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Anticancer potential of methanol extract of seeds of *Artocarpus hirsutus* in human breast cancer cell lines

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Artocarpus hirsutus, belonging to the family Moraceae, is used traditionally for the treatment of skin problems, diarrhoea, snake bite and ulcers. In the present study, the methanol extracts of fruit seeds of *A. hirsutus* (MAH) (Wild jack) were investigated for the presence of phytochemicals and anticancer activities against human breast cancer cell lines *in vitro*. Methanolic extraction was done using soxhlet. The phytochemicals were determined by conventional qualitative tests and GC-HRMS. Cytotoxicity study was conducted using MTT assay. Apoptotic changes produced by the plant extract in the treated cells were determined by acridine orange ethidium bromide and hoechst staining. Apoptotic pathway was analysed using JC-1 staining and molecular expressions of Bcl-2. Protein and gene expressions of Bcl-2 were studied by performing western blot and RT-qPCR respectively. In the cytotoxicity study, MAH exhibited significant ($p<0.05$) reduction in per cent cell viability. The IC_{50} value was found to be 65.47±9.51 µg/mL in MDA-MB-231 cells and 64.05±9.3 µg/mL in MCF-7 cells. Microscopic studies in both the cells after MAH treatment revealed morphological and nuclear alterations which were characteristics of intrinsic pathway of apoptosis. Significant ($p<0.05$) down regulation in the expression of anti-apoptotic gene and protein Bcl-2 was observed which confirmed the intrinsic pathway of apoptosis. The present study revealed a concentration dependent anticancer activity of methanol extract of seeds of *A. hirsutus* mediated through intrinsic pathway of apoptosis.

Keywords: *Artocarpus hirsutus*, Apoptosis, Bcl-2, GC-HRMS, Intrinsic pathway, MTT assay

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India has great resources of traditional medicinal plants with exclusive diversity among them. It would be beneficial to use these medicinal plants to develop new anticancer drugs as a novel approach towards cancer treatment[1,2]. *Artocapus hirsutus* belonging to family Moraceae is commonly known as 'Wild jack' or 'Jungle jack'. This tropical evergreen plant species is native to India and primarily found in Kerala as well as in Maharashtra, Karnataka and Tamil Nadu. It prefers moist, deciduous to partially evergreen woodlands. They are endemic to Western Ghats and found in evergreen forests. Lengthwise, tree can reach to 35 m height, 4.5 m girth and produces small jackfruit like fruits which are edible. It is 10-15 mm thick, with dull, smooth, brown, warty, brown surface, fibrous and red peeled surface, sticky milky white exudations and hairy branchlets. Leaves are alternate, simple, lateral, broadly obovate, ovate or elliptic. Flowers are unisexual in axillary inflorescence ranging in length upto 15 cm. Fruits are sweet syncarp, becomes orange hue on ripening. Seeds are white, oval ranging in length from 16-18 mm. Fried seeds are often used as snack. Traditionally, *A. hirsutus* has been used for treatment of skin problems, diarrhoea, snake bite, ulcers[3]. In folk medicine, bark is used against snake bite, dhobi's itch and ringworm. Leaves are used to treat joint pain, chronic haemorrhage, seeds used as laxative and appetite stimulant. Ayurvedic records show evidence that it pacified vitiated vata and pitta, anorexia, burning sensation of extremities while the unripe fruit caused vitiation of Tridosha. Studies had proven its use

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as antioxidant, antimicrobial, anti-inflammatory, cytotoxic, antibacterial, anti-hyperglycaemic, anti-melanogenesis and anti-alzheimer agent[4-7]. With the above background, the present study was aimed to identify the phytochemicals and anticancer potential of seeds of *A. hirsutus* using *in vitro* methods.

Materials and Methods

Chemicals and drugs

Acridine orange, Agarose, Acrylamide, Ammonium acetate, Dihydrogen sodium phosphate, Ethylene diamine tetraacetic acid (EDTA), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), Methyl thiazolyl diphenyl-tetrazolium bromide (MTT), Nonidet P-40, N1,N1- Dimethyl bisacrylamide, Nicotinamide adenine dinucleotide (NADH), Propidium Iodide, Proteinase K, RNAse A, Sodium pyrophosphate, Tween 20, Triton-X-100, Dimethyl sulphoxide (DMSO), Antibiotic-antimycotic solution (100x), Doxorubicin, Monoclonal Anti-Bcl-2 clone Bcl-2-100. Monoclonal anti beta actin antibody were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640, Fetal bovine serum (FBS), Trypsin/EDTA were purchased from Invitrogen Life Technologies, USA. Horse radish peroxidase-conjugated secondary anti-mouse and anti-rabbit antibodies were purchased from Cell Signalling Technology, Danvers, MA, USA. Glycerine, secondary goat anti-rabbit antibody- HRP conjugated, cDNA synthesis kit.

Collection of plant material and authentication

The whole seeds of *A. hirsutus* were collected from Mannuthy region, Thrissur district, Kerala, India. The plant material was taxonomically identified and authenticated by Botanical Survey of India, Southern Regional Centre, T.N.A.U. Campus, Coimbatore, Tamil Nadu, India as Collection No. BIS/SRC/ 5/23/2019/Tech./2931. A voucher specimen of the plant has been deposited at the Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India (HERB/VPT/ CVASMTY/2/2018).

Methanol extraction

Since the previous study had shown methanol extract to be cytotoxic[6], methanol extract was preferred in the present study. For methanol extraction, the seeds of *A. hirsutus* were air dried at room temperature, coarsely powdered and extracted using Soxhlet apparatus using methanol (99.9%) at 55°C. Further it was concentrated using a rotary vacuum evaporator (Evator, Equitron EV11.ABI.029, India) under reduced pressure and temperature (55°C). After complete evaporation of the solvent, methanol extract of seeds of *A. hirsutus* (MAH) was kept under refrigeration in an airtight container.

Sample preparation

The methanol extract was solubilized in dimethyl sulphoxide (DMSO) at a concentration of 1 mg/mL, further this stock solution was diluted with sterile PBS to required concentrations. The final concentration of DMSO in the well was maintained less than one per cent w/v.

Culturing of cell lines

MDA-MB-231 and MCF-7 cell lines were procured from the Cell Repository, National Centre for Cell Sciences, Pune, India. The cells were cultured in RPMI-1640 supplemented with 10% foetal bovine serum and 1% antibiotic antimycotic solution containing penicillin-streptomycin and amphotericin B. The cells were maintained in a humidified incubator at 37°C with 5% CO_2. The cells were subcultured by enzymatic digestion with 0.25% trypsin and 1 mM ethylene diamine tetraacetic acid solution after attaining 70% confluency. Trypsinized cells were used for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Qualitative phytochemical analysis

MAH was tested for the presence of various active phytoconstituents namely steroids, alkaloids, tannins, phenolic compounds, flavonoids, diterpenes, triterpenes and saponins[8].

GC-HRMS analysis

GC-HRMS system of Sophisticated Analytical Instrument Facility (SAIF), Indian Institute of Technology (IIT), Bombay, Maharashtra, India was used to detect the active phytoconstituents of MAH. Gas Chromatography (Agilent, USA) with a mass resolution of 6000, FID detector, EI/CI source and time of flight analyser with a mass range of 10-2000 amu was utilized for the study. Carrier gas was helium at flow rate of 1 mL/ min. The oven temperature was stabilized at 70°C for 1 min and then

raised to 200°C in 5 min with injector temperature 250°C and total analysis time 50 min. Extract aliquot of 1 µL was injected into the chromatographic column (capillary column, length – 30 m, inner diameter: 0.25 mm, film thickness: 0.25 µm) after a clear baseline had been obtained. NIST MS search 2.0 mass spectrum library was used to identify the major constituents[9].

***In vitro* cytotoxic study of MAH**

Cytotoxic potential of MAH was assessed using 3-(4,5- dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay in MDA-MB-231 and MCF-7 human breast carcinoma cell lines as per the method of Naidu *et al.* (2013)[10]. The cells were seeded at a density of 5 x 10^3 cells per well, in 200 µL medium and were allowed to attach for overnight in a CO_2 incubator. Initially, a pilot MTT assay was conducted in both the cells at concentrations of 10, 20, 40, 80, 160 and 320 µg/mL for 48 h to find out the concentrations at which MAH produced cytotoxicity. The concentrations were selected in geometric progression. Since the concentrations used for the pilot study showed cytotoxicity, the same concentrations were used for the main study. In the main study, the cells were treated with MAH at concentrations of 10, 20, 40, 80, 160 and 320 µg/mL for a period of 48 h. After the treatment, 20 µL of MTT (5 mg/mL) in 150 µL medium was added and incubated at 37°C for 4 h after removing the medium with extract of *A. hirsutus*. Then the media with MTT was removed and the formed purple formazan crystals were dissolved in 200 µL of DMSO and read at 570 nm in an ELISA plate reader (Varioskan flash, Thermo Fischer Scientific, Finland). The per cent cell viability and per cent cell inhibition were calculated using following formulae: Per cent cell viability = (Average absorbance of treated cells /Average absorbance of untreated cells) × 100% cell inhibition was calculated as 100% cell viability. The net absorbance from the control wells was taken as 100% viable. The IC_{50} value of extract was calculated by plotting the concentration against per cent cell inhibition using the online software "very simple IC_{50} tool kit". "Very simple IC_{50} tool kit" allows biological dose-response data to be plotted and fitted to the curve to give midpoint ligand concentration c, d-hill coefficient using the following formula:

$$\frac{y= a+(b-a)}{[1+(x/c)^d]}$$

Microscopic studies

Microscopic studies using acridine orange ethidium bromide

Trypsinized cells at concentration of 1x 10^5 cells were seeded into a six well plate and allowed to grow for 24 h. Based on the MTT assay, IC_{50} concentration of the plant extract was selected for the study. The selected concentrations of MAH for MDA-MB-231 and MCF-7 cells were 65 and 64 µg / mL respectively. Doxorubicin was used as positive control at 0.58 µg/mL concentration. After treatment of cells with IC_{50} concentration of extract for 24 h, the acridine orange ethidium bromide (AO/EB) staining procedure was followed to differentiate the live, apoptotic and necrotic cells. After discarding media from wells of treated or untreated cells, they were stained with 200 µL of acridine orange (10 µg/mL) and ethidium bromide (10 µg/mL) and analysed under fluorescence inverted microscope (Axio Vert. A1 FL-LED, Carl Zeiss, Germany) with blue excitation (488 nm) and emission (550 nm) filters[11].

Analysis of morphological changes in nucleus

Morphological changes in nucleus were studied with modified method. Both MDA-MB-231 and MCF-7 cells (1x10^5 cells per well) were seeded in six well plates and after 24 h of growth, cells were treated with IC_{50} concentration of extract and doxorubicin at 0.58 µg/mL concentration for 24 h. Then cells were trypsinized and collected in eppendorf tube and centrifuged at 1000 rpm. After discarding supernatant, cell pellet was washed thrice with PBS and the cells were transferred to glass slide and fixed with warm air flow. Then staining was done with 5 µL of Hoechst 33258 stain (Invitrogen 35 µg/mL) for 30 min and images were captured by trinocular research fluorescence microscope (DM 2000 LED, Leica, Germany) with blue excitation (352 nm) and emission (461 nm) filters[12].

***Analysis of mitochondrial transmembrane potential* (*MMP*)**

Both MDA-MB-231 and MCF-7 cells were plated at a seeding density of 1 x 10^5 cells per well in six well plates. After 24 h of treatment with extract at IC_{50} concentration and doxorubicin at 0.58µg/mL concentration, cells were incubated with 5 *µM* fluoroprobe 5, 5', 6, 6'- tetrachloro- 1, 1', 3,3'-tetra ethyl benzimidazol-carbocyanine iodide (JC-1) for 30 min at room temperature in the dark. The cells were analysed using fluorescent inverted microscope (Axio Vert. A1 FL-LED, Carl Zeiss, Germany) with filters having blue excitation/ emission of 540/ 570 nm and red excitation/ emission of 590/ 610 nm filters[13].

B cell lymphoma -2* (*Bcl-2*) *gene expression study

The *Bcl-2* gene expression analysis in cell culture samples were performed using real time – quantitative polymerase chain reaction (RT-qPCR). The cells were initially treated with the extract at its IC_{50} dose for 24 h. Using Gen Elute mammalian total ribonucleic acid (RNA) kit, RNA was isolated from control and extract treated cells as per manufacturer's instruction with slight modifications. Complementary deoxyribonucleic acid (cDNA) synthesis was carried out from total RNA (500 ng) using Revert Aid first strand cDNA synthesis kit as per manufacturer's protocol. The reaction mixture was then subjected to polymerase chain reaction (PCR) for amplification of *Bcl-2* gene using specifically designed primers by online Primer three primer design software (*Bcl-2* Forward: 5'-TGGATCCAGGATAACGGAGG-3', *Bcl-2* Reverse: 5'-CAAACAGAGGTCGCATGCTG-3'). The housekeeping gene *GAPDH* (Forward: 5'-CAACGAATTTGGCTACAGCA-3', Reverse: 5'-AGGGGAGATTCAGTGTGGTG-3') was co-amplified in each reaction as an internal control. The Quantitative real time PCR (Applied Biosystems, USA) was carried out in a final volume of 20 µL containing 250 ng of template cDNA, Maxima SYBR green qPCR master mix (12.5 µL) and 10 pM/µL of each primer. In negative control, template cDNA was replaced by diethylpyrocarbonate (DEPC) water. Separate PCR reactions were set up for target gene and housekeeping gene/reference (*GAPDH*). Each sample was run in triplicate in 20 µL reaction. The cycling conditions were 4 min initial incubation at 95°C and 40 cycles of amplification cycle with denaturation at 95ºC, annealing 61°C (*Bcl-2*) and 58°C (*GAPDH*) for 40 sec, extension 72°C for 35 sec, and terminated with an additional extension step for 1 min at 72°C. The relative change in expression of *Bcl-2* gene was analysed by comparative C_T (Cycle threshold) method[7] and was expressed as 'n' fold change up/ down regulation of the transcripted gene in relation to untreated control group. Fold change = $2^{-\Delta\Delta C}{}_T$, where $\Delta\Delta C_T$ = **(**C_T target gene - C_T GAPDH**)** treatment-(C_T target gene-$C_{T,}$ GAPDH) control[14].

Western blot analysis

After treatment of cells with extract at its IC_{50} concentration for 24 h, cells were collected and washed twice in 1X PBS, then lysed in radio immunoprecipitation assay (RIPA) buffer with protease and phosphatase inhibitors on ice for 1 h. Cell lysates were then centrifuged for 15 min at 18,728 g at 4°C. Proteins were separated using 12% sodium dodecyl sulphated- polyacrylamide gel electrophoresis (SDS-PAGE) using GeNei electrophoretic appartus, Merck India Private Limited, Mumbai, India. The gel after electrophoresis was immersed in transfer buffer for 15 min. The proteins were subsequently transferred to polyvinylidene difluoride (PVDF) membrane (0.45 µm) (Hoefer Semi dry transfer apparatus, USA) as per manufacturer's instructions. The blots were blocked with 5% bovine serum albumin (BSA) in tris buffered saline tween -20 (TBST) at room temperature for 1 h. Then the membranes were washed thrice with TBST for 10 min. After overnight incubation at 4°C with respective primary antibodies, the membrane was washed thrice in TBST for 10 min each. After washing with TBST, the blots were incubated with horse radish peroxidase conjugated secondary antibody for 1 h. The unbound conjugate was removed by washing thrice with TBST. Colour reaction was carried out with 10 mL of 3,3' diaminobenzidine (DAB) substrate buffer with an incubation of 10 min. The reaction was stopped by washing the membrane in triple distilled water. To ensure equal protein loading, β actin was used as an internal control. The strength of western blotting bands was determined by Image J density Measurement program (http://imagej.en.softonic.com)[15].

Statistical analysis

All results were expressed as Mean ± SE with 'n' equal to number of replicates. The half maximal inhibitory concentration (IC_{50}) value of extract was calculated using the online software "Very Simple IC_{50} Tool Kit". All the statistical analysis was conducted using SPSS software version 21. Analysis of variance (ANOVA) in a completely randomized design followed by Duncan's multiple range tests were used to compare any significant differences between extract concentrations. Expression fold change in gene and protein expression was assessed using one sample t test.

Results

Extract preparation

The yield obtained after extraction was 14.79% with reference to dry starting material.

Qualitative phytochemical analysis

On phytochemical analysis, MAH revealed the presence of flavonoids, phenols, tannins, steroids,

saponins and diterpenes. The results are provided in Table 1.

GC-HRMS analysis of MAH

The phytoconstituents obtained on GC-HRMS analysis of MAH are given are listed in Table 2. Methanol extract of seeds of *A. hirsutus* on GC-HRMS analysis indicated the presence of 12 compounds, out of which 2,3-dihydro-5,6-dimethyl-1,4-dioxin; glyceraldehyde; 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one(DDMP); 1,2-benzenediol; 5-hydroxymethylfurfural (HMF); levoglucosan; palmitic acid β-monoglyceride; cholesterol; β- tocopherol and vitamin E were found to be the major compounds.

Cytotoxicity studies: MTT assay

The results of MTT reduction assay after 48 h treatment with MAH in MDA-MB-231 and MCF-7 tumour cell lines are presented in Table 3. The per cent cell viability of both cells treated with plant extract showed significant ($p<0.05$) reduction after 40 µg/mL. The half maximal inhibitory concentration (IC_{50}) for MAH was found to be 65.47±9.51 µg/mL for MDA-MB-231 cells and 64.05±9.3 µg/mL for MCF-7 cells.

Table 1 — Phytochemical analysis of methanol extract of seeds of *A. hirsutus*

Test	Methanol extract of seeds of *A. hirsutus*
Steroids - Salkowski's test	+
Alkaloids	
a. Dragendorff's test	-
b. Mayer's test	-
c. Wagner's test	-
d. Hager's test	-
Glycosides	
a. Sodium hydroxide test	-
b. Benedict's test	-
Tannins	
a. Ferric chloride test	+
b. Gelatin test	+
Flavonoids	
a. Lead acetate test	+
b. Ferric chloride test	+
Diterpene detection test	+
Triterpenes	
a. Salkowski's test	-
b. Liberman Burchart's test	+
Saponins - Foam test	+
Phenolic compounds	+

Table 2 — Gas chromatography- high resolution mass spectroscopy (GC-HRMS) analysis of phytochemicals in methanol extract of *A. hirsutus*

Sl No.	RT (min)	Name of compound	Molecular formula	MW (g/mole)	Class	Probability %
1	4.82	2,3-Dihydro-5,6-dimethyl-1, 4-dioxin	$C_6H_{10}O_2$	114	Dioxin	59.6
2	5.41	D-Alanine, N-proparglyoxycarbonyl-, isohexyl ester	$C_{13}H_{21}NO_4$	255	Ester	14.7
3	6.37	Glyceraldehyde	$C_3H_6O_3$	90	Aldehyde	52.5
4	6.5	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H- pyran-4-one (DDMP)	$C_6H_8O_4$	144	Flavonoid fraction	94.6
5	7.48	1,2- Benzenediol	$C_6H_6O_2$	110	Phenols	46.2
6	7.72	5-Hydroxymethylfurfural (HMF)	$C_6H_6O_3$	126	Aryl-aldehyde	89.8
7	12.6	Levoglucosan	$C_6H_{10}O_5$	165	Hexose	46.3
8	13.95	1,2,3,5- Cyclohexantetrol	$C_6H1_2O_4$	148	Cyclic Tetrol	38.6
9	26.8	Palmitic acid β-monoglyceride	$C_{19}H_{38}O_4$	330	Palmitic acid	68.2
10	28.08	β- Tocopherol	$C_{28}H_{48}O_2$	416	Tocopherol	53.1
11	30.13	Cholesterol	$C_{27}H_{46}O$	386	Sterol	33
12	30.33	Vitamin E	$C_{29}H_{50}O_2$	430	Tocopherol	53.6

Table 3 — The per cent cell viability of MDA-MB-231 and MCF-7 cells after 48 h treatment

Cells	Extracts	Conc. (µg/mL)	10	20	40	80	160	320	IC_{50} (µg/mL)
MDA-MB-231 cells	MAH	% cell viability	115^a ±7.02	112.75^a ±5.98	108.63^a ±6.82	62.81^b ±9.55	38.8^b ±2.97	52.13^b ±9.23	65.47 ±9.51
MCF-7 Cells	MAH	% cell viability	80.41^a ±13.20	76.86^a ±1.84	74.95^a ±4.90	52.26^b ±5.75	39.65^b ±0.91	46.10^b ±5.92	64.05 ±9.3

Values are expressed as Mean ± SE (n = 3). Means bearing the different superscript (a-c in rows) vary significantly at $p<0.05$.

Microscopic studies

Acridine orange/ethidium bromide dual (AO/EB) staining

In the present study, upon AO/EB staining, MAH treated MDA-MB-231 cells showed uniform number of early (yellowish green fluorescence) and late apoptotic cells (orange to red fluorescence). In MAH treated MCF-7 cells, majority were in the late apoptotic stage with orange to red fluorescence. Nuclear fragmentation was also observed. Most of the doxorubicin treated cells were in in early apoptotic stage for both cell lines but number was higher in MCF-7 cells. Fig. 1 and Fig. 2 depict representative images of cells treated with AO/EB staining.

Analysis of morphological changes in nucleus

After Hoechst 33258 staining in MDA- MB-231 and MCF-7 cell lines, the control cells were live releasing uniform blue fluorescence. Apoptotic features like fragmented, marginalised nuclei and condensed chromatin was seen in positive control doxorubicin and extract treated MDA-MB-231 and MCF-7 cells evident by bright blue fluorescence (Fig. 3 and Fig. 4).

Analysis of mitochondrial transmembrane potential (MMP)

JC-1 aggregates with red/ orange fluorescence were observed in both control cells. Fluorescence emission shift from orange to green was obtained in both the MAH treated MDA-MB-231 and MCF-7 cells indicating loss of mitochondrial membrane potential (Fig. 5 and Fig. 6).

Western blot analysis

The relative expression of Bcl-2 protein in MDA-MB-231 and MCF-7 cell lines in response to addition

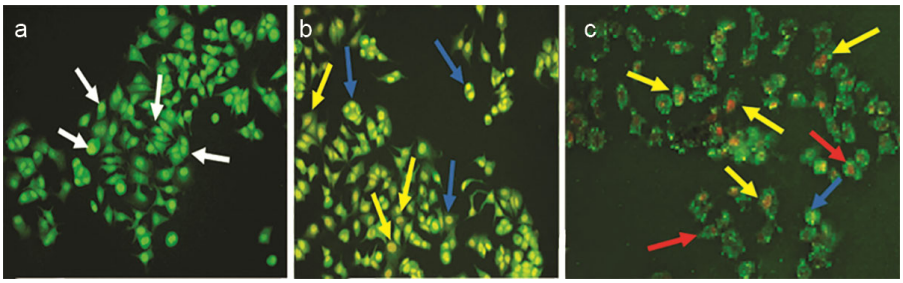

Fig. 1 — Morphological changes of MDA-MB-231 cells by acridine orange/ethidium bromide staining, 20X. A- Control cells; B- Cells treated with doxorubicin 0.58 µg/mL; C- Cells treated with MAH IC_{50} concentration. White arrow- normal cells, Blue arrow - early apoptotic cells, Yellow arrow-late apoptotic cells, Red arrow - nuclear fragmentation

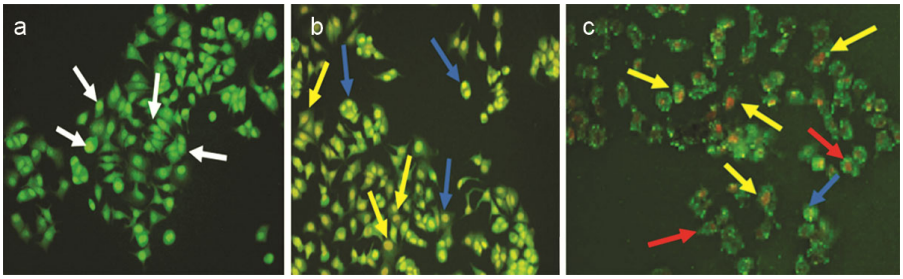

Fig. 2 — Morphological changes of MCF-7 cells by acridine orange/ethidium bromide staining, 40X. A- Control cells; B- Cells treated with doxorubicin 0.58 µg/mL; C- cells treated with MAH IC_{50} concentration. White arrow- normal cells, Blue arrow - early apoptotic cells, Yellow arrow-late apoptotic cells, Red arrow - nuclear fragmentation

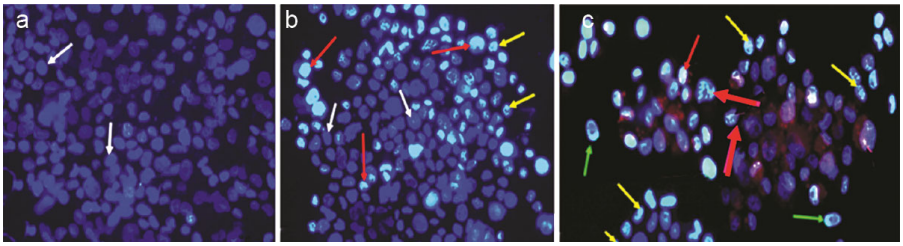

Fig. 3 — Morphological changes of MDA-MB-231 cells by Hoechst staining, 40X. A- control cells; B- Cells treated with doxorubicin 0.58 µg/mL; C- cells treated with MAH IC_{50} concentration. White arrow – live cells, Red arrow- fragmentation of nuclei, Yellow arrow-chromatin condensation, Green arrow – marginalization of nucleus

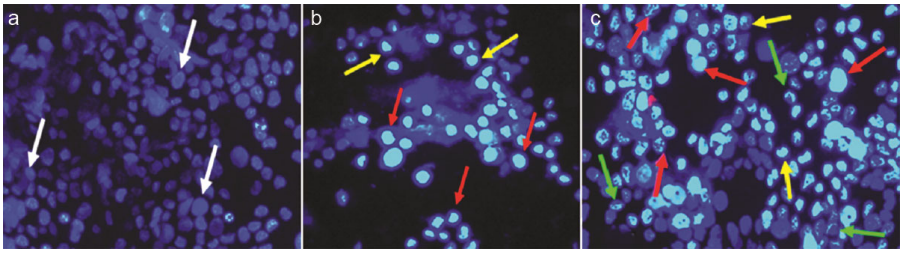

Fig. 4 — Morphological changes of MCF-7 cells by Hoechst staining, 40X. A- control cells; B- Cells treated with doxorubicin 0.58 µg/mL; C- cells treated with MAH IC_{50} concentration. White arrow – live cells, Red arrow- fragmentation of nuclei, Yellow arrow- chromatin condensation, Green arrow – marginalization of nucleus

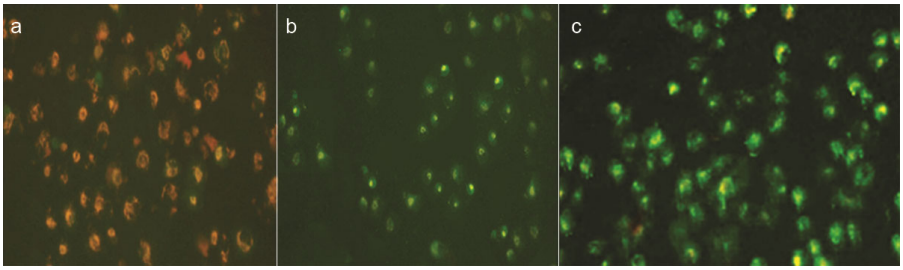

Fig. 5 — Morphological changes of MDA-MB-231 cells by JC-1 staining, 40X. A- control cells; B- Cells treated with doxorubicin 0.58 µg/mL; C- cells treated with MAH at IC_{50} concentration

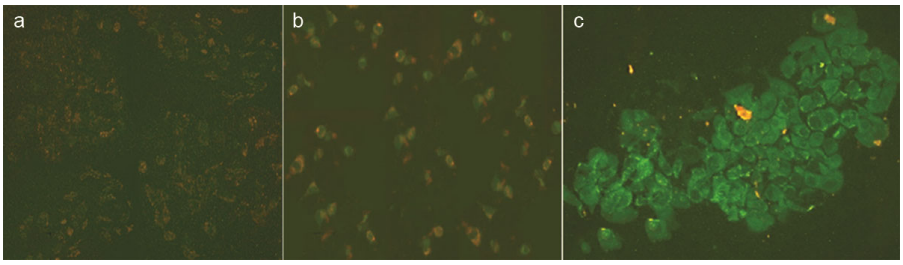

Fig. 6 — Morphological changes of MCF-7 cells by JC-1 staining, 40X, A- control cells; B- Cells treated with doxorubicin 0.58 µg/mL; C- cells treated with MAH at IC_{50} concentration

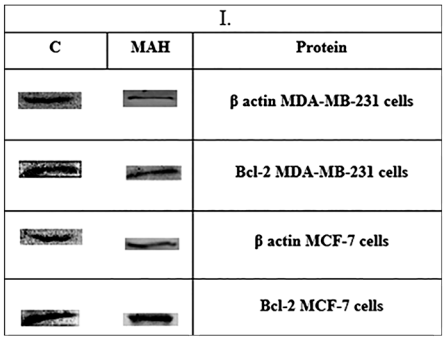

Fig. 7 — Relative Bcl-2 protein expression in MDA-MB-231 and MCF-7 cells in response to treatment with MAH and western blot images of β- actin and Bcl-2 proteins in MDA-MB-231 and MCF-7 cells. C are control cells, MAH are cells after treatment with MAH at IC_{50} concentration)

of MAH at IC_{50} concentration was compared with control cells and the results are presented in Fig. 7 and Table 4. Western blot images of β- actin and Bcl-2 proteins in MDA-MB-231 and MCF-7 tumour cells and plant extract treated cells respectively are presented in Fig. 7. The results revealed significant ($p<0.01$) down regulation in Bcl-2 protein level for the plant extract treated with MDA-MB-231 and MCF-7 cells when compared with control cells.

Bcl-2 gene expression study

The *Bcl-2* gene expression between control MDA-MB-231 and MCF-7 cells and the cells treated with IC_{50} concentration of MAH was compared and is depicted in Table 4. Significant ($p<0.01$) reduction in *Bcl-2* gene expression was obtained for plant extract treated MDA-MB-231 and MCF-7 cells when compared with control cells. Down regulation of *Bcl-2* gene was significantly ($p<0.05$) higher in MCF-7 cells than in MDA-MB-231 cells, after treatment with the plant extract.

Table 4 — The relative *Bcl-2* gene and protein expression in MDA-MB-231 and MCF-7 cells in response to treatment with MAH

Cells		Fold change in *Bcl-2* RNA expression	Normalized protein levels
	Control cells	1	1
MDA-MB-231 cells	MAH	0.40 ± 0.05**[a]	0.80 ± 0.04**[a]
MCF-7 cells	MAH	0.71 ± 0.20**[b]	0.85 ± 0.04**[b]

Values are expressed as Mean ± SE (n = 3); ** denotes significant ($p<0.01$) difference compared with control. Means carrying different superscript ([a,b]) differ significantly ($p<0.05$).

Discussion

Extremely fatal and non-communicable disease among humans is cancer. Many external and internal risk factors contribute for cancer development but basically all cancers develop as a lack of reaction from natural growth inhibitors. Programmed cell death is a vital process to maintain cell homeostasis in the development of body. But some injured or mutant cells get away with apoptosis and they are allowed to grow and multiply uncontrolled, leading to cancer. Breast cancer is the second biggest killer after lung cancer. One in ten women gets breast cancer at some stage of her life. Several studies using breast cancer cell lines have suggested that various natural plant products and antioxidants decrease or inhibit the carcinogenesis. Nutraceuticals are gaining researchers' interest because of their therapeutic potential, less side effects and as adjunct to chemotherapy. Yet many herbal sources like seeds of *A. hirsutus* lack scientific validation and mechanistic insight.

In the present investigation, upon phytochemical analysis, MAH was detected for the presence of flavonoids, phenols, tannins, steroids, saponins and diterpenes. Similar results were found in methanol extract of fruits of *A. hirsutus*[16].

GC-HRMS analysis indicated the presence of compounds belonging to the class of dioxins, aldehydes, flavonoids, phenols, hexoses, tetrols, palmitic acids, sterols and tocopherols. The results of GC-HRMS analysis are in accordance with the previous work[17]. Among the major compounds, vitamin E (α-tocopherol) has been found to have excellent antioxidant activity[18]. Anti-cancer, antioxidant and pesticidal activities were observed for the phenolic compound - 1, 2-benzenediol[19]. Glyceraldehyde has been reported to possess anticancer effect[20]. Phenols and flavonoids were reported for their role in antioxidant activity[1,2]. Flavonoids isolated from *A. hirsutus* have been reported to have significant antioxidant and cytotoxic potential[21].

Cytotoxicity of methanol extract of *A. hirsutus* was assessed in MDA-MB-231 and MCF-7 cell lines using MTT assay. The per cent cell viability of plant extract treated MDA-MB-231 and MCF-7 cells showed significant ($p<0.05$) reduction after 40 µg/mL. The IC_{50} of MAH for MDA-MB-231 and MCF – 7 cells were in accordance with previous studies[21,22]. Even though MAH cannot be used for the purification of active compounds as per the criteria laid down by National Cancer Institute[23], it can be observed that the plant extract is capable of producing cytotoxicity and the cytotoxicity produced might be due to the synergistic interactions of the various phytochemicals with respect to antioxidant and anti-cancer properties[24]. And also, out of the two breast cancer cells lines, MDA-MB-231 cells are highly aggressive, invasive and lack oestrogen, progesterone and HER2/neu receptors while MCF-7 cells are non-invasive, luminal and ductal origin and they lack only HER2/neu receptors. Hence the present study noted that MAH could be beneficial for both hormone dependent and hormone independent breast cancers.

The morphological alterations produced by MAH on MDA-MB- 231 and MCF-7 cells were assessed using acridine orange/ ethidium bromide and hoechst 33258 staining techniques. In AO/EB staining, AO dye is permeable to intact live cells and emits green fluorescence following intercalation in DNA. While EB dye enters only in the cells with damaged cell membrane and emits red fluorescence[25]. Hoechst 33258 stain is very common DNA specific dye which works by intercalation between adenine and thymine and visualisation of uniform blue fluorescence. It was observed that, in the present study, MAH was capable of inducing apoptosis evident by cellular and nuclear changes. The changes observed in acridine orange/ethidium bromide staining was in accordance with previous studies stating that in early apoptotic stage, the cells were crescent shaped with bright yellowish-green fluorescence and in late stage, there were condensation and irregular localized orange coloured nucleus while untreated cells were uniformly green in colour with intact cell membrane[26,27]. After Hoechst 33258 staining, the extract treated cells showed morphological changes in nucleus like, chromatin condensation, fragmentation and

marginalization similar to that of positive control. The results were in accordance with the previous studies[28-30]. Thus it could be inferred that the cytotoxicity produced by MAH triggered apoptosis.

In early apoptosis, the mitochondrial transmembrane potential (MMP) reduction and depolarization of membrane leads to DNA fragmentation and nuclear chromatin condensation. Fluorescence probe (5,5′,6,6′ tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide; JC-1) targeting MMP has been a great tool for detection of such apoptotic cells. A lipophilic cation like JC-1 which particularly enters mitochondria and in healthy mitochondria, accumulated JC-1 called as J-aggregates emitting orange/red fluorescence and in apoptotic cells, JC-1 aggregates get converted to JC-1 monomer indicating loss of membrane potential and fluorescence emission get shifted from red/orange to green[31]. The involvement of mitochondria and drop in MMP were classical features of intrinsic pathway of apoptosis[31]. After analysing the results of MDA-MB-231 and MCF-7 cells treated with extract using JC-1 staining, shift in fluorescence from red to green was observed which indicated drop in MMP suggestive of mitochondria dependent intrinsic pathway of apoptosis. The results of the present study is in accordance with the previous studies[30,32] and this was more distinct with the hormone dependent breast cancer cells. The reduction in MMP could be correlated with the DNA fragmentation and nuclear chromatin condensation which was evident with the staining techniques.

B-cell lymphoma 2 (*Bcl-2*) belongs to BCl-2 family of proteins and it is found in humans as compressed form of *Bcl-2* gene. Chromosomal translocation between 14^{th} and 18^{th} chromosome induces strong transcriptional *Bcl-2* expression which further give rise to tumerogenesis by insuring survival of cells[33]. Oestrogen regulates *Bcl-2* gene expression in breast epithelial cells and in ER^{+ve} breast cancer cell lines. Studies suggested that marked downregulation in *Bcl-2* gene expression after treating breast cancer cell lines MDA-MB-231 and MCF-7 with various plant extracts[34-36]. Similar results were also obtained in the present study where significant ($p<0.01$) reduction in *Bcl-2* gene expression level was obtained for plant extract treated MDA-MB-231 and MCF-7 cells. Expression of *Bcl-2* gene was down regulated significantly ($p<0.05$) higher in MCF-7 cells than in MDA-MB-231 cells, after treatment with the plant extract since MCF-7 cells being ER^{+ve} breast cancer cell line. The results could explain the underlying reason for apoptosis induced by MAH as *Bcl-2* is a key gene regulating apoptosis. And also it could be observed that MAH could be more beneficial in hormonal dependent breast cancers.

The gene expression studies on *Bcl-2* were further supported by protein expression studies on *Bcl-2*. B-cell lymphoma 2 is one of the anti-apoptotic/guardian protein from *Bcl-2* family members. Generally the protein is located on mitochondria, endoplasmic reticulum (ER) and nuclear membranes. It specifically fuses with outer membrane of mitochondria and so involved in intrinsic pathway of apoptosis. Lethal pore formation on outer membrane of mitochondria (permeabilization) is prevented by *Bcl-2* and thus inhibition of cytochrome C release culminating in apoptosis[37]. Previous studies revealed that decreased *Bcl-2* expression led to induction of intrinsic pathway of apoptosis[38-42]. In the present study, significant ($p<0.01$) down regulation in *Bcl-2* level was observed for the plant extract treated with MDA-MB-231 and MCF-7 cells. The results of *Bcl-2* protein and gene expressions along with JC-1 staining concluded that MAH produced apoptosis mediated through intrinsic pathway.

Conclusion

Phytochemical analysis of test plant extract upon qualitative studies revealed the presence of flavonoids, phenols, diterpenes, cardiac glycosides, saponins tannins and steroids in fruit seeds of *A. hirsutus.* GC-HRMS analysis showed high levels of phenols, phytols, flavonoids, palmitic acid, diterpenes, ester, aldehyde, pyrans, vitamin E, glycerides and fatty acids. Acridine orange ethidium bromide (AO/EB) dual staining, JC-1 staining and Hoechst staining showed that MAH triggered intrinsic mitochondrial pathway to induce apoptosis in breast cancer cells and apoptosis was more distinct in MCF-7 cells. Real time-qPCR and western blot analysis suggested that *Bcl-2* gene and Bcl-2 protein expressions was significantly down regulated on treatment with MAH where MCF-7 showed more significant downregulation. The anticancer effect produced by MAH might be due to the presence of various phytochemicals present in the extract as evident by GC-HRMS and qualitative phytochemical analysis. Thus, the present study revealed the anticancer activity of methanol extract of seeds of

Artocarpus hirsutus against hormonal dependent and hormonal independent breast cancer cell lines. The study also suggested that methanolic extract of seeds of *A. hirsutus* might be more beneficial for hormonal dependent breast cancers. Further in-depth studies in both types of breast cancers are warranted.

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Conflict of Interest

The authors declare no conflict of interest.

Authors' Contributions

HMG: executed the research work; BJK - conceptualized and designed the experiment; RJ - assisted the research work and prepared the manuscript; UPTA: guidance for conceptualization, designing and execution of research work

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