Research Paper

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

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 cencer cell lines.

METHODS: 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 IC_{50} values of 0.42, 0.70 and 0.88 M concentrations for 293 T, HeLa and PANC-1 cells respectively. This is an indicating the potentials of KA in the treatment of cervical and pancreatic cancers.

CONCLUSIONS: Kaurenoic acid (KA) a terpenoid, possesses antiproliferative effect against HeLa, PANC-1 and 293 T 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.

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

1.0 Introduction

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 [1, 2]. The disease caused about 0.6 million deaths in the United States in 2011 [3]. Globally, there is an obvious increase in prevalence, mortality and health burden of various malignancies. World Health Organization (WHO) report [4], projected that cancer prevalence rates could further increase by 50% to 15 million new cases in the year 2020. In Nigeria, the burden of cancer is enormous and quite on the increase and it has been projected that by 2020, cancer incidence for Nigerian males and females may rise to 90.7/100,000 and 100.9/100,000, respectively, with mortality rates of 72.2 and 76/100,000 for males and females respectively [4]. At the early stage diagnosis and detection, cancer is usually managed by surgery and radiotherapy while advanced cases of cancer could be treated with chemotherapeutic agents. Although chemotherapeutic agents are effective, they are often associated with serious adverse effects and drug resistance [5]. Additionally, the exorbitant costs, non-affordability and unavailability of effective anticancer agents in some parts of world, have paved way for herbal therapies as alternatives in the management of cancer in rural communities. Herbal therapies employing plants extracts and plant bioactive compounds have long been used in the treatment of cancer [6, 7]. The fact that herbs and plant-derived products lack much of the toxicity in the synthetic chemicals enhances their appeal fro treating cancer and for long term preventive strategies [8]. Therefore, other therapeutic options that are devoid of serious adverse effects at the same time cheap and affordable are obviously desired. The use of natural sources can provide an opportunity for the isolation and chemical characterization of phytoconstituents that could be the desired source of lead compounds for the introduction of novel chemotherapeutic agents. Hence the urgent need for novel therapeutic compounds (or leads) with

potent anticancer effects and minimal toxicities to normal cellular system. The plant *Annona senegalensis* Pers. (Annonaceae) popularly known as African custard apple or wild custard apple [9], has been reported to possess cytotoxic and anticancer effects [10, 11, 12]. A diterpenoid compound, kaurenoic acid, isolated from the root bark of *A. senegalensis* was reported to possess antimicrobial [13] and anticonvulsant [14] effects. Also from other sources, kaurenoic acid has shown to possess anti-inflammatory [15], anticonvulsant [16] and antimicrobial activities [17]. Among the naturally isolated phytoconstituents used in cancer chemotherapy are the diterpenoid compounds known as the taxanes (paclitaxel and docetaxel), vinca alkaloids (vincristine and vinblastine) and podophyllotoxins (etoposides, teniposides and etopophos) [18]. Therefore, due to the reported ethnomedicinal use of *A. senegalensis* root bark extract cancer treatment [10, 12], we serendipitously evaluated the cytotoxic effects of kaurenoic acid isolated from its root bark using some selected human cancer cell lines.

2.0 Materials and Methods

2.1 Plant material

Fresh roots of *A. senegalensis* were collected from Enugu-Ezike, Enugu State, Nigeria in the month of June and authenticated by Mr. A. O. Ozioko of the International Centre for Ethnomedicine and Drug Development (InterCEDD), Nsukka, Enugu State, Nigeria where a voucher specimen was deposited (specimen number: BDCP/INTERCEDD 64). The root-bark was peeled off, cut into pieces and allowed to dry. The dried root-bark was pulverized into coarse powder. The dried powdered root-bark (2.95 kg) was extracted with a mixture of methanol: methylene chloride (1:1) using Soxhlet extractor to obtain the *Annona senegalensis*

root bark extract (MME). This was evaporated using a rotary evaporator at reduced pressure to obtain a yield of 375 g (12.71% w/w).

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 ThermoFinningan 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% CO_2 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 10^4 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% CO_2 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% CO_2 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 (TC_{50}) 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 Dunnet test as *post hoc*. Differences between means were regarded significant at $P < 0.05$.

3.0 Results

3.1 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 ^{1}H and ^{13}C NMR, the molecular formula of AS2 was deduced as $C_{20}H_{30}O_2$. 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. 6). 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].

3.2 Cytotoxicity studies

The A2 exhibited cytotoxicity effects against the three cell lines tested (Figs. 1, 2 and 3), with estimated IC_{50} values of 125.89, 211.35, 266.07 µg/ml for 293 T, HeLa and PANC-1 cells respectively (Figs. 4 and 5). In molars concentrations, the IC_{50} values of kaurenoic acid against the 293 T, HeLa and PANC-1 cells were estimated to be 0.42, 0.70 and 0.88 M respectively. Hence the cytotoxic activity of A2 activity against the cells in the order of decreasing activity was; 293 T > HeLa cells > PANC-1 cells.

4.0 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 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 293-T cells showed the possible antitumor effect of A2 against cancer of the kidney. 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]. In addition to kaurenoic acid, other naturally isolated terpenoid compounds have shown to possess anticancer effects [27]. Similarly, betulinic acid, a triterpenoid, isolated from plants of Cactacea family has shown to possess potent anticancer effect against HeLa cells [28]. 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 [29,15]. Kaurenoic acid was shown to significantly inhibit inflammatory mediators in lipopolysaccharide-induced RAW264.7 macrophages, inhibit the production of nitric oxide and reduced the secretion of prostaglanding E (PGE_2), which are all potent mediators of inflammation [29]. There is the possibility that the mechanism of antiproliferative activity of KA might be likely through modulation of inflammatory mediators. In other reported studies, the mechanism of anticancer effects of kaurenoic acid has been attributed to its inhibition of DNA topoisomerases I and II [24] as well as the stimulation of p53 tumor suppressor gene [25]. However, the likely specific mechanism of action of kaurenoic acid against these cancer cells at this stage of the work is not yet elucidated. Hence further work is encouraged into the underlying anti-cancer mechanism of kaurenoic acid.

4.1 Conclusion

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

5.0 Acknowledgements

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6.0 Conflict of Interest

The authors declare that there are no conflicts of interest.

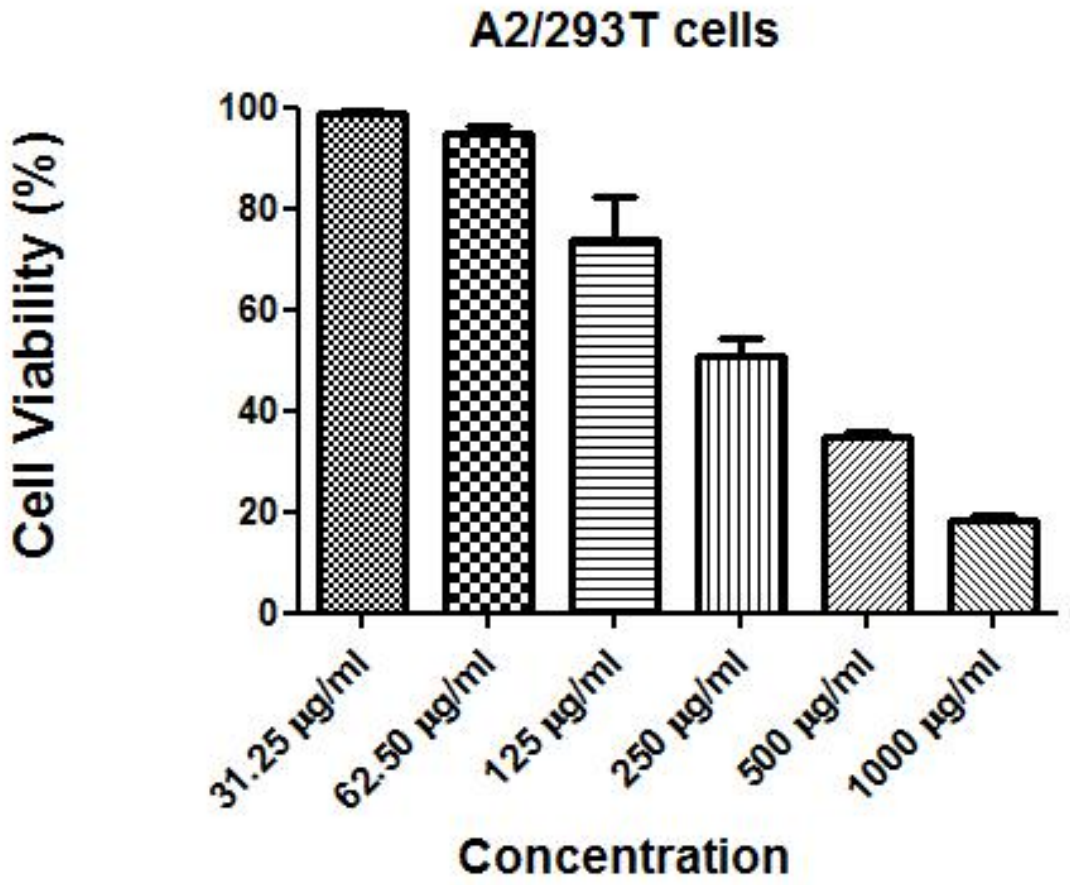

Fig. 1: Effect of A2 on 293-T cells

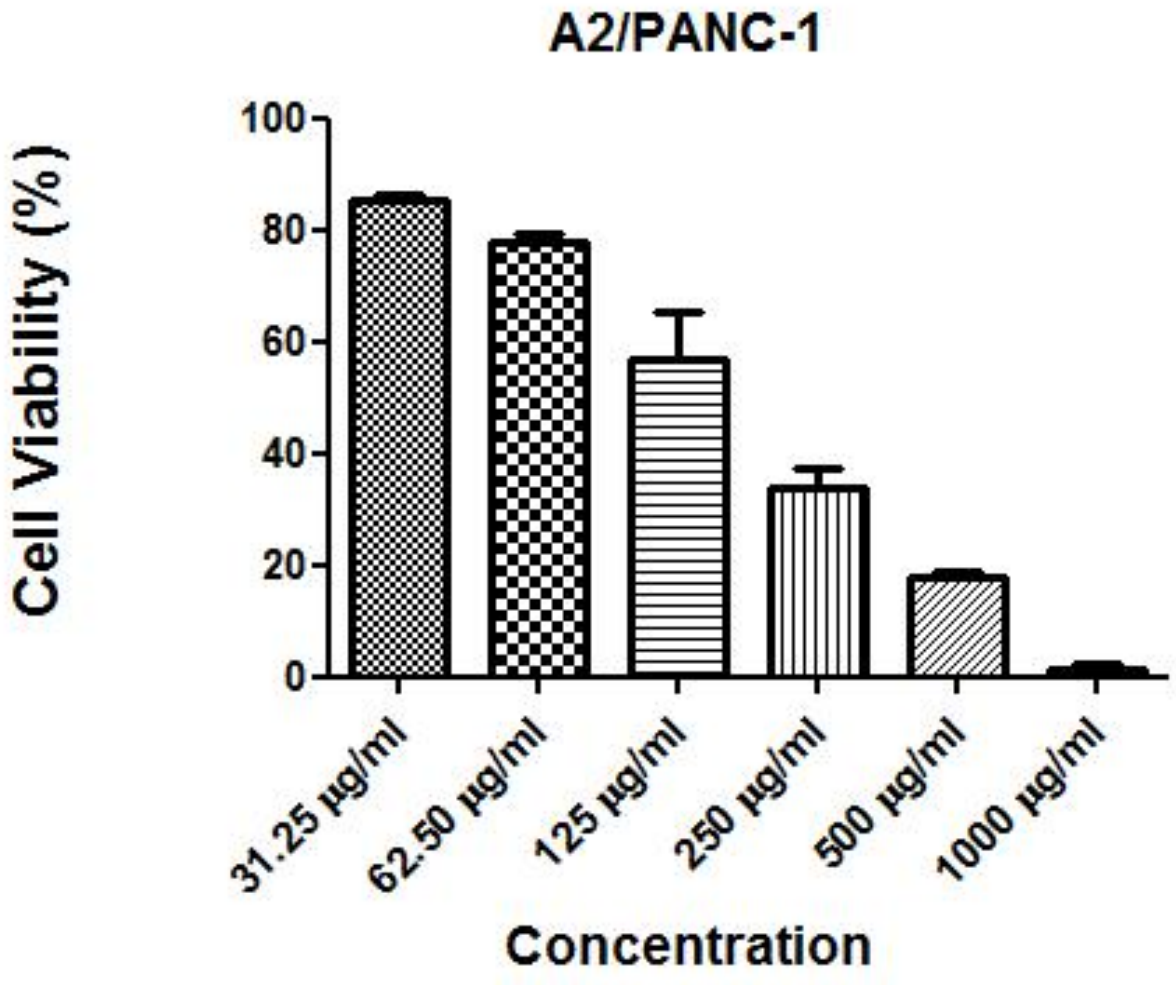

Fig. 2: Effect of A2 on PANC-1 cells

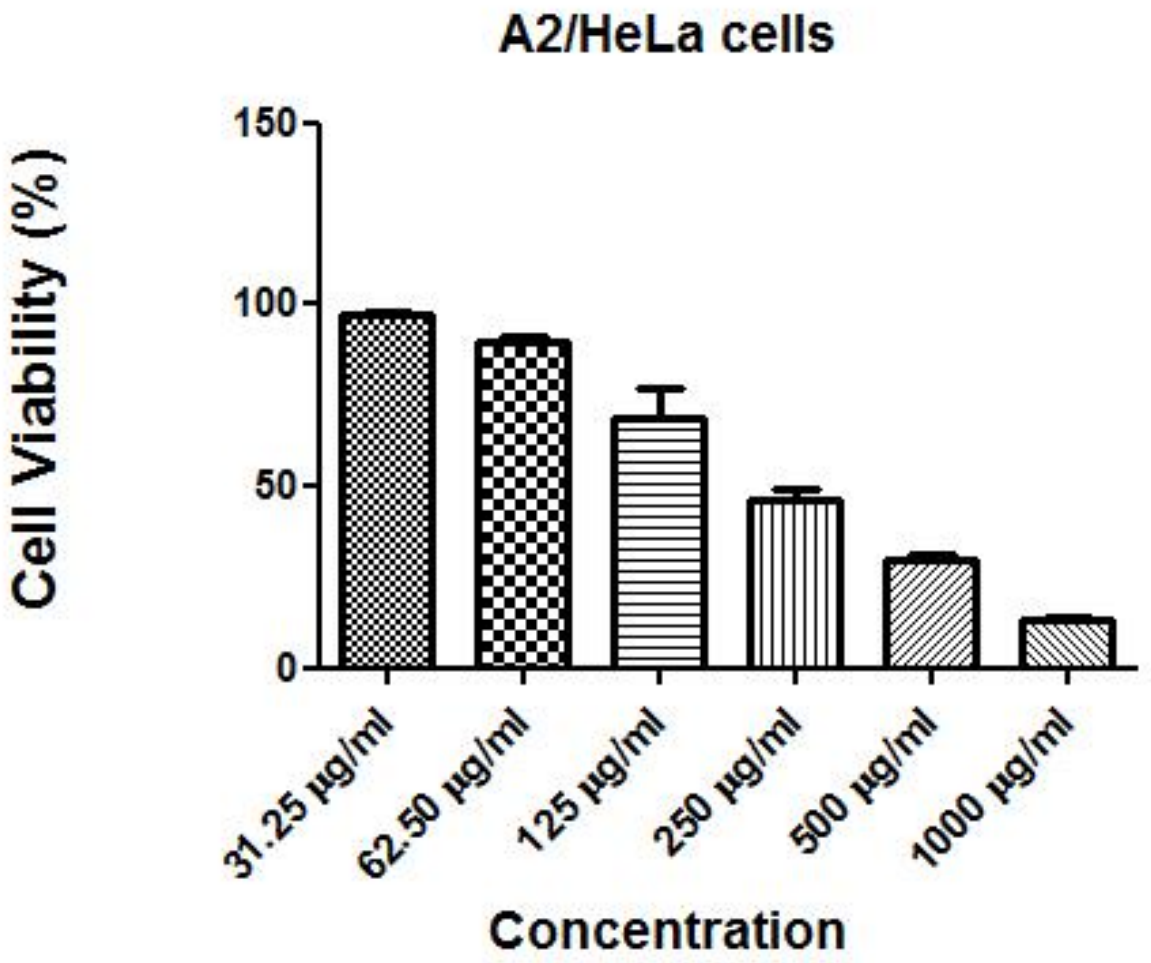

Fig. 3: Effect of A2 on HeLa cells

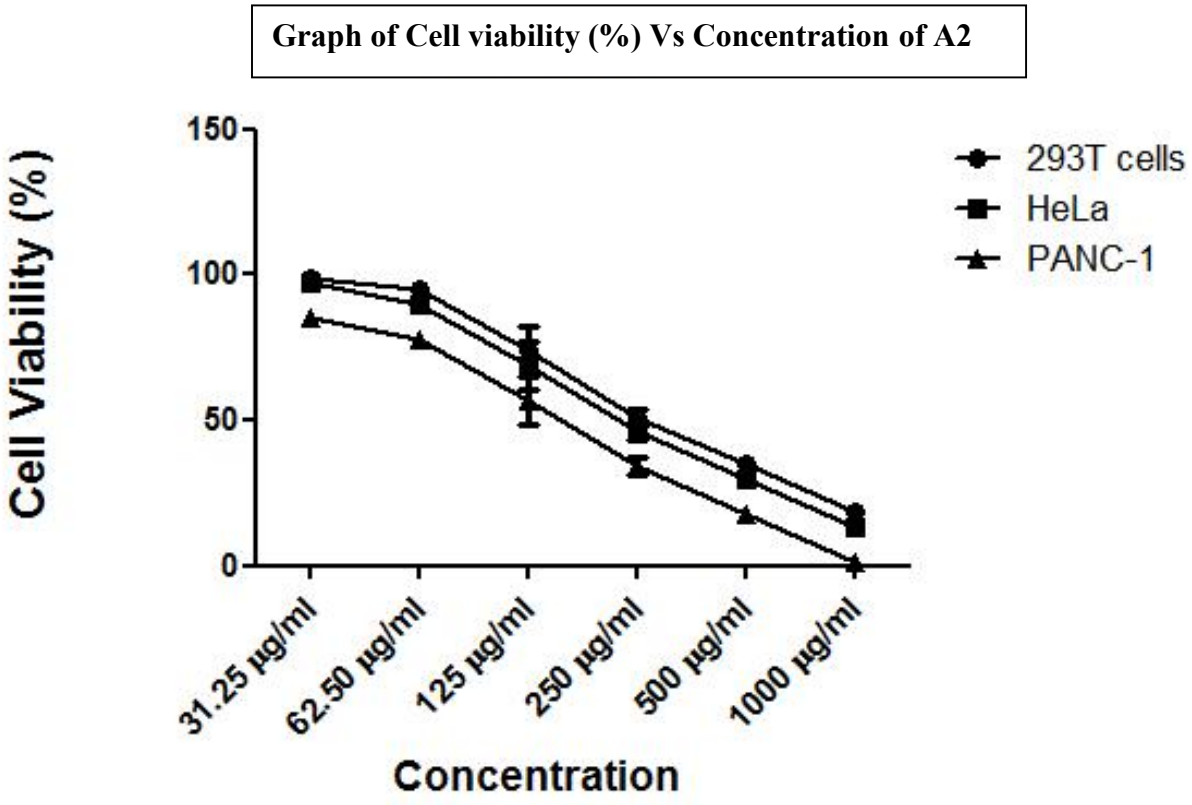

Fig. 4: Cytotoxic effects of A2

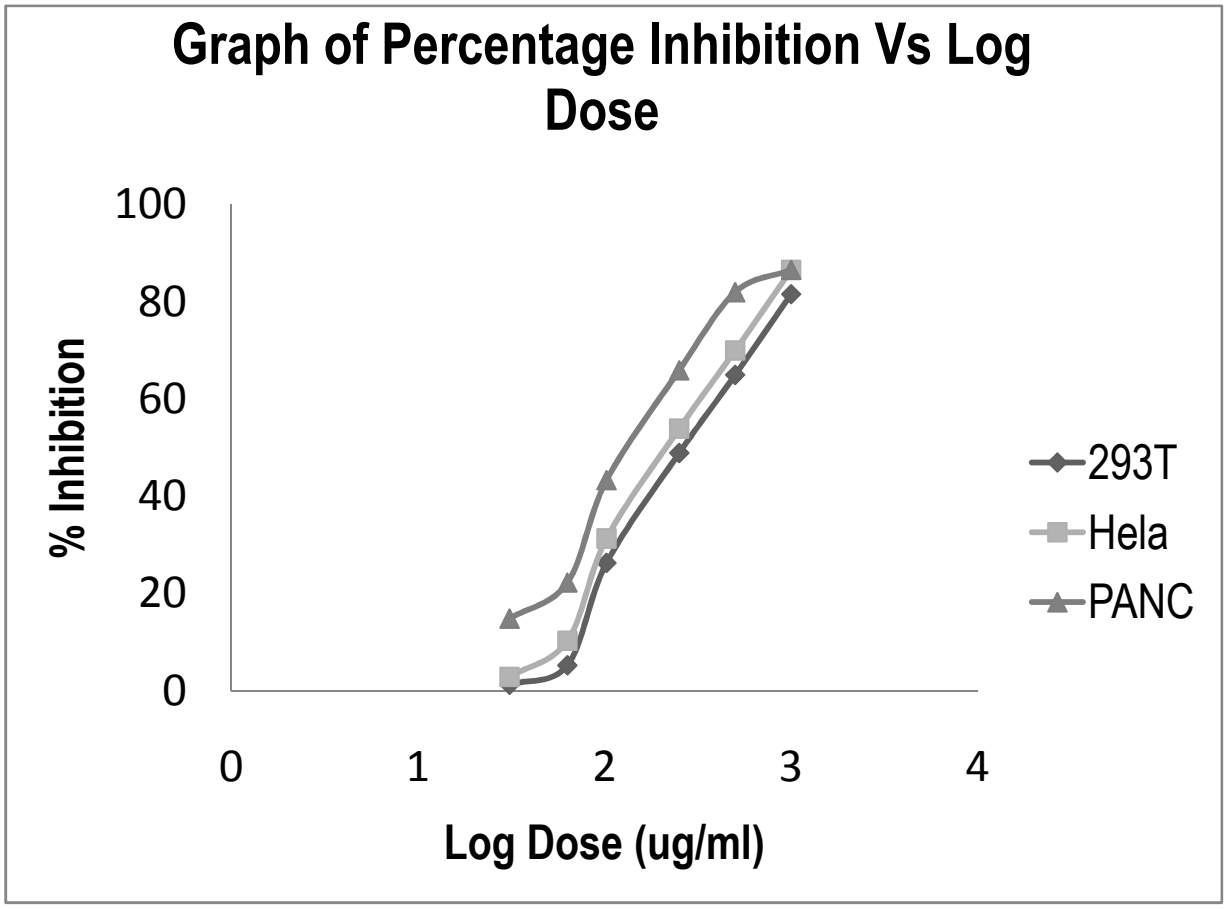

Fig. 5: Effects of A2 on TC_{50} of cell lines

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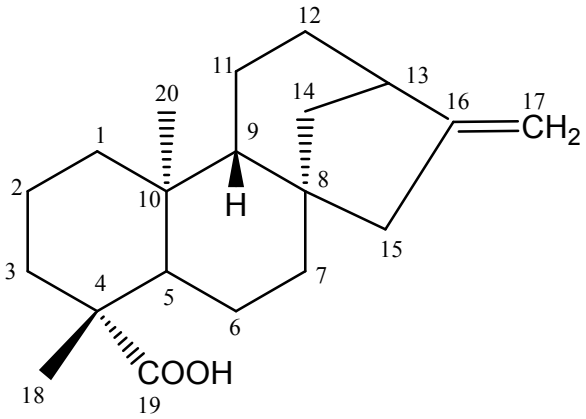

Fig. 6: Chemical structure of Kaur-16-en-19-oic acid

TABLE 1: NMR spectroscopic data of A2[a]

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

[a]Spectra were measured in $CDCl_3$ at 500 (^1H) and 150 (^{13}C) MHz. Assignments were made on the basis of DEPT, ^1H-^1H COSY, HMQC and HMBC experiments. The detailed NMR data are available with the author for correspondence and will be readily supplied on request.

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