	4.60	15.00	20.45	60.89	171.89

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ᐞAML= *A. muricata* leaf extract, AMSt= *A. muricata* stembark, IC_{50} = concentration tested sample to inhibit 50% growth of cell line

Table8: Cytotoxicity test result for methanol fraction from *A. muricata* leaves after 24h of treatment

Fraction type	Concentration (ppm)	No of cell death (x10 cells) Repeat#1	Repeat#2	Repeat#3	Mortality(%)
Control	0	0	0	0	0
F1	200	10	10	10	100*
	100	0	1	1	3.33
	10	0	0	0	0
F2	200	10	10	10	100*
	100	1	3	1	16.66
	10	0	0	0	0
F3	200	8	10	9	90
	100	0	0	1	3.33
	10	0	0	0	0
F4	200	8	10	6	80
	100	0	0	1	0
	10	-	-	-	-

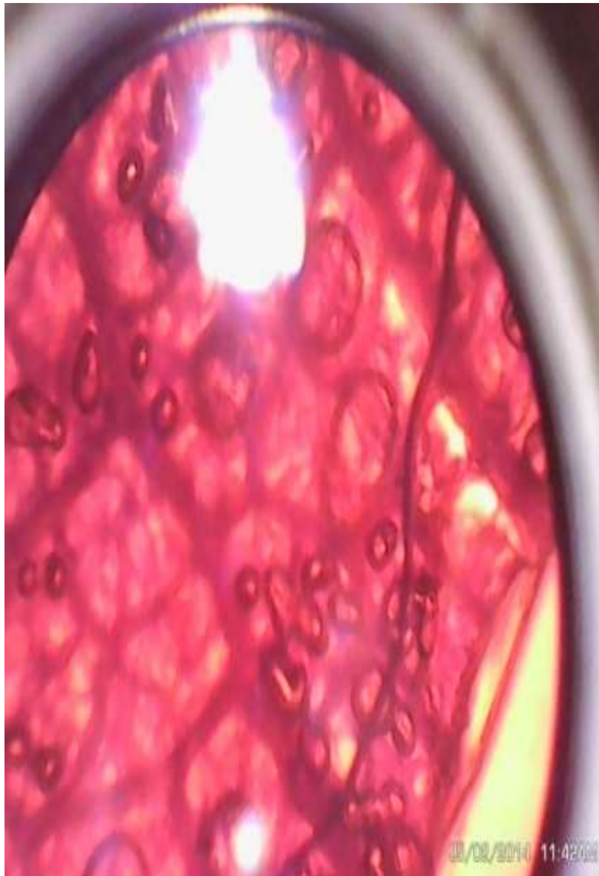
- no cell deaths, * highest cytotoxicity

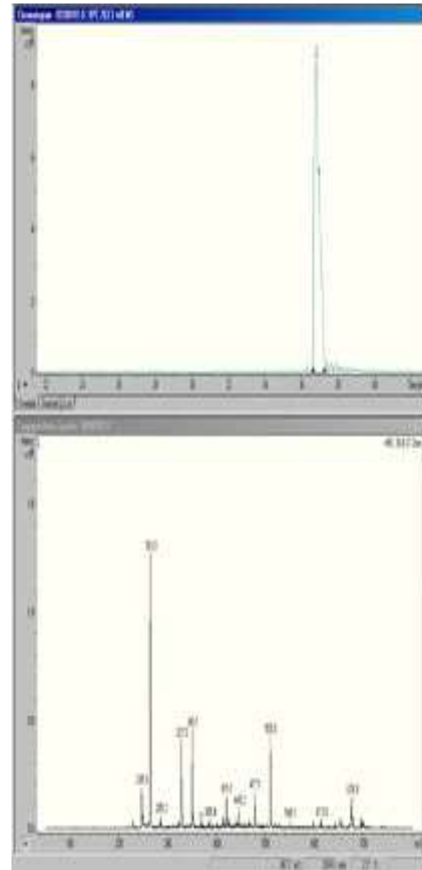
Table 9: Composition of compounds contained in F2 fraction from GC-MS examination

Peak (#)	Retention time (min)	Abundance (%)	Possible compound
1	17.416	1.45	2-cyanoethyl-1-(2-methyl-2-nitrocyclohexane)acetate
2	19.883	39.93	Methyl palmitate*
3	20.467	0.79	2-cyanoethyl-2-(iminomethylene)dec-2-enoate
4	20.942	0.66	Vinyl-1-(iminomethylene)non-2-enoate

5	21.649	25.05	Methyl oleate
6	21.880	25.71	Methyl stearate
7	23.135	6.43	5-nonadecanol

* Most abundance compound .





a. b.

figure. 1: Tumor cell as viewed from the microscope (a), and HPLC Standard curve for *A. muricata* (b)

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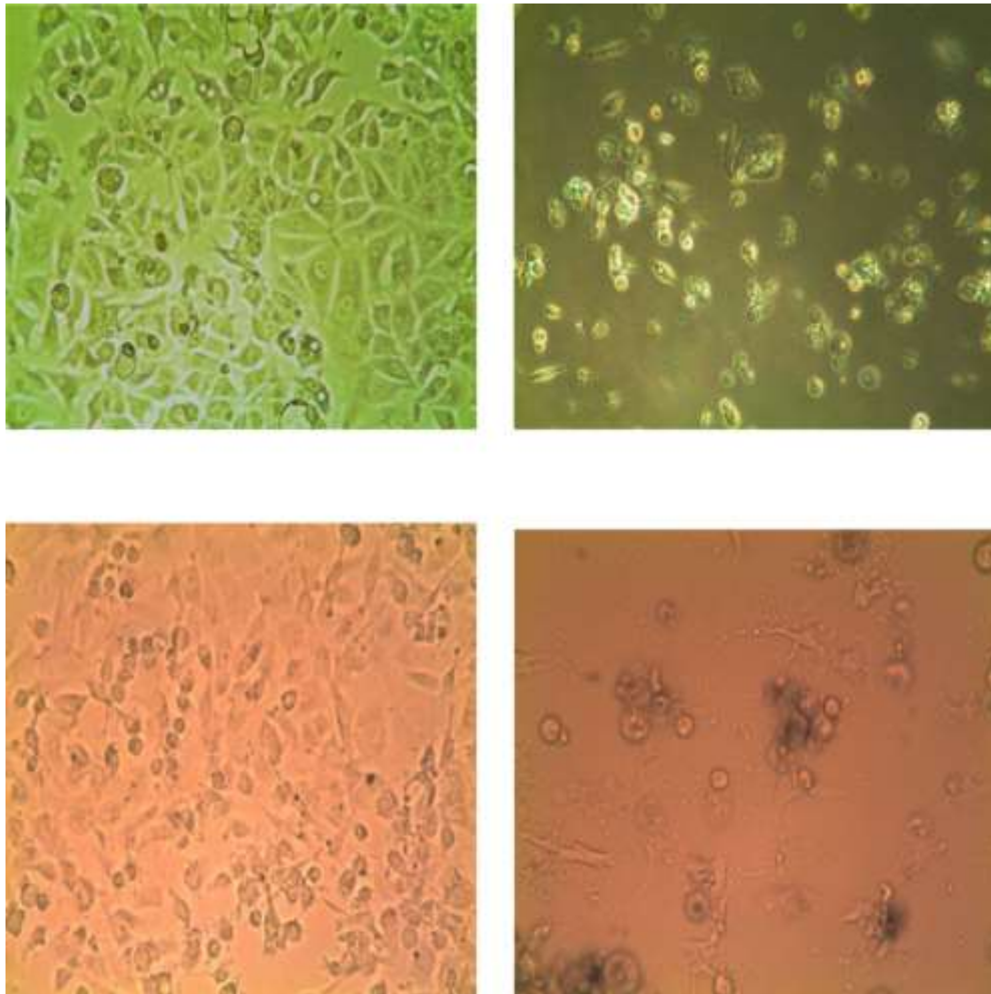

Figure 2: Cancer cell healing after treatment with plant extracts, AML*(A. muricata* leaf), ASL and AMS(*A. muricata* stembark) 1000x

Discussion

Acquired resistance has been a serious problem in cancer therapy. Nanoparticle-based chemotherapy with other anticancer drugs has been an effective way to improve therapeutic outcome .*Annona muricata* has been a folk medicine for centuries, and its extract has been shown to have cytotoxic and antiproliferative effects in different systems based on its inhibition of diverse cellular events associated with tumor pathogenesis. Ovarian cancer disease is a

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major problem which still remain unaddressed. Studies on the anti-cancer activities began after the first cytotoxic acetogenin, uvaricin, which was isolated in 1982 [12]. Since then, exponential zeal has been attached to the acetogenins that showed potential to inhibit tumor cells that are resisted by many anti-cancer drugs.

Importantly, *Annona muricata* inhibit tumor growth in a mouse model with intraperitoneal metastasis ascites formation. With *Annona muricata* treatment, a remarkable tumor inhibition of > 90% was achieved. Bioactive compounds are often found in medicinal plant and herbal mixtures, making them a superb source for the discovery of novel drug leads.

A. muricata have the ability to treat wide-ranges of human diseases including twelve types of cancer [13]. There are many studies with evidences to support this claim. Extracts from various parts of *A. muricata* have been reported to show selective cytotoxic effects against various cancer cell lines such as pancreatic cancer, cervical cancer, lung cancer, and breast cancer [14-16].

Following the vast number of *A. muricata* preliminary anti-cancer studies, it was discovered that the major bioactive components known as Annonaceous acetogenins were the major contributor to anticancer activity. Acetogenins are derivatives of long chain (C_{35} to C_{37}) fatty acids derived from the polypeptide pathway. These fatty acids are found connected to a variable number of tetra-hydrofuran or tetra-hydropyran rings and a terminal lactone moiety[17]. The compound isolated from the leaf of *A. muricata* is not different structurally from other acetogenins in terms of number of carbons. The pure compound isolated Murisolin A had shown very potent cytotoxicity against ovarian cancer cell line *in vitro* and *in vivo* than the crude extract and the first line anti-cancer drug adriamycin. The compound isolated from the leaf exhibited anti-ovarian cancer activity with IC_{50} value of 11.29 μg/mL after 24 h (Table 7), and this

value was significantly different from that obtained from the control group and the crude extract at $p \leq 0.05$. In table 8, fraction 2 with ethyl acetate showed most cytotoxicity on the cell line with 100% inhibition. Some studies have shown the anti-tumor and anti-cancer property of other Annona species.
Ukwubile and Bibinu 2015[18] carried out a work on the anti-cancer/anti-tumor activity of *Annona senegalensis*, a plant used in folklore medicine for the treatment of several diseases, and showed that the plant also possess anti-tumor property in mice. A good anti-tumor agent is also a good anti-cancer agent. This is because tumor especially malignant type most often give rise to cancers [19]. GC-MS analysis identified other compounds in the leaf of *A. muricata*, but of these Murisolin A from NMR elucidation was shown to be responsible for the property investigated *in vitro* by MTT assay and *in vivo* mice models. From the results in tables 1-7, tumor parameters and haematological indices such as Hb,RBC,WBC, PCV, NVCC, etc, showed dose dependent activity in the animal groups, and this suggested very strongly that *A. muricata* possessed anti-ovarian cancer property.
Our study therefore showed that *Annona muricata* isolated compound Murisolin A possess anti-tumor and anti-ovarian cancer cell activities in mice.

Conclusion

Annona muricata leaf offers significantly greater medicinal value but the fruits were reported to have more anti-cancer property[20]. Apart from its various medicinal properties, acetogenins in *Annona muricata* showed potent anti-ovarian cancer activity which selectively attack cancerous cells without harming healthy cells and preventing metastasis. Apoptosis of granulosa cells of the ovary has been initiated by this acetogenin in many organs of cancerous cells of humans. The plant had proved to be an effective anti-tumor and anti-

cancer medicinal plant, and thus represents a source for new anti-cancer drug discovery.

Conflict of interests

We have no any competing interests.

Authors contribution

The authors have prepared this manuscript with the detailed perusal of the literature work, and information represented have been thoroughly examined and approved by all authors in the preparation of this manuscript.

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