

Kaurenoic acid isolated from the root bark of *Annona senegalensis* induces cytotoxic and antiproliferative effects against PANC-1 and HeLa cells

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ABSTRACT

Aims: Cancer is one of the leading causes of death worldwide with an estimated 6.7 million deaths and 24.6 million people living with cancer in 2002. Presently, there is a global increase in prevalence, mortality and health burden of various malignancies. World Health Organization (WHO) report projected that cancer prevalence rates could further increase by 50% to 15 million new cases in the year 2020. The bioactivity guided isolation of the bioactive constituent, and its characterization, responsible for the anticonvulsant effects of the root bark extract of *A. senegalensis* yielded kaur-16-en-19-oic acid (KA). Therefore, the aim of this study was to evaluate the anti-proliferative activity of kaurenoic acid from *A. senegalensis* on selected cancer cell lines.

Study design: The study was designed to ascertain the antiproliferative and cytotoxic effects of kaurenoic acid, a terpenoid isolated from the root bark of Nigerian *Annona senegalensis* (Annonaceae).

Place and duration of study: Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Nigeria, between October 2010 and June, 2012.

Methodology: Human embryonic kidney cells expressing SV40 Large T-antigen (293 T), Pancreatic tumour (PANC-1) and Henrietta Lacks' cervical (HeLa) cell lines were used in the study using standard MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide, assay method.

Results: Kaurenoic acid (KA) exhibited cytotoxic effects against the cells with estimated IC₅₀ values of 0.93, 0.74 and 0.52 M concentrations for 293 T, HeLa and PANC-1 cells respectively. This is an indication of the possible potentials of KA in the treatment of cervical and pancreatic cancers.

Conclusions: Kaurenoic acid (KA), a diterpenoid, possesses antiproliferative effect against HeLa and PANC-1 cell lines, and could be the anticancer constituent in the root bark extract of *A. senegalensis* with potentials as a lead in the chemical synthesis of standard anti cancer agents.

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Keywords: Kaurenoic acid, *Annona senegalensis*, antiproliferative and cytotoxic

21 1. INTRODUCTION

22

23 Cancer is one of the leading causes of death worldwide with an estimated 6.7 million deaths
24 and 24.6 million people living with cancer in 2002 [1, 2]. The disease caused about 0.6
25 million deaths in the United States in 2011 [3]. Globally, there is an obvious increase in
26 prevalence, mortality and health burden of various malignancies. World Health Organization
27 (WHO) report [4], projected that cancer prevalence rates could further increase by 50% to 15
28 million new cases in the year 2020. In Nigeria, the burden of cancer is enormous and quite
29 on the increase and it has been projected that by 2020, cancer incidence for Nigerian males
30 and females may rise to 90.7/100,000 and 100.9/100,000, respectively, with mortality rates
31 of 72.2 and 76/100,000 for males and females respectively [4]. At the early stage diagnosis
32 and detection, cancer is usually managed by surgery and radiotherapy while advanced
33 cases of cancer could be treated with chemotherapeutic agents. Although chemotherapeutic
34 agents are effective, they are often associated with serious adverse effects and drug
35 resistance [5]. Additionally, the exorbitant costs, non-affordability and unavailability of
36 effective anticancer agents in some parts of world, have paved way for herbal therapies as
37 alternatives in the management of cancer in rural communities. Herbal therapies employing
38 plants extracts and plant bioactive compounds have long been used in the treatment of
39 cancer [6, 7]. The fact that herbs and plant-derived products lack much of the toxicity in the
40 synthetic chemicals enhances their appeal for treating cancer and for long term preventive
41 strategies [8]. Therefore, other therapeutic options that are devoid of serious adverse effects
42 at the same time cheap and affordable are obviously desired. The use of natural sources
43 can provide an opportunity for the isolation and chemical characterization of
44 phytoconstituents that could be the desired source of lead compounds for the introduction of
45 novel chemotherapeutic agents. Hence the urgent need for novel therapeutic compounds (or
46 leads) with potent anticancer effects and minimal toxicities to normal cellular system. The
47 plant *Annona senegalensis* Pers. (Annonaceae) popularly known as African custard apple or
48 wild custard apple [9], has been reported to possess cytotoxic and anticancer effects [10, 11,
49 12]. A diterpenoid compound, kaurenoic acid, isolated from the root bark of *A. senegalensis*
50 was reported to possess antimicrobial [13] and anticonvulsant [14] effects. Also from other
51 sources, kaurenoic acid has shown to possess anti-inflammatory [15], anticonvulsant [16]
52 and antimicrobial activities [17]. Among the naturally isolated phytoconstituents used in
53 cancer chemotherapy are the diterpenoid compounds known as the taxanes (paclitaxel and
54 docetaxel), vinca alkaloids (vincristine and vinblastine) and podophyllotoxins (etoposides,
55 teniposides and etopophos) [18]. Therefore, due to the reported ethnomedicinal use of *A.*
56 *senegalensis* root bark extract cancer treatment [10, 12], we serendipitously evaluated the
57 cytotoxic effects of kaurenoic acid isolated from its root bark using some selected human
58 cancer cell lines.

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60 2. MATERIAL AND METHODS

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62 2.1 Plant material

63 Fresh roots of *A. senegalensis* were collected from Enugu-Ezike, Enugu State, Nigeria in the
64 month of June and authenticated by Mr. A. O. Ozioko of the International Centre for
65 Ethnomedicine and Drug Development (InterCEDD), Nsukka, Enugu State, Nigeria where a
66 voucher specimen was deposited (specimen number: BDCP/INTERCEDD 64). The root-
67 bark was peeled off, cut into pieces and allowed to dry. The dried root-bark was pulverized
68 into coarse powder. The dried powdered root-bark (2.95 kg) was extracted with a mixture of
69 methanol: methylene chloride (1:1) using Soxhlet extractor to obtain the *Annona*
70 *senegalensis* root bark extract (MME). This was evaporated using a rotary evaporator at
71 reduced pressure to obtain a yield of 375 g (12.71% w/w).

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2.2 Isolation and purification of A2

The kaurenoic acid, A2, was isolated from the separation and activity guided fractionation of MME. The detailed methods of isolation and purification of A2 from the root bark extract and fractions of *A. senegalensis* have been documented [13,14].

2.3 Identification and characterization of A2

The purity of A2 was assessed by analytical HPLC using a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, Dionex Softron GmbH, Germering, Germany). Detection was at 235, 254, 280 and 340 nm. The separation column (125 × 4 mm; length × internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent. The molar mass was determined by Liquid Chromatography-Electrospray Ionization Mass Spectroscopy (LC-ESI-MS) using a ThermoFinnigan LCQ-Deca mass spectrometer (Germany) connected to an UV detector. Complete structural characterization of the pure crystals of A2 was achieved by 1D (HNMR, ¹³CNMR, DEPT) and 2D (HHCOSY, HMQC, HMBC) NMR spectroscopy using a Bruker ARX-500 and X-ray crystallography. Spot detection was done with ultra-violet (UV) light at 254 nm and spraying with vanillin sulphuric reagent. The melting point of A2 was also determined using a melting point apparatus (Electrothermal®, Cat. No.: IA 6304, England).

2.4 Cell lines

Human embryonic kidney cells expressing SV40 Large T-antigen (293 T), Pancreatic tumour (PANC-1) cell line and Henrietta Lacks' cervical (HeLa) cancer cell line were propagated in D-10 medium, consisting of Dulbecco's modified Eagle's medium (DMEM) with high glucose, 2 mM L-glutamine and supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Tissue culture medium and supplements were purchased from Invitrogen (Karlsruhe, Germany). The cell cultures were maintained in a humidified 5% CO₂ atmosphere at 37°C.

2.5 Cytotoxicity studies

The cytotoxicity assay was performed in parallel to the antiviral screening using the MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide], assay method as previously described [19] on 293T, PANC-1, and HeLa cell lines. In the MTT assay, cells were seeded onto a 96-well plate at a concentration of 10⁴ cells/well and a volume of 100 µl per well. Different concentrations of the test extracts (31.5-1000 µg/ml) were added to culture wells in triplicate. Culture medium without any drug was used as the "no-drug" control. After incubation at 37°C under 5% CO₂ for 2 days, a solution of MTT (3 mg/ml, 50µl per well) was added to each well and further incubated at 37°C + 5% CO₂ for 4 h to allow formazan formation. Subsequently, the medium was removed and 150 µl of DMSO was used to dissolve the resulting blue formazan crystals in living cells. The optical density was determined at 550 nm using a multi-well microtitre plate reader (Tecan, Austria). Each single value of the triplicates was expressed as percent of the mean of triplicates of the "no-drug" control cultures and the mean and standard deviation of the percent values were calculated for each triplicate. The concentration of 50% cellular toxicity (TC₅₀) of the test extracts was calculated by non-linear regression.

2.6 Statistical analysis

Data were analyzed using One Way Analysis of Variance (ANOVA, SPSS Version 16) and expressed as mean ± SEM and multiple comparisons was done using Dunnett test as *post hoc*. Differences between means were regarded significant at *P* < 0.05.

3. RESULTS AND DISCUSSION

3.1 RESULTS

3.1.1 Identification and characterization of A2

The A2 was isolated as a white crystalline compound. The melting point range was estimated to be at 170-172 °C and in methanol it exhibited a UV maximum at 214 nm, which is typical of an unconjugated compound. It had a strong peaks at 303.2 (M + H), 650.2 (2M + 2Na) in the positive mode of LC-ESIMS and a corresponding peak at 301.6 (M - H) in the negative mode, which are consistent with the molar mass of 302. Based on this, and the analysis of ¹H and ¹³C NMR, the molecular formula of AS2 was deduced as C₂₀H₃₀O₂. The analyses of the HNMR, HHCOSY, C-13 NMR, DEPT, HMQC and HMBC (Table 1) and comparison of data with literature reports [20, 21] established the structure of A2 to be kaur-16-en-19-oic acid (Fig. 5). The absolute configuration as shown was based on the observed HNMR coupling constants, HMBC and X-ray crystallography and comparison with literature report [22, 13, 14].

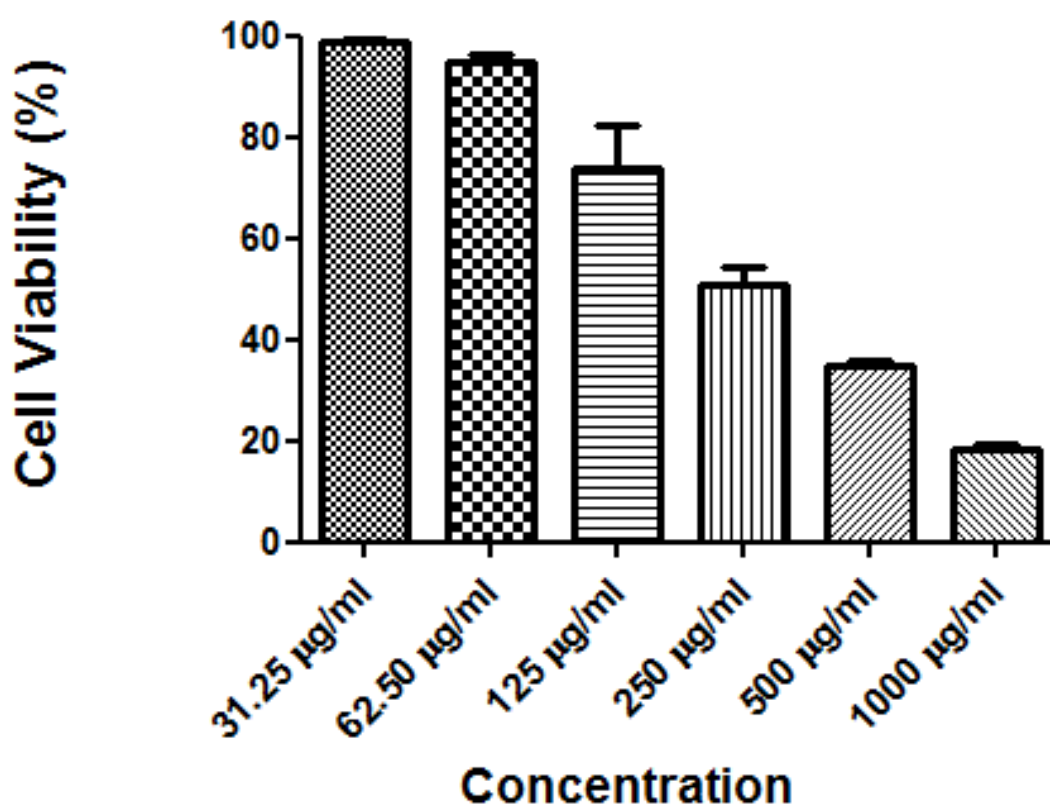
3.1.2 Cytotoxicity studies

The A2 exhibited cytotoxicity effects against the three cell lines tested (Figs. 1, 2 and 3), with estimated IC₅₀ values of 281.85, 223.65, 156.35 µg/ml for 293 T, HeLa and PANC-1 cells respectively (Fig. 4). In molar concentrations, the IC₅₀ values of kaurenoic acid against the 293 T, HeLa and PANC-1 cells were estimated to be 0.93, 0.74 and 0.52 M respectively. Hence the antiproliferative activity of A2 against the cells in the order of increasing activity was; 293 T > HeLa cells > PANC-1 cells.

3.2 DISCUSSION

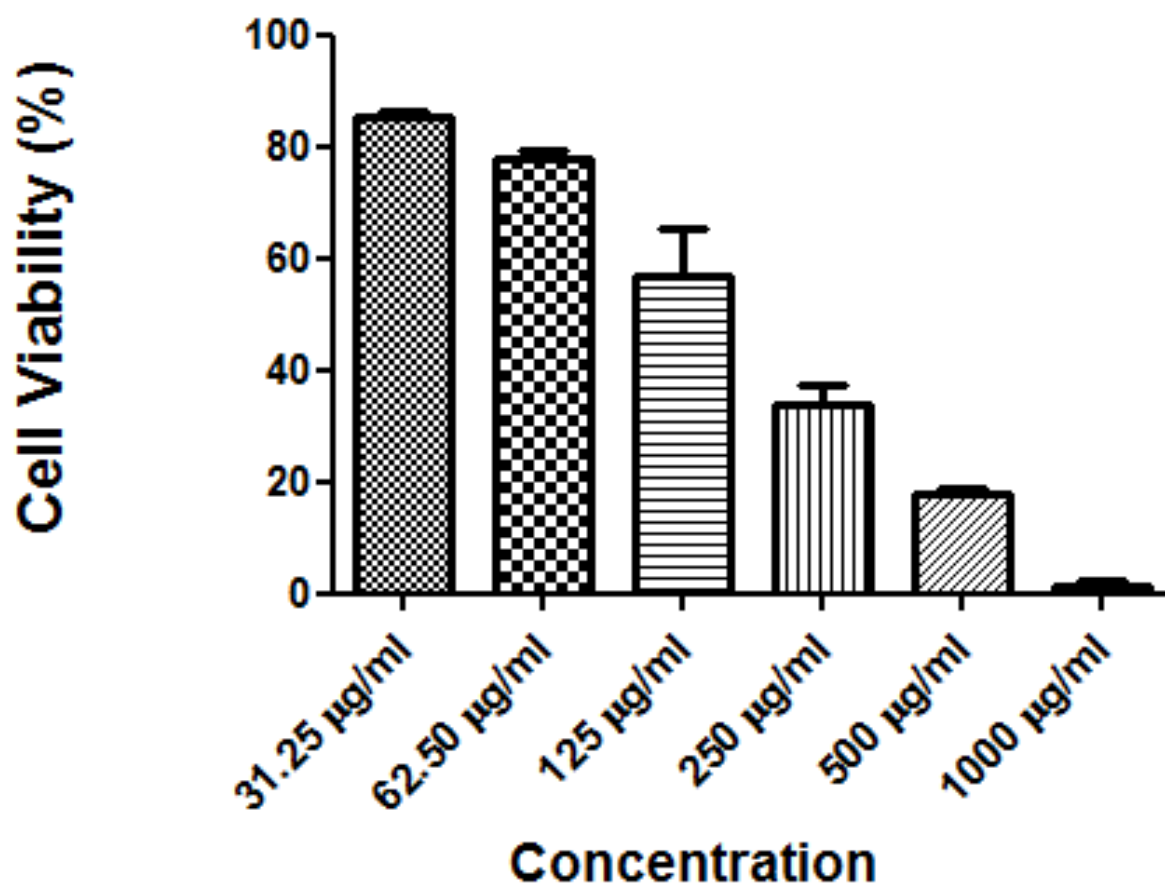
Kaurenoic acid, A2, exhibited cytotoxic activity against the 293 T, HeLa and PANC-1 cancer cell lines treated. It exhibited better cytotoxic and antiproliferative activity against HeLa cells than the PANC-1 cells, an indication of the potential anticancer effects of this diterpenoid on cervical and pancreatic cancers. On a separate reported work, kaurenoic acid has been identified to possess cytotoxic effects against HeLa cell lines [23]. However, the most potent cytotoxic effect on the PANC-1 cells showed the possible antitumor effect of A2 against cancer of the pancreas. Therefore, A2 could serve as a lead compound in the development of novel anticancer agents. Anticancer effect of kaurenoic acid on breast, leukemia and colon cancer cells has also been documented [24, 25, 26, 27]. Recently kaurenoic acid has exhibited anticancer activity on human glioblastoma [28], murine and human melanoma [29, 30] cell lines. Additionally, reports have indicated that KA, its derivatives and other naturally isolated terpenoid compounds have shown to possess anticancer effects [31, 32, 33]. Similarly, betulinic acid, a triterpenoid, isolated from plants of Cactacea family has shown to possess potent anticancer effect against HeLa cells [34]. Documented studies revealed that terpenoids exhibited antitumor activities by inducing apoptosis in various cancer cells by activating various pro-apoptotic signaling cascades and by the inhibition of metastatic progression and tumor-induced angiogenesis [26]. The molecular mechanisms involved in these activities include the inhibition of various oncogenic and anti-apoptotic signaling pathways and suppression of nuclear translocation of various transcription factors including nuclear factor kappa B (NF-κB) [26]. The anti-inflammatory effects of kaurenoic acid isolated from different sources have variously been reported [35,15]. Kaurenoic acid was shown to

178 significantly inhibit inflammatory mediators in lipopolysaccharide-induced RAW264.7
 179 macrophages, inhibit the production of nitric oxide and reduced the secretion of
 180 prostaglandin E (PGE₂), which are all potent mediators of inflammation [35]. There is the
 181 possibility that the mechanism of antiproliferative activity of KA might be likely through
 182 modulation of inflammatory mediators. In other reported studies, the mechanism of
 183 anticancer effects of kaurenoic acid has been attributed to its inhibition of DNA
 184 topoisomerases I and II [24] as well as the stimulation of p53 tumor suppressor gene [25].
 185 However, the likely specific mechanism of action of kaurenoic acid against these cancer
 186 cells at this stage of the work is not yet elucidated. Hence further work is encouraged to
 187 determine the possible anti-cancer mechanism of kaurenoic acid.



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 195 **Fig. 1: The effect of kaurenoic acid on the viability of 293-T cells**

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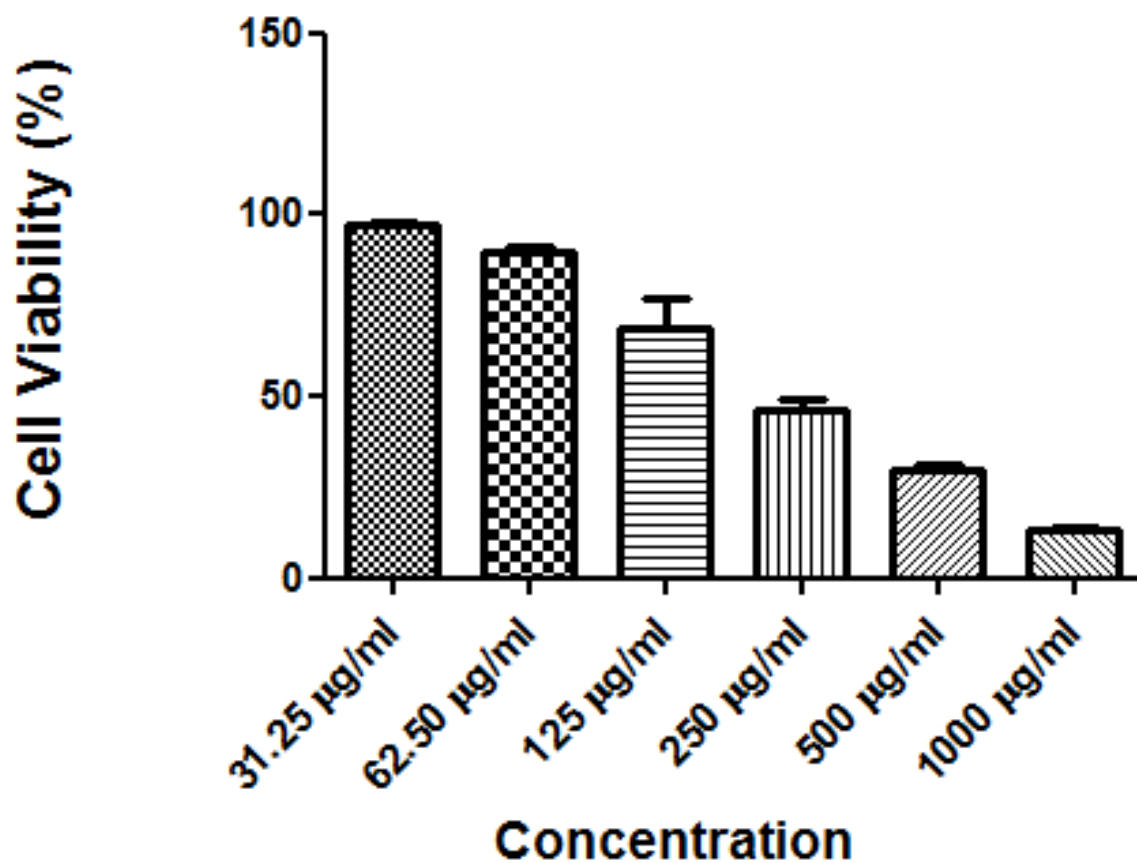


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Fig. 2: Antiproliferative effect of kaurenoic acid on pancreatic cancer (PANC-1) cell line

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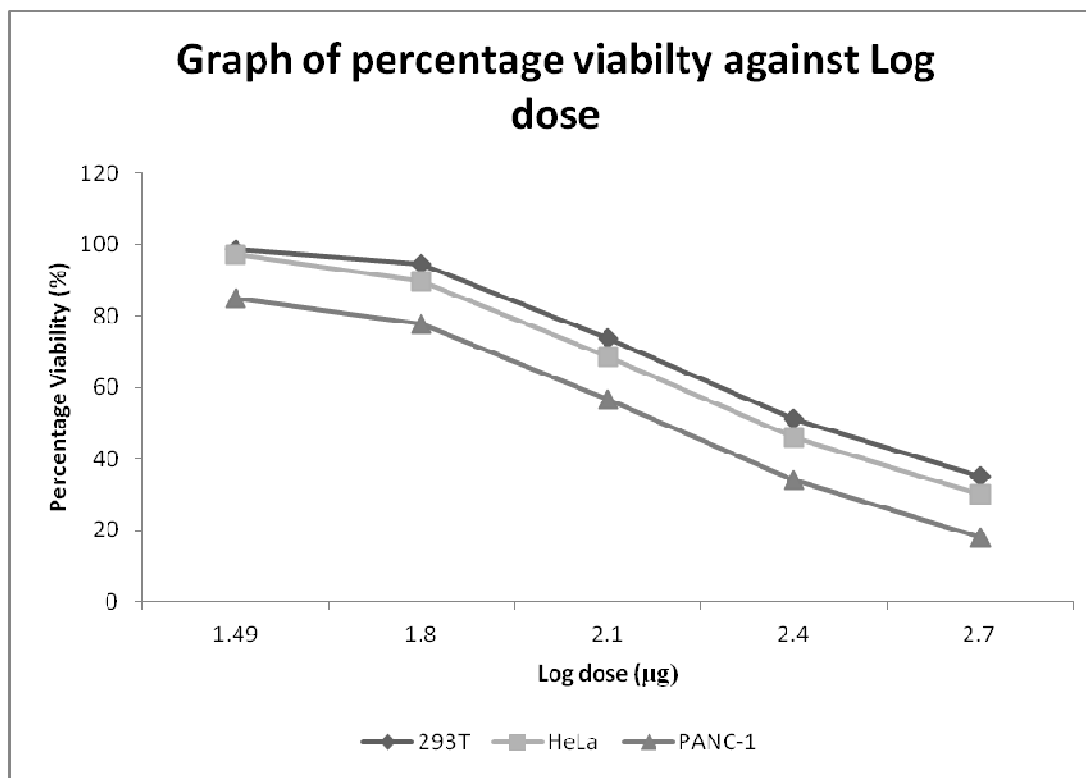


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Fig. 3: Antiproliferative effect of kaurenoic acid on cervical cancer (HeLa) cell line

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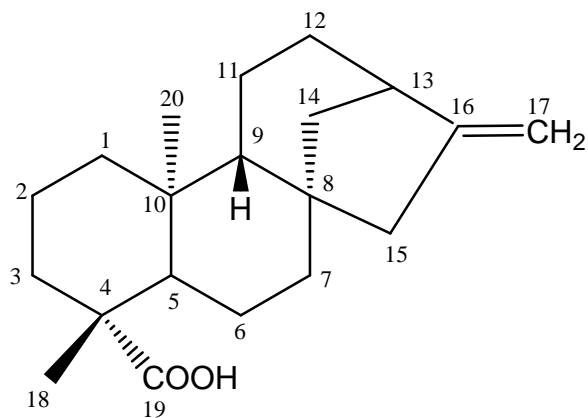
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253 **Fig. 4: A comparison of the effects of kaurenoic acid on the viability of 29T**
254 **cells, PANC-1 cells, and HeLA cells**

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260 **Fig. 5: Chemical structure of Kaur-16-en-19-oic acid**
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263 **TABLE 1: NMR spectroscopic data of A2^a**
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Position	δH	δc	HMBC
1	Ha 0.82 m Hb 1.54 m	41.48	Ha C-20 Hb C-2, C-3
2	1.42 m (2H)	19.29	1, 3, 4, 10
3	Ha 1.01 m Hb 2.17 m	37.97	1,2,4,5,19
4		43.95	
5	1.07 m	57.26	4, 6, 7, 10, 20
6	Ha 1.62 m Hb 1.84 m	22.03	4, 5, 7, 8, 10
7	Ha 1.44 m Hb 1.54 m	40.90	5, 6, 6, 8, 9, 15
8		44.05	
9	1.05 m	55.29	5,, 8, 10, 11, 14, 15, 20
10		39.85	
11	Ha 1.60 m Hb 1.88 m	18.64	9, 10, 12, 13
12	Ha 1.46 m Hb 1.62 m	33.97	11, 13, 16, 14
13	2.61 bs	43.95	C-8, C-12, C-14, C-16, C-17
14	Ha 1.16 Hb 2.02	39.9	
15	2.06	48.48	C-8, C-9, C-16, C-17
16		156.12	
17	Ha 4.72 s Hb 4.78 s	103.21	
18	1.22 s (3H)	29.18	C-2, C-3, C-4, C-5, C-19
19		184.94	
20	0.93 s (3H)	15.79	C-1, C-5, C-9, C-10

265 ^aSpectra were measured in CDCl₃ at 500 (¹H) and 150 (¹³C) MHz. Assignments were made
266 on the basis of DEPT, ¹H-¹H COSY, HMQC and HMBC experiments. The detailed NMR data
267 are available with the author for correspondence and will be readily supplied on request.
268

271 4. CONCLUSION

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273 Kaurenoic acid, a diterpenoid, isolated from the root bark extract of *A. senegalensis* Pers.
274 (Annonaceae) possesses cytotoxicity and anticancer effect against PANC-1 and HeLa
275 human cell lines while the specific mechanism of anticancer activity is a point for further
276 research.
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280
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COMPETING INTERESTS

The authors declare that there are no conflicts of interest.

AUTHORS' CONTRIBUTIONS

Okoye TC and Akah PA designed, performed the experiment on the isolation of the compound and prepare the manuscript. Nworu CS performed the cytotoxicity studies while Ezike AC contributed in the manuscript preparation. All authors read and approve the final manuscript.

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