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Apoptosis mediated cytotoxic potential of *Erythrina variegata* L. stem bark in human breast carcinoma cell lines

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Erythrina variegata L. (Fam. Fabaceae) is traditionally known for antitumour, expectorant, febrifuge, antibacterial and antioxidant activities. In the present study, we did cytotoxic evaluation of the methanol extract of *E. variegata* stem bark (MEV) using 3-(4,5-dimethyl thazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay in MCF-7 and MDA-MB-231 human breast carcinoma cell lines. The cytological and metabolic alterations due to MEV was evaluated using acridine orange/ethidium bromide (AO/EB) dual staining, hoechst 33258 and fluoroprobe, benzimidazol-carbocyanine iodide 5,5',6,6'-tetrachloro-1,1',3,3'-tetra ethyl (JC-1) staining. The antiapoptotic gene and protein, Bcl-2 expression was analysed using real time PCR and Western blotting, respectively. The methanol extract was investigated for the presence of various phytochemicals using gas chromatography high resolution mass spectrometry (GC-HRMS). The results revealed a dose dependent cytotoxic activity in both MDA-MB-231 and MCF-7 breast carcinoma cell lines *in vitro*. The plant extract was effective in inducing apoptotic cell death via mitochondrial dependent intrinsic pathway and may be considered as a potent source for isolating therapeutic molecules against cancer.

Keywords: Anticancer, Antitumour, B-cell lymphoma-2, GC-HRMS, Indian coral tree, Mitochondria

Cancer is emerging as a key barrier to rising life expectancy and a prominent cause of death in almost all parts of World[1]. The prevalence and death rates of cancer is undergoing a swift growth worldwide and affects the socioeconomic development of population also[2]. Breast cancer, a complex, heterogeneous and invasive disease, is the most diagnosed cancer subtypes among other cancers with an occurrence of 23 lakh new cases globally[3]. According to WHO, breast cancer is the second leading cause of cancer death in women[4]. Among the various types of breast cancers, about 70 to 80% are hormone-dependent[5] and 10 to 14% are hormone-independent[6]. The hike has been attributed to reproductive, lifestyle risk factors and increasing trend of deliberate mammographic diagnosis. Wider gap between menarche and menopause, higher age of first birth, less breast-feeding, menopausal hormone therapy are the predisposing reproductive factors involved. Lifestyle changes like increased consumption of alcohol, obesity and lack of exercise are also a contributing factor for the transition[7]. Majority of novel anticancer medications are cytotoxic agents or targeted biologicals which inflict cell viability, inhibit cellular growth and angiogenesis and elevates associated immune response[8].

Herbal drugs are gaining popularity and researchers look into natural resources for safe alternative drugs, particularly for anticancer compounds. Medicinal herbs and their by-products could bestow upon conventional and subsidiary treatment modalities affirmed by the current research in cancer subpopulations. They are also believed to elevate the potency of existing drugs which might diminish their adverse effects in persons undergoing cancer treatment. Henceforth, the phyto drug development requires a consistent research to sequester novel effective compounds with least toxicity[9-15].

The study concentrates on the discovery of novel herbal derivatives which can contribute as a trustworthy alternative for cancer treatment. *Erythrina variegata*, Linn. commonly known as Indian Coral Tree, is known for its antitumour, expectorant, febrifuge, antibacterial and antioxidant activities[16,17]. Although there are meagre reports on antitumour activity of the plant, a comprehensive understanding on how the plant acts at a cellular and molecular level is not known. Hence, in the present research work

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focuses is on evaluation of anticancer properties of *E. variegata* in breast carcinoma cell lines and developing discernment towards the phytoconstituents involved in the action.

Materials and Methods

Chemicals and drugs

Acridine orange, agarose, acrylamide, dihydrogen sodium phosphate, ethylene diamine tetra acetic acid (EDTA), methyl thiazolyl diphenyl-tetrazolium bromide (MTT), N1,N1-dimethyl bisacrylamide, proteinase K, RNAse A, Tween 20, Triton-X-100, dimethyl sulphoxide (DMSO), antibiotic antimycotic solution (100X), doxorubicin, monoclonal anti-Bcl-2 clone Bcl-2-100 and monoclonal anti beta actin antibody were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). RPMI 1640, FBS and Trypsin/EDTA were purchased from Invitrogen Life Technologies, USA. Horse radish peroxidase-conjugated secondary antimouse antibodies were purchased from Cell Signalling Technology, Danvers, MA, USA.

Collection of plant material and authentication

The stem bark of *E. variegata* was collected from the premises of Rabbit Breeding Station, College of Veterinary and Animal Sciences, Mannuthy, Thrissur district, Kerala. The plant material was taxonomically identified and authenticated by Research and P.G. Department of Botany, St. Thomas College (Autonomous), Thrissur, Kerala, India and the voucher specimen has been deposited in the Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy, with accession no. HERB/VPT/CVASMTY/1/2017.

Methanol extraction

The stem bark of *E. variegata* was air dried at room temperature and coarsely powdered using an electric pulverizer. The powder obtained was extracted using a Soxhlet apparatus with methanol at 55°C. The methanol extract was then concentrated using a rotary vacuum evaporator under reduced pressure and temperature (55°C) and kept under refrigeration after complete evaporation of the solvent in an airtight container.

Sample preparation

The methanol extract was solubilized in dimethyl sulphoxide (DMSO) at a concentration of 1.0 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 4% 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.

In vitro cytotoxic study of MEV

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was done to assess the cytotoxicity of MEV as per the method of Naidu *et al*.[18]. The MCF-7 and MDA-MB-231 breast cancer cell lines were seeded at a density of 5×10^3 cells per well in 200 µL medium, and were allowed to attach for overnight in a CO_2 incubator. Cells were treated with methanol extract at concentrations of 2.5, 5, 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 *E. variegata*. 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 was calculated using the formula:

Per cent cell viability = (Average absorbance of treated cells /Average absorbance of untreated cells) × 100.

The half maximal inhibitory concentration (IC_{50}) values of extract were calculated by plotting the concentration against per cent cell viability using the online software "very simple IC_{50} tool kit".

Selection of concentrations

Based on the MTT assay, three concentrations of the plant extract was selected for the study: one below IC_{50}, IC_{50} and one above IC_{50} which were 7.5, 15, 30, and 11.5, 23, 46 µg/mL for MDA-MB-231 and MCF-7 cells, respectively.

Microscopic studies

Trypsinized cells at concentration of 1×10^5 cells were seeded into a six well plate and allowed to grow for 24 h. Based on the MTT assay, three concentrations of the plant extract was selected for the study: one below IC_{50}, IC_{50} and one above IC_{50}. Acridine orange/ethidium bromide[19], hoechst 33258[20] and fluoroprobe, benzimidazol-carbocyanine iodide 5,5',6,6'-tetrachloro-1,1',3,3'-tetra ethyl (JC-1)[21] staining techniques were used for staining. Doxorubicin at a dose of 0.58 µg/mL was adopted as positive control.

Microscopic studies using acridine orange ethidium bromide

The acridine orange ethidium bromide (AO/EB) staining procedure was followed to differentiate the live, apoptotic and necrotic cells. 25 µL of the treated or untreated cells were stained with 5 µL of acridine orange (10 µg/mL) and ethidium bromide (10 µg/mL) and analysed under Trinocular Research fluorescence Microscope, DM 2000 LED, Leica with blue excitation (488 nm) and emission (550 nm) filters at 20X magnification.

Analysis of morphological changes in nucleus

MCF-7 and MDA-MB-231 cells (1×10^5 cells per well) were seeded in six well plates and after 24 h of growth, cells were treated with the above mentioned concentrations of extract for 24 h. Cells were fixed with methanol for 30 min after washing with PBS and then stained with Hoechsht 33258 stain (Invitrogen 35 µg/mL) for 30 min. Images were captured by Trinocular Research fluorescence Microscope, DM 2000 LED, Leica microscope with blue excitation (352 nm) and emission (461 nm) filters at 20X magnification. The per cent apoptotic cells were assessed by counting number of apoptotic cells in six different microscopic fields after hoechst 33258 staining.

Analysis of mitochondrial transmembrane potential (*MMP*)

MCF-7 and MDA-MB-231 cells were plated at a seeding density of 1×10^5 cells per well in six well plates. After 24 h of treatment with extract at the above mentioned concentrations, cells were incubated with 5 µM JC-1 for 30 min at 30°C in the dark. The cells were analysed using fluorescent microscope with filters having blue excitation/ emission of 540/570 nm and red excitation/ emission of 590/610 nm filters (DM 2000 LED, Leica).

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

The technique of real time quantitative polymerase chain reaction (RT-qPCR) was employed for studying the gene expression of *Bcl-2* gene in cell culture samples. Briefly, the cells were treated with the extract at its respective IC_{50} concentration for 24 h. RNA was obtained from control and extract treated cells using Gen Elute mammalian total RNA kit (Sigma- Aldrich, USA) as per manufacturer's instruction with slight modifications. The obtained RNA was DNase treated using DNase kit (Sigma-Aldrich, USA). Complementary DNA (cDNA) synthesis was carried out from total RNA (500 ng) using Revert Aid first strand cDNA synthesis kit (Thermoscientific, USA) as per manufacturer's protocol. The reaction mixture was then subjected to PCR for amplification of *Bcl-2* gene using specifically designed primers by online Primer three primer design software (Primer three, http://bioinfo.ut.ee/primer3/) (Bcl-2 Forward: 5'-TGG ATCCAGGATAACGGAGG-3', Bcl-2 Reverse: 5'-CAAACAGAGGTCGCATGCTG-3'). The house-keeping gene GAPDH (Forward: 5'-CAACGAATTT GGCTACAGCA-3', Reverse: 5'-AGGGGAGATTCA GTGTGGTG-3') was co-amplified in each reaction as an internal control. The Quantitative real time PCR was carried out in a final volume of 20 µL containing 250 ng of template cDNA, Maxima SYBR green qPCRmastermix (12.5 µL) and 10 pM/ µL of each primer. In negative control, template cDNA was replaced by 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 s, extension 72°C for 35 s, and terminated with an additional extension step for one min at 72°C .

The relative change in expression of *Bcl-2* gene was analysed by comparative C_T (Cycle threshold) method[22] and was expressed as 'n' fold change up/ down regulation of the transcripted gene in relation to untreated control group.

$$\text{Fold change}=2^{-\Delta\Delta C_T}$$

where $\Delta\Delta C_T$ = **(**C_T target gene - C_T GAPDH**)** treatment-(C_T target gene-C_T, GAPDH) control.

Western blot analysis

Western blot analysis was done as per the method of Shrivasthava *et al*.[23]. After treatment of cells with extract at its respective IC_{50} concentrations for 24 h, cells were collected and washed twice in 1X PBS, then lyzed in radio immunoprecipitation assay (RIPA)

buffer with protease and phosphatase inhibitors on ice for 1.0 h. Cell lysates were then centrifuged for 15 min at 18,728 g at 4ºC. Proteins were separated using 12% sodium dodecyl sulphated- polyacrylamide gel electrophoresis (SDS-PAGE). The gel after electrophoresis was immersed in transfer buffer for 15 min. The proteins were subsequently transferred to PVDF (polyvinylidene difluoride) membrane (0.45 µm) using Hoefer Semi dry transfer apparatus 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 1.0 h. Then the membranes were washed thrice with TBST for 10 min. After overnight incubation at 4ºC 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 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).

Gas chromatography-high resolution mass spectroscopy (GC-HRMS) analysis

The active phytochemical principles of MEV was analysed using gas chromatography- high resolution mass spectrometry (GC-HRMS) system of Sophisticated Analytical Instrument Facility (SAIF), Indian Institute of Technology (IIT), Bombay. Gas chromatography (Agilent, USA, 7890) with FID detector, EI/CI source and time of flight analyser with a mass range of 10-2000 amu and a mass resolution of 6000 was used. Helium was used as the carrier gas at flow rate of 1.0 mL/ min. The oven temperature was maintained at 70ºC for one min and then increased to 200ºC in five min. The injector temperature was 250ºC and total analysis time was 50 min. 0.4 µL aliquots of extracts were injected into the chromatographic column after a clear baseline had been obtained. Major constituents were identified by using mass spectrum library (NIST MS search 2.0 library)[24].

Statistical analysis

All results were expressed as Mean ± SE with n equal to number of replicates. The half maximal inhibitory concentration (IC_{50}) values of extract were calculated using the online software "Very Simple IC_{50} Tool Kit". All the statistical analysis was conducted using SPSS software version 21. The variation between groups for apoptotic cell per cent were assessed by one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test. Expression fold change in gene and protein expression was assessed using one sample t test.

Results

In vitro cytotoxic study of MEV

The data of MTT reduction assay of *E. variegata* extract in MDA-MB-231 and MCF-7 tumour cell lines are presented in Fig. 1. The per cent cell viability of MEV showed a drastic reduction in cell viability after 20 µg/mL and was in a constant range in both MDA-MB-231 and MCF-7 tumour cells for further concentrations in a similar pattern. The half maximal inhibitory concentration (IC_{50}) for methanol extract of *E. variegata* were found to be 14.99±0.59 and 22.94±3.19 µg/mL for MDA-MB-231 cells and MCF-7 cells, respectively.

Microscopic studies

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

After treatment with extract, live, necrotic, early and late apoptotic cells were detected. Figs 2 and 3 depicted representative images of cells of treatments after AO/EB staining. Control cells were live with centrally distributed circular nucleus and were emitting greenish fluorescence. Below IC_{50} concentrations of extract in both cell lines were early apoptotic cells with localized crescent-shaped or

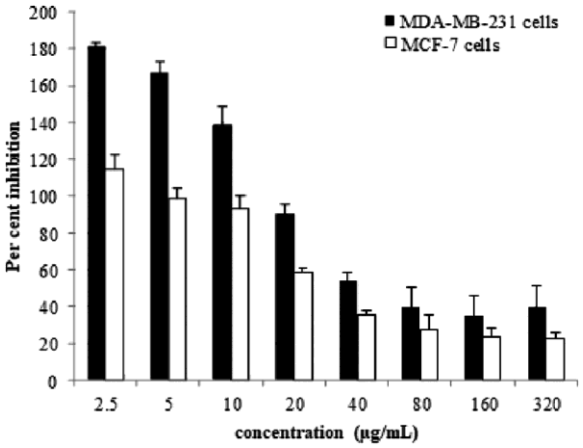

Fig. 1 — The per cent cell viability of MDA-MB-231 and MCF-7 cells after 48 h treatment with methanol extract of *Erythrina variegata* determined by MTT reduction assay. [Values are expressed as Mean ± SE (n=3)]

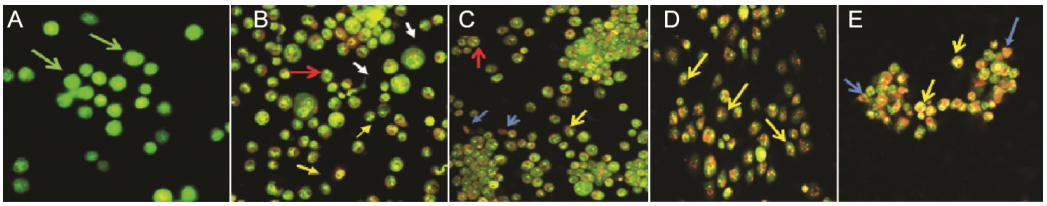

Fig. 2 — Morphological changes of MDA-MB-231 cells by acridine orange ethidium bromide staining, 200X. (A) control cells; (B) Cells treated with doxorubicin 0.58 µg/mL; (C-E) cells treated with methanol extract of *Erythrina variegata* at concentrations 7.5, 15 and 30 µg/mL, respectively. [Green arrow- normal cells White arrow - early apoptotic cells, Yellow arrow-late apoptotic cells, Blue arrow indicates necrotised cells, Red arrow- nuclear fragmentation]

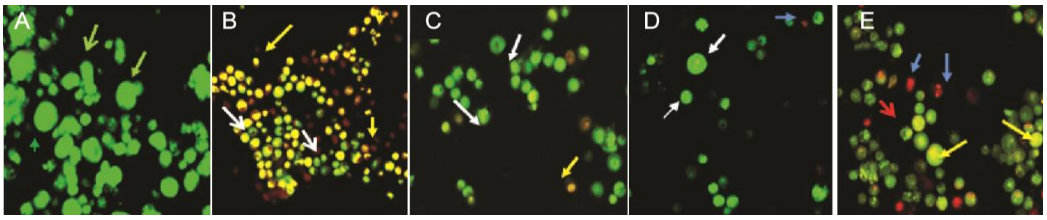

Fig. 3 — Morphological changes of MCF-7 cells by acridine orange ethidium bromide staining, 200X. (A) control cells; (B) Cells treated with doxorubicin 0.58 µg/mL; (C-E) cells treated with methanol extract of *E. variegata* at concentrations 11.5, 23 and 46 µg/mL, respectively. [Green arrow- normal cells White arrow - early apoptotic cells, Yellow arrow-late apoptotic cells, Blue arrow indicates necrotised cells, Red arrow- nuclear fragmentation]

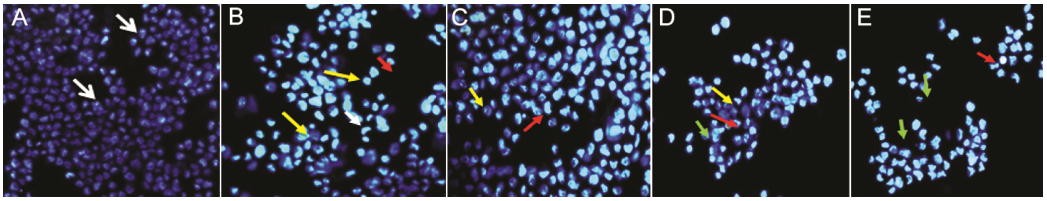

Fig. 4 — Morphological changes of MDA-MB-231 cells by hoechst staining, 200X. (A) control cells; (B) Cells treated with doxorubicin 0.58 µg/mL; (C--E) cells treated with methanol extract of *E. variegata* at concentrations 7.5, 15 and 30 µg/mL, respectively. [White arrow – live cells, Red arrow- apoptotic cells, Yellow arrow- chromatin condensation, green arrow-marginalisation of nucleus, orange arrow-fragmentation of nuclei]

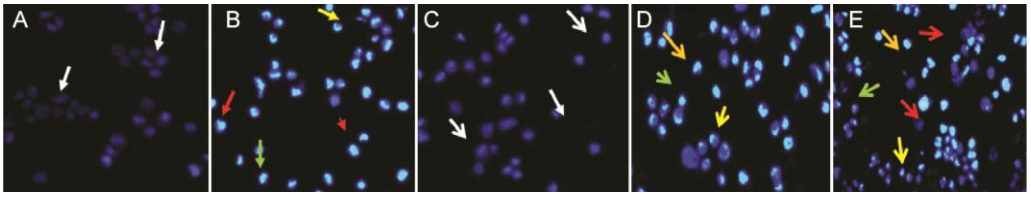

Fig. 5 — Morphological changes of MCF-7 cells by Hoechst staining, 200X. (A) control cells; (B) Cells treated with doxorubicin 0.58 µg/mL; (C-E)- cells treated with methanol extract of *E. variegata* at concentrations 11.5, 23 and 46 µg/mL, respectively. [White arrow – live cells, Red arrow- apoptotic cells, Yellow arrow- chromatin condensation, green arrow-marginalisation of nucleus, orange arrow-fragmentation of nuclei]

granular yellow-green stained nucleus. At IC_{50} and above IC_{50} concentrations, majority of cells were in late apoptotic stage emitting orange to red fluorescence. A few necrotic cells and some early apoptotic cells were present. Cellular changes like membrane blebbing, nuclear fragmentation, chromatin condensation and apoptotic bodies were also detected. Majority of doxorubicin control cells also were in late apoptotic stage.

Analysis of morphological changes in nucleus

Both control cells were live emitting uniform blue fluorescence. Positive control cells and cells treated with various concentrations of plant extract were showing apoptotic features like fragmented and marginalised nuclei and condensed chromatin (Figs 4 and 5). Apoptotic cell per cent increased significantly (P <0.01) in a concentration dependent manner (Fig. 6).

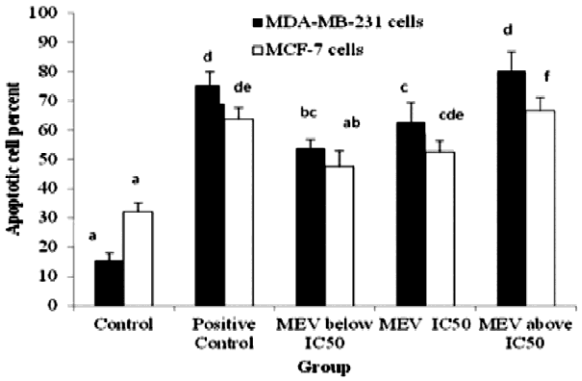

Fig. 6 — The apoptotic cell per cent of MDA-MB-231 and MCF-7 cells after treatment with methanol extract of *E. variegata* determined by Hoechst staining. [Values are expressed as Mean ± SE (n=6) Means with different superscripts differ significantly (P <0.05)]

Analysis of mitochondrial transmembrane potential

In both control cells, JC-1 aggregates with reddish fluorescence were obtained indicating a higher mitochondrial membrane potential. A concentration dependent rise in green and fall in red fluorescence for the plant extract in both cell lines after 24 h indicated a concentration dependent mitochondrial membrane potential drop (Figs 7 and 8).

Bcl-2 gene expression study

The relative *Bcl-2* gene expression in MCF-7 and MDA-MB-231 cell line in response to addition of MEV at their respective IC_{50} concentrations as compared to control is presented in Fig. 9. In MDA-MB-231 cells, *Bcl-2* gene expression was decreased significantly (P <0.01) for the plant extract with 0.36 ± 0.04 fold change in expression respectively when compared with the control. In MCF-7 cells, *Bcl-2* gene expression when compared with the control decreased significantly (P <0.01) for MEV, with 0.89 ± 0.02 expression fold change respectively. Hence, it could be concluded that *Bcl-2* gene expression was downregulated in both the cell lines after treatment with the plant extract.

Western blot analysis

Fig. 10 (A-D) represent the Western blot images of β-actin and Bcl-2 proteins in MDA-MB-231 and MCF-7 tumour cells, respectively. The expression of Bcl-2 protein in the control cells was normalized to unity. In comparison with the control, expression of Bcl-2 protein in MDA-MB-231 cells was decreased significantly (P <0.01) for the plant extract with 0.39±0.01 fold change in expression. In MCF-7 cells, expression of Bcl-2 protein when compared with the control decreased significantly (P <0.01) for MEV, with 0.87±0.006 expression fold change. Hence, it could be concluded that expression of Bcl-2 protein was downregulated in both the carcinoma cell lines after treatment with the plant extract.

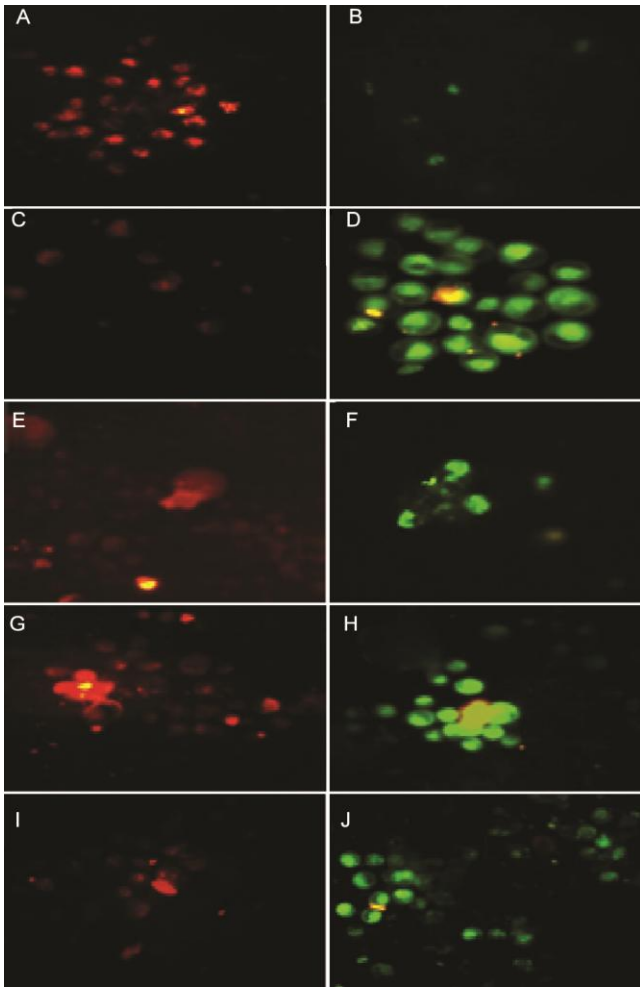

Fig. 7 — Morphological changes of MDA-MB-231 cells studied by JC-1 staining 200X. A2, C2,E2,G2 and I2- JC-1 Red; B2, D2, F2, H2 and J2- JC-1 Green A2, B2- control cells; C2, D2- Cells treated with doxorubicin 0.58 µg/mL; E2, F2, G2,H2,I2, J2- cells treated with methanol extract of *E. variegata* at concentrations 7.5, 15 and 30 µg/mL, respectively .

Gas chromatography- high resolution mass spectroscopy (GC-HRMS) analysis

Phytoconstituents obtained on GC-HRMS analysis of MEV are listed in Table 1. Methanol extract of *E. variegata* upon GC-HRMS analysis showed the presence of spathulenol, asarone, 3,7,11,15-tetramethyl 2-hexadecen-1-ol, 17 octadecynoic acid, 1-hexadecanol, 2-methyl, palmitic acid, heptacosanoic acid, methyl ester, 1-tricosanol, hexacosanoic acid methyl ester, octadecanoic acid, ethyl ester, and hexadecanoic acid, ethyl ester. The compounds were broadly belonging to terpenoids and fatty acid analogues.

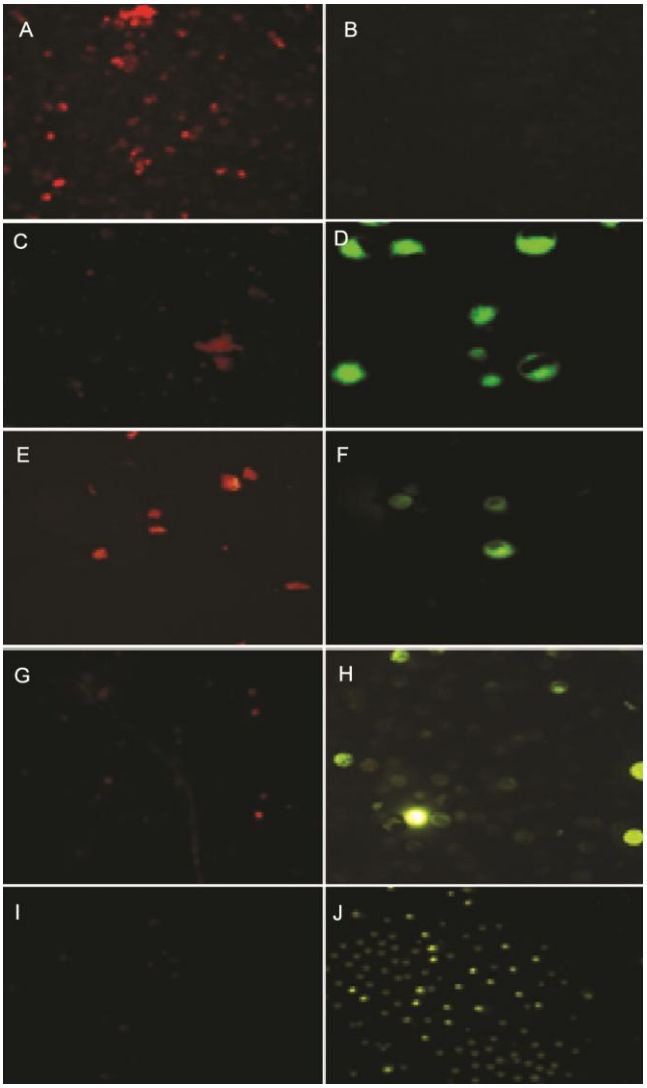

Fig. 8 — Morphological changes of MCF-7 cells studied by JC-1 staining, 200X. K2, M2,O2,Q2 and S2- JC-1 Red; L2, N2, P2, R2 and T2- JC-1 Green K3, L2- control cells; M2, N2- Cells treated with doxorubicin 0.58 µg/mL; O2, P2, Q2, R2, S2, T2- cells treated with methanol extract of *E. variegata* at concentrations 11.5, 23 and 46 µg/mL, respectively .

Discussion

Breast carcinoma, being one of the most frequently occurring types of carcinomas in humans, is responsible for majority of carcinoma related deaths[3]. Receptors for hormones like oestrogen and progesterone may either be present or absent in breast cancers owing the treatment options to be categorized broadly into hormone reactive and nonreactive types[25]. Comparing the types, hormone nonresponsive cancers are invasive, belligerent, and require

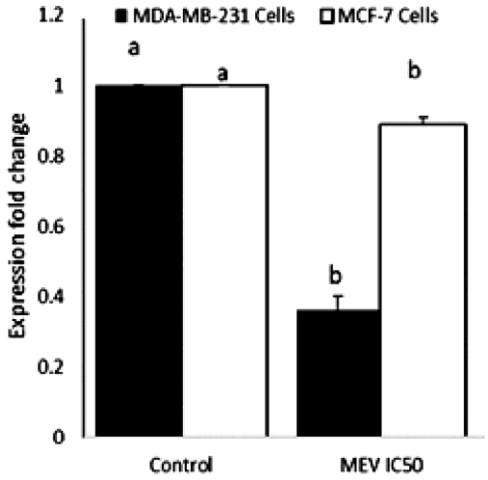

Fig. 9 —The relative Bcl-2 gene in MDA-MB-231 and MCF-7 cells in response to treatment with methanol extract of *E. variegata*. [Values are expressed as Mean ± SE (n=3), Means with different superscripts differ significantly (P <0.01)]

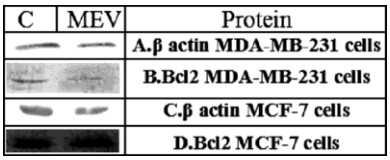

Fig. 10 — (A-D) Western blot images of β- actin and Bcl-2 proteins in MDA-MB-231 and MCF-7 cells. C and MEV are control cells and cells after treatment with MEV at its respective IC_{50} concentration.

Table 1 — Gas chromatography- high resolution mass spectroscopy (GC-HRMS) analysis of phytochemicals in methanol extract of *Erythrina variegata*

RT (min)	Name of compound	Molecular formula	MW (g/mole)	Peak Area (%)	Class	Probability %
17.32	Spathulenol	$C_{15}H_{24}O$	220	873820.24	Tricyclic sesquiterpene alcohol	8.48
18.66	Asarone	$C_{12}H_{16}O_3$	208	4266166.76	Ether	27.5
21.86	3,7,11,15-Tetramethyl 2-hexadecen-1-ol	$C_{20}H_{40}O$	296	2181822.81	Acyclic diterpene alcohol	13.4
24.42	Palmitic acid	$C_{16}H_{32}O_2$	256	1120403.81	Saturated fatty acid	42
28.33	17 Octadecynoic acid	$C_{18}H_{32}O_2$	280	910486.45	Fatty acid analogue	8.48
37.11	1-Hexadecanol,2- methyl	$C_{17}H_{36}O$	256	4683005.50	Fatty alcohol	5.26
37.55	Heptacosanoic acid, methyl ester	$C_{29}H_{56}O_2$	454	780541.78	Fatty acid ester	14.5
38.3	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284	730614.78	Fatty acid ester	18.9
39.82	1-tricosanol	$C_{23}H_{48}O$	340	5689181.5	Fatty alcohol	7.88
40.27	Hexacosanoic acid methyl ester	$C_{27}H_{54}O_2$	410	18921282.00	Fatty acid ester	32.4
41.28	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	312	2294417.55	Fatty acid ester	26.8

strenuous diagnosis demanding the availability of therapeutics effective in both the types. The current study was carried out in MDA-MB-231 and MCF-7 cells to obtain apprehension on both hormone mediated and non-mediated carcinomas.

Apart from the ancient records about *E. variegata*, there were also some preliminary scientific studies reported on the utility of *E. variegata* against certain cancers[26]. Nevertheless, an explicit understanding on how the plant acts has not been found out.

In vitro antitumour potential of MEV was assessed initially in the cell lines using MTT assay. Viable cells alone are capable of reducing yellow MTT to insoluble purple formazan whose absorbance is measured[28]. According to NCI guidelines, substances having an IC_{50} less than 30 µg/mL could be considered as having potent cytotoxic action[28]. The IC_{50} values of the extract in both the cell lines are coming under this range. Henceforth, the extract could be chosen and scrutinized further as a potential antitumour material[29].

MTT assay is incapacitated to detect the mode of inhibition of cell growth. Drugs mostly destroy cancer cells by apoptosis where apoptotic levels are positively regulated based on their sensitivity[30]. Apoptosis plays a pivotal role in the normal physiological growth and development stages by selective destruction of cells. Various therapeutic agents used against cancer activate signal transduction pathways associated with apoptosis[31]. The mechanism by which apoptosis is initiated by a therapeutic agent is not well understood. However, morphological and biochemical alterations like cellular shrinkage, nuclear fragmentation and marginalisation, chromatin condensation are characterised[32]. Apoptotic mediation of cell death was assessed using AO/EB dual staining which was reported to be used as an easier and economic tool for detecting apoptotic cells[33]. A marked variation between live, early, late apoptotic and necrotic cells could be identified. AO penetrates normal and early apoptotic cells, stains the nuclei green by binding to DNA while EB stains the nucleus of late apoptotic and necrotic cells red by securing to DNA fragments and apoptotic bodies[34].

Hoechst 33258 staining was performed to detect nuclear morphological changes due to MEV in MDA-MB-231 and MCF-7 cells. Hoechst 33258 is a popular nuclear counter stain which binds to double stranded DNA, intercalates between adenine and thymine residues and causes emission of blue fluorescence. Apoptotic cells could be recognized among viable cells by the emission of bright blue fluorescence whereas latter emits uniform blue fluorescence[6]. Nuclear changes like marginalization, chromatin condensation, and nucleosomal ladder formation are detected during apoptosis by the action of caspases and other mitochondrial factors[35]. Evidences from the nuclear details along with dose enhanced apoptotic cell rise obtained in our study imply that MEV induces apoptosis[36].

In intrinsic pathway, apoptosis occurs initially via Bcl-2 family protein activators localized in endoplasmic reticulum, nuclear and mitochondrial membranes. It leads to functional alteration in mitochondria due to fall in transmembrane potential. Apoptotic factors like cytochrome c will be released from intermembrane space leading to activation of caspases[37].

Apoptosis when occurs via intrinsic pathway causes reduction in mitochondrial membrane potential by effusion of apoptotic proteins[38]. JC-1 a cationic dye, accumulates in mitochondrial matrix to form red fluorescence aggregates owing to high mitochondrial membrane potential. As mitochondrial membrane potential lowers, red to green signal ratio decreases and per cent of green monomers increase. Cytotoxicity related to mitochondrial damage could be easily detected based on assessment of red to green signal ratio[39]. In the present study, MEV instigated a concentration relied lowering of $\Delta\Psi m$ by JC-1 suggestive of mitochondria dependent intrinsic pathway of apoptosis.

Bcl-2, an antiapoptotic gene belonging as second member of a group of proteins, is involved in release of proteins from intermitochondrial membrane[40]. It is an antiapoptotic protein occurring in association with β subunit of F_1F_0 ATPase in inner mitochondrial membrane[40]. It inhibits certain proapoptotic proteins which are involved in release and movement of cytochrome c and ROS involved in apoptotic cascade. Tawfik *et al.*[41] reported 81 and 29% expression of Bcl-2 gene in triple negative and non-triple negative breast cancers, respectively. They can either be proapoptotic proteins like Bax and Bak, antiapoptotic molecules like Bcl-2, Bcl-xL. Bcl-2 acts by obstructing the outpour of cytochrome c from mitochondria, inhibits caspases activation and thereby subdues apoptosis[42].

Expression of Bcl-2 gene and protein was down regulated significantly due to MEV in MDA-MB-231

and MCF-7 cells[43]. The results depict the first report of the cellular mechanism of *E. variegata* extract behind the anticancer potential.

GC-HRMS have evolved as a mainstream tool for qualitative and quantitative analysis of organic compounds. A combination of gas chromatography and mass spectrum division patterns with a database is utilized to detect phytoconstituents and relative peak area per cent method for quantitative estimation. Previous studies[44] reported the presence of flavonoids, glycosides, alkaloids, tannins, steroids, phenolics and diterpenes in methanol extract of *E. variegata* upon analysing phytochemical constituents using biochemical tests.

Out of the major chemicals detected in GC-HRMS, 3,7,11,15-tetramethyl 2-hexadecen-1-ol, octadecanoic acid, eicosanoic acid and palmitic acid were previously reported[45]. Palmitic acid has potent antioxidant and 5α-reductase activity. Hexadecanoic acid ethyl ester has antioxidant, antiandrogenic and 5α-reductase activity. According to Li *et al.*[46], terpenoids possessed potent anticancer activity.

Conclusion

The results indicate that the methanol extract of stem bark of *Erythrina variegata* (MEV) possess anticancer potential *in vitro* against human breast cancer cell lines. The extract has been found to induce cytotoxicity through intrinsic apoptotic pathway as noted with AO/EB, Hoechst 33258 and JC-1 staining. The extract also downregulated Bcl-2 expression, the antiapoptotic gene and protein, which substantiated the observed apoptosis *in vitro*. Terpenoids obtained on GC-HRMS analysis be a contributing factor for the depicted anticancer activity *in vitro*.

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Conflict of interest

Authors declare no competing interests.

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