SUPPLEMENTARY MATERIAL

Unmodified Household Coffee Maker Assisted Extraction and Purification of Anticancer Agents from *Dillenia indica* Fruits

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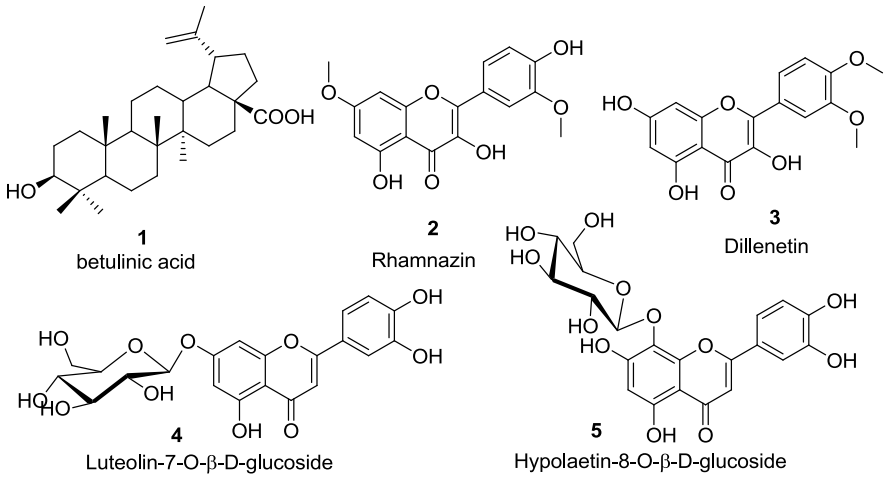
ABSTRACT

Bioassay targeted 80% aqueous ethanol crude extract of the fruits of *Dillenia indica* Linn using unmodified household coffee maker, afforded five compounds namely betulinic acid (**1**), rhamnazin (**2**), dillenetin (**3**), luteolin-7-*O*-β-D-glucoside (**4**) and hypolaetin-8-*O*-β-D-glucoside (**5**). Crude extract, fractions and purified compounds were tested against MDA MB-231, A549 and HeLa cancer cell lines by MTT assay. Compound **3** showed the best activity against A549 (IC_{50} = 26.60±2.5 µM) and HeLa cancer cell lines (IC_{50} =19.35±0.9 µM) whereas compound **5** was found to show the best activity against MDA MB-231 (IC_{50} = 34.62±5.2µM) cancer cell line. These results highlight the utility and efficacy of this eco-friendly procedure to obtain highly potent anticancer compounds from the fruits of *D. indica* that may be suitable for herbal drug development and formulations.



Keywords: *Dillenia indica;* Household coffee maker; Betulinic acid; Anticancer agents

Experimental

General experimental procedures

Infra red (IR) spectrum was obtained by Perkin-Elmer RX-1 spectrophotometer using Br pellets or in neat. ESI-MS and HR-ESIMS were recorded on Jeol SX 102/DA-6000 spectrometer at 70 eV with direct inlet system. 1D and 2D NMR spectra were recorded on AVANCE, Bruker DRX 300 MHz spectrometers using TMS as internal standard for recording chemical shift. Silica gels of different mesh size (60-120 and 230-400 mesh) wereused for normal as well as flash chromatography. HPLC was run on Shimadzu, UV SPD-10 AVP system, using RP-18 (Shim-pack RRC-ODS 20 mm x 25 cm) columns. TLC was run on precoated silica gel 60 F_{254} and RP-18 F_{254}(Merck). Detection was done under UV light, by iodine vapours or by spraying with10% methanolic sulphuric acid followed by heating. All the solvents were purified prior to use. Plant material was grounded using a spice grinder. Pressurized hot water extraction was undertaken employing household coffee maker of Inalsa Company (Model Cafe Aroma)

Plant material

The ripe fruits of *Dillenia indica* were collected from the campus of University of Lucknow, Lucknow UP India. The plant was identified by Dr Alka Kumari, Department of Botany, University of Lucknow, Lucknow, UP India, where a voucher specimen (No. 4785) was deposited.

Extraction and isolation of compounds

The air-dried fruits of *Dillenia indica* (100 gm) were finely grounded using a spice grinder. The obtained powder material (25 gm) was extracted with 70% water/ ethanol (250 ml solution) using unmodified espresso coffee machine. This step of extraction was repeated four times (4 x 25 gm). The extracts were combined, dried ($MgSO_4$), filtered and evaporated on a rotary evaporator to obtain a brown color crude extract which was then fractionated with solvents of varying polarity viz. hexane, $CHCl_3$ and n-butanol. The ensuing fractions were dried ($MgSO_4$), filtered and evaporated to dryness under vacuum to afford three samples viz. hexane fraction (1 gm), $CHCl_3$ fraction (3 gm) and n-butanol fraction (7 gm). The $CHCl_3$ fraction was dissolved in ethanol and kept for the crystallization process. After 2-3 days a white crystalline solid was obtained as betulinic acid (**1**) (700 mg). Likewise 6 gm of the *n*-butanol fraction was subjected to column chromatography using chloroform containing increasing polarity of methanol (1-0 to 0-1) to yield rhamnazin (25 mg) (**2**), dillenetin (56 mg) (**3**), luteolin-7-O-β-D-glucoside (22 mg) (**4**) and hypolaetin-8-*O*-β-D-glucoside (15 mg) (**5**).

Precautions during extraction process

The following precautions should be taken while using household coffee machine for extraction process

- Thoroughly mixed 80% H_2O/EtOH (250 mL) solvent should be taken in beaker
- Steam of 80% H_2O/EtOH solvent mixt in boiler should be made prior to starting the extraction process
- Boiler cap should be properly tighten to prevent the leakage of steam

- Experiment time should be limited to 5 min to ensure the correct ratio of H_2O/EtOH mixture is not disturb in the boiler

Betulinic acid (1)

Off white amorphous powder ($CHCl_3$); HRESI-MS *m/z* 457.3734 $[M+H]^+$, IR (υ_{max}, KBr): 5 1712, 1688, 1640, 1600, 1581, 1289 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 300 MHz)): δ (ppm) 4.76 (1H, brs, H-29a); 4.62 (1H, brs, H-29b); 3.51 (1H, brt, J = 7.8 Hz, H3); 3.23 (1H, m, H-18); 1.82 (3H, s, H-30), 1.21, (3H, s, H-23); 1.09 (3H, s, H-27); 1.06 (3H, s, H-26); 1.02 (3H, s, H-24); 0.81 (3H, s, H-25). ^{13}C-NMR (CDCl3, 75 MHz): δ 179.7 (C28); 150.4 (C-20); 109.7 (C-29); 79.0 (C-3); 56.2 (C-17); 55.3 (C-5); 50.5 (C-9); 49.2 (C-19); 46.8 (C-18);); 42.4 (C-14); 40.6 (C-8); 38.8 (C-4); 38.7 (C-1), 38.3 (C-13); 37.2 (C-10); 37.0 (C-22); 34.3 (C-7); 32.1 (C-16); 30.5 (C-15); 29.7 (C-21); 27.9 (C-23); 27.3 (C-2); 25.4 (C-12); 20.8 (C-11); 19.3 (C-30); 18.2 (C-6); 16.1 (C-25); 16.0 (C-26); 15.3 (C-24); 14.7 (C-27).

Rhamnazin (2)

Yellow amorphous powder (MeOH); HRESI-MS *m/z* 331.1142 $[M+H]^+$, 353.1423 $[M+Na]^+$, IR (υ_{max}, KBr): 3450, 1645, 1605, 1581 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm), 12.44 (1H, s, OH-5), 10.86 (1H, s, OH-4′), 9.56 (1H, s, OH-3), 7.78 (1H, d, J = 1.9 Hz, H-2'), 7.14 (1H, dd, J=1.9, 8.4 Hz, H-6'); 6.19 (1H, d, J = 1.9 Hz, H-6), 6.45 (1H, d, J = 1.9 Hz, H-8), 6.49 (1H, d, J = 8.7 Hz, H-5'), 3.80 (6H, s, OCH_3)^{13}C-NMR (DMSO-d_6, 75 MHz) δ (ppm), 176.0 (C-4), 164.0 (C-7), 160.8 (C-5), 156.3 (C-9), 150.4 (C-2), 148.4 (C-4'), 146.3 (C-3'), 136.1 (C-3), 123.3 (C-1'), 121.5 (C-6'), 115.5 (C-5'), 114.1 (C-2'), 103.1 (C-10), 98.3 (C-6), 93.6 (C-8), 55.6 (OCH_3), 55.4 (OCH_3)

Dillenetin (3)

Yellow amorphous powder (MeOH); HRESI-MS *m/z* 353.1047 $[M+Na]^+$. IR (υ_{max}, KBr): 3454, 1649, 1610, 1585 cm^{-1}, ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm), 12.46 (1H, s, OH-5), 10.89 (1H, s, OH-7′), 9.57 (1H, s, OH-3), 7.71 (1H, d, J = 1.8 Hz, H-2'), 7.19 (1H, dd, J=1.8, 8.7 Hz,

H-6'); 6.16 (1H, d, J = 1.9 Hz, H-6), 6.41 (1H, d, J = 1.9 Hz, H-8), 6.49 (1H, d, J = 8.7 Hz, H-5'), 3.90 (6H, s, OCH_3)^{13}C-NMR (DMSO-d_6, 75 MHz) δ (ppm), 178.0 (C-4), 163.3 (C-7), 160.6(C-5), 156.1 (C-9), 150.3 (C-2), 148.9 (C-4'), 146.7 (C-3'), 136.5 (C-3), 123.6 (C-1'), 121.2(C-6'), 115.7(C-5'), 114.3 (C-2'), 103.3 (C-10), 98.2 (C-6), 93.4 (C-8), 55.5 (OCH_3), 55.3 (OCH_3).

Luteolin-7-O-β-D-glucoside (4)

Yellow powder (MeOH); HRESI-MS *m/z* 449.3661 $[M+H]^+$, IR (υ_{max}, KBr): 3450, 2923, 2850, 1650, 1605, 1490, 1259. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 12.83 (1H. s, OH-5), 7.70 (1H, dd, J = 8.4, 2.0 Hz, H-6′); 7.57 (1H, d, J = 2.0 Hz, H-2′), 6.92 (1H, d, J = 8.4 Hz, H-5′), 6.84 (1H, d, J = 2.0 Hz, H-8), 6.71 (1H, s, H-3), 6.27 (1H, d, J = 2.0 Hz, H-6), 5.08(1H, d, J = 7.6 Hz, H-1″), 4.80-3.01 (5H, m, sugar protons). ^{13}C NMR (DMSO-d6, 75 MHz) δ 182.6 (C-4); 167.4 (C-2), 163.3 (C-7), 161.7 (C-5), 157.6 (C-9), 149.7 (C-4′), 146.1 (C-3′), 122.1 (C-1′), 119.7 (C-6′), 116.3 (C-5′), 113.9 (C-2′), 106.9 (C-10), 102.9 (C-3), 100.3 (C-1″), 99.3 (C-6), 98.5 (C-8), 77.5 (C-5″), 76.7 (C-3″), 72.9 (C-2″), 69.6 (C-4″), 63.5 (C-6″).

Hypolaetin-8-O-β-D-glucoside (5)

Yellow powder (MeOH); HRESI-MS *m/z* 465.1355 $[M+H]^+$, IR (υ_{max}, KBr): 3390, 2928, 2840, 1652, 1600, 1491, 1259. ^{1}H NMR (DMSO-d_6, 300 MHz) δ 12.82 (1H. s, OH-5), 7.70 (1H, dd, J = 8.5, 2.0 Hz, H-6′); 7.56 (1H, d, J = 2.0 Hz, H-2′), 6.94 (1H, d, J = 8.4 Hz, H-5′), 6.70 (1H, s, H-3), 6.27 (1H, d, J = 2.0 Hz, H-6), 4.68 (1H, d, J = 7.7 Hz, H-1″), 4.80-3.17 (5H, m, sugar protons). ^{13}C NMR (DMSO-d_6, 75 MHz) δ 181.8 (C-4); 167.0 (C-2), 164.1 (C-7), 161.3 (C-5), 157.2 (C-9), 149.9 (C-4′), 145.6 (C-3′), 121.5 (C-1′), 125.4 (C-8), 120.0 (C-6′), 116.0 (C-5′), 113.7 (C-2′), 106.9 (C-10), 103.5 (C-3), 102.5 (C-1″), 98.9 (C-6), 77.3 (C-5″), 75.9 (C-3″), 74.1(C-2″), 69.0 (C-4″), 60.3 (C-6″).

Quantification of Betulinic acid by HPLC

Precisely weighed samples of extract and Betulinic acid were separately dissolved in HPLC grade acetonitrile and filteredin 0.45 mm filterprior to injection. The protocol was based on previouslydescribed methods. Portions (10 ml) of both samples were injected on to the HPLC column (Waters C18RP column (250×4.6 mm, 5 μm particle sizes, maintained at 25 °C). The

mobile phase consisted of a mixture of acetonitrile (Solvent A) and methanol (Solvent B) which was applied as a gradient for 30 min. The injection volumes were 15.0 µl and flow rate was set at 1 mL/min for both samples. The mobile phase was applied in the following gradient for 30 min: 90% A, 10% B for 0-5 min, 10% A, 90% B for 15 min, 50% A, 50% B for 10 min. The wavelength for absorbance was set at 254 nm. Each solution was prepared and injected three times and the curve was plotted c was expressed in terms of mean ± SD (mg/g).

Cytotoxicity assay

Reagents

Dulbecco's Modified Eagle's Medium DMEM/F-12 (1x), 0.4% trypanblue, PBS (pH ¼ 7.2, 1x), 0.25% trypsine EDTA (1x), and antibiotic/antimycotic solution (100x) were purchased from Gibco, Life Technologies; whereas fetal bovine serum (FBS) and MTT were purchased from Himedia. DMSO from Calbiochem

Cell lines

Three cell lines MDA-MB-231 (human breast carcinoma), HeLa (cervical cancer) and A549 (Adenocarcinomic, human alveolar basal epithelial cells), were obtained from the National Centre for Cell Science (Pune, India), and maintained by sub-culturing in 25 cm and 75 cm cell culture flask at 37°C. Cells were incubated in 5% CO_2 incubator at 95% humidity, maintain in DMEM media with 5% FBS at the Tissue Cell Culture Lab in Era's Lucknow Medical University, Lucknow, India.

MTT assay

The anticancer property of extract and fraction as well as the pure compounds isolated from *D. indica* extract were determined by the MTT {3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay against MDA MB-231, HeLa and A549 cell lines. The exponentially growing cells were trypsinized and seeded, 200 µl in each well of 96 well plates with cell density 5×10^{-4} cells/ml. The 96 well plates were incubated in Form a Steri-cycle CO_2 Incubator (Thermo Fisher Scientific) at 37°C and 5% CO_2 atmosphere for 24 hour and the cells were allowed to grow. After an incubation of 24 hour, the cells were treated by the extract and

fractions in different concentrations (100µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, and 500 µg/ml) and by pure compounds in different concentration (10µM, 25µM, 50µM and 100µM) for 48 hours. After the incubation, 20µl MTT was added in each well at a concentration of 5 mg/ml and again incubated for 2-4 hours. In this incubation formazone crystals were formed. Afterward, DMSO was added to dissolve the crystal. The absorbance was measured at 570 to 650 nm on a Microplate Absorbance Reader (Bio-Rad). The % cell viability was calculated as:

$$\% \text{ cell viability} = \{(A_T - A_B)/ (A_C - A_B)\} * 100$$

where

AT = Absorbance of the treatment well

AB = Absorbance of the blank

Ac = Absorbance of the control well

$$\% \text{ cell inhibition} = 100 - \text{Cell Survival}$$

Table S1: *In vitro* anticancer activity against MDA MB-231, A549 and HeLa cancer cell lines of crude extract and fractions isolated from *D. indica* fruits

Samples	Cell lines	Percentage inhibition					IC_{50} (µg/mL)
		100 (µg/ml)	200 (µg/ml)	300 (µg/ml)	400 (µg/ml)	500 (µg/ml)	
EE	MDA MB-231	39.75	55.88	69.92	77.72	90.33	165.21±6.1
	A549	31.44	50.64	76.82	83.88	92.68	190.51±15.9
	HeLa	6.81	23.22	24.54	34.55	54.72	437.38±28.0
HF	MDA MB-231	03.88	05.61	20.72	50.84	95.61	363.65±23.42
	A549	14.72	25.54	31.35	36.52	52.03	496.65±46.5
	HeLa	07.08	15.84	16.63	23.16	35.07	765.99±199.7
CF	MDA MB-231	38.26	54.37	81.71	86.37	99.04	157.48±30.4
	A549	40.15	68.52	86.88	92.58	97.25	107.98±9.0
	HeLa	04.61	08.15	15.75	22.85	38.88	523.16±55.8
MF	MDA MB-231	39.45	57.90	65.99	67.03	99.80	176.71±7.3
	A549	46.62	52.84	62.88	77.28	88.39	152.57±49.5

HeLa	06.85	24.99	29.19	35.59	52.77	479.31±52.5

EE: Ethanol Extract; CF: Chloroform Fraction; EF: Ethyl acetate Fraction; ME: Methanol Fraction

Table S2: *In vitro* anticancer activity against MDA MB-231, A549 and HeLa cancer cell lines of pure compounds isolated from *D. indica* fruits

Compound No.	Cell lines	Percentage inhibition				IC_{50} (µM)
		10 µM	25 µM	50 µM	100 µM	
1	MDA MB-321	30.88	52.35	83.77	94.84	24.04±1.2
	A549	46.66	76.00	88.14	96.61	11.72±0.5
	HeLa	38.58	80.85	92.85	87.93	13.57±1.9
2	MDA MB-321	25.62	38.61	57.12	93.18	40.86±5.8
	A549	21.73	39.81	60.12	70.25	38.67±2.45
	HeLa	06.79	19.22	42.20	62.07	75.40±11.0
3	MDA MB-321	27.27	40.14	70.45	90.00	36.30±1.0
	A549	33.76	56.87	64.68	84.88	26.60±2.5
	HeLa	33.20	65.72	83.54	90.22	19.35±0.9
4	MDA MB-321	11.78	22.99	33.90	61.96	78.88±5.8
	A549	06.91	21.12	30.04	46.99	103.75±7.8
	HeLa	05.45	11.87	21.23	30.42	167.82±15.5
5	MDA MB-321	31.11	38.80	70.51	92.02	34.62±5.2
	A549	16.40	33.07	50.97	84.89	51.42±3.9
	HeLa	13.57	20.20	47.95	61.57	72.27±4.3

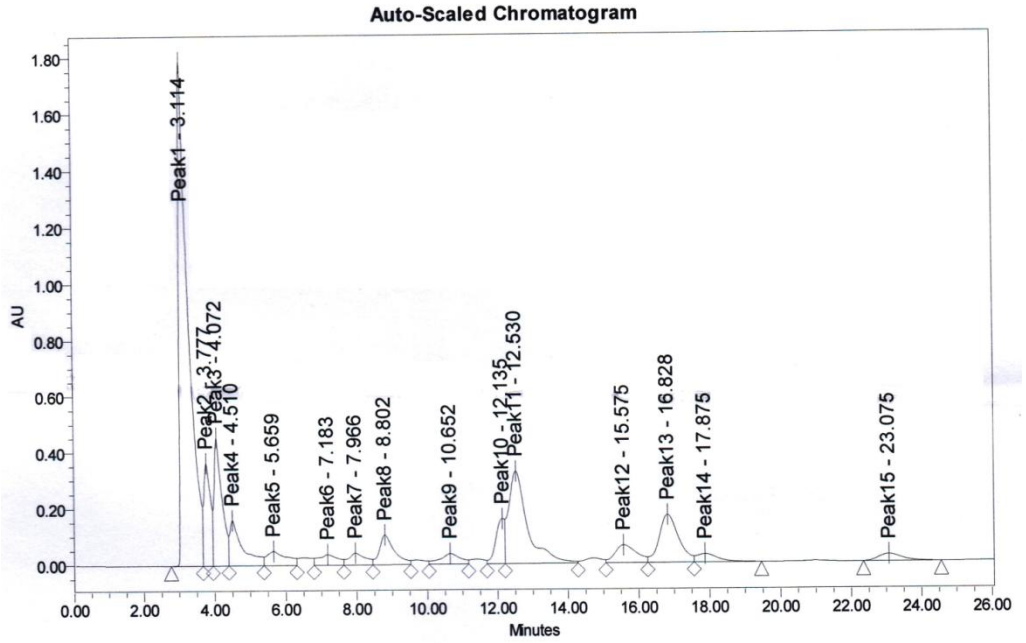


Figure S1: HPLC chromatogram for extract; Eluent:acetonitrile: methanol (9:1); flow rate:1ml/min;UVdetection:210nm. Longest peak in extract and fraction (RT 3.114) indicates peak for Betulinic acid

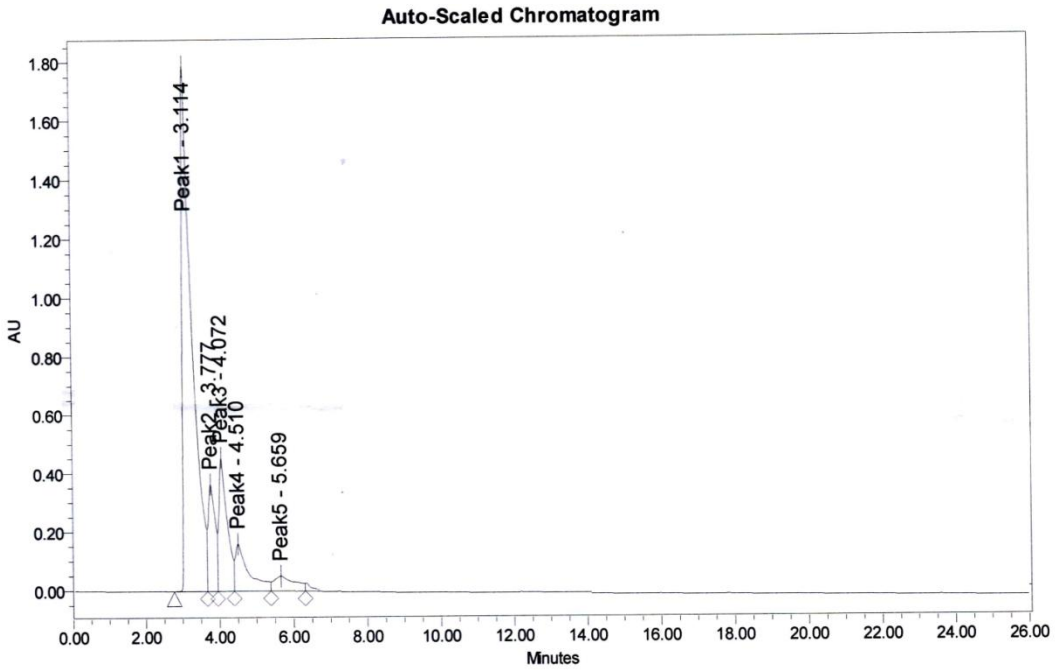


Figure S2: HPLC chromatogram of chloroform fraction; Eluent:acetonitrile: methanol (9:1);flow-rate:1ml/min;UVdetection:210nm. Longest peak in extract and fraction (RT 3.114) indicates peak for Betulinic acid

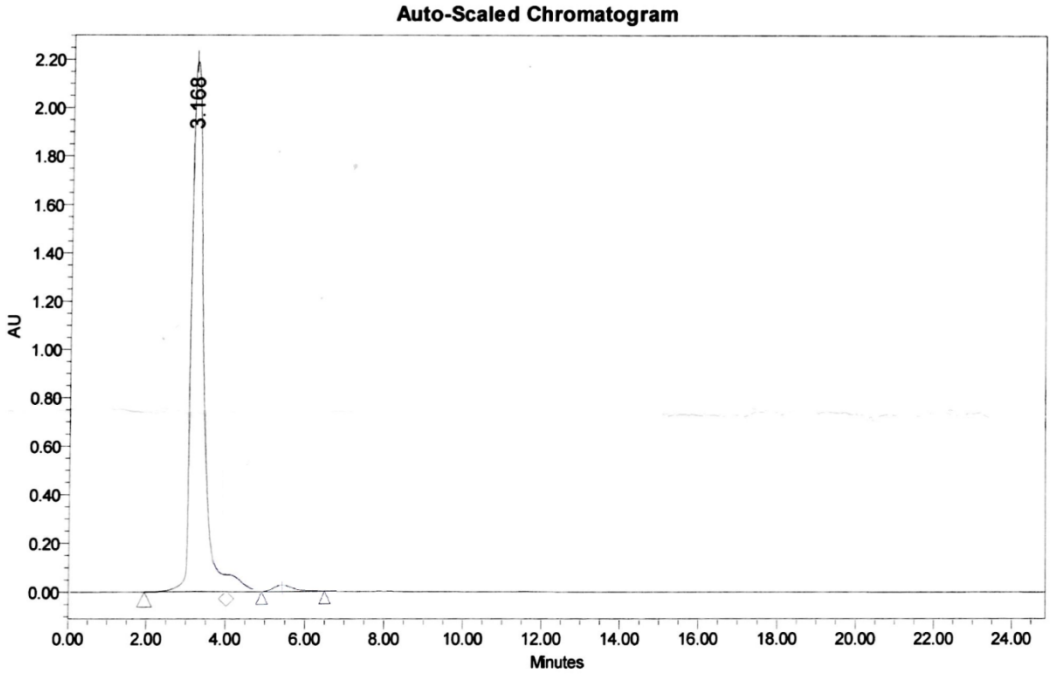


Figure S3: HPLC chromatogram of purified betulinic acid; Eluent:acetonitrile: methanol (9:1);flow-rate:1ml/min;UVdetection:210nm. Longest peak in extract and fraction (RT 3.114) indicates peak for Betulinic acid

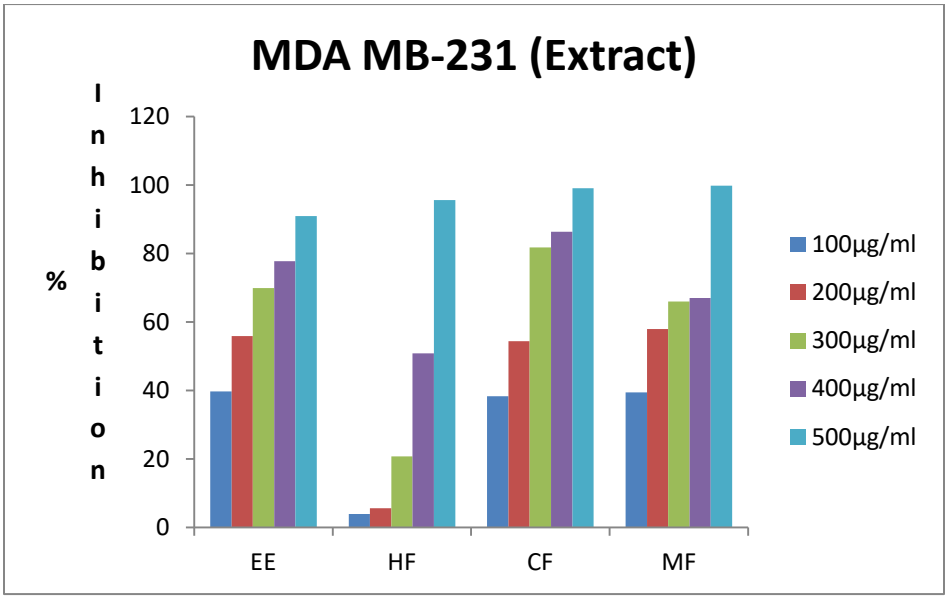


Figure S4: Anticancer activity of extract and fractions against MDA MB-231 cell lines at different concentrations

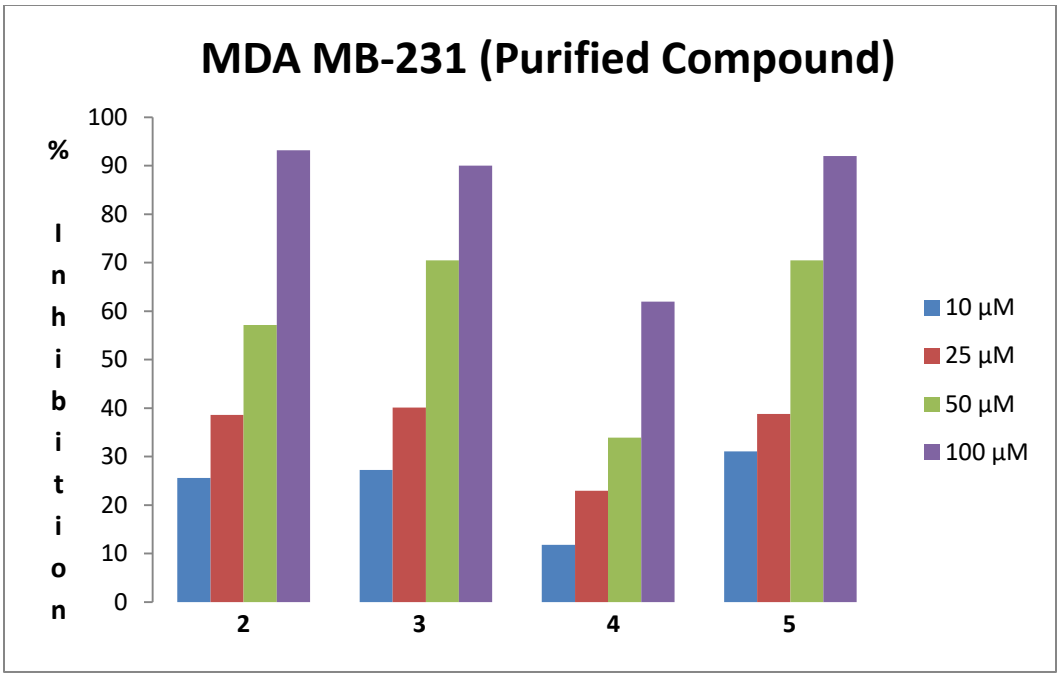


Figure S5: Anticancer activity of purified compounds against MDA MB-231 cell lines at different concentrations

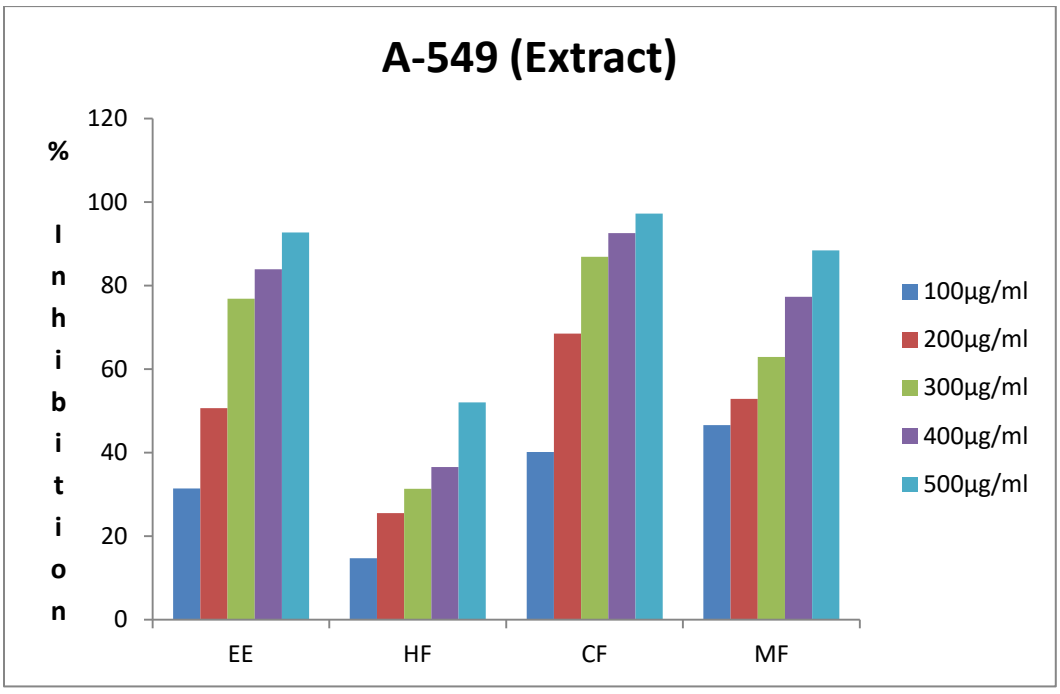


Figure S6: Anticancer activity of extract and fractions against A-549 cell lines at different concentrations

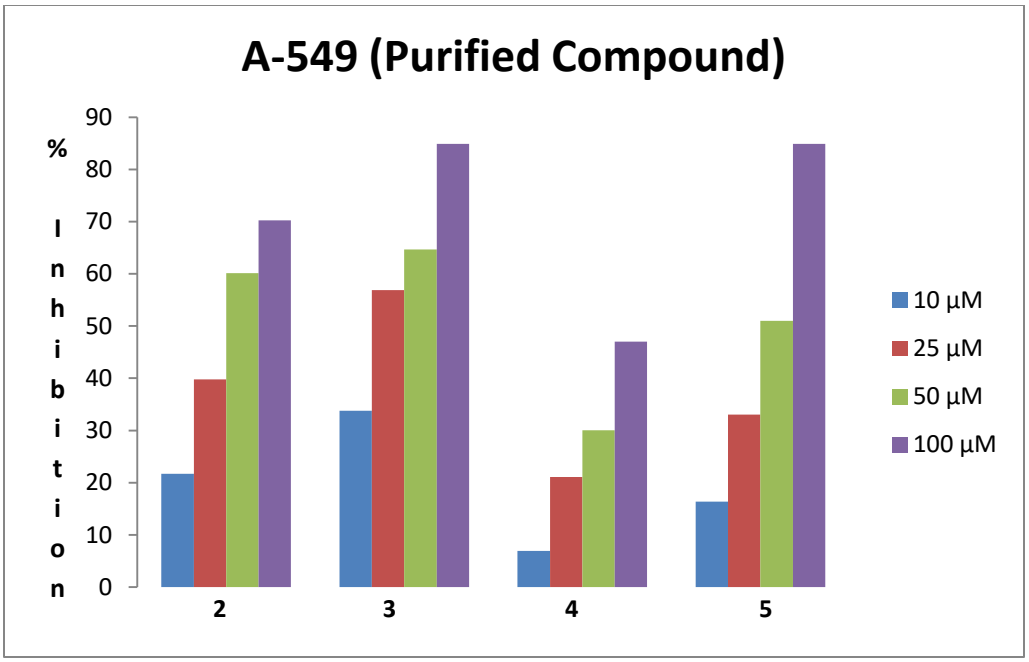


Figure S7: Anticancer activity of purified compounds against A-549 cell lines at different concentrations

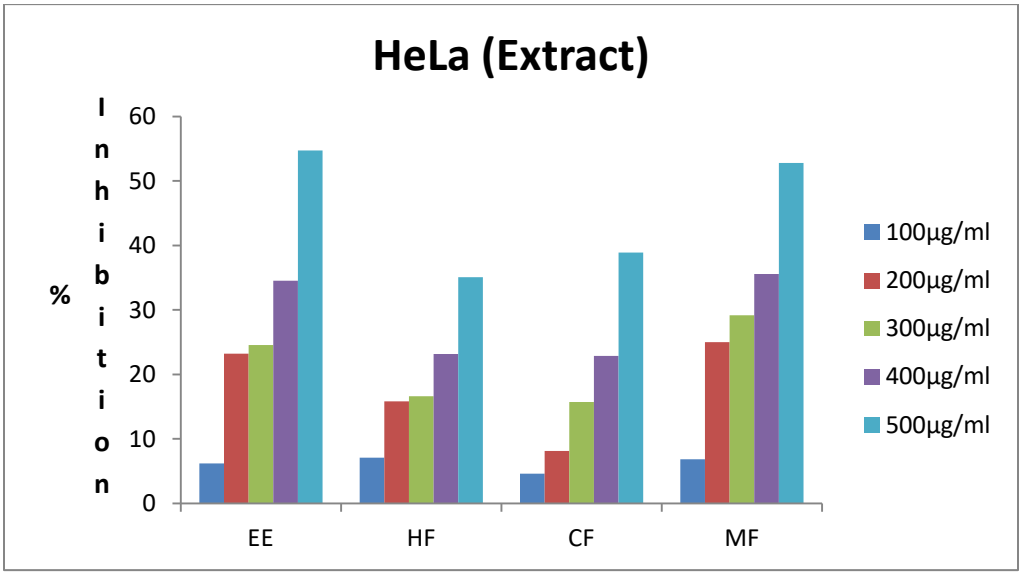


Figure S8: Anticancer activity of extract and fractions against HeLa cell lines at different concentrations

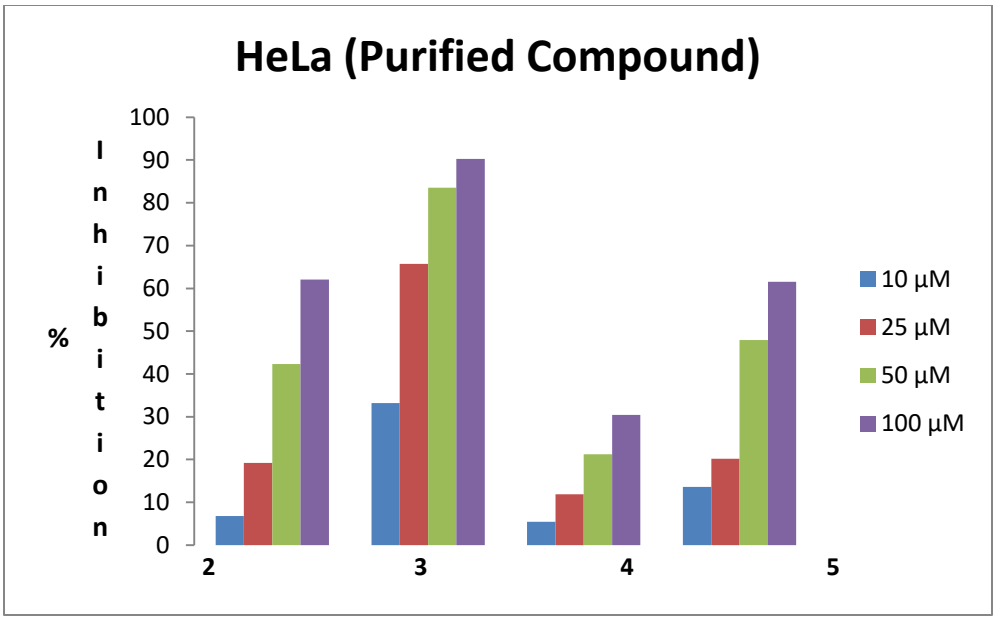


Figure S9: Anticancer activity of purified compounds against HeLa cell lines at different concentrations