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IN VITRO CYTOTOXIC ACTIVITY OF TRITERPENE ISOLATED FROM BARK OF *RANDIA DUMETORUM LAMK*

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ABSTRACT

Randia dumetorum, family Rubiaceae is highly reputed ayurvedic medicinal tree commonly known as the Mainphal. A large deciduous thorny shrub grows upto 5 meters of height. It occurs in almost throughout India upto 4,000 ft attitude. It is found in Himalaya from Jammu east ward's ascending to 1300 and from the Kashmir to east ward's upto 4000 ft. Present study aimed at isolation of phytoconstituents from titled medicinal plant so as to validate its cytotoxic potential. α-L-Arabinosyl (1→3)-β-galactopyranosyl (1→3)-3-β-hydroxyolean-12-en-28-methyloate (compound 1) was isolated from the bark of this plant. The structure was characterized by using spectroscopic methods including 1D- ^{1}H NMR, ^{13}C-NMR, FABMS, experiments and hydrolytic studies. **Compound (1)** was subjected to cytotoxic activity against MDA-MB-231 (Breast cancer cell line) and SK-MEL-2 (Human Skin Melanoma cell line), BE(2)C (Neuroblastoma cell line derived from human bone marrow) and U87MG (Human Neuronale Glioblastoma (Astrozytom) cell-Line showed appreciable cytotoxic effect with IC_{50} value 93.33 µg/mL concentration for SK-MEL-2 (Human Skin Melanoma cell line).

Key words: *Randia dumetorum*, Rubiaceae, Triterpene, Cytotoxic activity.

INTRODUCTION

Randia dumetorum is a large deciduous thorny shrub belonging to family Rubiaceae. It is also known as *Catunaregam spinosa* (Thumb.). It leaves are simple, obovate, wrinkled, shiny and pubescent. Flowers are white, fragrant, solitary, seen on at the end of short branches. Fruits are globose, smooth berries with longitudinal ribs, yellow when ripe. Seeds many, compressed, embedded in the dark fetid pulp[1]. *Randia dumetorum Lamk.* has been reported as an emetic, antidysentric agent. It has anti-bacterial, anti-allergic, anti-inflammatory, analgesic and immunomodulatory activity[2]. Different workers[3-10] have isolated saponins and iridoid from the fruits and barks of this plant. Herein, we report the isolation of α-L-Arabinosyl (1→3)-β-galactopyranosyl (1→3)-3-β-hydroxyolean-12-en-28-methyloate **(Compound 1)**. The compound (1) at 600 µg/mL concentration against MDA-MB-231 (Breast cancer cell line) showed cell cytotoxicity of 56.26%, IC_{50} value 354.8 µg/mL, though the cytotoxic effect of compound (1) was also studied against SK-MEL-2 (Human Skin Melanoma cell line) showed 47.88% cell cytotoxicity at 80 µg/mL, which increased to 52.98% at 120 µg/mL concentration with IC_{50} value 93.33 µg/mL, BE(2)C (Neuroblastoma cell line derived from human bone marrow) showed 45.87% cell cytotoxicity at 160 µg/mL concentration which increased to 53.62% cell cytotoxicity with 320 µg/mL concentration with IC_{50} value 281.8 µg/mL and U87MG (Human Neuronale Glioblastoma (Astrozytom) cell-line) showed 40.88% cell cytotoxicity at 140 µg/mL concentration, which increased to 51.87% at 180 µg/mL with IC_{50} value of 166.0 µg/mL compound concentration.

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EXPERIMENTAL

Material and method

Plant bark (3 Kg) was collected from Ghansali (Tehri Garhwal U.K.) and was air dried in shade. After complete drying of the bark of the plant, it was subjected to defatting and defatted bark of the plant was exhaustively extracted with 100% absolute ethanol solvent (5 Litre) until the extract become colourless. Ethanol extract was subjected to distillation under reduced pressure to liberate the crude extract from ethanol solvent. Ethanolic crude extract was then evaporated to near dryness by leaving overnight. Crude ethanloic extact (80 g) was adsorbed on silica gel and subjected to column chromatographic separation using $CHCl_3$ followed by increasing proportions of MeOH in $CHCl_3$ (v/v) as elutant, collecting 150 mL fractions, $CHCl_3$: MeOH (80 : 20 v/v) elutant gave sample(1). The ^{1}H-NMR spectrum showed that the samples were not pure compounds although TLC showed single spot. For this reason, samples were subjected to further purification by RP-HPLC with a water 590 series pumping system equipped with a water R401 refractive index detector, a μ-Bondapack C18 column (300 × 7.8 mm i.d) and a U6k injector using MeOH-H_2O (9 : 1) as mobile phase (flow rate-2.0 mL/min.) giving compound (1) (t_R 17.3 min).

NMR experiments, a Bruker DRX-600 NMR spectrometer using the UXNMR software package, NMR 600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C-NMR, values relative to TMS reference. Chemical shift expressed in δ (parts per million) values; coupling constant (*J*) are in Hz. Melting points are uncorrected. FABMS: solvent DMSO: glycerol, accelerating voltage 2KV, gas Xe. Column chromatography was on silica gel (Merck) and TLC on Kieselgel 60G (Merck), spot on TLC was visualized by spraying with 20% H_2SO_4 and heating at 120^{o}C for a few minutes.

Compound (1)

Crystallized from methanol as colorless needles. M.P.: 180-185^{o}C; IR ν_{max}^{KBr} (cm^{-1}): 3.4, 1690; ^{1}H NMR (δ_H ppm): 0.74, 0.80, 0.86, 0.91, 1.0, 1.1, 1.18 (each CH_3, 7 x tet-Me), 5.25 (1H, d, J = 7Hz, C-1-H of galactose), 4.41 (1H, d, J = 6.5 Hz, C-1-H of arabinose), 5.11 (1H, brs, 12-H of aglycone); ^{13}C NMR: carbons of sugars moiety: Arabinopyranosyl-104.5 (C-1), 71.7 (C-2), 75.0 (C-3), 70.1 (C-4), 67.5 (C-5); Galactopyranosyl-106.4 (C-1), 74.7 (C-2), 86.8 (C-3), 71.7 (C-4), 78.1 (C-5), 62.5 (C-6); Carboxy part of aglycone (C1-C30): 39.7, 28.53, 97.04, 40.1, 56.9, 19.2, 33.5, 40.5, 48.1, 37.8, 23.9, 23.6, 145.09, 42.8, 28.6, 23.9, 47.2, 42.6, 47.6, 31.5, 34.8, 33.7, 16.9, 15.9, 17.7, 26.4, 28.8, 181.7, 33.9, 24.4, 56.6 (-OCH_3); FABMS (negative ions) m/z: 763, 631, 469, 437, 365, 297, 248, 208.

Acidic hydrolysis

Compound (1) (50 mg) was hydrolyzed in MeOH (10 mL) with 6% H_2SO_4 for 4 h, after addition of water (15 mL); a white precipitate was obtained which was filtered. The residue (25 mg) was crystallized from MeOH as fine needles m.p. 195-200^{o}C and identified as oleanolic acid methyl ester in all respect (m.m.p., cotlc, superimposable IR). The aqueous hydrolysates were separately concentrated on a water bath and neutralized with Ag_2CO_3 chromatographed on Whatmann filter Paper No 1 using system (n-BuOH-AcOH-H_2O, 4:1:5) and identified as L-arabinose and D-galactose. The presence of sugar in filtrate was confirmed by paper chromatography and comparing R_f value.

Cell viability assay preparation

Cells were grown in a 96-well plate in Delbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum and anti-biotic (Streptomycin, Penicillin-G, Amphotericin).

About 1 mL of each cell suspension type (10^5 cells/mL) was seeded in each well and incubated at 37°C for 48 hours in 5% CO_2 for the formation of confluent monolayer. The monolayer of cells in the plate was exposed to various dilutions of extract. The cell viability was measured using MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) with MTT (5 mg/mL) and DMSO. This tetrazolium salt is metabolically reduced by viable cells to yield a blue insoluble Formazan product measured at 540 nm spectrophotometrically. Controls were maintained throughout the experiment (untreated wells as control) the assay was performed in triplicate for each of the concentration. The mean of the cell viability values was compared to the control to determine the effect of compound on cells and % cell viability was plotted against concentration values.

IC_{50}, the concentration of compound required to inhibit 50% cell growth, was determined by plotting a graph of log (concentration of compound) *vs* % cell inhibition. A line drawn from 50% value on the *Y* axis meeting the curve and interpolated to the *X* axis. The X axis value gave log (concentration of compound). The antilog of that value gave IC_{50} value. Percentage inhibition of compound against all cell lines was calculated using the following formula:

$$\% \text{ Cell survival} = (A_t\text{-}A_b) \times 100/(A_c\text{-}A_b)$$

Where, A_t = Absorbance of test,

A_b = Absorbance of blank (Media) and

A_c = Absorbance of control (cells)

% Cell inhibition = 100 − % Cell survival.

RESULTS AND DISCUSSION

Compound (1)

It was crystallized from methanol as colorless needles. M.P. 180-185°C. On hydrolysis with 7% HCl it gave an aglycone identified as oleanolic acid methyl ester by comparison with an authentic sample (m.m.p., co-tlc, co-ir and EIMS). The hydrolysate was monitored on paper chromatography to show the presence of L-arabinose and D-galactose Compound (1) was permethylated by the method[11] of the completion of the reaction was checked by IR. The permethylate of compound (1) on acidic hydrolysis afforded methylated monosaccharides which were identified as 2, 3, 4-tri-O-methyl-L-arabinose and 2, 4, 6-tri-O-methyl-D-galactose (PC). The identities of permethylated sugars were confirmed by comparison with available authentic sample[12] and $NaIO_4$ oxidation results.

The mass spectrum (FABMS and FDMS negative ion) of compound (1) was helpful in determining the molecular weight and the sugar sequence in the glycone part of the molecule. FABMS and FDMS showed its molecular mass to be as 764. The peak at m/z 631 arose by the loss $[M\text{-}H\text{-}132]^+$ of a pentosyl unit (arabinosyl) from the molecular ion. Subsequently loss of a hexosyl unit (galactosyl) gave a peak at m/z 469 $[M\text{-}H]^+\text{-}(132 + 162)^+$. These peaks showed that the aglycone (m/z 469) was aglycosylated with galactose, which was further glycosylated with a terminal arabinosyl unit. The sequence of sugars was also confirmed by the partial hydrolysis. It afforded only one prosapogenin designated as PS_1. Acid hydrolysis of PS1 afforded oleanolic acid methyl ester and D-galactose. Its permethylate on acid hydrolysis showed the presence of 2, 3, 4, 6-tetra-O-methyl-D-galactose. Thus we concluded that methyl ester of oleanolic acid was glycosylated at its C-3 hydroxyl group with a molecule of L-arabinose. The types of linkage at the

glycosidic points were found to be β-in D-galactose and α- in case of L-arabinose by ^{1}H NMR and ^{13}C NMR spectrum.

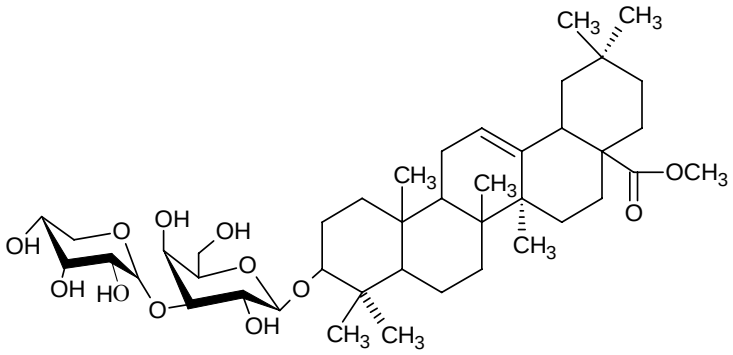

Fig. 1: Compound (1)

The ^{13}C NMR is shown in (Fig. 3) showed the signals recognized for all CH_3, CH_2, CH and quaternary carbon atoms. The signals of C-3 and C-5 of outer arabinose unit occur at δ 75.0 and δ 67.5, respectively (74.3 and 66.1 ppm in case β-linked[13] units), which confirmed the α-linked, α-arabinose. Similarly, the signals of C-2 and C-5 of galactose unit appeared at δ 74.7 and 78.1, respectively (73.5 ppm and 77.6 ppm) in case of β-linked, 70.5 and 72.5 ppm in α-linked galactoside, which confirmed the β-linked D-galactoside unit. The ^{13}C NMR values of α- and β-linked methylpyranosides of L-arabinose and D-galactose have been reported[13].

The ^{1}H NMR spectra further confirmed the type of linkages at glycosidic points. It is shown in (Fig. 2). The anomeric signals of D-galactose units was recorded at δ 5.32 (^{1}H, d, J = 8 Hz) which shown the presence of β-linked galactose while ^{1}H of α-linked L-arabinose was recorded at δ 4.59 (^{1}H, d, J = 6.5 Hz). The exact inter-glycosidic points of linkage were confirmed by ^{13}C NMR studies. ^{13}C NMR chemical shift values of oleanolic acid[14] and methyl pyranosides of β-D-galactose and α-L-arabinose[13] reported. The glycosidation shifts of compound (1) indicated that the inner galactose was glycosylated at its C-3 (86.6 ppm) with an arabinose. Thus the compound (1) was characterized as α-L-Arabinosyl (1→3)-β-galactopyranosyl (1→3)-3-β-hydroxyolean-12-en-28-methyloate.

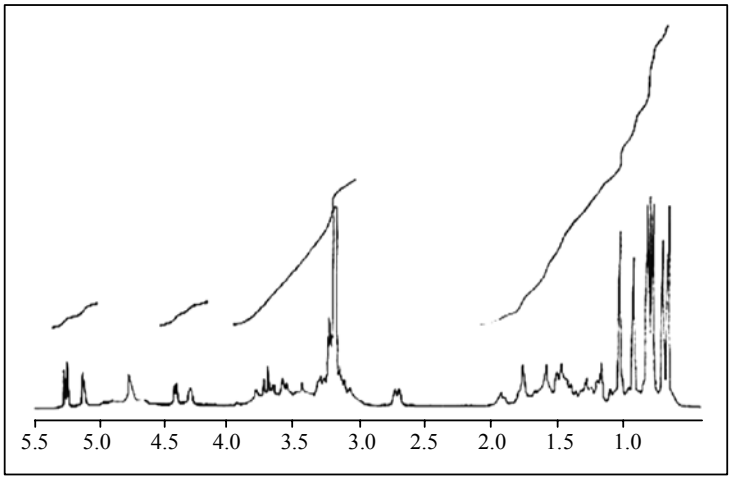

Fig. 2: ^{1}H NMR spectrum of compound (1) "α-L-Arabinosyl(1→3)-β-galactopyranosyl(1→3)-3-β-hydroxyolean-12-en-28-methyloate"

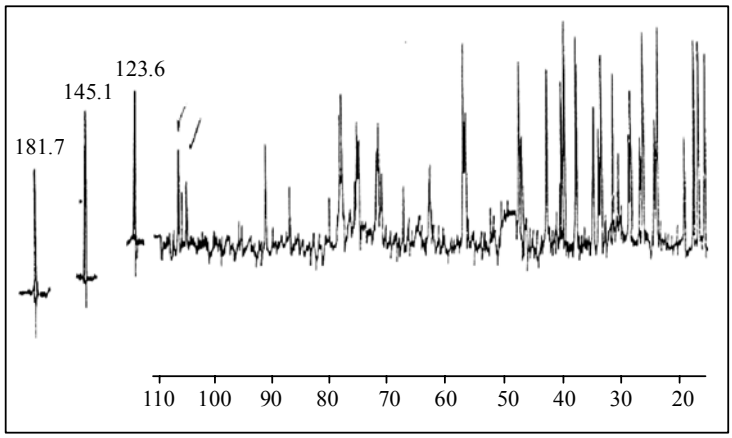

Fig. 3: ^{13}C NMR spectrum of compound (1) "α-L-Arabinosyl(1→3)-β-galactopyranosyl(1→3)-3-β-hydroxyolean-12-en-28-methyloate"

Cytotoxic activity

Compound (1) was subjected to cytotoxic activity against MDA-MB-231 (breast cancer cell line) and the results are shown in Table 1. The percentage viability was 72.17% on treating cell line at 100 µg/Ml, which decreased to only 43.74% with further increasing concentration to 600 µg/mL with IC_{50} value 354.8 µg/mL (Fig. 4).

Table 1: Cytotoxic activity of compound (1) on MDA-MB-231 (Breast cancer cell line)

Concentration	Log_{10} concen.	O.D. Average	% Cell viability	S.D.	% Cell cytotoxicity
Control	-	0.426	100	3.642	00
50 µg/mL	1.698	0.038	90.03	4.929	9.97%
100 µg/mL	2.0	0.307	72.17	3.803	27.83%
200 µg/mL	2.30	0.227	53.38	2.429	46.62%
400 µg/mL	2.60	0.204	48.06	3.456	51.94%
600 µg/mL	2.78	0.186	43.74	4.132	56.26%

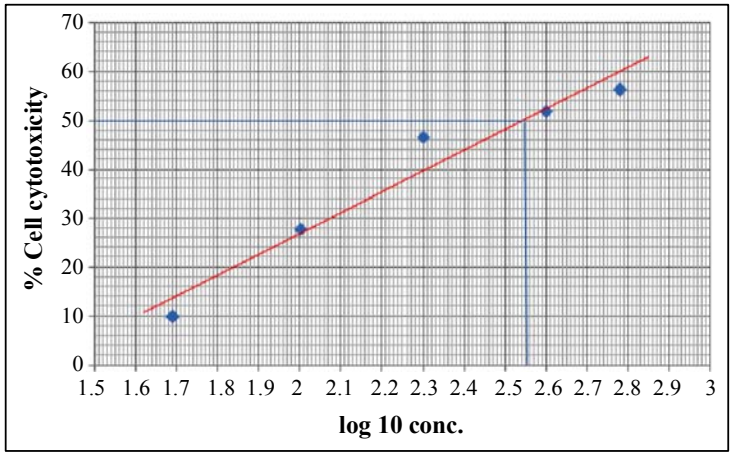

Fig. 4: Compound (1) against MDA-MB-231 (Breast cancer cell line). (IC_{50} = Antilog 2.55 = 354.8 µg/mL)

Compound (1) subjected against SK-MEL-2 (Human Skin Melanoma cell line) and its Table 2 showed % cell cytotoxicity of 18.28% at 10 µg/mL of concentration which increased to 52.98 % at 120 µg/mL concentration with IC_{50} value of 93.33 µg/mL (Fig. 5).

Table 2: Cytotoxic activity of Compound (1) on SK-MEL-2 (Human Skin Melanoma cell line)

Concentration	Log_{10} concen.	O.D. Average	% Cell viability	S.D.	% Cell cytotoxicity
Control	-	0.956	100	3.401	0
5 µg/mL	0.698	0.899	94.13	6.562	5.87%
10 µg/mL	1	0.781	81.72	3.669	18.28%
20 µg/mL	1.30	0.701	73.29	5.77	26.71%
40 µg/mL	1.60	0.593	62.08	5.949	37.92%
80 µg/mL	1.90	0.498	52.12	3.212	47.88%
120 µg/mL	2.07	0.449	47.02	4.351	52.98%

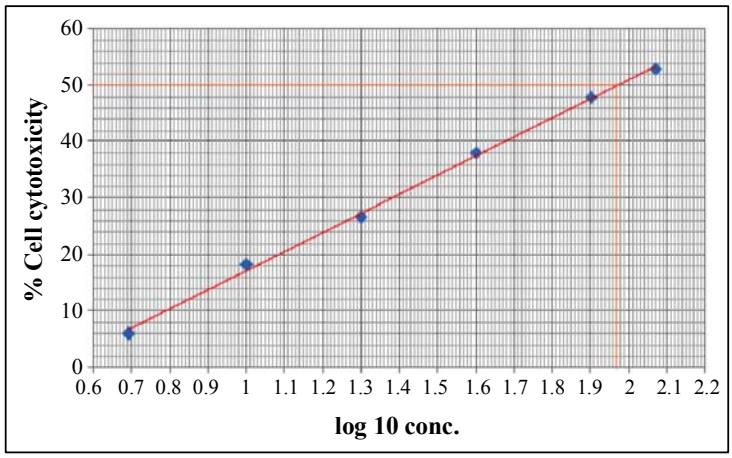

Fig. 5: Compound (1) against SK-MEL-2(Human Skin Melanoma cell line). (IC_{50} = Antilog 1.97 = 93.33 µg/mL)

Compound (1) subjected to BE (2)C (Neuroblastoma cell line) and its Table 3 showed cell cytotoxicity of 28.94% at 80 µg/mL; 53.62 % at 320 µg/mL with IC_{50} value of 281.8 µg/mL (Fig. 6).

Table 3: Cytotoxic activity of Compound (1) on BE(2)C is an Neuroblastoma cell line

Concentration	Log_{10} concen.	O.D. Average	% cell viability	S.D.	% cell cytotoxicity
Control	-	0.488	100	2.869	**0**
10 µg/mL	1	0.434	89.13	0.392	10.87%
40 µg/mL	1.60	0.384	78.73	1.453	21.27%
80 µg/mL	1.90	0.346	71.06	1.356	28.94%
160 µg/mL	2.20	0.334	68.38	3.652	31.62%
240 µg/mL	2.38	0.264	54.13	3.532	45.87%
320 µg/mL	2.50	0.226	46.38	2.343	53.62%

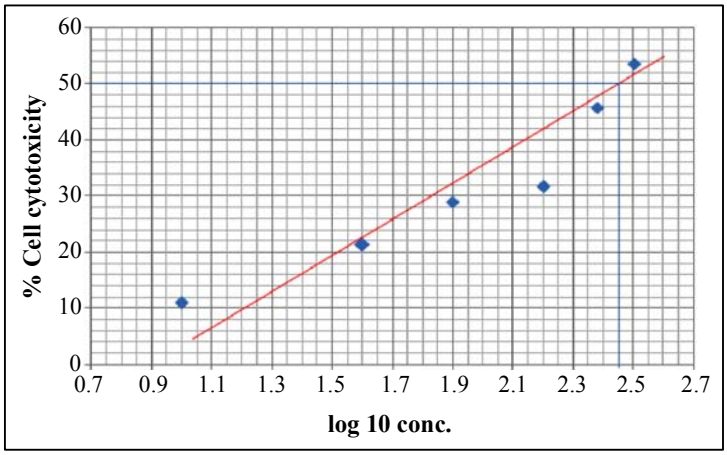

Fig. 6: Compound (1) against BE(2)C is an Neuroblastoma cell line. (IC_{50}=antilog 2.45=281.8 µg/ml)

Compound (1) against U87MG (Human Neuronale Glioblastoma (Astrozytom) cell line and its Table 4 showed 10.19% cell cytotoxicity at 50 µg/mL concentration and 51.87% cell cytotoxicity at 180 µg/mL with IC_{50} value of 166.0 µg/mL concentration (Fig. 7).

Table 4: Cytotoxic activity of Compound (1) on U87MG (Human Neuronale Glioblastoma (Astrozytom) cell-Line

Concentration	Log_{10} concen	O.D Average	% cell viability	S.D.	% cell cytotoxicity
Control	-	0.378	100	5.006	0%
50 µg/mL	1.69	0.339	89.81	3.543	10.19%
80 µg/mL	1.90	0.305	80.91	1.037	19.09%
110 µg/mL	2.04	0.259	68.71	5.435	31.29%
140 µg/mL	2.14	0.223	59.12	2.703	40.88%
180 µg/mL	2.25	0.182	48.13	4.981	51.87%

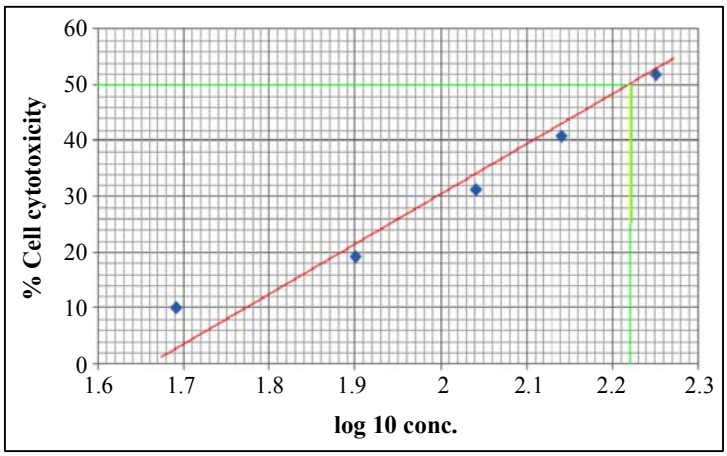

Fig. 7: Compound (1) against U87MG (Human Neuronale Glioblastoma (Astrozytom) cell-Line (IC_{50} = Antilog 2.22 = 166.0 µg/mL)

From cytotoxic study, it was found that % cytotoxicity against various cell lines increased with increase in compound concentration and comparing the IC_{50} value for different cell line, compound showed potant activity against SK-MEL-2 (Human Skin Melanoma cell line) with minimum IC_{50} value of 93.33 µg/mL (Fig. 8).

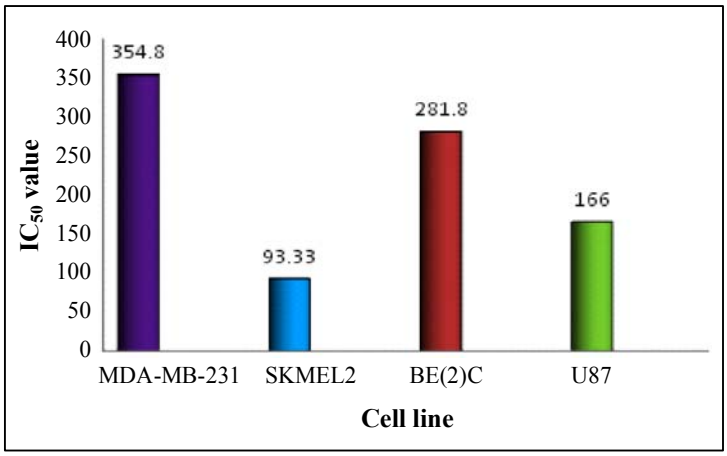

Fig. 8: Showing IC_{50} values of Compound (1) for different cell lines

CONCLUSION

The isolation of compounds from *Randia dumetorum Lamk.* confirms the presence of triterpenes in genus *Randia*. The natural occurance of this compound can be conclusive for the chemotaxonomic characterization of this genus. Tiled ayurvedic medicinal plant have triterpenes beside glycosides, iridoids, iridoid glycosides etc phytoconstituents. The appreciable cytotoxic effect exhibited by triterpene isolated and characterized from studied plant validate the cytotoxic effects exhibited by crude extracts of this indigenous medicinal plant of India.

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